Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN
"SCIENZE BIOMEDICHE, ENDOCRINOLOGICHE E NEUROFISIOLOGICHE"

CICLO
XXI

COORDINATORE Prof. Alessandro Martini

INVOLVEMENT AND ROLE OF THE PI3K/AKT NETWORK IN DIFFERENT TYPES OF LEUKEMIA CELLS BEFORE AND AFTER IN VITRO TREATMENT WITH CONVENTIONAL OR NEWLY DEVELOPED DRUGS.

Settore Scientifico Disciplinare BIO/16

Dottorando	**Tutore**
Dott.ssa Silvia Missiroli	dott. Luca Maria Neri
(firma)	*(firma)*

Anni 2006/2008

INDEX

1 Introduction

1.1 The PI3K/Akt signal transduction pathway

1.1.a The PI3K family

The pathways regulated following activation of the phosphoinositide 3-kinase (PI3K) family of enzymes have been intensively studied in recent years. In the 1980s, investigators observed phosphoinositide kinase enzymatic activity that modified a specific site on phosphatidyl-inositides (PtdIns), showing that it was associated with tyrosine-phosphorylated proteins. Much of this work was initiated in the laboratories of Tom Roberts and Lewis Cantley [Whitman et al 1985, Kaplan et al 1986, Whitman 1987]. In 1988, Whitman et al. provided biochemical proof that the kinase activity was able to specifically phosphorylate the 3 position on the inositol ring, thereby initiating the age of PI3K investigations. Subsequent characterization and molecular cloning revealed that a p110 catalytic subunit was bound with a regulatory subunit containing Src homology 2 (SH2) domains, p85, which was responsible for the association with tyrosine-phosphorylated proteins initiating PI3K activation. The p85 subunit, which was later shown to be one of the several forms of regulatory subunit, associates with a p110 subunit responsible for the catalytic activity. Studies over several years revealed that the PI3K family consists of at least nine genes in mammalian systems, corresponding to various isoforms that are categorized into class I, II and III. The class I subgroup is the most relevant when discussing growth-factor- or cytokine-activated PI3K.

1.1.b Class I, II and III PI3Ks

Unlike the other PI3K isoforms, class I PI3Ks are thought to exclusively phosphorylate PtdIns(4,5)P2 to generate PtdIns(3,4,5)P3 *in vivo*, even though they can utilize other lipids such as PtdIns and PtdIns4P when assayed *in vitro*. The class I enzymes consist of p110α, p110β, p110γ and p110δ catalytic subunits. The α, β and δ subunits are referred to as class IA and associate with regulatory subunits of the p85 type, which contain SH2 domains. Following activation of tyrosine kinases, the SH2 domains of p85 mediate activation of PI3K by binding to a consensus YXXM motif, when the tyrosine residue is phosphorylated [Cantley et al 1991]. In an alternative pathway of class IA PI3K activation, activated p21-Ras has been shown to directly bind and activate the p110 catalytic subunits [Rodriguez-Viciana et al 1994]. The potential importance of Ras-mediated regulation of PI3K was recently reinforced in Downward's laboratory using a murine model to

show that Ras-driven tumorigenesis requires the Ras-binding site on p110α [Gupta et al 2007]. The less-well-characterized p110γ isoform of PI3K associates with a regulatory subunit known as p101, and is activated in response to G-protein-coupled receptor (GPCR) stimulation [Stephens et al 1997, Stoyanov et al 1995]. To date, the exact molecular mechanism involved in regulation by the p101 subunit is not fully defined, but it has been shown to have sites of interaction with $\beta\gamma$ subunits of the trimeric G-proteins [Voigt et al 2005]. On the other hand, earlier studies suggested that, *in vitro*, the $\beta\gamma$ subunits may activate p110γ directly [Brock et al 2003]. Knockout studies showed that the function of p110γ was most important for T-cells and neutrophils and was essential for GPCR-mediated generation of PtdIns(3,4,5)*P*3 in these cells [Sasaki et al 2000, Li et al 2000, Hirsch et al 2000]. The p110α and p110β forms of PI3K are essential for normal mammalian development, as well as p85α [Vanhaesebroeck et al 2005]. Interestingly, loss of the p85β subunit has a milder phenotype, resulting in increased sensitivity to insulin receptor. Therefore, although there are unique roles for the various isoforms of PI3K, it has also become clear that this family plays a very important role in normal mammalian development. Key functions for the various PI3K enzymes have been demonstrated in numerous model systems, utilizing cellular responses to different agonists. In fact, virtually every hormone and growth factor investigated has been shown to have effects on PI3K activity. There are two major lipid products of class I PI3Ks: PtdIns(3,4,5)*P*3 which results from phosphorylation of PtdIns(4,5)*P*2, and PtdIns(3,4)*P*2 that is a product of 5-phosphatases, such as SH2-containing inositol phosphatase-1 (SHIP-1) or SHIP-2, removing one phosphate residue from the 5 position on the inositol ring of PtdIns(3,4,5)*P*3 [Erneux et al 1998]. The mechanism by which the lipids mediate their cellular effects was clarified by the identification of pleckstrin homology (PH) domains in a variety of proteins as docking modules for these lipids [Lemmon et al 2000]. Thus the lipid products of PI3K can be considered as second messengers, representing the internal chemical signal that is generated in response to an external signal. These lipids transmit information thus orchestrating the recruitment of cytosolic proteins to the plasma membrane where they can dock to the polyphosphorylated lipids and are activated.

Class II PI3Ks can also be activated by tyrosine-kinase receptors (RTKs), cytokine receptors and integrins; the specific functions in response to these activators are not understood [Urso et al 1999].

Class III PI3Ks are heterodimeric enzymes of catalytic (Vps34, 100 kDa) and adaptor (p150) subunits, and use only phosphatidylinositol as a substrate. Class III PI3Ks are implicated in the regulation of mammalian target of rapamycin (mTOR) activity in response to aminoacid availability and the regulation of autophagy in response to cellular stress, indicating the importance of class III PI3K in controlling cell growth and survival [Cantley 2002]. The most extensively

investigated PI3Ks are class I, especially class IA PI3Ks. Figure 1 reports the scheme of the PI3K classes and their modular structure.
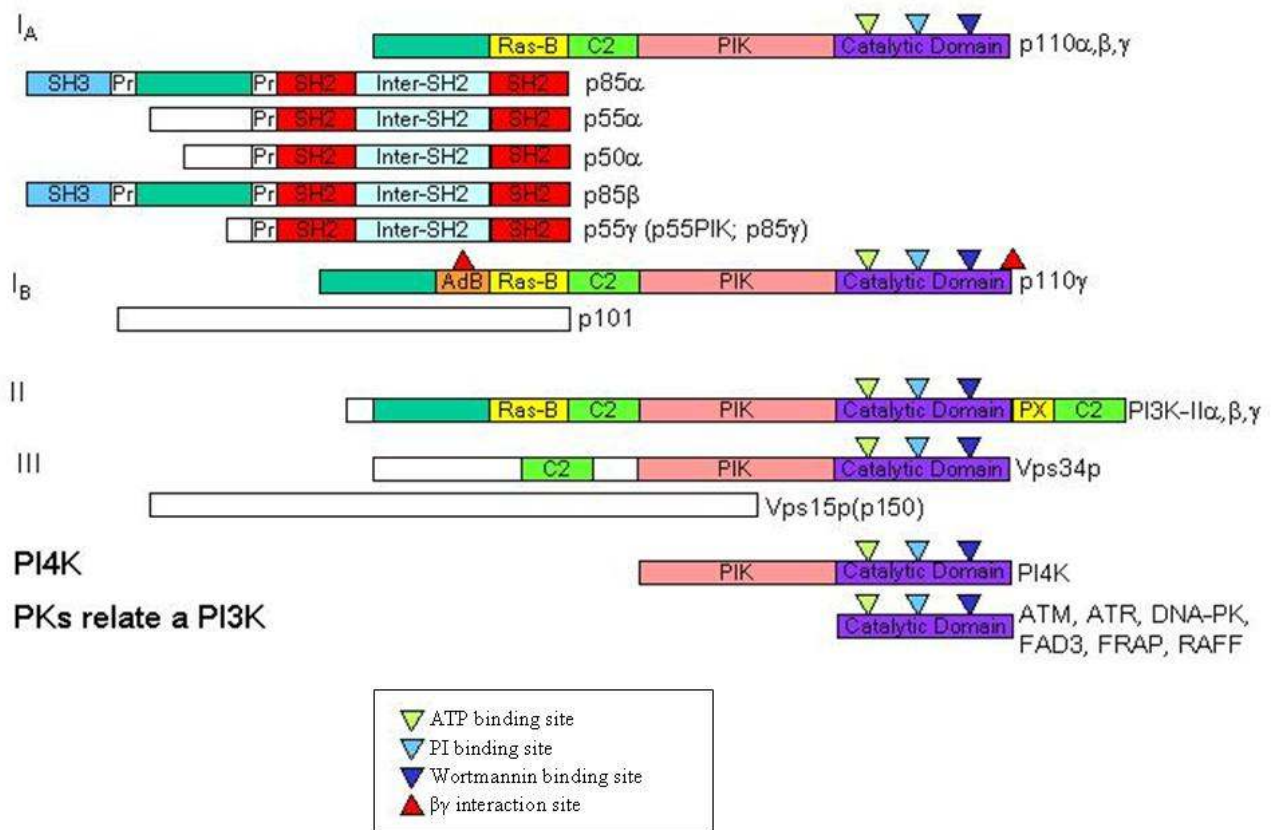
Figure 1. Scheme of the PI3K classes and modular structure.

1.1.c The main actors: PI3K, PDK1, Akt

The initial signaling cascade that occurs following PI3K activation (Figure 2) sets in motion the activation of one of the key kinases acting downstream of PI3K at the plasma membrane, phosphoinositide-dependent kinase 1 (PDK1) [Alessi et al 1997]. Interestingly, PDK1 has a PH domain that allows binding to PtdIns(3,4,5)P3 or PtdIns(3,4)P2, and thus to be recruited to the plasma membrane at sites of PI3K activation; lipid binding to PDK1 does not appear to be required for activation of the enzyme. PDK1 has an activating phosphorylation site that is likely site of autophosphorylation [Wick et al 2003]. PDK1 activity is directed toward the phosphorylation site in the activating loop of virtually all kinases of the AGC family, which includes PDK1 itself, Akt, protein kinase C (PKC) and p70-S6 kinase. Even before the characterization of PDK1, the dependence of Akt activity on upstream regulation by PI3K has been demonstrated in numerous

studies [Burgering et al 1995, Franke et al 1995, Marte et al 1997, Coffer et al 1998, Gold et al 1999, Scheid et al 2001, Yang et al 2002]. When both PDK1 and Akt are recruited to the plasma membrane via their PH domains, the kinase activity of PDK1 phosphorylates the Thr308 residue on Akt, dependent upon the presence of PtdIns(3,4,5)$P3$ or PtdIns(3,4)$P2$ [Alessi et al 1997, Mora et al 2004, Vanhaesebroeck et al 2000]. The AGC kinase family is also characterized by an hydrophobic motif that must be phosphorylated to fully activate the kinase [Yang et al 2002]. In Akt, the residue in the hydrophobic motif which is phosphorylated is Ser473, which allows intramolecular binding to occur, resulting in activation of the kinase. Although earlier studies had suggested that PDK1 might also serve to phosphorylate Ser473, many lines of evidence have now shown that a distinct kinase activity is responsible for what is often referred to as 'PDK2' activity [Dong et al 2005]. In particular, it was shown that in PDK1-knockout cells, Ser473 phosphorylation of Akt is retained [Williams et al 2000]. A recent work has demonstrated that the target of rapamycin (TOR), when bound to the adaptor protein rector [rapamycin-insensitive companion of mTOR (mammalian TOR)], which makes it rapamycin-insensitive, functions as a Ser473 kinase [Sarbassov et al 2006, Jacinto et al 2008]. In previous studies, other kinases that may phosphorylate Ser473 have been proposed, including the integrin-linked kinase (ILK) [Delcommenne et al 1998], DNA-dependent protein kinase (DNA-PK) [Feng et al 2004] or even Akt itself acting by autophosphorylation [Chan et al 2001]. The initial report of DNA-PK as a Ser473 kinase did not provide a possible explanation of the potential physiological functions, but Bozulic et al have reported that Akt is activated in a DNA-PK-dependent manner following double-strand breaks. This study showed not only that DNA-PK was required for Ser473 phosphorylation, but also that PDK1 was necessary for Thr308 phosphorylation of Akt following irradiation of cells to induce DNA damage [Bozulic et al 2008]. Intriguingly, Akt activation was correlated with pro-survival function, which could be a necessary event in allowing repair of damaged DNA to proceed. Another important aspect of this study is that it may explain the important function previously attributed to nuclear localized PDK1 [Scheid et al 2005]. Nuclear PI3K-generated PtdIns lipids have not been described, and thus PDK1 and Akt in the nucleus probably function independently of the PtdIns lipids. The difficulties in defining the precise events involving the phosphorylation of Akt at the hydrophobic domain also extends to how the PI3K lipid products are involved in the regulation of this event. The above-mentioned study suggests that PDK1 and DNA-PK-dependent regulation of Akt, at least under specific circumstances, may proceed independently of PI3K activation [Bozulic et al 2008]. However, it is clear that, under most conditions, inhibition of PI3K potently inhibits phosphorylation of both Akt Thr308 and Ser473 sites. Considering the more classical PI3K-dependent events, although Thr308 phosphorylation is most closely tied with the levels of PtdIns(3,4,5)$P3$ - or a combination of

PtdIns(3,4,5)$P3$ and PtdIns(3,4)$P2$ -, phosphorylation of Ser473 is linked to the levels of PtdIns(3,4)$P2$ only [Scheid et al 2002, Ma et al 2008]. Furthermore, the levels of PtdIns(3,4)$P2$ and Ser473 phosphorylation were found to be more accurate predictors of Akt activation state than PtdIns(3,4,5)$P3$ and Thr308 phosphorylation status [Ma et al 2008]. The crystal structure of Akt has demonstrated that its PH domain can bind either PtdIns(3,4)$P2$ or PtdIns(3,4,5)$P3$, since the phosphate at the 5-position does not fit into the binding pocket [Thomas et al 2002]. Thus either the two lipids can serve to recruit Akt to the plasma membrane. On the other hand, none of the reported Akt Ser473 kinases, with the exception of Akt itself, has been linked directly to the level of PtdIns(3,4)$P2$. Thus it is tempting to speculate that Akt autophosphorylation at Ser473 may be regulated either directly or indirectly by PtdIns(3,4)$P2$ levels, but more work will certainly be required to define the molecular events involved in this process. Although Akt translocates to the plasma membrane following activation of PI3K as a result of the elevated levels of 3-phosphorylated PtdIns lipids, Akt kinase activity targets are present both in the cytosol and nucleus.

In summary, at the cell membrane PI3K has the ability to phosphorylate phosphatidylinositol 4,5-biphosphate (PIP2) at the 3-position of the inositol ring, generating phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 acts as second messenger and serves as membrane anchor for proteins with a pleckstrin homology (PH) domain, such as Akt and PDK1. The colocalization of Akt and PDK1 at the cell membrane favours Akt phosphorylation and consequent activation [Cantley 2002].

Akt associates with a number of proteins that may regulate its activity and/or localization, one of which is the T-cell leukaemia 1 (TCL1) family of proteins. TCL1 enhances Akt activity with oncogenic properties. The TCL1–Akt complex forms at the cell membrane, but it has also been shown to be transferred to the nucleus [Pekarsky et al 2000, Teitell et al 2005]. The potential importance of TCL1 in nuclear localization of Akt in early stages of embryo development has been recently suggested [Fiorenza et al 2008]. As noted above, nuclear localization of PDK1 has also been reported [Scheid et al 2005], which allows an additional regulation of Akt and its nuclear targets. A recent study proposed another potential regulatory event that may control Akt release from the plasma membrane: calmodulin/Ca2+ was shown to directly bind to the PH domain of Akt, which would potentially enhance the release of active Akt from membranes, or prevent its re-association with membranes [Dong et al 2007]. Some of the details of Akt phosphorylation and the complete details of all PDK1's functions remain to be fully explored, both at the plasma membrane/cytoplasm and in the nucleus. However, the vast majority of Akt targets are not at the plasma membrane and thus the cytosolic and nuclear targets of Akt are playing a major role in regulation of several cellular events.
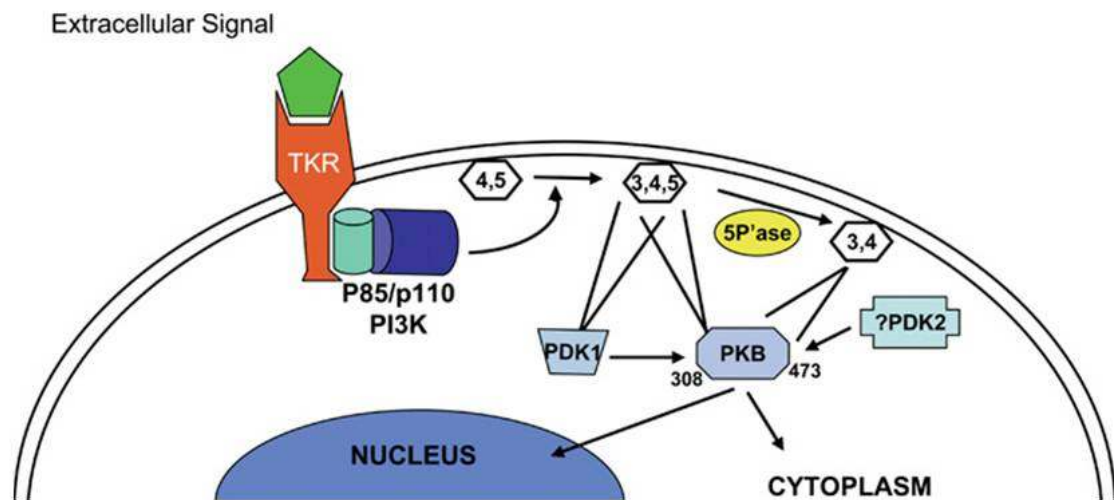
Figure 2. Scheme of the PI3K/Akt signaling pathway.

1.1.d The antagonist: PTEN-mediated inhibition of the pathway

The PI3K/Akt activity is negatively modulated by the Phosphatase and Tensin homolog deleted on chromosome 10 (PTEN) and SH2 Inositol 5-Phosphatase (SHIP) inhibitors. PTEN is a 3'-phosphatase which terminates the PI3K signaling in cells and was found to be inactivated in several human cancers, thus resulting in PI3K/Akt signaling constitutively activated. In particular, PTEN is a dual lipid and protein phosphatase. Its primary target is PIP3 [Maehama et al 1998], the direct product of PI3K. Since PTEN dephosphorylate PIP3, it acts as negative regulator of the PI3K/Akt pathway [Cantley 2002]. Loss of PTEN function, either in murine embryonic stem cells or in human cancer cell lines, results in accumulation of PIP3 mimicking the effect of PI3K activation and triggering the activation of its downstream effectors. PDK1 contains a C-terminal pleckstrin homology domain, which binds the membrane- bound PIP3 triggering PDK1 activation. Activated PDK1 phosphorylates Akt at Thr308 activating its serine–threonine kinase activity (100-fold over the basal). Once phosphorylated in Thr308, further activation of Akt occurs by PDK2 (the complex rictor–mTOR or DNA-PK) phosphorylation at Ser473. It is known that Akt activation stimulates cell cycle progression, survival, metabolism and other crucial events through phosphorylation of many physiological substrates [Dahia 2000, Kandel et al 1999, Downward 2004, Vivanco et al 2002]. Activation of Akt results in the suppression of apoptosis induced by a number of stimuli including growth factor withdrawal, detachment of extracellular matrix, UV irradiation, cell cycle discordance and activation of FAS signaling [Kandel et al 1999, Downward 2004, Plas et

al 2005]. Hyperactivated Akt has been also shown to promote cell proliferation, possibly through down-regulation of the cyclin-dependent kinase inhibitor p27 as well as up-regulation and stabilization of cyclin D1 (see below in the text) [Blume-Jensen et al 2001]. Different genetic approaches have been used to directly assess the role of Akt in PTEN null-induced phenotype. Deleting Akt reversed the cell survival phenotype in PTEN-null cells and reversed its growth advantage [Stiles et al 2002]. Similarly, inactivation of Akt by dominant-negative mutants inhibits the survival advantage provided by activated class I PI3K [Link et al 2005]. These and other results point out the essential role of PTEN in modulating and turning off the PI3K/Akt network [Chen et al 2005, Samuels et al 2006, Toker et al 2006, Mayo et al 2002, Brazil et al 2001].

SHIP 5'-phosphatase dephosphorylates PtdIns(3,4,5)P3 to PtdIns(3,4)P2; SHIP-1 isoform is prevalently expressed in hematopoietic cells, while the ubiquitary SHIP-2 isoform co-localises with nuclear components [Deleris et al 2003].

1.1.e Human Akt-1

RACK (related to A and C kinase), which was discovered based on its similarity to both PKA (protein kinase A) and PKC [Coffer et al 1991, Jones et al 1991] was discovered as a retroviral oncogene referred to as *Akt* [Bellacosa et al 1991]. The connection between PI3K and Akt was established in several independent studies, first using the PI3K inhibitor Wortmannin, but eventually validating the connection by various alternative means of activating and inhibiting PI3K and/or Akt [Burgering et al 1995, Franke et al 1995, Kohn et al 1995, Franke et al 1997, Marte et al 1997, Coffer et al 1998, Franke et al 1997]. As noted above, details of PI3K-mediated regulation of Akt have subsequently been established.

Akt belongs to the AGC protein kinase family and three isoforms, Akt-1, Akt-2 and Akt-3, plus a fourth isoform defined Akt-γ1, have been identified in humans. They are codified by different genes with 80% sequence homology. Akt-1 is the predominant isoform in the major part of tissues, Akt-2 is present in insulin sensitive tissues and Akt-3 has not been completely localized, but it is absent in central nervous system. Akt is constituted by three distinct modules: the pleckstrin homology (PH) domain in the amino-terminal region able to bind phospho-lipids; the central kinase domain which contains an highly conserved activation loop, called T-loop, with threonine residue important for the enzyme activation; a regulative carboxyl-terminal extension of about 40 amino acids containing the hydrophobic F-X-X-F/Y-S/T-Y/F motif. The crystallographic structure of the Akt-1 PH domain and the Akt-2 catalytic domain have recently been solved and have helped understanding the activation mechanisms of the kinase [Thomas et al 2002, Yang et al 2002].

Akt activation is a complex process constituted by subsequent phosphorylation events on different residues [Bellacosa et al 1995]. Akt, after binding the just formed PIP3, translocates to the cellular membrane where it undergoes phosphorylation on two sites important for the activation, threonine 308 in Akt-1 (309 in Akt-2 and 305 in Akt-3) and serine 473 in Akt-1 (474 in Akt-2 e 472 in Akt-3). Threonine 308 is located within the T-loop and is phosphorylated by PDK1, producing a conformational change which promotes the second phosphorylation on serine 473, on the carboxyl-terminal hydrophobic extension of kinase domain. It is still not clear which protein phosphorylates serine 473 in Akt; most of authors identify this kinase with PDK2 [Chan et al 2001], but other candidates are integrin-linked kinase (ILK), mitogen activated protein kinase-activated kinase 2 (MAPKAPK2) and PDK1, also if silencing of Pdk1 gene inhibited phosphorylation on 308 residue but not on 473 [Neri et al 2002]. It was recently reported that a complex of the mammalian target of rapamycin (mTOR) kinase mediates Akt serine 473 phosphorylation in vivo [Sarbassov et al 2005]; some author has also hypothesized that Akt auto-phosphorylation occurs on 473 residue [Toker et al 2006]. Akt activity is maximal when the kinase is phosphorylated on both residues, increasing substrates affinity and greatly powering the catalytic potential. Beside these essential activation sites, serine 124 and threonine 450 residues are constitutively phosphorylated [Alessi et al 1998], while tyrosine 315 and 326 can be phosphorylated by Src kinase, maybe regulating Akt activity. The interaction between Akt and the oncogene TCL1 further increases the kinase activity [Chen et al 2006].

Activated Akt is able to translocate from cytoplasm into the nucleus, where signaling events appear independent from those on the plasmatic membrane [Borgatti et al 2003]. Akt translocation into nucleus has been demonstrated in several cell lines in response to stimuli as after insulin like growth factor (IGF-I) treatment of NIH3T3 cells, nerve growth factor (NGF) stimulation of PC12 cells [Borgatti et al 2003], erythropoietin (EPO) in K562 cells and IGF-I or platelet derived growth factor (PDGF) mitogen factors in MC3T3 [Neri et al 2002]. Also if Akt contains a sequence for nuclear export rich in leucine and some protein may have a role of localization signal for Akt intranuclear migration, a nuclear localisation sequence have not yet been identified and Akt intranuclear translocation mechanism remains to be elucidated.

Akt exerts its kinase activity toward proteins containing the consensus sequence R/K-X-R/K-X-X-S/T, where S or T are the phosphorylable residues. Several Akt substrates that mediate crucial events as apoptosis, cell cycle progression, growth or metabolism have been identified, but it is unlikely that we currently know all the critical Akt substrates or that their Akt dependent phosphorylation is completely characterized. The identification of more than 400 different proteins containing the consensus sequence for Akt phosphorylation makes presume that in the future other

Akt substrates will be characterized [Nicholson et al 2002]. The heterogeneity of proteins potentially phosphorylated by Akt supports the key role of this kinase in different fundamental cell processes. Notwithstanding it is still to be completed, the study of the role of Akt in the nucleus is revealing important information. The regulation of Akt intranuclear translocation in response to different stimuli and the panel of molecules targeted by Akt is going to be elucidated.
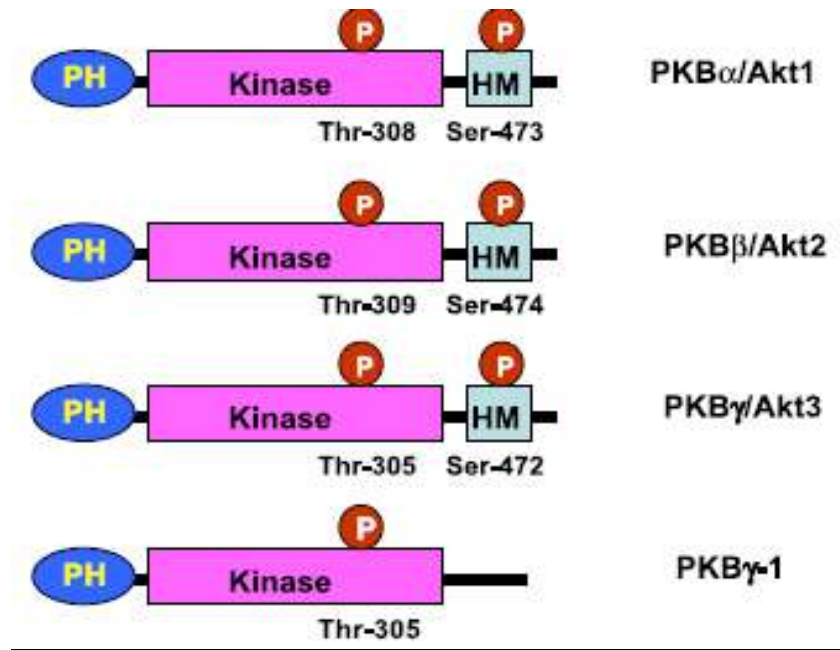
Figure 3. Scheme of the Akt isoforms structure.

1.2 The PI3K/Akt network regulates important cell functions

The activation of important biological functions in the cell by the PI3K/Akt network is outlined in Figure 4.
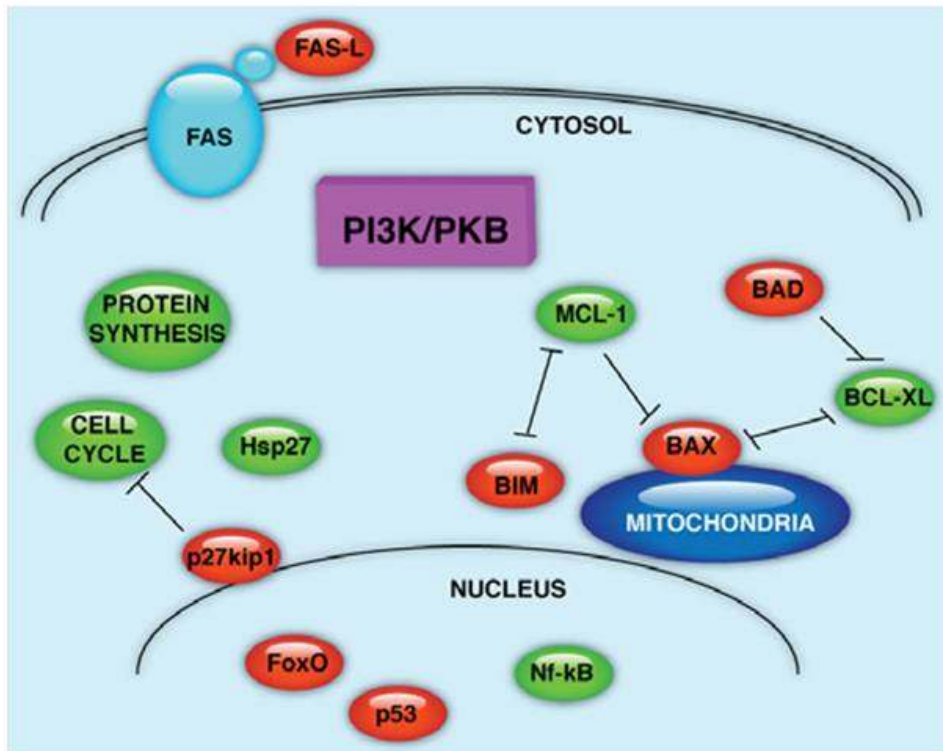
Figure 4. Scheme of the biological functions activated by the PI3K/Akt network in the cell.

Akt is well known to enhance **cell survival** through the inhibition of pro-apoptotic proteins such as FOXO and Bcl2-Antagonist of cell Death (BAD). FOXO regulates transcription of pro-apoptotic proteins like Fas-ligand (FasL) and Bim; BAD inhibits the function of pro-survival molecule BclXL for inducing apoptosis. Akt can also block the apoptosis through the induction of survival proteins such as Bcl2, I-KappaB Kinase (IKK), and human double minute 2 (HDM2) [Engelman et al 2006, Stephens et al 2005].

The PI3K/Akt network has been demonstrated to play a key role in regulating **cell cycle progression**. PI3K axis increases cyclin D1, cyclin-dependent kinase 4 (Cdk4) and cdc25A expression and Rb phosphorylation at Ser780, Ser795, and Ser807/811 [Gao et al 2003]. PI3K mediates the cell cycle progression and cyclin expression through activation of the Akt/mTOR/p70S6K1 axis. Akt promotes the G1-S phase transition by blocking FOXO-mediated transcription of p27/Kip1, or directly phosphorylates and inactivates p27/Kip1. Akt induces cell proliferation by phosphorylating the tuberous sclerosis complex 2 (TSC2) protein tuberin, and by inhibiting the GAP (GTPase-activating protein) activity of the TSC1-TSC2 complex. Moreover, Akt seems to be directly linked to activation of its downstream target p21CIP1/WAF1 important for the cell cycle progression.

The PI3K/Akt mediated activation of the mTOR-raptor kinase complex, in turn mediates p70S6K1 activation and the phosphorylation of eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) to regulate **protein synthesis** [Engelman et al 2006]. Importantly, PI3K/Akt axis is involved in **transcription modulation** through the FOXO transcription factor family, which in particular regulates transcription of pro-apoptotic proteins.

Akt can increase **glucose metabolism** in the cell and in particular glycogen synthesis through the inactivation of forkhead (FHKH) family transcription factors and glycogen synthase kinase 3 (GSK3) [Summers et al 1999, Cong et al 1997].

Moreover, Akt-mediated activation of FAK protein produces the **cytoskeleton reorganization**.

The essential functions activated by the PI3K/Akt network are mainly carried out by Akt downstream targets. A wide panel of the characterized Akt substrates has been identified and reported (Table 1), some of which are cytoplasmatic while others reside or translocate into the nucleus.

CYTOPLASM	MW	REFERENCE	NUCLEUS	MW	REFERENCE
GSK3β (S9, S21)	84	Jope RS et al, 2004	GSK3β (S9, S21)	84	Jope RS et al, 2004
eNOS (S1177/9)	240	Iantorno M et al, 2007	eNOS (S1177/9)	240	Iantorno M et al, 2007
TBC1D1 (T590)		Zhou QL et al, 2008	acinus (S422, S573)	220,98, 94	Hu Y et al, 2005
PFK2, ACL		Hammerman PS et al, 2004	p21 (T145, S146)	32	Rossig L et al, 2001
caspase 9 (S196)	53	Donepudi M et al, 2002	p27 (T157)	40	Shin I et al, 2002
bad (S136)	20	Downward J, 1999	MDM2	56	Vousden KH et al, 2002
PRAS40 (T246)	40	Kovacina KS et al, 2003	nucleolin (T597)	109	Borgatti P et al, 2003
TSC2 (S939, T1462)		Manning BD et al, 2002	FKHR (T32, S253, S256, S315)	131	Nicholson KM et al, 2002
mTOR (S2448, S2481)	40	Varma S, 2007	IKK	23	Ozes ON et al, 1999
Pdcd4 (S67, S457)		Palamarchuk A et al, 2005a	AR factors	/	Lin HK et al, 2001
ASK1 (S83)	154	Autret A et al, 2008	tal1 (T90)	66	Palamarchuk A et al, 2005b
tau (S214)	151	Kyoung Pyo H et al, 2004	GATA-2	95	Menghini R et al, 2005
MLK3 (S674)	92	Barthwal MK et al, 2003	AHNAK (S5535)	628	Sussman J et al, 2001
YAP (S127)	65	Basu S et al, 2003	SRK	96	Koh H et al, 1999

Table 1. Characterized Akt substrates.

1.2.a Apoptosis

PI3K/Akt signal transduction pathway plays a critical role in cell survival by activating anti-apoptotic events. Apoptosis is a complex set of events initiated within a cell by proteolytic enzymes known as caspases that eventually leads to cellular destruction [Earnshaw et al 1999, Budihardjo et al 1999]. The onset of apoptosis has been well studied, and many of the key pathways are now well understood. Many different regulatory events involved specifically in promoting cell survival are modulated as a result of PI3K/Akt activation. The PI3K/Akt network blocks many pro-apoptotic proteins, or have a positive effect on multiple prosurvival components, several categories of which are summarized in Figure 5.
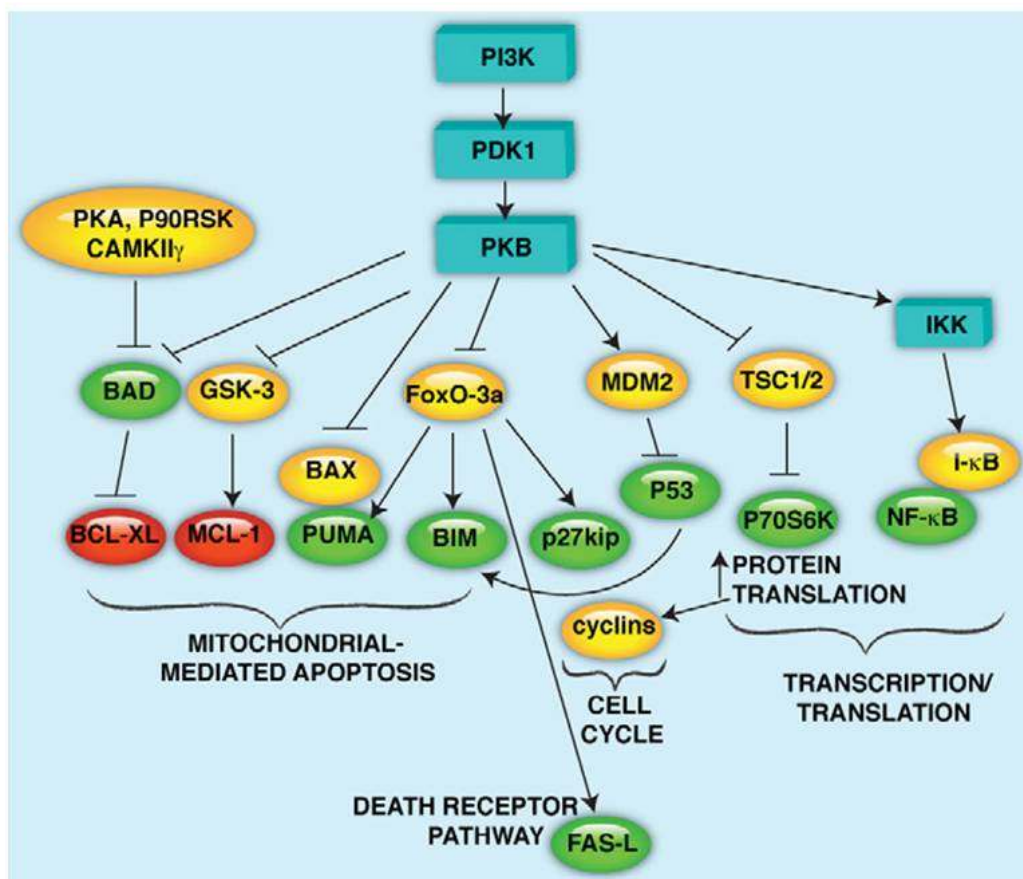

Figure 5. Scheme of the Akt positively and negatively activated proteins involved in apoptosis.

Potential pro-apoptotic proteins may be increased in their level of expression, or they become activated as a result of the loss of suppressive phosphorylation events. In some cases, maintenance of normal levels of anti-apoptotic proteins in healthy living cells requires the PI3K/Akt

regulation. Akt generates anti-apoptotic signals through the inhibitory phosphorylation of Bad, GSK3, Forkhead (FOXO1) and caspase-9, through the activating phosphorylation of mTOR, and through the release of transcriptional factors such as NF-κB [Cantley 2002, Barata et al 2005].

The first evidence suggesting PI3K involvement in cell survival appeared in 1995 [Yao et al 1995, Scheid et al 1995]. These authors showed that, in rat phaeochromocytoma PC12 cells, blocking PI3K caused death of cells and that survival required a PDGF receptor that could activate PI3K activity. PI3K function in promoting cell survival subsequently became a very important area of research [Webb et al 2000, Minshall et al 1996] and haemopoietic cell survival (apoptosis inhibition) had long been known to depend on specific cytokines [Park 1996, Lotem and Sachs 1996, Brach et al 1992]. The next obvious direction in this field came with the demonstration that activation of Akt was a key event in the promotion of cell survival that was dependent upon PI3K [Marte et al 1997, Alessi et al 1998]; this knowledge partially explained how Akt could function as an oncogene. Much of the attention in this field was initially focused on Akt-mediated phosphorylation of the pro-apoptotic protein Bad [Yang et al 1995, Zha et al 1997, Zha et al 1996]. Subsequently, several Akt substrates were observed to be involved in apoptosis regulation as caspase-9, which after Akt dependent phosphorylation promotes cell survival [Franke et al 2003, Donepudi et al 2002, Downward et al 1999]. Phosphorylation at Akt consensus site threonine 246 of the Akt substrate PRAS40 appears to protect neuronal cells from apoptosis after stroke [Kovacina et al 2003]. Phosphorylated PRAS40 has also been proposed to promote cell survival in cancer cells [Huang et al 2005]. When Akt specifically phosphorylates serine 67 and 457 of another critical target, programmed cell death 4 (Pdcd4), nuclear translocation of Pdcd4 and interference with the transactivation of AP-1-responsive promoter by c-Jun occur [Palamarchuk et al 2005]. Also the apoptosis signal-regulating kinase 1 (ASK1) is a key regulator of c-Jun amenable to inhibition by Akt-mediated phosphorylation at serine 83 in different cell models, reducing apoptosis [Autret et al 2008]. Microtubule-associated protein tau contains a consensus motif for Akt encompassing the double phospho-epitope (T212/S214), a specific marker for Alzheimer's disease (AD). Akt dependent phosphorylation of tau occurs in vitro at both threonine 212 and serine 214 and may play specific roles relevant to AD and other neurodegenerations [Ksiezak-Reding et al 2003]. In primary cultures, Akt selectively phosphorylates tau at serine 214, raising the possibility that 214 residue may participate in Akt-mediated anti-apoptotic signaling [Kyoung Pyo et al 2004]. Mixed lineage kinase 3 (MLK3) is a mitogen-activated protein kinase kinase kinase (MAPKKK) that can induce cell death in neurons. Akt phosphorylates MLK3 on serine 674 and suppresses MLK3-mediated neuronal apoptosis in a manner dependent on serine 674 phosphorylation [Barthwal et al 2003]. The yes-associated protein (YAP), which can be phosphorylated by Akt at

serine 127, normally binds to the 14-3-3 protein [Basu et al 2003]. Akt promotes YAP localization to the cytoplasm, resulting in loss from the nucleus where it functions as a coactivator of transcription factors. Akt phosphorylation of YAP may suppress the pro-apoptotic gene expression response following DNA damage. Amongst the Akt substrates identified into cell nucleus, acinus is a nuclear factor required for chromatin condensation which induces resistance to caspases proteolysis and to apoptosis when phosphorylated by Akt on serine 422 and 573 [Hu et al 2005].

Bcl-2 family - Cell survival can be promoted by Akt phosphorylation of Bad at Ser136 producing Bad inactivation and association to cytosolic 14-3-3 proteins [del Peso et al 1997, Dudek et al 1997, Datta et al 1997, Blume-Jensen et al 1998], also if thise point is controversed [Scheid and Duronio 1998, Hinton and Welham 1999, Ekert et al 2006]. Many studies have provided compelling evidence that endogenous levels of Bad may be regulated by PI3K/Akt-mediated phosphorylation and thus this pathway may be important in some specific cell types. Other kinases are known to phosphorylate Bad at other sites, including PKA [Lizcano et al 2000, Tan et al 2000] and p90rsk [Tan et al 1999], and thus Bad's activity in cells is normally very well suppressed. Most recently, the γ isoform of Ca2+/calmodulindependent protein kinase II (CaMKIIγ) was identified as further regulator of Bad pro-apoptotic activity. However, since the Bad-knockout mice developed normally and had a very mild phenotype, it might be expected that the profound pro-survival effect of PI3K/Akt must be functioning through other proteins.

Another Bcl-2 family pro-apoptotic protein regulated by PI3K/Akt is Bax, which is a key regulator of mitochondrial permeability leading to apoptosis. Bax is phosphorylated by Akt at the inhibitory Ser184 site, which contributed to suppression of Bax-mediated death of neutrophils [Gardai et al 2004]. A subsequent study reported that protein phosphatase 2A (PP2A)-mediated dephosphorylation of Bax contributed to its activation; phosphorylation of Bax at Ser184 could be mediated by a PKCζ-dependent event [Xin et al 2007]. GSK-3, which is inhibited by Akt-mediated phosphorylation, has been reported to phosphorylate Ser163 on Bax, promoting Bax translocation to the mitochondria [Linseman et al 2004]. Thus there are at least two possible ways in which Akt can suppress the apoptotic activity of Bax: by inhibiting it directly phosphorylating Ser184 residue, or by suppressing Ser163 phosphorylation due to Akt-mediated inhibition of GSK-3 activity. A recent study investigated the role of the stress-induced heat-shock protein Hsp27 in regulating the activity of Bax [Havasi et al 2008], according to many studies that have suggested that stress response proteins can provide protection against apoptosis [Garrido et al 2003, Calderwood and Ciocca 2008, Arrigo 2007]. In particular, regulation of the forkhead pathway can suppress expression of the pro-apoptotic members Bim and p53-Up-regulated Modulator of Apoptosis (PUMA) [Bauer et al 2006, You et al 2006], whereas the NF-κB survival pathway can promote

expression of Bcl-XL. Bim and PUMA are two Bcl-2 family proteins thought to promote apoptosis as a result of binding to the pro-survival family members [Willis and Adams 2005]. However, Bim has also been reported to mediate direct activation of Bax [Kuwana et al 2005], while PUMA function has been reported to be due to its association with Bcl-XL and p53 [Chipuk et al 2005].

Forkhead transcription factors - A very important set of Akt targets involved in apoptosis regulation are the forkhead transcription factors [Tang et al 1999, del Peso et al 1999, Arden et al 2002]. Their phosphorylation leads to sequestration and degradation in the cytoplasm [Brunett et al 2004], thus their activity increases when PI3K/Akt is not active, causing increases in multiple potential regulators of cell death, including the Fas death receptor ligand, FasL [Suhara et al 2002], as well as the BH3 (Bcl-2 homology domain 3)-only protein Bim, and the cell cycle inhibitor, p27kip1 [Dijkers et al 2002, Dijkers et al 2000, Dijkers et al 2000, Behzad et al 2007]. PUMA, a key mediator of apoptosis, seems to be regulated in a FoxO3a-dependent manner [You et al 2006. Ekert et al 2006. Behzad et al 2007].

NF-κB - The role of NF-κB in regulating multiple genes involved in cell survival is well established [Sonenshein 1997], and forms the basis upon which NF-κB alterations may play a role in development of many types of cancer [Dutta et al 2006, Ravi and Bedi 2004]. NF-κB is an important regulator of many antiapoptotic or pro-survival genes, the most important of which are the anti-apoptotic Bcl-2 and Bcl-XL proteins [Bharti and Aggarwal 2002, Beinke and Ley 2004, Sevilla et al 2001, Konishi et al 2006, Chen et al 2000, Jones et al 2000, Hundal et al 2003]. In addition, the inhibitor of caspase 8, FLIP {FLICE [FADD (Fas-associated death domain)-like IL-1β-converting enzyme]-inhibitory protein}, which is a natural inhibitor of the death receptor pathways, can be induced by NF-κB [Kreuz et al 2001]. The Inhibitor of Apoptosis Proteins (IAPs) have been shown to function in cell survival by blocking multiple caspases and NF-κB can regulate the expression of several IAPs [Hong et al 2000]. The regulation of NF-κB transcription factor activity occurs primarily by regulation of its associated inhibitory molecule, IκB [Karin and Ben-Neriah 2000]. IκB is phosphorylated by a specific kinase complex consisting of IκB kinase (IKK) α and β, which target the protein for ubiquitination and degradation, thereby releasing the active transcription factor. IKKα can be activated by Akt, thus providing one of the multiple ways in which NF-κB activity can be enhanced in cells [Ozes et al 1999, Romashkova and Makarov 1999].

GSK-3 - A well known target of Akt is the GSK-3 kinase [Moule et al 1997, Cook et al 1996, Ikeda et al 1998]. GSK-3α and GSK-3β isoforms have been shown to be negatively regulated by numerous signaling pathways [Doble et al 2003]. GSK-3 activity is normally suppressed in proliferating cells by Akt-dependent phosphorylation on the N-terminal serine residue (Ser21 in GSK-3α, and Ser9 in GSK-3β) [van Weeren et al 1998, Cross et al 1995]. As a result, enhanced

apoptosis can be observed in cells in which active forms of GSK-3 are expressed [Pap and Cooper 1998]. Several possible ways in which GSK-3 may contribute to cell death have been suggested: one involved c-Jun N-terminal kinase (JNK) and Mixed-Lineage Kinase-3 (MLK-3) activation [Mishra et al 2007, Eom et al 2007]; recent reports suggested that GSK-3 could promote apoptosis by phosphorylating myeloid cell leukemia-1 (Mcl-1) and promoting Mcl-1 degradation [Maurer et al 2006. Ding et al 2007], also if the latter mechanism is controversial [Vilimek and Duronio 2006].

p53 - PI3K/Akt block apoptotic events by regulating the well-known tumor suppressor, p53. p53 regulates apoptosis, most notably when chromosomal aberrations due to drugs or radiation-induced DNA damage are detected, by a combination of events including the up-regulation of pro-apoptotic molecules such as Bax and PUMA [Wu and Deng 2002, Roos and Kaina 2006]. The transcriptional regulator p53 may also have apoptotic effects independently from its transcriptional activity and resulting from its function in the mitochondria [Moll et al 2005]. p53 activity is negatively controlled by murine double minute 2 (Mdm2), which can translocate into the nucleus and promote the ubiquitination and subsequent inactivation of p53. It was shown that Akt could mediate Mdm2 phosphorylation promoting nuclear translocation [Mayo and Donner 2001]. Recently, Mdm2 has been shown to be associated with MdmX, a direct target of Akt, stabilizing Mdm2 [Lopez-Pajares et al 2008]. Akt activity is thus important in keeping Mdm2 active and in suppressing p53 activity [Duronio 2008].

Notch - The transmembrane Notch receptor binds the Delta/Serrate ligand of a neighboring cell. This interaction activates a cascade of proteolytic cleavages that ultimately leads to activation of target genes and changes in the transcriptional pattern. In mammals, four Notch receptors (Notch1-4) and five Notch ligands (Delta-like-1,3 and 4 and Jagged1-2) are described [Leong and Karsan 2006, Zweidler-McKay and Pear 2004]. Interaction with ligands induces a conformational change in the Notch receptor that leads to two successive proteolytic cleavages; consequently, the intracellular Notch domain is released and translocates into the nucleus, where it is capable of binding proteins leading to the transcriptional activation of Notch-target genes [Leong and Karsan 2006]. A recent work showed that down-regulation of Notch-1 by transfection of a small interfering RNA could cause cell proliferation inhibition by inducing cell cycle arrest and apoptosis. The apoptotic effect resulting from down-regulation of Notch-1 may be mediated through regulating the expression of cell cycle regulatory proteins cyclin D1, CDK2 and p21 and the activity of Akt signaling [Guo et al 2008].

Jak/STAT - The evolutionarily conserved Janus Kinase/Signal Transducer and Activator of Transcription (Jak/STAT) receptor plays an important role in apoptosis as well as in other biological processes such as proliferation, differentiation and cellular immune responses, mediated

by growth-factors and cytokines [Dreesen et al 2006]. Binding of cytokines results in cell surface receptor oligomerization and tyrosine kinases activation. Activated Jaks phosphorylate the cytoplasmic domain of the receptor, thereby creating docking sites for STATs, which are phosphorylated by Jaks and consequently dimerize and migrate into the nucleus where they regulate gene transcription [Valentino et al 2006]. A recent work showed that cardiomyocytes possess an apoptosis-resistant property as a cytoprotection mechanism which is likely conferred by mutual transactivation between AKT/NF-kappaB and JAK2/STAT3, a novel crosstalk between the two signaling pathways within the networking governing the cell fate [Lu et al 2008].

1.2.b Cell cycle progression

The PI3K/Akt network plays a pivotal role in regulating the cell cycle progression and proliferation. Akt promotes the G1-S phase transition by blocking FOXO-mediated transcription of cell-cycle inhibitors, including p27/Kip1, or directly phosphorylates and inactivates p27/Kip1. Akt induces cell proliferation by phosphorylating the tuberous sclerosis complex 2 (TSC2) protein tuberin, and by inhibiting the GAP (GTPase-activating protein) activity of the TSC1-TSC2 complex. Akt can also indirectly stabilize the cell-cycle protein c-Myc and cyclin D1 by inhibiting GSK3 [Engelman et al 2006]. Akt may regulate cell proliferation promoting cell cycle progression by phosphorylating the cyclin inhibitors p21CIP1/WAF1 and p27/Kip1 [Rossig et al 2001, Shin et al 2002, Viglietto et al 2002]. Moreover, phosphorylation of the murine double minute 2 (MDM2) oncogene by Akt promotes a change in intracellular p53 localization and subsequent modification of the cell cycle in relation to DNA repair mechanisms [Vousden 2002, Mayo et al 2005]. Experiments in ovarian cancer cells demonstrates that PI3K inhibition blocked proliferation inducing cell cycle arrest in G1 phase [Gao et al 2004]. This effect was accompanied by decreasing expression of G1-associated proteins including cyclin D1, cyclin-dependent kinase 4 (Cdk4), cdc25A, and Rb phosphorylation at Ser780, Ser795, and Ser807/811 [Gao et al 2003]. The inhibition of PI3K also inhibited the activation of Akt and of its downstream molecules p70S6K1 and p21CIP1/WAF1. PI3K transmits the mitogenic signal through Akt, the mammalian target of rapamycin (mTOR) and p70S6K1. The mTOR inhibitor rapamycin has inhibitory effects on G1 cell cycle progression and expression of cyclin D1, Cdk4, cell division cycle 25A (cdc25A), and retinoblastoma protein (Rb) phosphorylation, indicating that PI3K mediates G1 phase of cell cycle progression and cyclin expression through activation of the Akt/mTOR/p70S6K1 axis [Gao et al 2004, Gao et al 2003]. Many authors agree indicating mTOR as a direct Akt substrate involved in

regulation of cell proliferation through translational control of several proteins [Starkman et al 2005, Varma et al 2007].

1.2.c Transcription modulation

Protein synthesis is a complex process that can be modulated by the PI3K/Akt network at different levels; Akt activates the mTOR-raptor kinase complex, which in turn mediates p70S6K1 activation and the phosphorylation of the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) responsible for protein synthesis [Engelman et al 2006]. PI3K/Akt mediated protein synthesis can be regulated also via tuberous sclerosis complex (TSC) and 1/2–Rheb–TOR–raptor [Marygold and Leevers 2002]. Previous studies have solidified the placement of TSC1/2 as direct downstream targets of Akt, which lie directly upstream TOR [Pan et al 2004]. Inhibitory phosphorylation of TSC2 by Akt allows TOR and p70S6K activation. A recent report has suggested that Akt-mediated phosphorylation of TSC2 can regulate nuclear/cytoplasmic localization of these proteins thus modulating nuclear as well as cytoplasmic p70S6K [Rosner et al 2007]. There are multiple targets within the protein translational machinery that are phosphorylated by p70S6K. The best known of these are the eukaryotic initiation factor 4E (eIF-4E) and the eIF4E-binding proteins (4E-BPs), which inhibit eIF-4E. The target of p70S6K, the ribosomal S6 protein, is important for terminal oligopyrimidine tract (5'-TOP)-dependent translation [Fingar and Blenis 2004, Jastrzebski et al 2007]. Together, these events result in an increased level of protein synthesis that is at least partially dependent on activation of the PI3K/Akt pathway, and downstream targets TOR and p70S6K.

A recently described protein highly expressed in cells undergoing apoptosis is programmed cell death protein 4 (PDCD4); PDCD4 has an inhibitory effect on protein synthesis and its expression is regulated in a PI3K/Akt/mTOR-dependent manner [Ozpolat et al 2007].

A recent review [White-Gilbertson et al 2008] has highlighted importance of protein translation regulation, pointing out that many of the anti-apoptosis proteins including Mcl-1, FLIP, X-linked IAP (XIAP) and survivin, have short half-lives (under 2 h), whereas many of the pro-apoptotic Bcl-2 family proteins and some caspases are much longer-lived. Thus the balance of pro- and anti-apoptotic proteins can be disrupted in favour of apoptosis if there is a loss of protein translation.

Some specific studies have highlighted ways in which apoptotic regulatory proteins can be specifically regulated at translational level by PI3K/Akt. PI3K activity contributes to increase translation of the pro-survival Bcl-2 family protein Mcl-1 [Huang et al 2000, Schubert and Duronio 2001, Adams and Cooper 2007].

Nucleolin, one of the most represented proteins into nucleoli of actively dividing cells, is an Akt substrate involved in processes important for protein synthesis as rRNA maturation and ribosomes biogenesis [Borgatti et al 2003, Ginisty et al 1999].

On the other hand, several Akt substrates are nuclear transcription factors which functions are modulated by the kinase activity: forkhead transcription factors (FKHR) drive the expression of pro-apoptotic genes; when phosphorylated by Akt, FKHR are sequestrated in the cytoplasm thus inhibiting apoptosis [Nicholson et al 2002]. The Akt-dependent activation of the FOXO transcription factor family regulates different transcription events, in particular of pro-apoptotic genes. The nuclear transcription factor NFκB, key regulator of the immunity response, is activated by Akt-phosphorylated IKK and degradation of Iκ-B inhibitor [Ozes et al 1999]. The nuclear androgen receptors (AR) play important role in apoptosis promotion in different cell lines; Akt dependent phosphorylation of AR seems to reduce their transcriptional activity thus inducing cell survival [Lin et al 2001]. The helix-loop-helix transcription factor tal1, required for blood cell development, is specifically phosphorylated by Akt at threonine 90, causing its nuclear redistribution [Palamarchuk et al 2005]. Akt regulates the adipogenesis through the substrate GATA2, a transcription factor expressed in adipose tissue which phosphorylation determinates exit from the nucleus and adipocytes differentiation [Menghini et al 2005]. Akt phosphorylates the nuclear protein p70S6K-related kinase (SRK), which possesses a carboxyl-terminal nuclear localization sequence [Koh et al 1999]. SRK is constitutively localized into the nucleus and may represent an important downstream component in the nuclear function of Akt. The enormous potentiality of the Akt target involved in the transcription function is supported by the huge number of substrates localizated into the nucleus.

1.2.d Glucose metabolism

The PI3K/Akt signaling pathway regulates essential metabolic functions of cells through Akt phosphorylation of its substrates. The Akt substrate glycogen synthase kinase 3-beta (GSK3β) is involved in metabolism regulation by decreasing glycogen synthesis and increasing glycolytic enzymes transcription [Jope et al 2004, Kohn et al 1996], relating the Akt activation with high glycolysis efficiency in cancer cells (Warburg effect). The recently reported TBC1D1 Akt substrate phosphorylated at threonine 590 may be involved in controlling the GLUT1 glucose transporter expression through the mTOR-p70 S6 kinase pathway [Zhou et al 2008]. It has been also proposed that Akt directly stimulates glycolysis through the phosphorylation of 6-phosphofructo-2-kinase (PFK2), and fatty-acid synthesis through the phosphorylation of ATP citrate lyase (ACL)

[Hammerman et al 2004]. Since it is involved in cell metabolism, we also report that endothelial nitric oxide synthase (eNOS) phosphorylation at Akt phosphorylation site, serine 1179, results in increased production of nitric oxide (NO) in vascular endothelium [Iantorno et al 2007].

1.2.e Cytoskeleton reorganization

Akt activation has been shown to be involved in remodeling and reorganization of the actin cytoskeleton of human podocytes, a cell type that composes the glomerular filtration barrier [Lennon et al 2008, Schlondorff et al 2008]. A recent work showed that phosphorylation of rat nephrin by the Fyn kinase markedly increased its interaction with a regulatory subunit of PI3K. Stable transfection of rat nephrin in the podocytes with podocin led to nephrin tyrosine phosphorylation, PI3K-dependent phosphorylation of Akt, increased Rac1 activity, and an altered actin cytoskeleton with decreased stress fibers and increased lamellipodia. These changes were reversed with an inhibitor of PI3K, thus Akt1 contributed to decrease stress fibers. These results suggest that PI3K is involved in nephrin-mediated actin reorganization in podocytes and that disturbed nephrin-PI3K interactions may contribute to abnormal podocyte morphology and proteinuria [Zhu et al 2008].

Akt-mediated activation of focal adhesion kinase (FAK) protein produces the cytoskeletal rearrangement and also the cell migration process on solid substrates. Activation of endogenous cardiomyocyte FAK leads to its increased association with the p85 regulatory subunit of PI3K and to concomitant activation of Akt; this activation affords a survival advantage, protecting cardiomyocytes from apoptotic insult induced by either hydrogen peroxide treatment or glucose deprivation [Del Re et al 2008].

1.2.f Differentiation

PI3K/Akt pathway is involved in many differentiation processes, such as erythroid differentiation. Binding of Erythropoietin (EPO) to its receptor (EPO-R), essential for erythroid cell proliferation and differentiation [Bunn 2007], triggers the phosphorylation and activation of EPO-R bound JAK2 tyrosine kinase, resulting in activation of several downstream signaling pathways that include PI3K/Akt pathway, MAP kinase, STAT5 and protein kinase C [Jelkmann 2007]. Several studies suggest that the PI3-kinase/AKT signaling pathway may have an important role in

supporting erythropoiesis [Klingmuller et al 1997, Huddleston et al 2003, Hammerman 2005] and AKT is rapidly phosphorylated and activated in response to EPO [Kashii et al 2000, Bouscary et al 2003].

EPO scarcely stimulates K562 human erythroleukemia cell proliferation, but induces erythroid differentiation [Kitanaka et al 1994]. Suppression of PI3K activity by the fungal metabolite Wortmannin reduces the expression of erythroid differentiation in K562 cells [Kubota et al 1996] and antisense src RNA expression interferes with hemoglobin synthesis, lying src immediately upstream of PI3K in the signal transduction of erythroid differentiation induced by EPO [Kubota et al 2001].

Neri et al previously demonstrated that, in addition to its well established role at the plasma membrane, PI3K translocates to the nucleus in response to EPO exposure of K562 cells, with increased PI3K activity and PtdIns(3,4,5)P$_3$ production that occurs early after treatment at the nuclear level [Neri et al 2002]. Indeed evidence collected from other leukemic cell types points to a key role played by PI3K [Marchisio 1998, Neri 1999] and by Akt [Matkovic 2006] in the transduction of differentiating signals from cell periphery to the nucleus.

Since we observed that PI3K plays a role in erythroid differentiation at the nuclear level, we felt that K562 could constitute a useful model to investigate the relevance of the PI3K downstream molecule Akt in erythroid differentiation induced by EPO. Here we show that PI3K activation is a prerequisite for the nuclear translocation of Akt, that Akt activation by phosphorylation is also critical for its nuclear translocation and that increased Akt activity rapidly occurs in the nucleus in response to the cytokine. These events appear to be essential for EPO-induced erythroid differentiation that is blocked by Akt inhibitors, indicating that nuclear Akt may play a crucial role triggering the signal transduction cascade necessary for EPO's erythroid differentiating signal from cell periphery to the nucleus.

1.3 Role of the PI3K/Akt network in disease

As detailed above, activation of the PI3K/Akt network is fundamental for regulating cell survival, proliferation and other important cellular events. Up-regulation or constitutive activation of PI3K/Akt signaling can have deleterious effects on cells leading to uncontrolled proliferation, enhanced migration and adhesion-independent growth. These events favor the development of inflammatory and autoimmune disease, and the formation of malignant tumors [Wymann and Marone 2005, Vivanco and Sawyers 2002, Engelman et al 2006].

1.3.a PI3K in chronic inflammation and allergy

Allergies arise as consequences of the development of a local inflammatory response, initiated by the combined effects of cytokines, chemokines, lipid-derived mediators, proteolytic enzymes, and vasoactive mediators that are secreted and/or released by activated tissue mast cells. The results reported below underlie the importance of PI3K in modulating these processes.

All cell types express PI3Kα and PI3Kβ, whereas PI3Kδ and PI3Kγ are mainly expressed in leukocytes. PI3Kγ is also present at low concentration in smooth muscle cells, endothelial cells and cardiomyocytes [Wymann and Marone 2005, Patrucco et al 2004, Vecchione et al 2005, Alloatti et al 2005, Okkenhaug et al 2003]. Mice lacking PI3Kα or PI3Kβ die during embryonic development [Bi et al 1999, Bi et al 2002], while mice without functional PI3Kδ or expressing a catalytically inactive version of PI3Kδ (knock-in) [Okkenhaug et al 2002, Clayton et al 2002, Jou et al 2002] or PI3Kγ [Hirsch et al 2000, Li et al 2000, Sasaki et al 2000] are viable, fertile, and show attenuated function of immune cells. PI3Kδ knock-out and knock-in mice present impaired development of the marginal zone and peritoneal B-cells; moreover, the B-cell receptor (BCR) downstream signaling is attenuated or completely abolished. In mice with non-functional PI3Kδ, peripheral T-cells have a more pronounced naïve phenotype, as when compared to PI3Kδ null animals. PI3Kδ knock-in T-cells have some mild defects in T-cell receptor (TCR) signaling, showing a reduced phosphorylation of the PI3K effector Akt upon stimulation. T-cells of PI3Kγ null mice display significant differentiation and signaling defects; TCR activation in peripheral T-cells leads to a reduced proliferation and cytokine production. New data are suggesting a role for PI3Kγ in the reduction of CD4+ memory T-cells survival [Barber et al 2006]. B-cells of PI3Kγ null mice do not have differentiation and functional defects. The number of B-cells was reduced to levels similar to those of the PI3Kδ single knock-in mice. PI3Kδ knock-in, but not PI3Kγ null mice, have reduced numbers of natural killer (NK) T cells. Importantly, macrophages, dendritic cells and granulocytes derived from PI3Kγ null mice show impaired migration towards chemokines and sites of

inflammation [Jones et al 2003, Del Prete et al 2004], and mast cells derived from PI3Kγ null and PI3Kδ knock-in mice do not show maximal reaction in allergic responses [Laffargue et al 2002, Ali et al 2004]. Allergy and inflammation involve the activation of tissue resident mast cells and macrophages, followed by the invasion of effector cells like monocytes, neutrophils and more mast cell precursors to the inflamed area. PI3Kγ is required for chemokine-dependent migration of neutrophils, macrophages [Hirsch et al 2000] and mast cells [Kitaura et al 2005] to the site of infection and consequent clearance of infection. As a consequence, PI3Kγ knock-out mice are completely protected against systemic anaphylaxis [Laffargue et al 2002, Wymann et al 2003].

Mice expressing a kinase-inactive version of PI3Kδ also display a partial impaired antigen-IgE inflammatory response and bone marrow mast cells derived from these mice have migratory defect in response to stem cell factor [Ali et al 2004]. However, in comparison to PI3Kγ null mice, PI3Kδ knock-in mice are only partially protected against anaphylactic allergic responses, attributing to PI3Kγ a central role in allergic response.

Rheumatoid arthritis (RA) is a chronic systemic inflammatory autoimmune disorder affecting about 1% of the population, and is characterized by the inflammation of the joints, causes cartilage and bone erosion terminating in joint destruction. Other tissues including skin, blood vessels, heart, lungs and muscles can also be affected. T-cells, mainly CD4+ memory cells, invade the synovial membrane and start to secrete IL-2 and IFN-γ leading to the activation of monocytes, macrophages and fibroblasts. These cells then produce pro-inflammatory cytokines such has TNF-α, IL-1 and IL-6, which are critical in the onset of the chronic inflammation [Firestein 2003]. A variety of cells contribute to the progression of RA: T-cells, B-cells and plasma cells, which produce auto-antibodies recognizing type II collagen and proteoglycans; macrophages, mast cells, dendritic cells, neutrophils and fibroblasts accumulate in high numbers in the synovial membrane. As described above, PI3Kγ and PI3Kδ are essential for T-cell and B-cell development and function, and they are required for neutrophil and mast cell migration as well as for mast cell degranulation in allergic responses. These results suggest that these PI3K isoforms may be valuable targets in inflammatory disorders such as RA.

Atherosclerosis is a chronic inflammatory disease affecting arterial blood vessels, is characterized by deposits of oxidized low-density lipoproteins (LDLs), and is the primary cause of heart failure and stroke [Lusis 2000, Glass and Witztum 2001]. Macrophages and T lymphocytes are the first cells involved in the inflammatory process in atherosclerosis. Macrophages take up oxidized LDLs and convert to foam cells. It is known that oxidized LDLs activate the PI3K/Akt network in macrophages/foam cells [Biwa et al 2000, Biwa et al 2000]. In macrophages derived from PI3Kγ knock-out mice, PI3K downstream signaling was not activated upon exposure to

oxidized LDLs. The amount of phosphorylated Akt, and other phosphorylated effector proteins (S6K, S6, GSK3β and FKHR), was found to be reduced in atherosclerotic lesions, illustrating that the members of the class IA PI3Ks were not able to compensate for the deletion of PI3Kγ. These results clearly demonstrate that PI3Kγ has the potential to stabilize atherosclerosis at a pre-symptomatic stage, and might contribute to the alleviation of cardiovascular disorders.

1.3.b PI3K in cardiovascular disease

More than 10 years ago, the first report on a role of the PI3K/Akt pathway in the control of cell and organ size was published: ectopic expression of the Drosophila class I PI3K Dp110 in wings, or in the eye marginal disks, resulted in enlarged cells and tissues [Leevers et al 1996]. To investigate if PI3Ks were involved in the control of organ size in vertebrates, Shioi et al. overexpressed constitutively active PI3Kα in the heart of mice [Shioi et al 2000]. Mice overexpressing the active PI3Kα had an enlarged heart. This phenotype was completely reverted following treatment with the mTOR inhibitor rapamycin, demonstrating that Akt signaling is relayed via mTOR to control heart size. The cardiomyocyte-specific inactivation of the lipid phosphatase PTEN also triggers heart hypertrophy and culminates in reduced cardiac contractility [Crackower et al 2002]. PI3Kγ is expressed in the heart at low levels; PI3Kγ deficient mice display no changes in heart size and rate. Interestingly, PI3Kγ targeting in PTEN deficient hearts leads a restoration of contractility. Mice expressing catalytically inactive PI3Kγ, however, developed less hypertrophy as compared to wild type mice, and did not show signs of apoptosis and fibrosis. During these studies it was found that the lipid kinase activity of PI3Kγ was not fundamental to control heart contractility, but that the PI3Kγ was required as a scaffold for phosphodiesterase 3B activity and cAMP breakdown [Patrucco et al 2004]. Recent studies demonstrated that PI3Kγ and PI3Kδ regulated infarct size after ischemia/reperfusion injuries. In agreement, animals treated with the PI3Kγ/δ inhibitor, developed diminished inflammatory responses and edema formation, while tissue repair processes like endothelial cell mitogenesis were left intact [Doukas et al 2006, Doukas et al 2007].

1.3.c PI3K signaling in metabolic disease

The mTOR cascade controls many steps of cellular metabolism, including protein biosynthesis, glucose homeostasis, and it has recently been shown that mTOR modulates fat metabolism [Wullschleger et al 2006, Dann et al 2007]. mTOR is present in two distinct complexes, mTORC1 and mTORC2. mTORC1 controls the activation of S6K and 4E-BP1, while mTORC2

phosphorylates Akt on Ser473, the so-called PDK2 site. Insulin, via its tyrosine kinase receptor, triggers phosphorylation of insulin receptor substrates (mainly IRS1 and IRS2). Specific phosphotyrosine motifs on IRS serve as docking sites for signaling molecules, such as the p85 adaptor subunits of class I PI3Ks. It has been demonstrated that insulin is not able to drive p70S6K activation in the absence of amino acids and glucose [Hay and Sonenberg 2004]. mTOR knock-out mice die shortly after implantation due to defects in trophoblast differentiation and embryonic stem cells proliferation [Gangloff et al 2004, Sarbassov et al 2006]. In contrast, S6K1 deficient mice are viable, but show developmental retardation, smaller pancreatic β-cell size, are hypoinsulinemic and mildly glucose intolerant [Abraham 2004, Um et al 2004]. Interestingly, S6K1 null mice are protected against diet-induced obesity, probably due to a rise in lipolysis in adipose tissue and an increased metabolic rate in muscle and fat [Um et al 2004]. The reported data demonstrate the importance of the PI3K/Akt network and in particular of the mTOR pathway in obesity and other metabolic diseases.

Diabetes type 2 is a metabolic disease starting with insulin resistance, developing towards variable insulin deficiency and hyperglycemia. It was shown that weight loss reduces the incidence of type 2 diabetes by 60%. Nutrients, such as glucose and amino acids are not only metabolic fuels, but also important signaling molecules in so called nutrient signaling pathways, which comprise the hexosamine signaling pathway, the mTOR signaling pathway, and the adenosine monophosphate-activated protein kinase (AMPK) signaling pathway [Marshall 2006]. They regulate energy metabolism, cell growth, proliferation, and survival. These pathways are interconnected at various levels and linked to the action of insulin, which signals to the PI3K/Akt pathway. Attenuation of the PI3K/Akt/mTOR axis contributes to a diabetic phenotype. Elevated levels of the regulatory subunit p85, associated with decreased PI3K activity, were found in muscles of type 2 diabetic patients [Bandyopadhyay et al 2005].

1.3.d PI3K signaling in Alzheimer

Alzheimer disease (AD) is an age-related pathology characterized by synaptic loss followed by massive neuronal death. Two main histopathological hallmarks have been found in AD brain: neuritic plaques composed by an extracellular core of the insoluble form of the 4 kDa β-amyloid peptide; and the intracellular neurofibrillary tangles, containing accumulations of anomalous filamentous structures composed mainly by abnormal hyperphosphorylated forms of tau protein. Hence, numerous studies have focused on the identification of the protein kinases and protein phosphatases regulating tau phosphorylation in vivo. The proline-directed serine/threonine kinase,

GSK-3β, also known as tau protein kinase I has been identified as one of the major candidates mediating tau hyperphosphorylation at the same residues as those found in brain tissues from patients showing neurodegenerative tauopathies, including AD. Thus GSK-3β, antagonized by protein phosphatase 2A (PP2A), regulates tau phosphorylation at many sites. Microtubule-associated protein tau contains also a consensus motif for Akt encompassing the double phospho-epitope (T212/S214), a specific marker for AD. Akt dependent phosphorylation of tau occurs in vitro at both threonine 212 and serine 214 and may play specific roles relevant to AD and other neurodegenerations [Ksiezak-Reding et al 2003].

Several kinases and phosphatases have been identified to regulate the phosphorylation of tau. GSK-3β has been identified as one of the major candidates mediating tau hyperphosphorylation at the same sites as those present in tau protein in brain from AD patients. However, the signal transduction pathways involved in the abnormal activation of GSK-3b, have not been completely elucidated. GSK-3β activity is modulated through the PI3K/Akt route; GSK-3β is regulated by phosphorylation at Ser-9, which is the target for several kinases, including Akt. Activated Akt maintains GSK-3β in a Ser-9-phosphorylated, inhibited state, whereas decreases in Akt activity lead to dephosphorylation and activation of GSK-3β. Modulators of the PI3K pathway might be reduced during aging leading to a sustained activation of GSK-3β, which in turn would increase the risk of tau hyperphosphorylation. The role of PI3K signaling inhibition on the extent of tau phosphorylation and neuronal morphology has not been completely elucidated [Mercado-Gómez et al 2008].

Tau is an important microtubule-stabilizing protein in neurons. In its hyperphosphorylated form, tau protein loses its ability to bind to microtubules and then accumulates and is part of pathological lesions characterizing tauopathies, as AD. Diabetes mellitus is linked to an increased risk of developing Alzheimer disease. This could be partially caused by dysregulated GSK-3β. The coupling of PI3K with mTOR signaling, in conjunction with a regulatory interaction between PP2A and GSK-3β, changed activities of both enzymes always in the same direction. These balanced responses seem to ensure the steady tau phosphorylation at GSK/PP2A-dependent sites observed over a long period of time. This may help in preventing severe changes in tau phosphorylation under conditions when neurons undergo transient fluctuations either in insulin or nutrient supply.

The main physiological function of the protein tau is to promote the assembly and stabilization of the microtubule structure in neurons. Tau is a protein with multiple phosphorylation sites. Phosphorylation is essential for its function, influencing the ability of the protein to bind microtubules and to promote polymerization. Tau protein is susceptible to hyperphosphorylation, which commutes it into an inactive form. Hyperphosphorylated tau aggregated into paired

helicalfilaments, which hallmark the pathohistology of several neurodegenerative diseases, denoted as tauopathies and of which AD is the most prominent.

It was hypothesized that the disease-related disturbances in either the insulin-receptor binding or transduction pathway leads to over-activated GSK-3β creating hyperphosphorylated tau protein in neurons. *In vitro* and *in vivo* studies dealing with this subject showed contradictory results. In a preliminary study we found that not only PI3K but also mTOR signaling participates in the regulation of GSK-3β mediated tau phosphorylation. This is important insofar as mTOR kinase influences the activity of the PP2A. PP2A is known to be the major tau phosphatase, antagonizing the phosphorylation mediated by tau kinases. To gain more insight into this regulation, either the insulin/PI3K signaling (LY294002 treatment and insulin boost) or the mTOR kinase signaling (AICAR and rapamycin treatment) were systematically influenced in cultured murine neurons. Sustained changes in both the specific activity of PP2A and in the phosphorylation of activity-regulating sites of Akt (Ser-473), GSK-3β (Ser-9), and mTOR (Ser-2448) were detected. PP2A and GSK-3β were simultaneously activated during an inhibition of PI3K and mTOR, whereas they were both repressed during a stimulation of PI3K with insulin. This equilibrium was established by the regulatory coupling of mTOR with the PI3K signaling and by a dephosphorylation of GSK-3β at Ser-9 mediated by PP2A. The changes in activities of PP2A and GSK-3β seemed to be well balanced, explaining why the phosphorylation of tau protein at GSK-3β/PP2A-dependent sites remained unchanged over a long period. The investigation of tau at Ser-262 showed that interferences in the insulin/PI3K and mTOR signaling potentially affects phosphorylation when only one of the enzymes, in this case the PP2A, is involved in the regulation [Meske et al 2008].

1.4 Role of the PI3K/Akt network in solid tumors

The PI3K/Akt network controls cell growth, proliferation and cell survival, which constitute critical events towards tumor formation and malignant cell dissemination. This network is more frequently activated by genomic alterations than any other signaling pathway across many cancer lineages; multiple components of this network are targeted by germline or somatic mutation, amplification, rearrangement, methylation, overexpression, and aberrant splicing [Hennessy et al 2005, Karni et al 2007, Kumar and Hung 2005, Manning and Cantley 2007]. A major challenge in determining the frequency and spectrum of mutations resides in the difficulty of differentiating the core PI3K pathway from the wider PI3K/Akt signaling network. The TSC1/2 tuberous sclerosis complex integrates information from the growth factor-sensing arm of the network, encompassing PI3K, PTEN, and Akt, and the energy sensing, passing the message to mTOR and other

downstream mediators. This represents a simplistic model, as each member of the network has multiple additional inputs and outputs [Hennessy et al 2005, Kumar and Hung 2005, Manning and Cantley 2007]. However, most human cancers exhibit alterations of the PI3K/Akt pathway, leading to the expectation that PI3K/Akt targeting will have broad antitumor activity.

The most frequent alterations are loss or attenuation of PTEN function and mutations in PI3Kα, both leading to elevated PtdIns(3,4,5)P3 levels [Cully et al 2006, Wymann et al 2005, Vivanco et al 2002, Engelman et al 2006]. Activating mutations, truncations and change in expression levels have been reported for the p85 regulatory subunit and the downstream effectors PDK1, Akt, S6K, TSC1/2, members of the forkhead family and in TCL1 [Hennessy et al 2005, Bader et al 2005]. Additionally, mutations have been identified in activators of PI3K as the epidermal growth factor receptor (EGFR), the platelet-derived growth factor receptor (PDGFR) and the Bcr-Abl kinase [Wymann et al 2005, Hennessy et al 2005, Shaw et al 2006]. A number of tumors have alterations in the level of expression of PTEN, changes in the methylation of the PTEN gene, or loss of heterozygosity. Sporadic mutations are found in more than 50% of glioma, melanoma, prostate, endometrial and ovarian cancers and to a lesser extent in breast cancer [Li et al 1997, Cairns et al 1997, Wu et al 2003]. Furthermore, some tumor cells down-regulate PTEN by promoter hyper-methylation. This occurs in more than 50% of breast and prostate tumors, as well as in melanoma, endometrial, colorectal cancer and leukemia [Khan et al 2004, Goel et al 2004, Stahl et al 2004, Mirmohammadsadegh et al 2006]. Loss of PTEN function is usually a late step in tumor progression occurring in advanced tumor stages or even after metastasis [Wymann and Marone 2005, Vivanco and Sawyers 2002, Abraham 2004].

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

Table 2. Alterations of the PI3K/Akt network in cancer.

1.4.a PI3K and cancer

The PIK3CA gene, encoding for the p110α catalytic subunit of PI3K, is amplified in different tumors [Shayesteh et al 1999, Zhang et al 2002]. PI3Kα mutations are found in more than 30% of solid tumors including colorectal, gastric, pancreatic, esophageal, breast, certain brain tumors, ovarian, cervix, thyroid, lung, and hepatocellular carcinomas [Bader et al 2005, Samuels and Ericson 2006, Parsons et al 2005, Bachman et al 2004, Campbell et al 2004, Kirkegaard et al 2005, Xing et al 2005, Nakayama et al 2006, Asano et al 2004, Okano et al 2003, Garcia-Rostan et al 2005, Broderick et al 2004].

The gain function of PI3K mutations has been frequently observed in many human cancers, in particular in ovarian, breast, gastric, and hepatocellular carcinoma [Lee et al 2005]. An increased copy number of PIK3CA, was observed in approximately 40% of ovarian cancer occurrences [Shayesteh et al 1999]. PIK3CA is also found to be amplified and over-expressed in several other types of cancers including cervical, gastric, ovarian, and breast cancers through a large-scale mutational analysis [Bertelsen et al 2006, Byun et al 2003, Pedrero et al 2005, Willner et al 2007, Wu et al 2006]. In addition, somatic mutations of PIK3CA were found in 25% to 32% of colorectal cancers, 27% of glioblastomas, 25% of gastric cancers, 3% to 8% of breast, and 4% of lung cancer [Samuels and Velculescu 2004]. The PIK3CA mutations were also found in ovarian, hepatocellular, thyroid, endometrial cancers, and acute leukemia, as well as in malignancies of the central nervous system [Campbell et al 2004, Gallia et al 2006, Oda et al 2005, Ollikainen et al 2007, Samuels et al 2004]. The mutations of PIK3CA constitutively increased PI3K activities in the cells. PI3K regulatory subunit p85 dimerizes with catalytic subunit p110, and inhibits PI3K activity in normal cells. The deletion and somatic mutations of p85α regulatory subunit (PIK3R1) were found in primary human glioblastoma, colon and ovarian cancers. The deletion of p85 protein that lacks the inhibitory domain, and loss of the autophosphorylation site at the p85 inhibitory domain, commonly increase PI3K activity [Mizoguchi et al 2004, Philp et al 2001]. The mutations of PI3KCA in human cancers are summarized in Table 2.

PI3Kα mutations are mainly localized in two hot-spots: the helical and the catalytic domain [Samuels et al 2004]. In the helical domain, residues Glu542 and Glu545 are often mutated to lysine, whereas His1047 residue localized in the kinase domain is changed to arginine. A rationale for the molecular mechanism on how the above mutations activate PI3Kα, has been provided on the basis of structural resolution of a co-crystallized p85 inter-SH2 domain interacting with the N-terminus of the PI3Kα catalytic subunit [Miled et al 2007]. The three PI3Kα mutant proteins

described above (Glu542Lys-PI3K, Glu545Lys-PI3K and His1047Arg-PI3K) display elevated lipid kinase activity as compared to wild type PI3Kα, and have the potential to transform chicken-embryo fibroblasts and NIH3T3 cells. Moreover, PIK3CA mutant cells have an increased migratory and invasive capacity in vitro and in vivo. Altogether, these data suggest that expression of a constitutive active form of PI3Kα allows cells to survive and even migrate in suboptimal environmental conditions, and that PI3Kα contributes to tumor formation and metastasis. Thus the importance of PI3K in cell transformation and cancer occurrence is now well established [Kang et al 2005, Osaki et al 2004, Bader and Vogt 2004] and is supported by the key role of PTEN, the PI3K antagonist, that acts as tumour-suppressor gene [Myers et al 1998, Stambolic et al 1998].

1.3.b PI3K upstream elements and cancer

PI3K can be activated by receptor protein tyrosine kinases (RTKs) in response to growth factors. RTKs include epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), insulin-like growth factor 1 receptor (IGF-1R), interleukin receptors, vascular endothelial growth factor receptor (VEGFR), interferon receptors, and integrin receptors. RTKs interact with the p85 regulatory subunit of PI3K, while Ras protein directly interacts with the p110 catalytic subunit of PI3K in a GTP-dependent manner. Activated receptors interact with p85 Src homology 2 (SH2) domains, and localize PI3K to the plasma membrane. In addition to RTKs, intracellular proteins such as protein kinase C (PKC), SHP1, Rac, Rho, and Src can also activate PI3K in the cells [Hennessy et al 2005]. Upon activation, PI3K phosphorylates the D3 hydroxyl of PI(4,5)P2 to produce PI(3,4,5)P3 as a second messenger which activates these downstream targets with lipid-binding domains at the membrane [Ward and Finan 2003, Engelman et al 2006, Hennessy et al 2005. Cantley 2002]. In addition, the mutations of PI3K upstream elements such as the epidermal growth factor (EGFR), ErbB2, and IGF-1R, as shown in Table 2, can also increase PI3K activity in various cancer cell types [Actor et al 2002, Hollestelle et al 2007, van Dam et al 1994, Douglas et al 2006, Livasy et al 2006].

1.3.c Akt and cancer

Akt is involved in tumor growth and metastasis [Duronio et al 1998, Jiang et al 2000]. Oncogenes such as Ras and Src, amplification or activated mutations of PI3K, and the somatic mutations or deletions of tumor suppressor PTEN can activate the Akt pathway [Hennessy et al 2005, Shayesteh et al 1999, Cantley et al 1999]. Akt gene amplification has been observed in a

number of human cancers (Table 2), including gastric carcinoma, glioblastoma and gliosarcoma [Actor et al 2002, Staal et al 1987]. Akt2 amplification was found in head and neck squamous cell carcinoma, pancreatic, ovarian, and breast cancers [Pedrero et al 2005, Cheng et al 1996, Nakayama et al 2006, Bellacosa et al 1995]. Increased Akt3 mRNA is correlated to breast and prostate cancers [Nakatani et al 1999]. In addition to amplification, recent studies have indicated that elevated Akt activities are detected in different kinds of cancers and are associated with a poor prognosis [Ermoian et al 2002, Yamamoto et al 2004]. Recent observations added the Glu17Lys mutation in the PH domain of Akt1 to the panoply of known PI3K pathway aberrations. This mutation results in PI3K-independent membrane recruitment of Akt1, which exhibits transforming activity in vitro and in vivo [Carpten et al 2007].

1.3.d The oncogenic potential of PTEN

Decreasing levels of PTEN expression are correlated with the progressive outcome of solid cancers, including ovarian, prostate, and cervical cancers [Harima et al 2001]. PTEN activity is lost by mutations, deletions or promoter methylation silencing at high frequency in many primary and metastatic human cancers [Vivanco et al 2002, Parsons et al 2005]. Germ line mutations of PTEN are found in Cowden, Bannayan-Riley-Ruvalcaba and a Proteus-like syndromes, all familial cancer predisposition syndromes [Liaw et al 1997, Marsh et al 1999, Nelen et al 1997, Schrager et al 1998]. Loss of PTEN function results in accumulation of activated Akt that plays a key role in PTEN-mediated tumorigenesis via multiple mechanisms including inhibition of apoptosis [Downward 2004, Plas et al 2005, Stiles et al 2004], over-expression of cyclin D [Weng et al 2001] and interactions with MDM2 that lead to increased p53 degradation [Mayo et al 2001, Oren et al 2002, Gottlieb et al 2002]. Mice with PTEN deletion and mutations are highly susceptible to tumor induction [Suzuki et al 1998]. Conditional knockout of PTEN leads to neoplasia in multiple organs such as the mammary gland, skin, and prostate [Backman et al 2004, Li et al 2002], demonstrating the key role of PTEN in cancer development.

Akt is necessary but not sufficient to trigger PTEN loss-dependent tumorigenesis [Chen et al 2006. Blanco-Aparicio et al 2008]. Mechanisms of PTEN-mediated tumorigenesis, possibly independent from Akt [Weng et al 2001, Chang et al 2004, Freeman et al 2003, Li et al 2006], involve p53, as PTEN directly associates with p53 increasing its stability, protein levels and transcriptional activity. Similarly, PTEN regulates cell cycle arrest via protein phosphatase-dependent interaction with cyclin D. The oncogenic potential of PTEN is further highlighted by roles in integrin signaling and ability to dephosphorylate focal adhesion kinase that reduce cell adhesion and enhance migration

[Gu et al 1998, Gu et al 1999, Tamura et al 1999]. The protein phosphatase activity of PTEN is necessary for its growth-suppressive effect. PTEN appears to inhibit cell cycle progression through the cooperation of its protein phosphatase activity, which leads to the downregulation of cyclin D1, whereas its lipid phosphatase activity leads to up-regulation of p27 [Weng et al 2001]. PTEN also abrogates insulin-stimulated ETS2 activation independently from PI3K, probably through the protein phosphatase activity [Weng et al 2001]. ETS transform NIH3T3 cells allowing tumor formation in mice. Inhibition of ETS2 can inhibit Ras-dependent transformation and abolish breast carcinoma cells anchorage independent growth and migration. ETS2 can also activate the cyclin D1 promoter. PTEN+/-;p53+/- double heterozygous mice show an onset of lymphoma development similar to that seen in p53-/- animals. p53 protein levels are dramatically reduced in Pten-/- cells due to PTEN-mediated stabilization of p53, which increases its half-life. Furthermore, ectopic expression of PTEN phosphatase-dead mutants also leads to a significant increase in p53 protein levels, and PTEN can stabilize p53, even in the absence of MDM2, suggesting that PTEN can regulate p53 levels in a phosphatase-independent and MDM2-independent manner. Moreover, PTEN physically associates with endogenous p53 and regulates the transcriptional activity of p53 by modulating its DNA binding [Freeman et al 2003].

The data of Blanco-Aparicio indicate that p53 does not cooperate with Akt [Blanco-Aparicio et al 2007]. Since it has been shown that p53 cooperates with PTEN loss and might be an essential bottleneck in PTEN loss-induced development of mammary tumors, our data suggest that these reported PTEN effects are not mediated by Akt. Therefore, PTEN loss might signal through another molecule cooperating with p53 in a lipid phosphatase-independent manner, that could be cyclin D1. PTEN contains a sequence motif that is highly conserved in the members of the protein tyrosine phosphatase family. PTEN has been shown to possess phosphatase activity on phosphotyrosyl and phosphothreonyl-containing substrates [Li et al 1997, Myers et al 1997, Myers et al 1998] in vitro and on phosphatidylinositol (3,4,5) trisphosphate, a product of PI3K, both in vitro and in vivo [Maehama et al 1998, Myers et al 1998, Stambolic et al 1998, Sun et al 1999].

The fact that naturally occurring mutations in the PTEN phosphatase domain (such as PTEN-C124S and PTEN-G129E mutants) are tumor causing indicates that the effects of PTEN phosphatase-independent activity in tumorigenesis may be more tissue specific or associated with a more aggressive phenotype upon loss of PTEN function. Given that many components of the PI3K pathway are also found in the nucleus [Deleris et al 2006], it is possible that PTEN is a nuclear PIP3 phosphatase; nuclear localization of PTEN may contribute to its tumor suppressor activity in several ways [Lindsay et al 2006. Li et al 2006, Lian et al 2005].

With few exceptions, PIK3CA and PTEN mutations are rarely seen together, suggesting that they mediate similar cellular functions. In contrast, PDK1 amplification and PTEN loss or mutations are frequently concordant, indicating that they mediate different cellular events and outcomes or act in concert to stimulate the PI3K/Akt pathway.

1.4.e The oncogenic potential of mTOR

As described above, the PI3K/Akt network via the downstream molecule mTOR has a pivotal role in regulating protein synthesis. In brief, mTOR is positively regulated by Akt, which phosphorylates the negative regulator TSC2 in the tuberous sclerosis complex (TSC) [Woods et al 2003]. mTOR controls the 5′ cap-dependent translation via phosphorylation of the translational repressor 4E-BP1. Hyper-phosphorylated 4E-BP1 releases bound eIF4E, which is required for translation initiation modulated by the eIF4F complex. In parallel, mTOR stimulates translation of ribosomal proteins and therefore ribosome biogenesis via the activation of p70S6K [Wullschleger et al 2006]. Elevated expression of eIF4E is present in a variety of cancers, and correlates often with aggressive disease and poor prognosis [Boudeau et al 2003]. The oncogenic potential of mTOR is thus very high and mTOR has been pointed as important target for cancer therapy.

1.4.f PI3K/Akt signaling regulates tumor angiogenesis

Angiogenesis is essential not only for embryo development, female reproduction, tissue repair and inflammatory diseases, but also for tumor growth and metastasis. The angiogenesis process includes dissolution of the basement membrane of the vessel, migration and proliferation of endothelial cells, formation of a new vessel lumen and vessel branches, maturation of the new vessels by the recruitment of pericytes, and the formation of the basement membrane [Risau 1997]. Tumor growth and metastasis require angiogenesis when the tumor reaches 1–2 mm in diameter [Folkman 2006]. Tumor angiogenesis occurs via the sprouting of new vessels from preexisting blood vessels or via the insertion of interstitial tissue columns into the lumen of pre-existing vessels (intussusception) [Carmeliet and Jain 2000]. Tumor angiogenesis can be triggered by extracellular signals such as growth factors, by genetic alterations such as activation of oncogenes, and by mutations of tumor suppressor genes such as PTEN and p53. The first direct evidence of PI3K and Akt involvement in regulating angiogenesis in vivo was observed by the forced expression of PI3K and Akt in chicken chorioallantoic membrane by retroviral vector [Jiang et al 2000]. In addition, it has been found that PI3K/Akt regulated VEGF and hypoxia-inducible factor 1 (HIF-1) expression

through HDM2 and p70S6K1 activation. VEGF and HIF-1 are the mediators that transmit PI3K-induced oncogenic signals for tumor growth and angiogenesis [Skinner et al 2004, Xia et al 2006]. VEGF is a potent inducer of angiogenesis, and HIF-1 is the major regulator of VEGF transcriptional activation through the binding to the hypoxia response element of VEGF promoter. HIF-1 is a heterodimeric transcription factor composed of two subunits: HIF-1α and HIF-1β [Jiang et al 1996, Wang et al 1995]. HIF-1α can be induced by hypoxia, growth factors, and oncogenes whereas HIF-1β is constitutively expressed in human cells. Various studies show that PI3K/Akt signaling is important for regulating HIF-1α and VEGF expression [Jiang et al 2000, Skinner et al 2004]. The expression of PI3K dominant negative mutants or the expression of PTEN inhibits tumor growth [Fang et al 2007]. Over-expression of PTEN mutants lacking phosphatase activity abrogates the inhibitory effect of PTEN on tumor growth, suggesting that PTEN phosphotase activity is required to inhibit tumor growth. Over-expression of active forms of PI3K induced angiogenesis, characterized by extensive sprouting of new blood vessels and enlargement of preexisting vessels [Jiang et al 2000]. Over-expression of the tumor suppressor PTEN and of dominant-negative constructs of PI3K in prostate cancer cells significantly inhibited angiogenesis, suggesting that PI3K/Akt signaling is required for tumor angiogenesis.

The potential PI3K downstream targets for regulating tumor growth and angiogenesis are outlined in Figure 6. Akt is the major target of PI3K for transmitting the oncogenic and angiogenic signals. Thus, the PI3K/Akt network has been demonstrated to be involved in growth factor- and hormone-induced tumor growth and angiogenesis, other than in tumor-induced angiogenesis through reactive oxygen species [Liu et al 2006, Carnesecchi et al 2006].
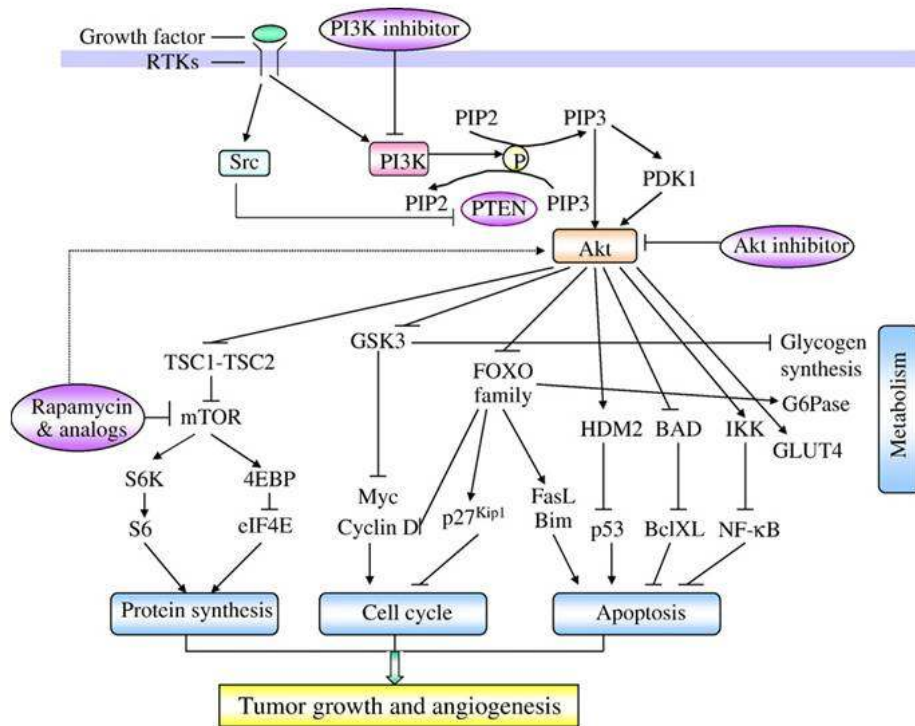
Figure 6. Elements of the PI3K/Akt network involved in tumor growth and angiogenesis

1.5 Role of the PI3K/Akt network in acute leukemia

Acute leukemias are one of the most common hematological malignancies in Europe and all over the world, with incidences (European standard population) estimated of 3-6/100,000 for the acute myeloid leukemia (AML) and 4/100,000 for acute lymphoblastic leukemia (ALL).

The general underlying mechanism of acute leukemias is the uncontrolled monoclonal proliferation of a lymphoid or myeloid progenitor / stem cell (Figure 7).
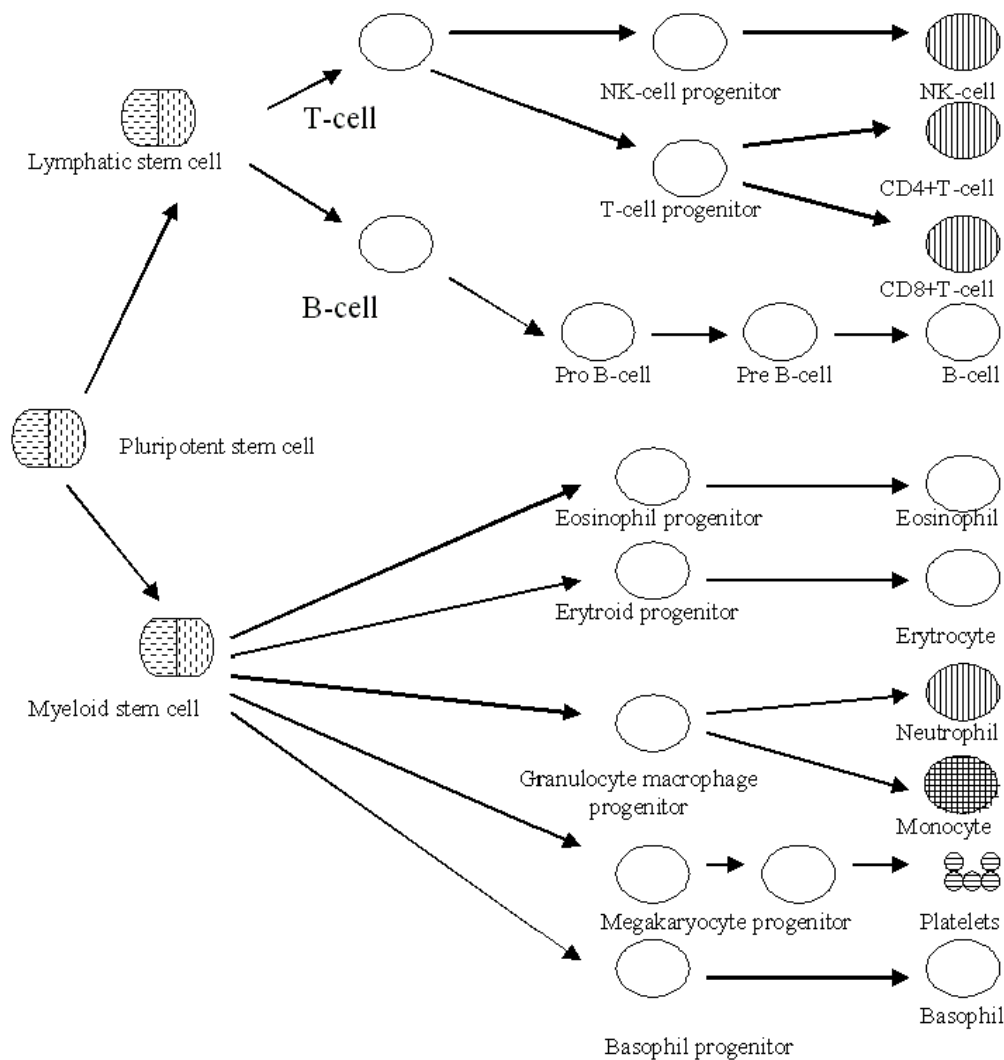
Figure 7. Scheme of myeloid and lymphoid cells maturation and alteration in acute leukemia.

1.5.a Acute Lymphoblastic Leukemia (ALL)

ALL comprises 20% of acute adult leukemias, but it is the most common leukemia in childhood, resulting from the clonal expansion of lymphoid progenitors that have undergone malignant transformation at distinct stages of differentiation. It has two incidence peaks: one is in the first decade of life and a second one with ages older than 60 years. Risk factors for the development of ALL include prior radiation therapy and chemotherapy, smoking and infections by the HTLV-virus. Diagnosis is established based on morphology, immunophenotype, cytogenetics and molecular investigations. Prognostic factors include genetic changes detected by cytogenetics and/or molecular biology as well as morphological/immunophenological subtype and white blood cell count at diagnosis [Kappelmayer J et al 2007]. Approximately 15% of the cases are of T-cell

origin (T-ALL) [Pui et al 1999]. Cell-autonomous lesions are certainly at the origin of T-ALL; however, microenvironmental factors are also believed to contribute to T-ALL expansion [Barata et al 2004]. Both cell-intrinsic defects and external stimuli frequently converge on the activation of key pro-oncogenic intracellular pathways. In particular, the PI3K/Akt and Notch signaling pathways have seen to be crucial for the survival and proliferation of the leukemic blasts.

PI3K/Akt signaling in T-ALL - In T-ALL, the PI3K/Akt pathway is activated by growth factors present in the leukemia milieu that signals through cytokine receptors [Barata et al 2004, Barata et al 2006]. Moreover, T-ALL cell lines present constitutively phosphorylated forms of Akt independently from external growth factors [Palomero et al 2007]. In addition, NF-κB, a downstream target of PI3K/Akt, is constitutively activated in some primary T-ALL samples [Kordes et al 2000]. Nevertheless, no activating mutations of PI3K and/or Akt have been described in T-ALL, suggesting that PI3K/Akt pathway over-activation results from PTEN downregulation and/or SHIP mutations [Maser et al 2007, Luo et al 2004]. However, results from Barata's lab show that non-deletional PTEN inactivation is a major contributor to hyper-activation of PI3K/Akt in primary T-ALL samples. PTEN mutations resulting in protein truncation have been identified more frequently in T-ALL cell lines established from relapsed patients (30.4%) than in diagnostic clinical specimens (5.2%), suggesting that PTEN deletion is a late event in human T-ALL [Maser et al 2007]. In contrast, recent studies in mice have shown that PTEN deregulation is important at early stages of leukemogenesis. In these models, PTEN expression was shown to be essential to maintain the hematopoietic stem cell pool and to prevent leukemia development [Yilmaz et al 2006, Zhang et al 2006]. As previously mentioned, PI3K activates several downstream targets including mTOR.

Notch signaling in T-ALL - Notch involvement in T-ALL was first described in three patients with t(7;9)(q43;q34.3), which juxtaposes the TCRß locus to the C-terminal coding region of the Notch1 gene. This translocation leads to the expression of a truncated, cytoplasmic form of Notch1 receptor with constitutive activity [Ellisen et al 1991]. Human truncated Notch1 was shown to be highly oncogenic, since transduction of mouse bone marrow cells with retroviruses encoding the corresponding sequence of Notch1 leads to the development of T-cell leukemia with high penetrance in the transplanted animals [Pear et al 1996], and the effect is synergistic with those of other known oncogenes, including c-Myc and E2A-PBX1 [Feldman et al 2000, Hoemann et al 2000]. The involvement of Notch1 in the pathogenesis of this disease was strongly supported by the discovery that more than 50% of T-ALL cases harbored mutations in the Notch1 alleles, resulting in the constitutive activation of the pathway [Weng et al 2004]. Similar mutations were found in T-ALL mouse models [Eguchi-Ishimae et al 2008]. In addition, non-mutational Notch1 activation seems to be an early event in the development of T-ALL in mouse models [Gothert et al 2007], and

recent work identified c-Myc as a Notch1-target gene highly up-regulated in T-ALL and critical for Notch1-mediated leukemogenesis [Weng et al 2006]. Notch3 receptor has also been implicated in T-cell leukemogenesis. It is not known how Notch3 can promote aberrant signaling responsible for leukemogenesis, since it is normally expressed throughout T-cell ontogeny. In contrast to Notch1 and -3, Notch2 and -4 have not been associated with T-ALL. Inhibition of Notch signaling by γ-secretase inhibitors, already available for treatment of Alzheimer disease, was shown to prevent growth and induce apoptosis of T-ALL cell lines in vitro, representing a promising therapeutic tool for T-ALL [Weng et al 2004, Lewis et al 2007, Kogoshi et al 2007, Palomero et al 2007].

1.5.b Acute Myeloid Leukemia (AML)

AML comprises 80% of acute adult leukemias. Its incidence increases with age reaching 30/100.000/year in patients older than 80 years. Risk factors for the development of AML include prior radiation therapy and chemotherapy, predisposing hematological diseases such as myelodysplastic syndrome as well as predisposing genetic disorders such as trisomy 21. Diagnosis is established based on morphology, immunophenotype, cytogenetics and molecular investigations. Prognostic factors include type of genetic changes detected by cytogenetics and/or molecular biology as well as biological factors such as age. An International Working Group on AML has adopted the World Health Organization (WHO) classification system of AML [Cheson et al 2003], dividing patients into categories based upon cytogenetic findings in combination with morphological, cytochemical and immunophenotypic characteristics [Stone 2007].

AML in younger individuals – In 50 to 70% of patients with AML an activation of the PI3k/Akt cascade can be found. Akt dependent alterations in the activity and expression of several transcription factors inhibit the normal myeloid differentiation. The overall survival time for patients demonstrating Akt activation was significantly shorter when compared to patients with no Akt activation, although the mechanisms that upregulate the PI3K/Akt signaling in AML cells still remain unclear [Kubota et al 2004]. However, no correlation was shown to exist between Akt phosphorylation levels and subtype of AML, percentage of blast infiltration of the bone marrow, cytogenetic anomalies, or when comparing untreated versus relapsed/refractory AML [Brandts et al 2005]. The PI3K/Akt signaling is thus an attractive target for the development of novel anticancer strategies.

Up to 20-25% of AML patients harbour internal tandem duplication of the juxtamembraen domain of the Fms-like tyrosine kinase 3 receptor (FLT3). This mutation results in ligand-independent dimerization of FLT3 and constitutive upregulation of its kinase activity, ensuing in stimulation of

downstream signaling pathways, including PI3K/Akt [Minami et al 2003, Kim et al 2006]. FLT3 and its ligand (FL) interaction plays an important role in the proliferation and differentiation of hematopoietic cells. FLT3 mutations, which result in unregulated cell proliferation, have been identified in up to one-third of AML cell lines [Knapper et al 2006]. AML patients with FLT3 mutations have a worse prognosis than those with normal FLT3 [Naoe and Kiyoi 2004]. Constitutively activated PI3K/Akt or FLT3 thus regulate AML cell survival, resistance to chemotherapy and significantly shorter survival.

AML in elder individuals - AML, the most common adult leukemia, is recognized as a disorder characterized by heterogeneous and distinct biological features, leading to a diagnosis based not solely on cellular morphology, but on the basis of immunologic, cytogenetic, and molecular features. Such characteristics have allowed for disease classification that results in more precise prognostic determinations, as well as the identification of specific therapeutic targets and subsequent novel treatment strategies. In parallel with an emerging understanding of the underlying molecular biology and genetics, AML in the elderly (age >60) has been recognized as a fundamentally different disease from AML in younger individuals.

AML is the most common type of acute leukemia occurring in adults; adults over age 60 comprise more than two-thirds of this group, [Stone et al 2004, Lowenberg et al 1999] with a median age of onset of about 65 [Stone et al 2004, Ries et al 2006]; the incidence of AML rises significantly with age [Ries et al 2006]. Retrospective data from the mid-1990's indicate that the vast majority of elderly AML patients are not referred to a subspecialist and do not receive chemotherapy. Additionally, of all elderly AML patients (treated or untreated), the prognosis is poor, with median survival estimated to be two months, and only six percent surviving at two years [Menzin et al 2002]. These observations heighten the need for more effective treatment approaches and the implementation of treatments.

Many cases of AML in the elderly are idiopathic, although a substantial portion are related to prior radiation therapy, chemotherapy, or arise from antecedent hematologic disorders such as myelodysplastic syndrome (MDS). Secondary AML (AML arising from MDS, myeloproliferative disorders, or therapies targeting previous malignancies) comprises 24 to 56% of AML diagnosed in elderly patients. Leukemia cells in older patients appear to be intrinsically more resistant to standard chemotherapy (e.g. higher expression of genes that mediate drug resistance) [Stone et al 2004], and aggressive treatments in the elderly have been associated with higher morbidity and inferior survival compared to younger patients [Lowenberg et al 1989, Tilly et al 1990, Disperati et al 2007]. Intensive chemotherapy may not be the best treatment for older patients who often have comorbid conditions, poor physiologic reserve, and decreased bone marrow reserve [Baudard et al

1994, Jabbour et al 2006]. Older age alone is a poor prognostic factor, with the annual incidence of AML rising with each decade [Lowenberg et al 1998, Jabbour et al 2006, Van Leeuwen 2006]. A number of other characteristics have been identified in older patients presenting with AML.

For the minority of elderly patients whose blast cells are characterized by one of the favorable balanced translocations t(8;21), t(15;17) or inversion 16, a relatively encouraging complete remission (CR) rate of 72%, and a five-year survival rate of 34% can be anticipated with conventional treatment. The term complete remission has historically been reserved for patients in whom, following induction therapy, residual bone marrow blasts have fallen to below five percent, together with recovery of peripheral blood neutrophils and platelet counts. Favorable cytogenetic abnormalities are more common in younger individuals, partly accounting for their better overall survival [Grimwade et al 2001]. The intermediate-risk group is comprised of patients without favorable cytogenetics and without an adverse karyotype, with adverse karyotypes including monosomies of chromosome 5 or 7, deletions of the long arm of chromosome 5, 3q abnormalities or multiple abnormalities. This intermediate-risk group of elderly patients comprises the majority of patients, having CR rates around 57%, and five-year survival between 10% and 15% with conventional treatment [Grimwade et al 2001, Grimwade et al 1998]. Patients in the poor-risk group have one of the aforementioned adverse karyotypes, CR rates of 26% and five-year survival of less than 5% with conventional treatment [Grimwade et al 2001]. This later group often includes patients with disease arising from MDS [Stone 2007]. These more recent retrospective data still require validation in an independent prospective study group before it may be generalized to all elderly patients with newly diagnosed AML.

1.6 Therapeutic strategies acting on PI3K/Akt network in leukemia

1.6.a Acute leukemia treatment

AML and ALL therapies are based on chemotherapies. Whereas for AML the backbone for chemotherapies consist of cytosine-arabinoside in combination with an anthracyclin, the backbone of ALL-chemotherapies consists of asparaginase, anthracyclins and corticosteroid.

Several courses of treatment are applied, with total treatment times ranging from 4-24 months.

Conventional therapeutic strategy is aiming at:

    i. complete remission induction

    ii. remission stabilization

iii. maintenance

However, for AML as well as for ALL patients risk factors have been identified that confer with a poor prognosis if the patient receives conventional chemotherapy only. Patients are therefore treated in a risk adapted manner. High risk patients receive in addition to their chemotherapies immunotherapeutics and/or allogeneic hematopoietic stem cell transplantation. However, relapses and chemoresistance continue to be hallmarks of adult ALL and AML [Pui et al 2000, Tzortzatou-Stathopoulou et al 2001, Cripe and Hinton 2000].

Cytogenetic findings permit AML patients risk to be categorized as favourable, intermediate or adverse, with very different cure rates [Byrd et al 2002]. Patients with poor risk have either =/> 3 chromosomal abnormalities or involvement of certain chromosomes such as chromosomes 7 or 5. Poor risk groups in ALL are also defined by cytogenetics with the translocation t(9;22) (Philadelphia chromosome) of special importance. However, in ALL also T-cell subtype as well as high leukocyte count, i.e. >30.000-50.000/mm³, are associated with poor prognosis and treated more intensively.

During last years, genome-wide gene expression profiling with DNA and RNA microarrays have found new risk groups within the AML and ALL patients and provided additional prognostic information [Bullinger et al 2005, Valk et al 2004]. Of importance, they highlighted the diversity of changes on the genomic, transcriptional, and protein level. Correlations of alteration found by these large scale experiments have been correlated with clinical outcomes in AML and ALL and led to the identification of new prognostic markers such as FLT3 tandem duplications in AML. The advantage of such screening approaches is the ability to also take into account cross interactions between different aberrantly expressed genes or activated signaling pathways. The PI3K/Akt network has so far not addressed in detail in this regards [Kornblau et al 2006].

Due to the heterogeneity in underlying alterations acute leukemia classification and the subsequent clinical management remains very difficult. It is necessary to identify novel genetic or metabolic elements which are altered in acute leukemia patients and could have value as:

i. diagnostic markers

ii. prognostic markers

iii. novel targets for treatment

In particular, it appears increasingly necessary to elucidate the molecular mechanisms underlying the enhanced survival capability displayed by tumor cells in the presence of anticancer treatments. The future of efficacious and safe therapies against acute leukemias is thus represented by finding new tools to lower the sensitivity threshold of cancer cells to molecules that induce apoptosis.

1.6.b Treatment of newly diagnosed acute leukemia

Younger patients do considerably better both with induction and consolidation regimens in the treatment of AML, whereas treatment in elderly patients remains challenging. Treatment options for newly diagnosed AML in the elderly include conventional intensive chemotherapy, low-intensity induction chemotherapy, non-myelosuppressive agents, colony-stimulating factors, supportive care, or clinical trials. However, previously documented results of holding with treatment of newly diagnosed AML in the elderly have yielded low survival rates and poor quality of life [Lowenberg et al 1999, Lowenberg et al 1989].

Conventional chemotherapy - Induction chemotherapy aims to restore normal bone marrow function. Conventional regimens that rely on an anthracycline in combination with cytarabine have long been the standard against which other therapies are compared. In the large Eastern Cooperative Oncology Group study of older adults, regimens containing daunorubicin, idarubicin or mitoxantrone for three days, combined with seven days of cytarabine, all showed similar CR (40–46%) and overall survival rates (median 7.5 months) [Rowe et al 2004]. In the Medical Research Council AML 11 trial, regarding primarily elderly patients, three regimens were compared: daunorubicin, cytarabine, thioguanine (DAT); daunorubicin, cytarabine, etoposide (ADE); and mitoxantrone and cytarabine (MAC). Use of colony-stimulating factors in this study also had no impact on survival [Goldstone et al 2001]. The most successful regimens appear to be those pairing cytarabine with an additional agent. Overall, conventional chemotherapy can be expected to induce complete remission in 35–60% of elderly patients, with no more than 10–20% in first complete remission living more than 3 years from their diagnosis [Lowenberg et al 1999, Stone et al 2007, Fey et al 2003].

Agents complementary to conventional chemotherapy - One complementary strategy used in treating elderly patients with AML who are already receiving chemotherapy, is to target the anti-apoptotic protein Bcl-2, which is over-expressed in many leukemia cells, resulting in an unregulated cell cycle.

Inhibitors of FLT3 activity have also been utilized as complementary agents. Although studies have demonstrated decreases in bone marrow and peripheral blast counts with these agents, most notably in patients whose leukemia is characterized by a FLT3 internal tandem duplication [Knapper et al 2006], CR rates have not been markedly improved, regardless of FLT3 mutation status [Stone 2007]. The majority of studies of FLT3 inhibitors presently underway utilize these agents as therapy complementary to conventional regimens; a notion that is supported by data

demonstrating a synergistic effect between FLT3 inhibitors and chemotherapy [Levis et al 2004]. Multidrug resistance (MDR) is a significant problem for many elderly patients with AML.

Expression of the MDR1 gene and its product, P-glycoprotein (Pgp) is one known mediator of MDR. AML cells expressing Pgp have been associated with lower CR in studies of elderly patients [Leith et al 1997, Van den Heuvel-Eibrink et al 2007]. One modulator of this glycoprotein has been studied in several randomized trials and unfortunately has not been shown to provide any clear benefit [Baer et al 2002, Van der Holt et al 2005, Greenberg et al 2004]. Expression of a number of other AML cell proteins, such as CD56 [Yang et al 2007], also correlate with MDR, and offer other potential avenues of clinical therapeutics. Additionally, it is evident from clinical trials that certain MDR1 genetic polymorphisms carry prognostic significance, and can likely be used to predict response to induction chemotherapy [Wang et al 2007]. The use of hematopoietic growth factors in addition to chemotherapy has been surrounded by some controversy. Many studies have suggested that the addition of hematopoietic growth factors (GM-CSF, G-CSF, and glycosylated G-CSF) as an adjunct to chemotherapy does not improve response to chemotherapy, decrease treatment-related mortality, or change overall survival [Rowe et al 2004, Stone et al 1995, Stone et al 2001].

Alternatives to conventional chemotherapy - Intensive chemotherapy may not be appropriate for many patients, largely due to treatment-related morbidity and mortality. As an alternative, low-dose cytarabine has been studied in elderly patients, producing superior CR rates when compared to hydroxyurea [Burnett et al 2007]. However, patients with adverse cytogenetics did not benefit from low-dose cytarabine. Low-dose cytarabine has the potential advantage of being administered subcutaneously, and some patients have been treated effectively as outpatients [Tilly et al 1985], although disposal of syringes and vials may be problematic. As an alternative to conventional-dose cytarabine, low-dose cytarabine has been suggested as the standard palliative therapy to which newer investigative agents be compared [Milligan et al 2006]. Clofarabine is a purine nucleoside analogue with several mechanisms of action that has shown promise in elderly patients with AML. Inhibition of ribonucleotide reductase, incorporation into DNA, and induction of apoptosis are all thought to underlie clofarabine's action [Kantarjian et al 2007]. Several other chemotherapeutic agents have also demonstrated clinical efficacy in specific populations. Idarubicin, an anthracycline given orally in one study, produced CR in 25% of poor-risk patients receiving the drug as induction therapy. Arsenic trioxide has been proposed as an alternative to intensive induction chemotherapy, as it has anti-proliferative and apoptotic effects. Its efficacy has been well-documented in the treatment of acute promyeocytic leukemia (APL) [Mathews et al 2006]. However, a small study of eleven patients with either newly diagnosed AML or relapsed or

refractory AML demonstrated very poor results, with median survival after first treatment of less than three months [Parmar et al 2004], although these results are difficult to interpret in such a heterogeneous population.

1.6.c Post-induction treatment

Even once induction therapy results in undetectable blasts in the bone marrow, it is likely that leukemia cells persist undetected, and, without post-induction (consolidation) therapy, relapse will occur. Traditionally, for adult patients tolerating intensive induction chemotherapy and achieving CR, the induction phase of treatment is followed by intensive consolidation chemotherapy, usually in the form of high-dose cytarabine, with or without an anthracycline. Following the consolidation phase, hematopoietic stem cell transplantation may be offered if patients are judged reasonable candidates. This may take the form of allogeneic or autologous hematopoietic stem cell transplantation, either from a bone marrow, peripheral blood, or cord blood source. Alternatives include less intensive chemotherapy, non-myelosuppressive therapy, clinical trials, or a combination of these approaches. For elderly patients tolerating intensive induction chemotherapy, and with favorable cytogenetics and functional status, it may be appropriate to pursue traditional consolidation therapy with two cycles of an anthracycline and cytarabine. Use of low-dose cytarabine is one alternative to intensive consolidation chemotherapy, and while it may prolong disease-free survival, it has not been shown to improve overall survival [Lowenberg et al 1998]. High-dose cytarabine is not tolerated well by many patients over the age of 60, with higher treatment-related morbidity and mortality observed in at least one study [Mayer et al 1994]. Another study demonstrated that high-dose cytarabine may be tolerated reasonably well by some elderly patients, but did not show any overall benefit in this age group [Schiller and Lee 1997]. Interleukin-2 (IL2) is currently being studied as an agent for maintenance therapy in patients over the age of 60. A recent study adding IL2 maintenance therapy to conventional chemotherapy regimens in older adults (age 50–70) did not demonstrate any benefit [Pautas et al 2007]. However, few patients in this study who were randomized to receive IL2 actually received it as a result of disease relapse or intolerance, a frequent problem with consolidation or maintenance therapy studies.

The concept of using immunotherapy (e.g. vaccines, donor lymphocytes) to target AML cells has been studied in various trials since the 1970's. The ability of lymphocytes to induce an anti-leukemia effect has been well demonstrated in trials of donor lymphocyte infusions (DLI) in patients with relapsed AML, following allogeneic transplantation [Kolb et al 2004]. This ability of donor lymphocytes to eradicate leukemia cells is referred to as the graft-versus-leukemia (GVL)

effect. While beneficial, this effect is often complicated by graft-versus-host disease (GVHD) and infections. Alternatively, vaccination trials have attempted to provide an antigenic stimulus for patients native lymphocytes to mount an immune response. Vaccine trials with elderly patients specifically have been limited, however; studies not limited to elderly patients have suggested some benefit of vaccinations containing leukemia-cell antigens [Alsabti et al 1979]. Vaccination with dendritic cells, functioning as antigen-presenting cells, generated from autologous leukemia blasts is also being investigated currently, with small cohort studies showing tolerability and enhanced cytotoxic T-cell function in some elderly patients [Berneman et al 2007, Li et al 2006].

Recently, the use of allogeneic hematopoietic stem cell transplantation (HCT) from matched unrelated and cord blood donors, as opposed to sibling donors, has become more popular. Retrospective data for patients over the age of 50 with intermediate or poor-risk cytogenetics suggest that unrelated allogeneic HCT should be considered equivalent to sibling allogeneic HCT in terms of survival [Schetelig et al 2007]. This observation greatly extends the pool of potential donors for transplant recipients. Autologous transplants have been studied in older individuals, and have the advantage of avoiding long-term immunosuppressive therapy. The disadvantages of autologous transplants include the absence of a graft-versus leukemia effect, as well as possible contamination of the autograft with leukemia cells, a hypothesis supported by gene-marking studies [Brenner et al 1993]. Thus it is important to inform patients of anticipated differences in physical and functional outcomes following these various therapies.

1.6.d Resistance to therapy and treatment of relapsed disease

Akt activation is found to correlate with the resistance to chemotherapy and radiation therapy. The combination treatment of Akt inhibitor with antitumor drugs renders cancer cells sensitive to the drug treatment [Brognard et al 2001, Martelli et al 2003]. Expression of the multi-drug resistance-associated protein 1 (MRP-1), which is a transmembrane pump for drug transport involved in resistance to therapy, has been shown to depend on PI3K activity in advanced prostate cancer and in human acute myelogenous leukemia blasts [Lee et al 2004, Tazzari et al 2007].

Recent studies have shown that low levels of PTEN expression are strongly associated with amplification of PIK3CA and with the increase of PI3K and Akt activities in OVCAR-3 cells, contributing to therapy resistance in ovarian cancer [Lee et al 2005, Liu et al 2007].

L-asparaginase is an important component of induction and consolidation multidrug chemotherapy in children and adults with ALL. The natural (native) forms of the enzyme, produced by Erwinia chrysanthemi and Escherichia coli bacteria strains are used in treatment, and the E. coli L-asparaginase derivative, covalently bound with mono methoxy polyethylene glycol, presented

prolongd half-life. In ALL adult patients longer overall survival and disease free survival have been reported. In patients in whom as a result of pegylated asparaginase administration an efficacious plasma elimination was achieved, overall survival was highest than in patients without administration [Piątkowska-Jakubas et al 2008].

Despite the advances, almost 20% of the ALL children either relapse or do not respond to treatment. This seems to be related to various parameters, including the presence of polymorphisms which may alter the activity of drug metabolizing enzymes, hence influencing the efficacy and the toxicity of therapy [Karathanasis et al 2009].

Relapsed AML in younger patients is often treated with chemotherapy plus allogenic HCT. However, as noted previously, transplantation in older adults is often associated with very-high treatment-related mortality [Wallen et al 2005]. For patients 60 years and older who are considered fit enough, it is recommended treating relapsed disease with salvage therapy, such as 1) clinical trial agents, 2) gemtuzumab, or 3) repetition of initial induction regimen, if they had a long initial remission. Best supportive care is always an option for those not fit for additional therapeutic regimens, or those not wishing to undergo further treatment. One group of clinical trial agents used in relapsed disease are inhibitors of VEGF, which plays a key role in leukemogenesis in AML. Empiric evidence suggests VEGF interaction with its receptors occurs in an autocrine/paracrine manner that likely mediates leukemia cell survival [Gerber et al 2002, Fiedler et al 1997, Zhou et al 2007]. Since approximately 90% of AML patients have blast cells that are CD33+, the drug theoretically is designed to selectively target AML cells, while limiting non-hematologic toxicity [Pagano et al 2007]. Notably, the CD33 protein is found on normal myeloid and monocytic progenitor cells as well, and the drug has a significant side effect profile, including veno-occlusive disease, hyperbilirubinemia, transaminitis, as well as respiratory side effects. Additional approaches to treatment of relapsed AML may employ one of the aforementioned methods utilized in treating newly diagnosed AML, or those used in consolidation regimens. Repetition of induction therapy, or an alternate induction regimen is a different strategy, although there are no good clinical data to support such an approach.

AML in the elderly poses significant treatment challenges. Poor physiologic reserve, unfavorable cytogenetic profiles, and poor responses to conventional chemotherapy all underlie the unfortunate outcome observed in these patients compared to their younger counterparts.

Many patients will be candidates for clinical trials rather than conventional therapy, largely based on initial cytogenetic testing or poor response to induction chemotherapy

1.6.e Recent developments in AML therapy

Three areas are considered in the treatment of AML: prognostic factors, which are improving risk discrimination; improvements in the availability and outcome of allogeneic bone marrow transplantation; and new and targeted therapies.

Prognostic Factors - One of the most important areas in the development of state of-the-art therapy for AML has been the delineation and clarification of prognostic factors, leading to much-improved risk determination. Over the past decade, virtually all major cooperative groups that have studied the treatment of AML in a prospective fashion have reported that the most important prognostic factor is the cytogenetics of patients at presentation. Research in defining prognostic factors in AML has moved from cytogenetics to an examination of molecular markers, beginning with enormous interest in the P-glycoprotein transmembrane transporter proteins, which are the product of the multidrug resistance gene (*MDR-1*). Unfortunately, most of the studies attempting to overcome *MDR-1* have been negative. More recently, various mutations resulting in over-expression of specific genes have been shown to be associated with specific prognoses. Unfavorable prognosis is associated with several of these, including: the Wilms tumor gene, *WT1*; the genes for the apoptosis regulators Bcl-2 and Bcl-2–associated X protein, *BAX*; the Brain and Acute Leukemia Cytoplasmic gene, *BALC*; the ectropic viral integration site 1 gene, *EVI1*; the FMS-like tyrosine kinase type 3 gene, *FLT3*; and *KIT*, *ERG*, and the Mixed-Lineage Leukemia gene, *MLL*. Some mutations of specific genes confer a more favorable prognosis; most notably, mutations in the genes for CCAAT enhancer binding protein-α(C/EBP-α), *CEBPA*, and nucleophosmin, *NPM1*. These prognostic determinants have been particularly important for patients with AML and a normal karyotype. The difficulty with using cytogenetic grouping as a risk factor is that most patients fall in the intermediate risk group, and the majority of these have a normal karyotype [Rowe et al 2006]. Between 40% and 50% of young adult AML patients in the U.S. and United Kingdom have been found to have a normal karyotype [Slovak et al 2000, Grimwade et al 1998, Byrd et al 2002, Rowe et al 2006]. The intermediate group, unlike the favorable or the unfavorable cytogenetics group, has a greater heterogeneous mix of patients, with some having a poor prognosis and others having a better prognosis. The mutation was associated with a lower complete remission rate, a higher risk for relapse, and lower disease-free survival, event-free survival, and overall survival rates. The occurrence of the *FLT3* mutation was the most significant prognostic factor linked with relapse risk and disease-free survival [Kottaridis et al 2001]. Other studies have shown that, in adult patients with AML and normal cytogenetics, the presence of the *MLL* partial tandem duplication is associated with a very poor prognosis [Döhner et al 2002]. Similarly, the expression of *WT1* has been shown to be of major prognostic significance and is associated with a lower disease-free survival rate in AML patients, including those with a normal karyotype [Barragan et al 2004]. The

KIT gene encodes a transmembrane glycoprotein that is part of the type 3 receptor tyrosine kinase family. A recent report demonstrated that, in 43 patients with the 8;21 translocation, the incidence of relapse was significantly higher among patients with *KIT* mutations than among those with wild-type *KIT*. The reports are in line with other published data for patients with the 8;21 translocation, indicating that CD56 expression [Baer et al 1997], trisomy 4 [Nishii et al 2003], and mutations in the receptor tyrosine kinase pathway [Nanri et al 2005] may each independently confer a poor prognosis, even among this group with so called favorable cytogenetics.

New and Targeted Therapies - A third area of recent progress in AML involves new and targeted therapies. The period between 1985 and 1995 was marked by the remarkable rediscovery of all-*trans* retinoic acid (ATRA) for use in acute promyelocytic leukemia [Huang et al 1988]. Arsenic was rediscovered a few years later. During the next decade, 1995–2005, one of the most important leaps in the treatment of myeloid leukemias occurred through translational research into the design of targeted therapy with inhibitors of breakpoint cluster region–Abelson (BCR-ABL) tyrosine kinase for the treatment of chronic myeloid leukemia, specifically the use of imatinib mesylate [Druker et al 2001]. It is fair to say that there have never been so many targeted and other novel agents with such enormous potential.

We may be moving into an era of combinations of multiple targeted therapies for the treatment of acute leukemia; therapy can even be tailored to several specific genetic subtypes of leukemia. One broad class of mutations associated with acute leukemia comprises the proliferation/survival mutations that do not affect differentiation. Examples include mutations that activate tyrosine kinases such as BCR-ABL or FLT3 and oncogenic *RAS* mutations that enhance the proliferative and survival advantage of cells. These mutations can be targeted by small-molecule inhibitors of the respective tyrosine kinases or, potentially, by farnesyl-transferase inhibitors.

A second class of mutation comprises loss-of-function mutations in hematopoietic transcription factors, as exemplified by *AML1ETO* or *PMLRARA* gene rearrangements, or point mutations in *AML1*. Treatments targeting this class of mutations can include agents that specifically induce differentiation and apoptosis of leukemic cells, for example, ATRA in acute promyelocytic leukemia, or the class of agents known as histone deacetylase inhibitors.

Members of a third class, consisting of genes and pathways that are responsible for the self-renewal potential of leukemic stem cells (e.g., *WNT*, Notch, *HOX*) are not known to be mutant in leukemia, but they may be candidates for molecularly targeted therapy [Tallman et al 2005]. Some authors suggest that these agents may have a role in combination with chemotherapy [Stone et al 2005, Smith et al 2006], in an adjuvant setting, for newly diagnosed patients with AML. Because over expression of the gene *BCL2* is associated with poor outcome in AML, the antisense

oligodeoxynucleotide oblimersen sodium was evaluated in conjunction with fludarabine, cytarabine, and G-CSF salvage chemotherapy in a phase I study of patients with refractory or relapsed AML or ALL [Marcucci et al 2003]. Finally, the agent clofarabine is a deoxyadenosine analogue intentionally designed to combine the favourable properties of fludarabine and cladribine, with multiple mechanisms of action, including inhibition of DNA replication and repair and disruption of mitochondrial function, leading to apoptosis. Clofarabine is active in both dividing and non-dividing cells. This agent has been the subject of phase II trials in patients with AML, and results have been encouraging when it is used as a single agent [Kantarjian et al 2003, Faderl et al 2005]. Several other active agents, some of which are in clinical trials, are awaiting correlation with specific genetic subtypes. Other studies are anticipated for agents that may be active in specific genetic subtypes of acute leukemia, for example, the tyrosine kinase inhibitors imatinib and dasatinib in *CKIT* AML. Both histone deacetylase inhibitors and DNA methyltransferase inhibitors will likely be evaluated in *MLL* partial tandem duplication variants of AML [Whitman et al 2005], and an inosine monophosphate dehydrogenase inhibitor may be evaluated in *BCR-ABL*−positive AML [Malek et al 2004, King et al 2007].

1.6.f PI3K/Akt network targeted therapy

Genetic alterations can provide important therapeutic targets as well as molecular markers for prognosis and therapy responsiveness [Hennessy et al 2005]. A network approach wherein the combinatorial effects of genomic alterations in the PI3K/Akt signaling are considered systematically defining "pathway activity" may provide superior prognostic and predictive power. A systems biology approach to development of robust PI3K/Akt network computational models in normal cells integrating the effect of alterations present in cancer patients will be required for efficient implementation of targeted therapeutics and, in particular, for combinatorial therapy. With the PI3K/Akt pathway representing a high-quality target, many different companies and academic efforts are developing drugs targeting this network components [Hennessy et al 2005]. Clinical trials are underway with inhibitors targeting PI3K, Akt and downstream targets. Thus, despite the importance of the PI3K/Akt network to normal cellular function, there may be a therapeutic index that allows implementation of PI3K/Akt pathway-targeted therapeutics. The inhibition of signaling molecules of this network would have very strong effects on inhibiting tumor growth and angiogenesis in human cancer.

PI3K inhibition for cancer treatment - Recent study shows that inhibition of PI3K suppresses angiogenesis and tumor growth [Xia et al 2006, Arbiser et al 2007]. Several therapeutic strategies targeting PI3K pathway are now in development. In a number of pre-clinical studies, the

use of specific PI3K pharmacologic inhibitors such as LY294002 and wortmannin, and natural compounds with PI3K inhibitory capacities such as resveratrol, increased apoptosis and arrested the cell cycle in T-ALL cells [Zunino et al 2006, Uddin et al 2004]. PI3K inhibition is also important to abolish chemoresistance to drugs used in current therapeutic regimens or that are being tested in clinical trials [Palomero et al 2007]. The PI3K inhibitors wortmannin and LY294002 are commonly used to inhibit cancer cell proliferation and tumor growth, and sensitize tumor cells to the treatment of chemotherapeutic drugs and radiation. Unlucky, the poor solubility and high toxicity of these inhibitors limit the clinical application. To overcome these shortcomings, derivatives of LY294002 and wortmannin are being developed. It has been reported that PX-866 (acetic acid (1S,4E,10R,11R,13S,14R)-[4-diallylaminomethylene-6-hydroxy-1-methoxymethyl-10,13-dimethyl-3,7,17-trioxo-1,3,4,7,10,11,12,13,14,15,16,17-dodecahydro-2-oxa-cyclopenta[a]phenanthren-11-yl ester), a wortmannin derivative, has more potent and less toxic effects than wortmannin. It potentiates the antitumor activity of the EGFR inhibitor gefitinib against tumor xenografts induced by non-small cell lung cancer in the early stages of treatment [Ihle et al 2005]. With the development of RNA interfering technologies, RNA interference, especially small interfering RNA (siRNA), has been demonstrated to be effective in vivo, providing the possibility for selectively targeting p110 or p85 isoforms using siRNA technology [Hennessy et al 2005].

Akt inhibition for cancer treatment - Akt is the major PI3K downstream target for regulating tumor growth and angiogenesis. In animal study, inhibition of Akt by siRNA technique is effective to decrease ovarian tumor growth and angiogenesis [Xia et al 2006].

Lipid-based inhibitors of Akt were the first group of inhibitors to be developed. These Akt inhibitors include perifosine, phosphatidylinositol ether lipid analogues, and D-3-deoxy-phosphatidylmyoinositol-1-[(R)-2-methoxy-3-octadecyloxyropyl hydrogen phosphate] (PX-316).

Perifosine [octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate] (Aeterna Zentaris) is the best-characterized Akt inhibitor, which inhibits the translocation of Akt to the cell membrane, blocking the growth of several different human cancers [Martelli et al 2003]. Moreover, perifosine treatment overcomes the cancer cell resistance to the treatment with chemotherapeutic drugs and radiation. It is a synthetic novel alkylphospholipid, a new class of antitumor agents which targets cell membranes, inhibits Akt activation, and induces apoptosis in PC-3 prostate carcinoma cells [Kondapaka et al 2003], epithelial carcinoma cell line A431, and HeLa cells [Ruiter et al 2003]. Importantly, perifosine does not directly affect activity of PI3K or PDK1 [Kondapaka et al 2003]. It induces p21Cip1 associated with G2/M cell phase accumulation, in head and neck squamous cell carcinoma cells [Patel et al 2002, De Siervi et al 2004]. Perifosine, 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. Most recently, a combination treatment of human leukemia cells with perifosine and histone deacetylase inhibitors was seen to inhibit Akt and trigger apoptosis [Rahmani et al 2005]. In phase-II studies of perifosine in tumors, dose-limiting toxicity was not reached, although gastrointestinal toxicity led to early treatment discontinuation at higher dose levels. Importantly, no hematologic toxicity was observed, and maximum-tolerated dose was defined at 200 mg/d, achieving plasma concentrations of perifosine of 7.5 μg/mL (16.2 μM) [Crul et al 2002]. About the effectiveness in hematological malignancies, perifosine induced significant cytotoxicity in both multiple myeloma cell lines and patient cells resistant to conventional therapies [Hideshima et al 2006].

Erucylphosphocholine (ErPC) (Aeterna Zentaris), is an alkylphospholipid-derivative with a 22 carbon atom chain and a cis-13, 14 double bond; it is the first derivative suitable for intravenous administration for distinctive reduced hemolytic activity [Erdlenbruch et al 1999]. ErPC exerts more potent anti-neoplastic effects in vitro and in vivo than other derivatives and presently is at preclinical developmental stage [Jendrossek et al 1999, Konstantinov and Berger 1999]. ErPC has been shown to reduce the number of viable tumor cells in vitro; inhibition of proliferation and induction of apoptosis contribute to its anti-neoplastic action. ErPC modulates specific intracellular signaling pathways leading to growth arrest in the G2/M phase of the cell cycle and the induction of programmed cell death even in chemoresistant astrocytoma/glioblastoma cell lines [Jendrossek et al 2001]. However, the exact molecular mediators of ErPC-induced apoptosis remain unclear.

ErPC mainly inhibits Akt, but also Akt downstream target (Bcl2, Bad, FOXO3A) and the Raf-Mek-Erk signaling pathway, triggering apoptosis via a Bcl-2-dependent mitochondrial pathway, in Jurkat and BJAB B-lymphoma cells [Jendrossek et al 2003]. ErPC interferes primarily with membranes of proliferating cells, acting toward tumor development and progression. This compound is of particular interest since it has been demonstrated that when one signaling pathway is inhibited by a drug the cancer cell by passes it by iper-activating a parallel and alternative signaling pathway.

A set of structurally modified phosphatidylinositol ether lipid analogues shows inhibitory effect on the Akt PH domain in vitro [Castillo et al 2004]. Other Akt inhibitors include peptide-based inhibitors of Akt, pseudopeptide substrates of Akt, a single-chain antibody against Akt, and an inhibitory form of Akt expressed by adenovirus virus system [Granville et al 2006].

The novel **Erk/PI3K inhibitor** (Aeterna Zentaris) is a new generation of anilino-substituted pyridopyrazine-urea derivatives showing highly selective PI3K-inhibition; it targets the active sites of PI3K and Erk, therefore displaying an ATP-competitive mechanism of action. This low molecular weight dual kinase inhibitor shows anti-proliferative potency in different tumor cell lines

and is involved in a preclinical development program (in vivo testing). No *in vivo* toxicity was observed from an initial tolerance study in nude mice; further efforts to optimize bioavailability parameters such as solubility, permeability and stability by medicinal chemistry are ongoing.

mTOR inhibition for cancer treatment - Rapamycin and its derivative RAD001 block mTOR complex 1 (mTORC1) activity very selectively. Rapamycin stimulates apoptosis of pediatric T-ALL cells [Avellino et al 2005, Barata et al 2001], reverses the chemoresistance of Notch1-overexpressing leukemic cells [Mungamuri et al 2006] and restores the normal function of hematopoietic stem cells from PTEN-deleted mice [Yilmaz et al 2006]. Various clinical trials were initiated using rapamycin and derivatives, mostly in patients with tumors displaying elevated PI3K signaling and hyperactive mTOR. Promising results were obtained in mantle cell lymphoma, endometrial cancer and renal cell carcinoma [Guertin and Sabatini 2007]. Rapamycin and its derivatives possess anti-angiogenic activity because they counteract the VEGF action [Guba et al 2002]. This opens avenues for combinatorial treatments with conventional chemotherapy [Beuvink et al 2005]. Preclinical studies with rapamycin and RAD001 indicate that these compounds have cytostatic activity as a single agent in animal models, and have synergistic effects for inhibiting tumor growth when they are used with conventional chemotherapy agent tamoxifen, or with radiation treatment. In clinical studies, these compounds have been shown to be effective against many types of solid cancers [Granville et al 2006]. However, the optimal dose, schedule, patient selection, and combination strategies using these mTOR inhibitors remain to be elucidated.

In addition, PI3K and mTOR inhibitors combined with other therapeutic drugs may have synergistic effects to inhibit tumor development. Although PI3K/Akt/mTOR signaling pathway is also required for normal cell proliferation and survival, low levels of these kinase activities may be sufficient for the normal cell survival. Moreover, these inhibitors preferentially induced the apoptosis in cancer cells, but not in normal cells. There are strong interests to develop the inhibitors of PI3K, Akt, or mTOR for the cancer therapy. The future challenges are to develop highly specific inhibitors of these kinases, and to determine the clinical efficacy and maximum tolerated doses of these inhibitors for clinical cancer treatment [Jiang et al 2008].

1.7 Concluding remarks and scope of the research

Alterations in the PI3K/Akt network play important roles in the etiology, maintenance and progression of acute leukemia. However, it is likely that specific inhibitor compounds will not be effective as single agents, and strategies involving their combination with drugs that inhibit other targets should be required. Additionally, data suggest that inclusion of inhibitors of the PI3K/Akt pathway into current leukemia therapeutic protocols may be of particular relevance. It is very plausible that the oncogenic signature of some acute leukemia cases embrace activation of this key pathway, and that those cases may benefit from tailor-made therapies involving the use of signaling-specific antagonists. Overall, the analysis of the intracellular signaling profile of leukemia patients could not only serve to reveal novel molecular targets for treatment of this disease, but also to identify critical biomarkers for accurate and clinically relevant diagnosis and prognosis [Cardoso et al 2008].

This research is aimed at investigating the signaling PI3K/Akt pathway in acute leukemia cells. This pathway has so far not been studied in detail in this disease, but preliminary data indicate that it contributes to genomic alterations, thereby contributing to leukemogenesis. We propose that a better understanding of PI3K/Akt alterations will allow the development of new specific drugs, the identification of prognostic markers and the better characterization of acute leukemia subtypes.

In order to achieve our goals we will use protein and genetic analyses combined with functional studies that are aimed at understanding mechanisms of PI3K/Akt induced alterations in leukemias. In particular, this work aims at investigating enzymatic and genetic alterations of the PI3K/Akt network in AML and ALL, which will be useful for diagnosis, getting light on the underlying mechanisms of alterations occurring and leukemia development and progression.

Scope of the present research is to determine the prognostic influence of PI3K/Akt variations for AML and ALL patients, and the influence of novel therapeutic agents on chemoresistance. The novel genetic and enzymatic alterations identified in acute leukemias will provide a basis for the diagnosis of the various type of AML and ALL and will be helpful as prognostic tool which will lead to guidelines for a better clinical management.

2 Materials and Methods.

RPMI-1640, IMDM (Iscove's Medium), alpha-MEM and fetal calf serum (FCS) were from Lonza (Swizzerland). Bovine serum albumin (BSA), normal goat serum (NGS), peroxidase-conjugated IgG, antibodies to β-tubulin and β-actin, ATP, acrylamide/bis-acrylamide 37:1, 1,4-diazabicycle-[2.2.2]octane and Doxorubicin were from Sigma (St. Louis, MO). Human recombinant erythropoietin (EPO) and Lumi-Light chemo-luminescence detection kit were from Roche Molecular Biochemicals (Germany). Anti CD71 FITC-conjugated was from BD Pharmingen, Milan, Italy. [γ-32P] ATP and Cy2 secondary antibodies were from Amersham Pharmacia Biotech (Uppsala, Sweden). Antibody to lamin-B, Akt inhibitor VI H-AVTDHPDRLWAWEKF-OH (TCL110-24), VII H-YGRKKRRQRRR-AVTDHPDRLWAWEKF-OH (TAT-TCL110-24), VIII 1,3-Dihydro-1- (1-((4-(6-phenyl-1H-imidazo [4,5-g] quinoxalin-7-yl) phenyl) methyl)-4-piperidinyl)-2Hbenzimidazol- 2-one, IX 9-Methoxy-2-methylellipticinium acetate, Wortmannin and Ly294002 were from Merck-Calbiochem (La Jolla, CA). Antibody to Akt was from Transduction Laboratories (Franklin Lakes, NJ) and anti p308Thr Akt, anti p473Ser Akt, anti-phosphorylated Akt substrate (PAS) antibodies from Cell Signaling Technology Inc. (Danvers, MA). Immunoprecipitation matrix was from Santa Cruz Biotechnology (Santa Cruz, CA); the kit for protein concentration detection was from Pierce (Rockford, IL). Histone H2B was from Boehringer Mannheim and recH2B was from UBI (Lake Placid, NY). Reagents for molecular biology were small interfering RNA (siRNA) targeting the human Akt1 gene (QIAGEN S.p.A., Milan, Italy), Taq polymerase, nucleotides, primers for PCR and sequencing and molecular weight markers (Fermentas, Canada).

Perifosine, Erucylphosphocoline (ErPC) and PI3K/Erk inhibitor were gently provided by Aeterna Zentaris, Frankfurt (Germany).

2.1 Isolation of lymphocytes and granulocytes.

Isolation of lymphocytes and granulocytes was obtained by using Ficoll-Paque solution (Amersham bioscience), briefly: separation media consisting of a mixture of Ficoll 400 and an iodinated density gradient medium such as sodium diatrizoate have been very widely used for purifying human lymphocytes following the publication of Bøyum's pioneering work in 1968. For lymphocyte separation, defibrinated or anticoagulant-treated blood is diluted with an equal volume of balanced salt solution and layered carefully over Ficoll-Paque PLUS (without intermixing) in a centrifuge

tube. After a short centrifugation at room temperature (typically at 400 gav for 30–40 min) lymphocytes, together with monocytes and platelets, are harvested from the interface between the Ficoll-Paque PLUS and sample layers. This material is then centrifuged twice in balanced salt solution to wash the lymphocytes and to remove the platelets.

Granulocytes sediment to the bottom of the Ficoll-Paque PLUS layer. This process is facilitated by an increase in their densities caused by contact with the slightly hypertonic Ficoll-Paque PLUS medium. Thus, on completion of centrifugation, both granulocytes and red blood cells are found at the bottom of the tube, beneath the Ficoll-Paque PLUS. Lymphocytes, monocytes, and platelets are not dense enough to penetrate into the Ficoll-Paque PLUS layer. These cells therefore collect as a concentrated band at the interface between the original blood sample and the Ficoll-Paque PLUS. This banding enables the lymphocytes to be recovered with high yield in a small volume with little mixing with the Ficoll-Paque PLUS medium. Washing and centrifugation the harvested cells subsequently removes platelets, any contaminating Ficoll-Paque PLUS and plasma. The resulting cell suspension then contains highly purified, viable lymphocytes and monocytes and is suitable for further studies.

2.2 Cell culture and hemoglobin detection.

HL60, MV4;11, NB4 (acute myeloid leukemia), REH (acute lymphocytic leukemia) and K562 (erythroleukemia) cell lines were cultured in RPMI-1640 medium with 10% FCS, SEM (acute lymphocytic leukemia) cells were cultured in IMDM (Iscove's medium) with 10% FCS and RS4;11 (acute lymphocytic leukemia) cells were cultured in alpha-MEM with 10% FCS. Chemotherapic drugs treatment was performed at different times and concentrations, as indicated for each drug protocol. EPO stimulation was performed at 3 U/ml. LY294002 was used at 25 μM, Wortmannin was used at 100 nM, and both these PI3K inhibitors were administered 1 h prior EPO stimulation; Akt inhibitor VI and VII were used at 50μM final concentration, Akt inhibitor VIII at 25 μM and IX at 20 μM; the Akt inhibitors were administered 12 h prior to EPO stimulation. The hemoglobin determination kit was from Vector Laboratories (Burlingame, CA); the benzidine staining solution was freshly prepared as described in the manufacturer's protocol. The expression of the specific erythroid antigen CD71 on K562 cell surface was determined by direct immunofluorescent staining with FITC-conjugated mouse monoclonal antibodies against human CD71. Analysis was performed with FACScan from Becton Dickinson. FITC conjugated mouse IgG1 was used as negative control.

2.3 Preparation of whole cell homogenates.

Samples (5.000.000 cells) were washed in PBS plus 100 μM Na_3VO_4 and resuspended in 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1 mM Na_3VO_4, 1mM phenylmethylsulfonyl fluoride (PMSF) plus the phosphatases and proteases inhibitors 30 mM sodium-fluoride (NaF), 60 mM sodium-pyrophosphate dibasic (NaPP), and 10 μM each of aprotinin and leupeptin. After incubation for 20 minutes at room temperature (RT), total lysates were briefly sonicated to shear DNA and reduce viscosity, and the protein concentration was determined according to the instruction of the manufacturer using the Pierce Protein Assay.

2.4 Isolation of nuclei.

Samples (100.000.000 cells) were washed in PBS plus 100 μM Na_3VO_4 and resuspended in 10 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 1mM PMSF plus the phosphatases and proteases inhibitors (TM2 buffer, 4°C). After 5 minutes of incubation at 0°C, 0.5 % (w/v) Triton X-100 was added to samples and cells were sheared by two passages through a 22-gauge needle. Nuclei were observed by a phase contrast microscope, centrifuged at 1000 g for 10 minutes and washed in TM5 buffer. Nuclei were incubated 90 minutes with 75 U DNase, washed twice and resuspended to 2 mg DNA/ml in 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 5 mM $MgCl_2$ plus the phosphatases and proteases inhibitors (TM5 buffer, 4°C). The purity of nuclear preparation was evaluated by Western blotting analysis using a monoclonal antibody to β-tubulin. The absence of immunoreactivity against this protein in isolated nuclear preparations confirmed that the procedure yielded nuclei of high purity and free of cytoplasmic contaminants.

2.5 Western blotting analysis.

Proteins separated on 0.1% SDS-PAGE were transferred to nitrocellulose sheets, stained by Ponceau S and saturated in TBS with 5% not-fat dry milk and 0.1% Tween-20 (T) 1 h at RT, then incubated 2 h in blocking buffer containing primary antibody 1:1000-1:5000 dilutions. After 3 washes in TBS with 0.1% T, samples were incubated 45 min at RT with secondary antibody and

washed as previously described. Bands were visualized by chemoluminescence method. Anti-actin antibody was used to ensure equal protein loading and anti β-tubulin antibody to assess purity of the nuclear fraction.

2.6 Immunoprecipitation (IP).

Total or nuclear homogenates obtained from 10.000.000 or cells were resuspended in 1 ml of 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM PMSF plus the phosphatases and proteases inhibitors at the final concentrations indicated above. The IP was performed according to the instruction of the manufacturer using the ExactaCruz IP Matrix. The immunoprecipitated proteins were loaded into a poliacrylamide mini-gel for Western blotting analysis or used for in vitro kinase activity assay.

2.7 Akt kinase activity assay.

Total Akt was immunoprecipitated from sample cells and resuspended in sample buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$). The reaction was started by mixing 10 μl of immunoprecipitated Akt, 100 μM ATP or 100 μCi/ml [γ-32P] ATP, 4 μg H2B or 1 μg GSK3 fusion protein (Celbio) and the reaction mixture was incubated under constant agitation 40 minutes at room temperature. The reaction was stopped by adding 1 M Tris pH 6.8, 8% SDS, 40% glycerol, 20% β-mercapto-ethanol and samples were loaded into 12.5-15% polyacrylamide mini-gels for electrophoresis. After protein separation, the fosforylated proteins were detected by Western blot or the gel was dried and phosphorylation induced by Akt was revealed by auto-radiography.

2.8 PI3K kinase activity assay.

The activity of PI3K was valuated by an ELISA kit from Echelon Bioscience, briefly: the assay is a competitive ELISA in which the signal is inversely proportional to the amount of PI(3,4,5)P3 produced. Once PIP3 has been extracted from cells samples, it is first incubated with a PIP3 detector protein, then added to the PIP3-coated microplate for competitive binding. A peroxidase-linked secondary detection reagent and colorimetric substrate is used to detect PIP3 detector protein

binding to the plate. The colorimetric signal is inversely proportional to the amount of PIP3 extracted from cells.

2.9 In situ immunofluorescence.

Cells plated onto 0.1% poly-l-lysine-coated glass slides were fixed in ethanol 10 min at RT, incubated in PBS with 2% BSA and 3% NGS for 30 min and washed with 5 mM $MgCl_2$ PBS. Antibody to Akt, p473Ser Akt and Cy2 were diluted 1:100, 1:75 and 1:1000 respectively, in PBS with 2% BSA, 3% NGS, 5mM $MgCl_2$. The coverslips were mounted in glycerol containing 1,4-diazabicyclo [2.2.2] octane to retard fading; slides were observed using a Zeiss Axiophot epifluorescence microscope coupled with a Photometric Cool Snap CCD camera for image acquisition.

2.10 RNA interference assays.

Small interfering RNA (siRNA) sequences targeting the human Akt1 gene (Gene accession number NM 001014431) were synthesized as follows: Hs Akt1 6 sense r(CCA GGA CCA UGA GAA GCU U)dTdT, antisense r(AAG CUU CUC AUG GUC CUG G)dTdT; Hs Akt1 5 sense r(UCA CAC CAC CUG ACC AAG A)dTdT, antisense r(UCU UGG UCA GGU GGU GUG A)dTdT; Ha Akt1 10 sense r(CGC UUG GUC CCG AGG CCA A)dTdT, antisense r(UUG GCC UCG GGA CCA AGC G)dTdG; Hs Akt1 11 sense r(CCA UGA GCG ACG UGG CUA U)dTdT, antisense r(AUA GCC ACG UCG CUC AUG G)dTdG. The lyophilized siRNA was dissolved in sterile Suspension Buffer (100 mM potassium acetate, 30 mM Hepes-KOH, 2 mM magnesium acetate, pH 7.4) to obtain a 20µM solution. Oligonucleotides were heated 1 min at 90 ∘C followed by 1 h at 37 ∘C. As a control, non-silencing fluorescein-labeled duplex siRNA, also purchased from QIAGEN, was used. Exponentially growing K562 cells were transfected with 6 µg of each siRNA duplex using Lipofectamine2000 (Invitrogen), according to the manufacturer's protocols. The day before transfection, cells were seeded without antibiotics at 1.000.000 cells/well in 6-well tissue culture plate. Cells were incubated with siRNA plus Lipofectamine2000 and Opti-MEM I or with Lipofectamine2000 and Opti-MEM I as control. After 5 h, cells transfected with fluorescein-labeled siRNA were subjected to cytofluorimetric analysis to determine transfection efficiency that resulted higher than 60%. After 48 h from transfection, cells were treated with EPO for 10 min and harvested for analyses.

2.11 MTT test.

The Cell Proliferation Kit (MTT, Roche) is designed to be used for the nonradioactive, spectrophotometric quantification of cell proliferation and viability in cell populations using the 96-well-plate format. It can be used for:

- measurement of cell proliferation in response to treatment
- analysis of cytotoxic and cytostatic compounds, such as anti-cancer drugs and other pharmaceutical compounds
- assessment of growth-inhibitory antibodies and physiological mediators.

MTT was the first tetrazolium salt described. It is mostly cleaved to formazan by enzymes of the endoplasmic reticulum. This bioreduction occurs intracellularly in viable cells only, and is related to NAD(P)H production through glycolysis. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture.

The assay is based on the cleavage of MTT salt in the presence of an electron-coupling reagent. Cells, grown in a 96-well tissue culture plate, are treated with different drugs at scalar concentrations and with the control samples, then are incubated with the MTT solution for approximately 4 hours. After incubation, a water-insoluble formazan dye is produced and must be solubilized in an additional step. After solubilization, the formazan dye is quantified using a scanning multi-well spectrophotometer (ELISA reader) at the wavelength of 550-600 nm. The measured absorbance directly correlates to cells number.

2.12 Determination of the 50% of cell growth inhibition value.

The 50% of cell growth inhibition calculation was in according with Vichai et al 2006, briefly:

Calculation of % of cells growth:

(Mean OD sample / Mean OD negative control) * 100

Calculation of % of growth inhibition:

100 - % of cells growth

Using the % of growth inhibition we defined the value of the inhibition of 50% of the cells.

2.13 Extraction of genomic DNA from cell lines.

Genomic DNA from cell lines was obtained by cells lysis, getting rid of phospholipid membranes and purifying DNA from contaminating proteins. Cells (3.000.000) were mixed with 9 ml lysis solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) and incubated 20 min on ice. The solution was then centrifuged at 1500 rpm for 10 min at RT to separate DNA-protein complex from phospholipid membranes. After discarding supernatant, precipitate was washed two times with 5 ml distilled water to eliminate the excess of heme. Then it was subjected to proteolytic digestion by adding 15 µl pronase at 20 mg/ml concentration (Boehringer Mannheim GmbH, Mannheim, Germany), 900 µl SE buffer (75 mM NaCl, 25 mM EDTA), 90 µl of SDS detergent 10% and incubating overnight at 37 °C. On the following day, 300 µl of NaCl saturated solution (6 M) were added to the tube, the mixture was vortexed and centrifuged 15 min at 2500 rpm and 4 °C to precipitate digested proteins. The supernatant was then transferred to a fresh tube and one volume of chloroform was added, for extracting residual proteins. After gently mixing by inverting the tube, the material was centrifuged again 5 min at 4500 rpm and at RT. Aqueous supernatant was brought to a fresh tube and two volumes ice-cold absolute ethanol were added. Delicate mixing caused DNA to condense into a tangle of whitish filaments, which were recovered with a glass pipet, washed in 70% ethanol, dried at air and resuspended in 300 µl TE buffer (10 mM Tris, 1 mM EDTA). Purity of extracted DNA was evaluated by reading DNA absorbance at 260 nm and comparing with that of proteins (280 nm), using a spectrophotometer. OD_{260}/OD_{280} ratio equal or greater than 1.8 indicates pure DNA. A rough estimate of DNA quality and quantity was obtained by running a few microliters on 0.8% agarose gel for electophoresis.

2.14 Polymerase chain reaction (PCR).

DNA amplification by PCR (Mullis 1987) takes advantage of a natural enzyme, the thermostable DNA-polymerase I of thermophilic bacterium Thermus aquaticus, known as Taq polymerase, to produce large copy number of target DNA. DNA polymerization reaction starts from a template, two single-stranded oligonucleotides that frame the target sequence and all four deoxyribonucleoside 5'-triphosphates (dNTPs) in presence of $MgCl_2$. Amplification is performed through 25-30 DNA replication cycles, each comprising three steps: i) denaturation (separation of the two template strands); ii) annealing (primers hybridization to complementary sequences on

template); iii) extension (primers elongation by DNA-polymerase activity). These steps require different temperatures: denaturation 95 °C, annealing 45-60 °C according to characteristics of primers, extension 70 °C. Temperature cycles are automatically provided by thermal cycler (MJ mini, Biorad, Milan).

Primers for PCR-amplification of some regions of PI3K and Akt genes were designed on the basis of wilde type gene sequences using the on-line available Oligo Analyzer software. Amplification reactions (50 µl total volume) were carried out using 1 unit of Taq polymerase (Dream Taq, Fermentas, Canada) in buffer provided by supplier. Reaction conditions were 20 ng DNA template, 272 nM each primer, 50 nM each nucleotide precursor and 1.5-2.0 mM $MgCl_2$, 4% DMSO was added to avoid formation of DNA loops. Negative control, consisting in reaction carried out in absence of template DNA, was always included to check for contaminations.

Qualitative and quantitative outcome of amplification reaction was checked by running 3 µl PCR product on 2% agarose gel for electrophoresis, in parallel to an appropriate molecular weight marker (Fermentas, Canada).

Primers:

Region Akt exon 4:

5' F-GCTGCCTCAGCCTGGAGTAG 3'

5' R-GGCCTGGGAGCACTGGGGAG 3'

Region Akt exon 9-10:

5' F-CGGTCTGAGAAACCCCAGC 3'

5' R-GGCACAGAGAGGACACAGC 3'

Region Akt exon 14:

5' F-GCTCATGACTGTCCCGTCTGC 3'

5' R-CCAAGCTATCGTCCAGCGC 3'

Region PI3K exon 9:

5' F-GATTGGTTCTTTCCTGTCTCTG 3'

5' R-CCACAAATATCAATTTACAACCATTG 3'

Region PI3K exon 20, part a:

5' F-TGGGGTAAAGGGAATCAAAAG 3'

5' R-CCTATGCAATCGGTCTTTGC 3'

Region PI3K exon 20, part b:

5' F-TTGCATACATTCGAAAGACC 3'

5' R-GGGGATTTTTGTTTTGTTTTG 3'

2.15 Gel electrophoresis.

DNA fragments were separated in electric field according to charge, length and supercoiling status. After the electrophoretic migration, DNA was visualized by ethidium bromide staining and using UV transillumination at 254 nm, with a GelDoc UV-gel camera (Bio-Rad Laboratories, Hercules, CA, USA).

Agarose gel, useful to separate fragments ranging from 200 bp to 50 kb, was prepared by dissolving agarose powder in TAE buffer (40 mM tris-acetate, 1 mM EDTA) and heating this mixture till complete clarification. 5 µl of 10 mg/ml ethidium bromide were added directly to 100 ml of the melted gel before casting. Migration was performed on horizontal electrophoretic apparatus (BioRad Laboratories Srl, Milan, Italy) in TAE buffer, applying 5 V/cm voltage.

2.16 PCR fragments purification.

PCR products were purified from contaminating primers, free nucleotides, Taq polymerase and salts using the Wizard PCR Preps DNA Purification System (Promega, Italy). 50 µl PCR reaction were mixed with 250 µl binding buffer (3 M guanidine-thiocyanate, 10 mM tris-HCl, 5% ethanol v/v, pH 6.6 at 25°C). The mixture was transferred into uppe r reservoir of assembled concentrating device, filter tube inserted in a collection tube, and spun at 13000 g for 30 sec. The filter was washed twice by adding 200 µl wash buffer (20 mM NaCl, 2 mM tris-HCl, pH 7.5 at 25 °C) and centrifugating at 13000 g for 30 sec. Finally, filter tube was applied to a clean collection tube and filled with 50 µl elution buffer (10m M tris-HCl, 1 mM EDTA, pH 8.5 at 25 °C), before spinning at 13000 g for 30 sec.

2.17 Automated sequencing.

DNA sequencing was performed according to dideoxy-mediated chain termination method (Sanger 1977) by BMR Genomics (University of Padua), using the novel Roche Genome Sequencer FLX with Life Science 454 technology. Sequencing reactions and all the steps required for samples processing were performed with robotic management. Data were analyzed by using the Finch TV 1.4 version software (Geospiza) and ClustalW programme for multiple sequence alignement on the web.

3 Results

3.1 Kinase assay of PI3K and Akt in AML and ALL patients.

The clinic of internal medicine, department of onco-hematology of the University of Rostock thanks to the collaboration of the "Vigoni project", sent to our department 20 samples of patients affected by AML and 4 samples of patients affected by ALL.

We performed a non-radioactive in vitro PI3K kinase assay, using 1000 μg of protein with an ELISA kit (Echelon bioscience). The calculation of the quantity of PIP_3 that it was produced indicate the different activity of the cell lines. We compared the activity of the patients with the activity of the healthy donor controls. All AML and ALL patient samples showed a higher PI3K activity when compared with the controls. In particular, the mean values of activity for AML and ALL patients showed that the activity of ALL patients is significantly higher than the activity of AML.

	Mean
AML	0,460976
ALL	0,647419
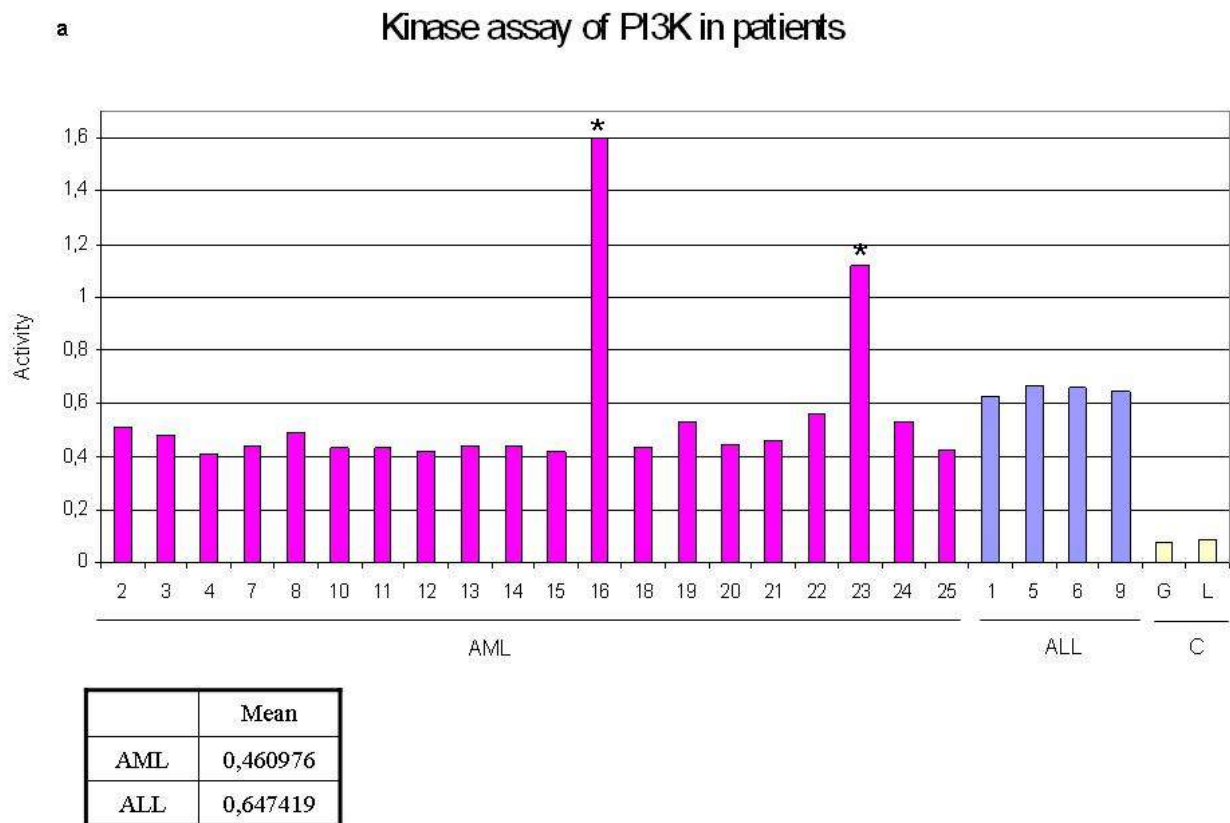
Figure 8. The histogram (a) shows the in vitro activity of PI3K. The graph represents 20 patients with AML, 4 patients with ALL and 2 controls: granulocytes and lymphocytes. The values of the graph are proportionally to the OD values of the reactions.

In the determination of mean values we excluded the values of patients 16 and 23 because they are out of the scale of the mean values and their activity is higher if compared with the other patients. The values of standard deviation indicate that the values of patients activity are similar, because lower than of 10%.

From the supernatants of PI3K immunoprecipitation, we immunoprecipitated Akt to make a radioactive in vitro kinase assay, on the direct substrate histone H2B. Also for Akt all the patients showed a very intense activity, when compared with the controls granulocytes and lymphocytes. Densitometric analysis of autoradiography indicated that ALL have higher activity than AML patients, as in PI3K assay.

Therefore nearly all the patients that showed an intense activity of PI3K showed also an intense activity of Akt.
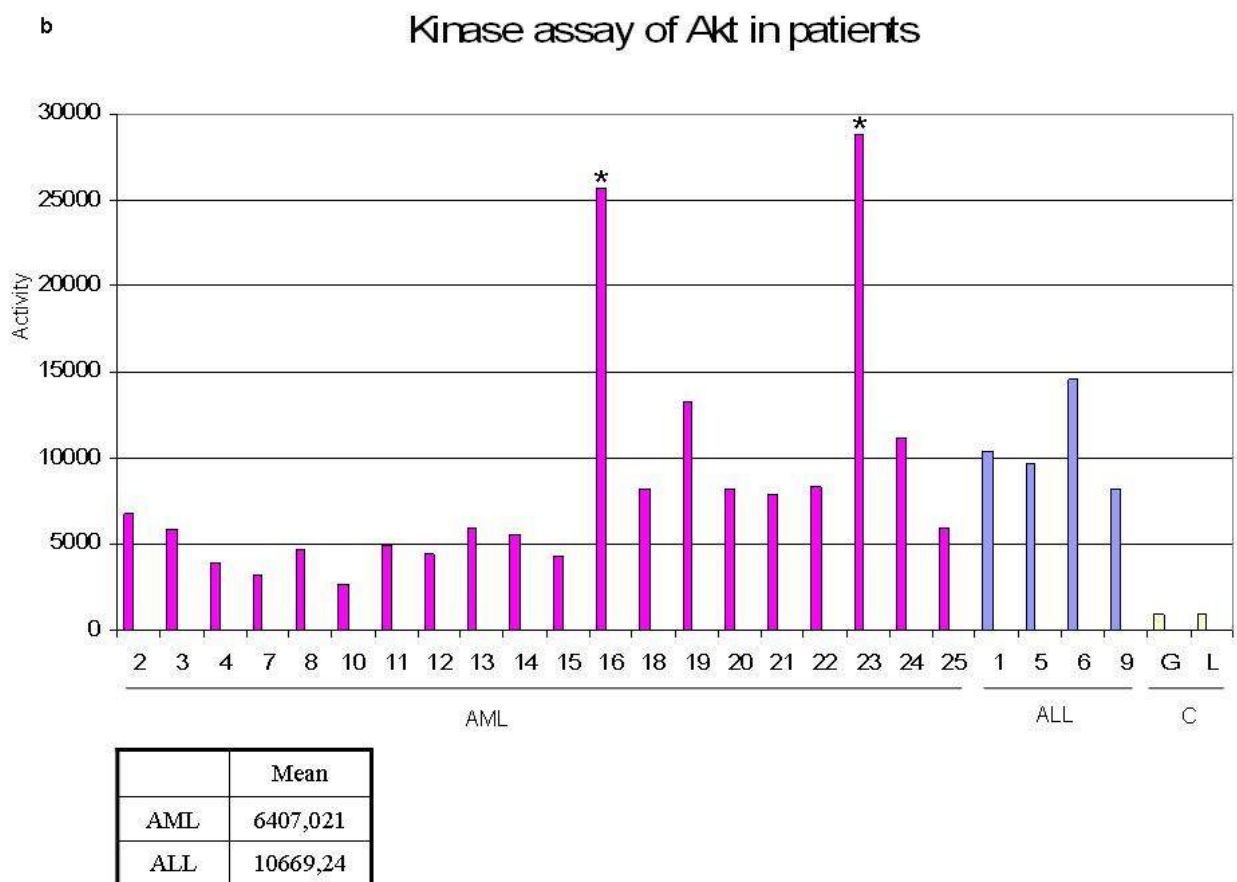

	Mean
AML	6407,021
ALL	10669,24

Figure 9. The histogram (b) shows the in vitro activity of Akt. In the histogram it is represented 20 patients with AML, 4 patients with ALL and 2 controls: granulocytes and lymphocytes. The values of the graph are proportionally to the OD values of the reactions.

Also the values of patients 16 and 23 were excluded, because out of the scale of the mean values and their activity is higher if compared with the other patients. The values of standard deviation indicates that the values are not so similar, because superior of 10%.

3.2 Expression and phosphorylation state of PI3K and Akt in AML and ALL cell lines.

We then studied the expression profile and phosphorylation level of different models of cell lines. We investigated 3 AML cell lines (HL60, MV4;11, NB4) and 3 ALL cell lines (SEM, RS4;11, REH).

We performed a Western blot analysis on lysates of untreated AML and ALL cell lines. The study of those experiments showed that PI3K have a similar quantity of total protein in the AML and ALL cell lines, whereas its phosphorylated form is more expressed in ALL.

About Akt we observed a different pattern, the ALL cell lines showed a stronger phosphorylation level, on the residues of serine 473, while the total protein Akt is nearly the same, when compared the AML to the ALL cell lines.

Western blot with anti β actin ensured that the same quantity of protein was loaded in all the lines.

Expression and phosphorylation state of PI3K and Akt in AML and ALL cell lines.
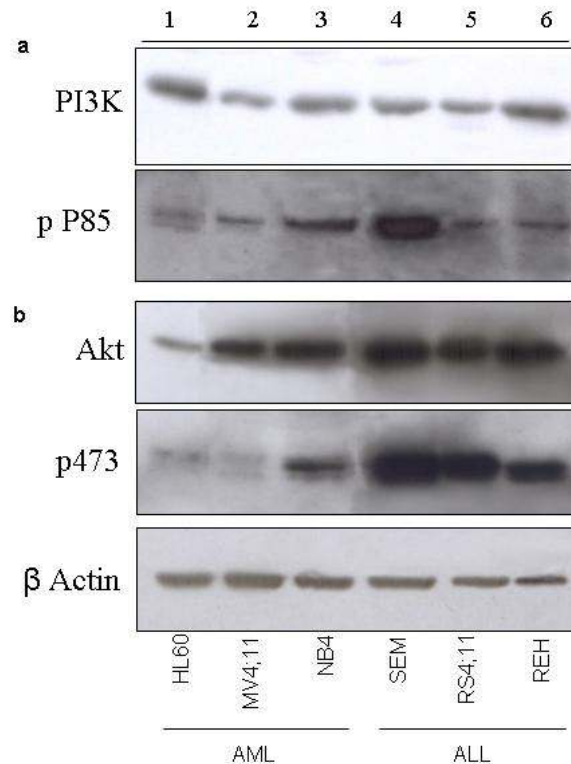

Figure 10. The panel (a) show Western blot analysis of PI3K total protein and phosphorylated protein on residue P85, the panel (b) show the analysis of the downstream target Akt and its phosphorylation state on serin 473. Both the analysis were made on 3 AML cell lines: HL60 lane 1, MV4;11 lane 2 and NB4 lane 3, and on 3 ALL cell lines: SEM lane 4, RS4;11 lane 5 and REH lane 6. Western blot with anti β actin shows the equal loading of all the lanes. All the experiment were done in triplicate.

3.3 Kinase assay of PI3K and Akt in AML and ALL cell lines.

The PI3K in vitro activity (panel a) was analyzed by testing its activity on the exogenous substrate PIP_2. Starting from 1000 μg of protein of all the AML and ALL cell lines, PI3K was immunoprecipitated and its activity was detected with the same ELISA kit used for the patients. From the histogram we can see that the ALL cell lines have an higher activity compared to the activity of the AML cell lines. Calculating the activity mean values for AML and ALL cell lines, we observed a strong difference between the activity of AML and ALL cell lines.

The activity of ALL cell lines is higher than the activity of AML, about 2 times more.

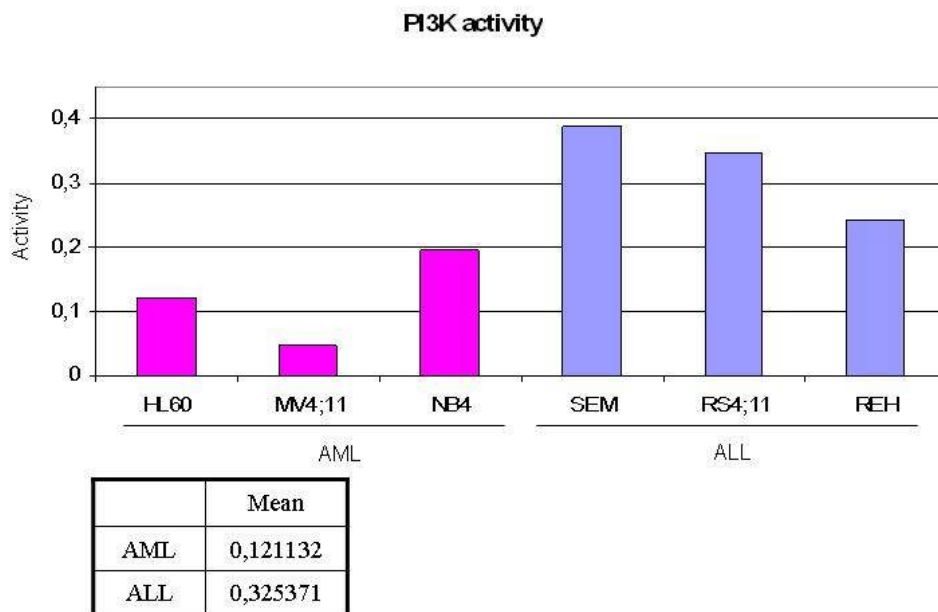**a** Kinase assay of PI3K in AML and ALL cell lines.

PI3K activity

	Mean
AML	0,121132
ALL	0,325371

Figure 11a. The figure shows the results of the PI3K activity assay and the product of PIP_3, lane 1: HL60, lane 2: MV4;11, lane 3: NB4, lane 4: SEM, lane 5: RS4;11, lane 6: REH. All experiments were done in triplicate with similar results.

We performed a kinase assay of Akt on the exogenous substrate of GSK3α/β (part b). Starting from the same quantity of cell lysates we immunoprecipitated total Akt protein. The kinase assay showed that the ALL cell lines have a more active Akt compared to the AML cell lines, as showed by the higher phosphorylation of the direct substrate GSK3α/β by ALL cell lines. Also for this kinase assay we made the densitometric analysis, as showed in the graph, and we determined the mean values of activity of the 2 groups of acute leukemia: it appears evident the higher activity of ALL cell lines, when compared with the mean value of AML cell lines. AML cell lines have 0,121132 of activity, ALL cell lines have 0,325371 of activity. The kinase assay was done in triplicate, with the same results.
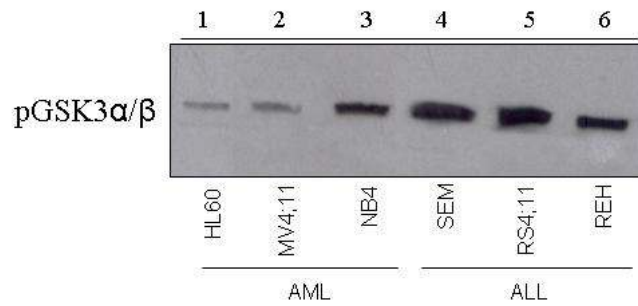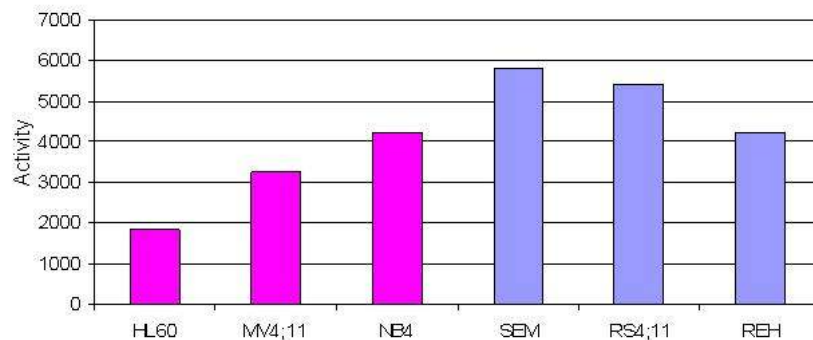
Figure 11b. The figure shows the results of Akt kinase activity assay on its direct substrate GSK3, lane 1: HL60, lane 2: MV4;11, lane 3: NB4, lane 4: SEM, lane 5: RS4;11, lane 6: REH. AML cell lines have 3112,897 of activity while ALL cell lines have 5159,31 of activity. All experiments were done in triplicate with similar results.

3.4 Expression of PTEN in AML and ALL cell lines.

We therefore sought to analyze by Western blot PTEN, a phosphatase that inhibits Akt by de-phosphorylating PIP_3, to explore if there is an altered expression of the enzyme. All the cell lines showed a similar expression of PTEN, suggesting that, if there is an impairment of function, it does not affect protein expression. The antibody recognize a sequence of aminoacid in the C- terminal residue, the highly conserved region in the protein.
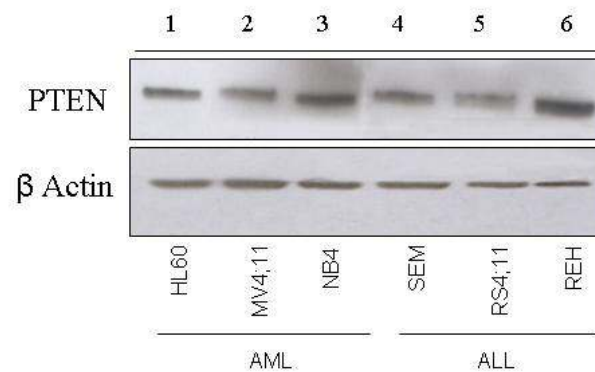
Expression of PTEN in AML and ALL cell lines.

Figure 12. Western blot of the expression of PTEN on 3 AML cell lines, lane 1: HL60, lane 2: MV4;11, lane 3: NB4, and on 3 ALL cell lines, lane 4: SEM, lane 5: RS4;11, lane 6: REH. The Western blot of β actin shows the equal loading of all the lane. The result was confirmed by 3 independents experiments.

3.5 Phosphorylation state of some Akt direct substrates: GSK3, mTOR and p21 in AML and ALL cell lines.

Some Akt direct substrates were analyzed to investigate some cellular processes in the cells, such as glucose metabolism by GSK3, protein synthesis by m-TOR and cell cycle progression by p21 in all the different cell lines of acute leukemia.

GSK3 in the subunits α and β did not show differences in the phosphorylation state between the AML and the ALL cell lines.

The substrate mTOR showed a different phosphorylation state between AML and ALL cell lines. The 2 ALL cell lines SEM and RS4;11 showed an intense phosphorylation when compared with the AML cell lines and even the REH cell line shows a higher staining than AML.

The third substrate analyzed is p21 and this protein is more phosphorylated in ALL, when compared with AML cell lines.
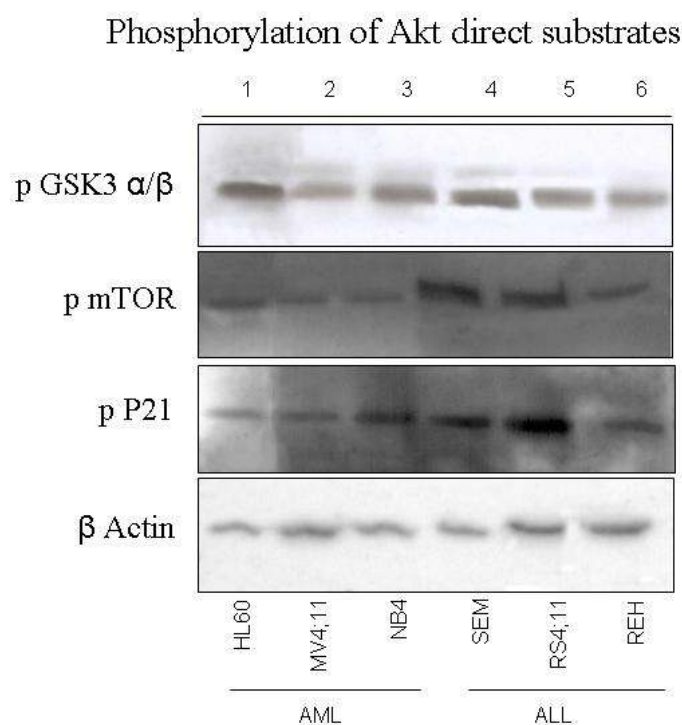
Figure 13. Analysis of phosphorylation state of GSK3 α/β, mTOR and p21 in AML and ALL cell lines. For all the Western blot, lane 1: HL60, lane 2: MV4;11, lane 3: NB4, lane 4: SEM, lane 5: RS4;11, lane 6: REH. As control for the correct loading of the protein in all the lanes, it was used β actin, as showed in the last panel.

3.6 DNA sequencing of selected regions of PI3K and Akt in AML and ALL cell lines and patient samples.

We performed DNA sequencing analysis of selected regions of PI3K and Akt of all AML and ALL cell lines and of some patients with acute leukemia. The regions that we sequenced are: the exon 9 and 20 of PI3K and the exons 4, 9, 10 and 14 of Akt. PI3K regions were selected by literature description of hot spot of mutations [Vogt et al 2007], while the regions of Akt are important regions for the phosphorylations or auto phosphorylations of the protein [Li et al 2006].

We have sequenced all the cell lines, 3 AML and 3 ALL, and 4 patients samples sent by the department of onco-hematology of the University of Rostock.

The results of DNA sequencing showed that 2 ALL out of 3 and all the patients have a heterozygous mutation in the intron 4 of Akt, at the position +30, with an exchange of a guanine with an adenine. Since this mutation is in the intron region, it does not affect directly protein

expression, but it could affect the splicing process. A silent mutation found in SEM line and in 1 patient. The mutation doesn't change the codified amino acid, because it is on the third nucleotide: GAG -> GAA, and it is an heterozygous mutation.

a

	Akt ex 4	Akt ex 9-10	Akt ex 14	PI3K ex 9	PI3K ex 20
HL60	wt	wt	wt	wt	wt
MV4;11	wt	wt	wt	wt	wt
NB4	wt	wt	wt	wt	wt
SEM	ivs 4 a+30g	E242E	wt	wt	wt
RS4;11	wt	wt	wt	wt	wt
REH	ivs 4 a+30g	wt	wt	wt	wt

b

	Akt ex 4	Akt ex 9-10	Akt ex 14	PI3K ex 9	PI3K ex 20
patient 2	ivs 4 a+30g	wt	wt	wt	wt
patient 5	ivs 4 a+30g	wt	wt	wt	wt
patient 9	ivs 4 a+30g	E242E	wt	wt	wt
patient 21	ivs 4 a+30g	wt	wt	wt	wt

Table 3. Summary of the regions analyzed and of the results of the sequencing, in cell lines (panel a) and in patients (panel b).

Akt ex 4 IVS4a+30g
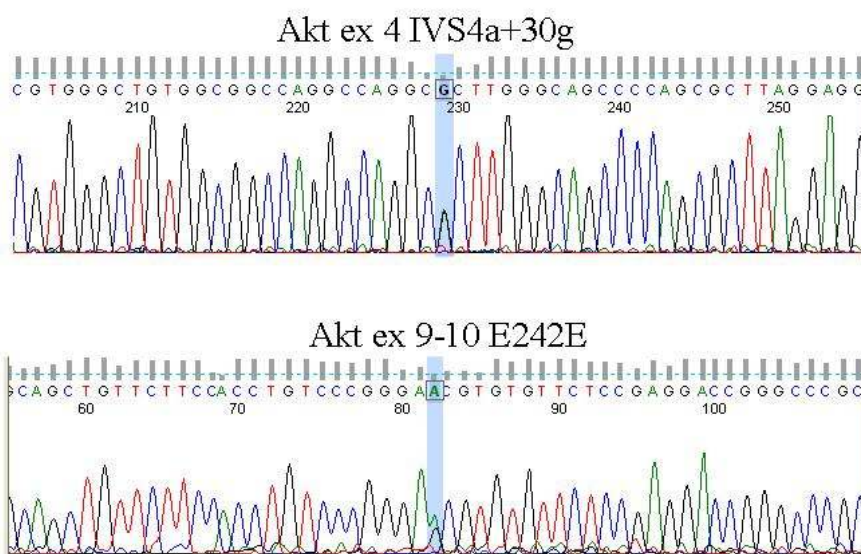

Akt ex 9-10 E242E

Figure 14. Representation of the histogram of the mutations found in the different cell lines and in the patients, both heterozygous mutation.

3.7 Determination of 50% of growth inhibition with treatment with conventional and new drugs.

We treated the cells with a conventional drug, Doxorubicin, and three drugs of new generation: Perifosine, ErPC and PI3K/Erk inhibitor, that were kindly give to us by the Aeterna Zentaris company. Perifosine, via its interference with the turnover and synthesis of natural phospholipids, disrupts membrane-linked signaling pathways at several sites including lipid rafts, thereby inhibiting PI3K/Akt survival network. ErPC (Erucylphosphocoline) inhibits Akt and Raf-Mek-Erk signaling pathways and interferes primarily with membranes of proliferating cells, acting on tumor development and progression. PI3K & Erk inhibitors target the active sites of PI3K and Erk, therefore displaying an ATP-competitive mechanism of action; it is highly potent dual mode inhibitors of both PI3K and Erk1/2, showing antiproliferative potency in different tumor cell lines. Doxorubicin is an anthracycline antibiotic and it intercalates DNA, inhibiting cell growth.

To define the values of the inhibition of 50% of cell growth of AML and ALL cell lines we performed an MTT assay on all the cell lines. We seeded 50.000 cells for each sample and we treated cells with 5 different drug concentrations. For Doxorubicin was employed a concentration suggested by the literature, while for the new drugs we used concentrations suggested by Aeterna Zentaris. The cells were treated for 24, 48 and 72 hours at the concentration of: 5, 10, 20, 40 and 80 μM with Perifosine, ErPC and PI3K/Erk inhibitor, while with Doxorubicin the cells were treated for the same time with 25, 50, 100, 200 and 400 nM of the drug.
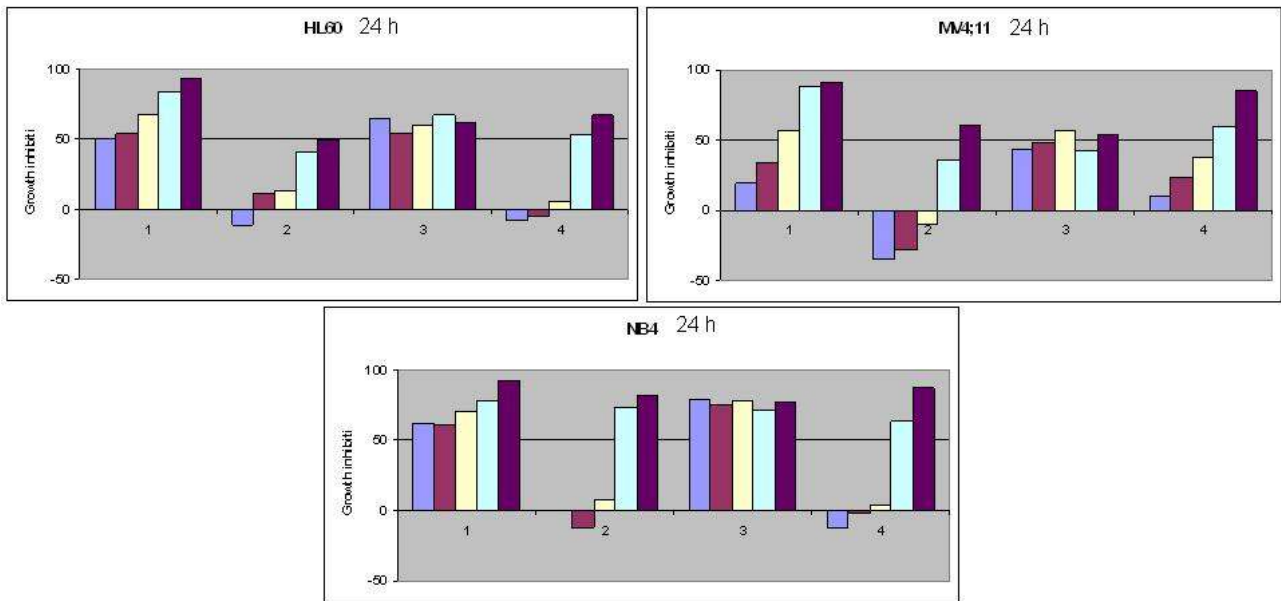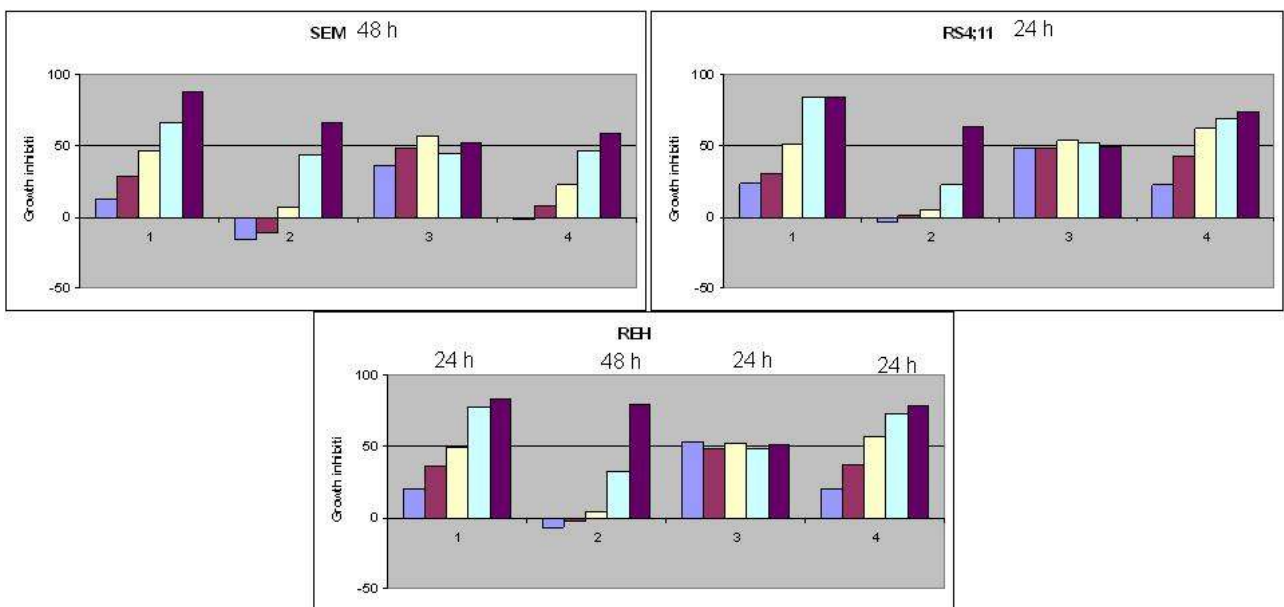
Figure 15. In the graphs are represented the cell inhibition effect of the different drugs. The drugs used are: 1 Perifosine, 2 ErPC, 3 PI3K/Erk inhibitor, 4 Doxorubicin. Perifosine, ErPC and PI3K/Erk inhibitor are used at the concentration of: 5, 10, 20, 40 and 80 μM, Doxorubicin is used at the concentration of: 25, 50, 100, 200 and 400 nM. In **a** are represented AML cell lines: HL60, MV4;11 and NB4, in **b** ALL cell lines: SEM, RS4;11 and REH.

We then determined the exact value necessary for inhibition of 50% of cell growth at selected time points. For all AML cell lines at 24 hours drugs are enough to inhibit the 50% of cell growth. At variance, the ALL SEM cell line requires higher concentration and longer time of treatment (48

hours) to obtain 50% of inhibition of cell growth with all the drugs. Similarly, in ALL REH cells ErPC needs 48 hours. The other time points in REH and all the time points in RS4;11 requires only 24 hours. The exact concentration that every cell line needs for every drug is summarized in the table below.

	Perifosine	ErPC	PI3K/Erk inh	Doxo
HL60	6,15 µM 24 h	75,88 µM 24 h	5 µM 24 h	247,06 nM 24 h
MV4;11	15,18 µM 24 h	68,55 µM 24 h	5 µM 24 h	129,20 nM 24 h
NB4	2,63 µM 24 h	34,79 µM 24 h	5 µM 24 h	176,76 nM 24 h
SEM	21,02 µM 48 h	55,79 µM 48 h	5 µM 48 h	274,41 nM 48 h
RS4;11	16,46 µM 24 h	75,73 µM 24 h	5 µM 24 h	79,55 nM 24 h
REH	17,47 µM 24 h	62,44 µM 48 h	5 µM 24 h	87,22 nM 24 h

Table 4. Concentrations and time necessary for each drugs to determine the 50% of growth inhibition.

3.8 Effects of conventional and new drugs on apoptosis and necrosis on HL60 and SEM cell lines.

Using the concentrations defined by calculating the 50% growth inhibition of the cells, we treated the cells and than performed a preliminary Western blot analysis and an Annexin V test on the AML and ALL cell lines.

The Western blot analysis shows that in the AML and ALL cell lines apoptosis is promoted by Perifosine, PI3K/Erk inhibitor and Doxorubicin, while ErPC does not promotes apoptosis in HL60 and SEM.
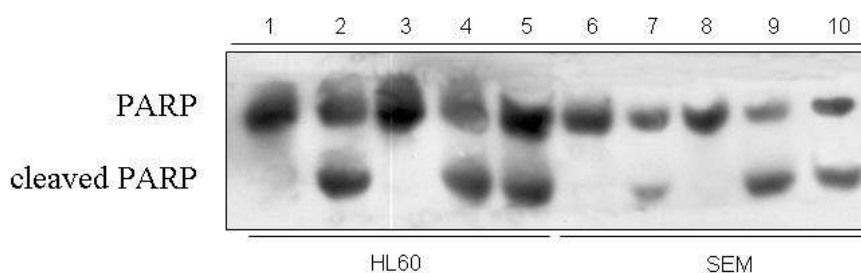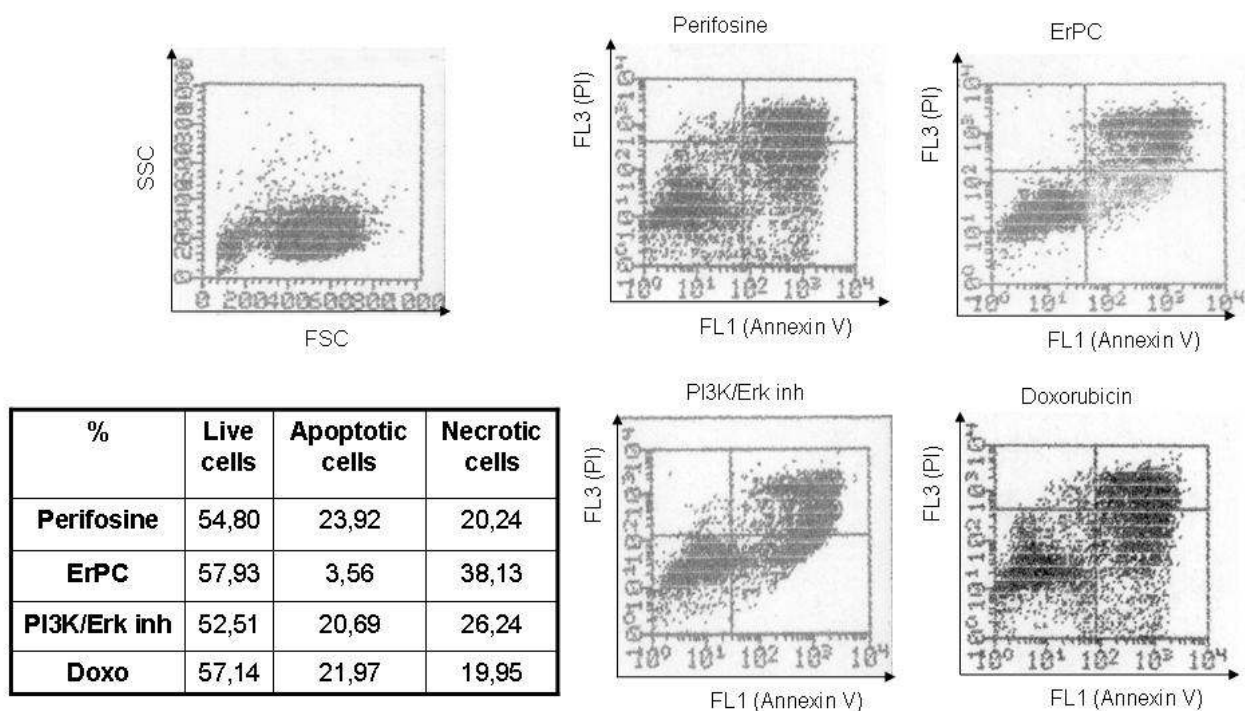
Phosphorylation of PARP after drugs treatment.

Figure 16. The Western blot shows the different cleavage of PARP apoptotic protein after treatment. The different lane indicates: lane 1 HL60 untreated, lane 2 HL60 + Perifosine, lane 3 HL60 + ErPC, lane 4 HL60 + PI3K/Erk inhibitor, lane 5 HL50 + Doxorubicin, lane 6 SEM untreated, lane 7 SEM + Perifosine, lane 8 SEM + ErPC, lane 9 SEM + PI3K/Erk inhibitor, lane 10 SEM + Doxorubucin.

HL60 and SEM cell lines are analyzed also by Annexin V assay. The assay divided the death cells in apoptotic cells and necrotic cells, thanks the marker with Annexin V and Propidium Iodide. In particular Annexin V is a marker for early stages of apoptosis while Propidium Iodide is a marker for necrosis. In the panel **a** is represented the FACS analysis of HL60 cell line and in the panel **b** is represented the analysis of SEM cell line. The graphs represented the different treatments, with the new drugs: Perifosine, ErPC and PI3K/Erk inhibitor and the conventional drug: Doxorubicin. In the tables are represented the different percentage of apoptosis and necrosis for the different treatments.

a

Analysis of apoptosis by Annexin V of HL60 cell line.

%	Live cells	Apoptotic cells	Necrotic cells
Perifosine	54,80	23,92	20,24
ErPC	57,93	3,56	38,13
PI3K/Erk inh	52,51	20,69	26,24
Doxo	57,14	21,97	19,95

b

Analysis of apoptosis by Annexin V of SEM cell line.

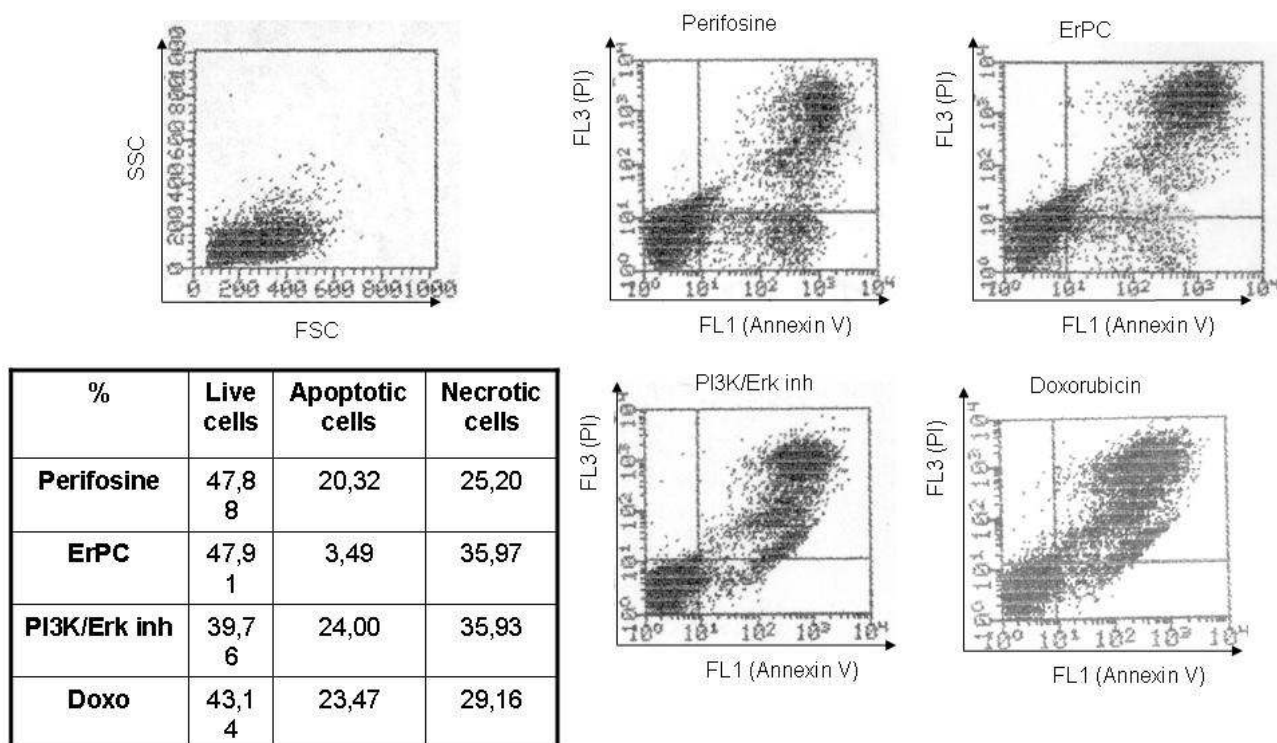| %	Live cells	Apoptotic cells	Necrotic cells
Perifosine	47,88	20,32	25,20
ErPC	47,91	3,49	35,97
PI3K/Erk inh	39,76	24,00	35,93
Doxo	43,14	23,47	29,16

77

Figure 17. Panel **a** shows the analysis of HL60 cell line, FACS analysis and table with the percentage of apoptosis and necrosis. Panel **b** shows the analysis of SEM cell line, FACS analysis and table with percentage of apoptosis and necrosis.

3.9 Phosphorylation state of Akt in HL60 and SEM cell lines after drug treatment.

The cell lines, HL60 and SEM, are treated with different concentration for 24 hours. We used the concentration determined by the inhibition of the proliferation of the 50% of the cells. The preliminary Western blot show the different phosphorylation state of Akt after drug treatments. The cell lines were treated with Perifosine, ErPC, PI3K/Erk inhibitor and Doxorubicin. As showed by the Western blot, all the treatments with the new and conventional drugs inhibit the phosphorylation of Akt, if compared with the control. For control of equal loading we used the Western blot with an antibody anti-β actin.
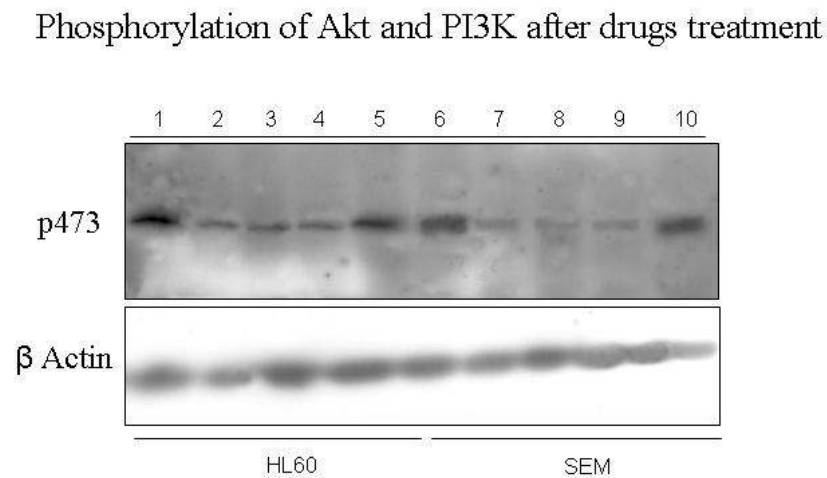

Figure 18. The Western blot shows the different phosphorylation levels of Akt after drug treatment. The different lane indicate: lane 1 HL60 untreated, lane 2 HL60 + Perifosine, lane 3 HL60 + ErPC, lane 4 HL60 + PI3K/Erk inhibitor, lane 5 HL60 + Doxorubicin, lane 6 SEM untreated, lane 7 SEM + Perifosine, lane 8 SEM + ErPC, lane 9 SEM + PI3K/Erk inhibitor, lane 10 SEM + Doxorubucin.

3.10 Kinase assay of Akt in HL60 and SEM after drugs treatment.

To verify the effect of the drugs, news and conventional, on the PI3K/Akt pathway, we performed an in vitro kinase assay of Akt on HL60 and SEM cells. We treated the cells with the different concentration of the drugs, defined by the quantity that inhibits the 50% of the growth of the cells. Than 1000 µg of protein was assayed with the direct substrate GSK3. The Western blot analysis shows that the drugs, Perifosine, ErPC and PI3K/Erk inhibitor, can act directly on the PI3K/Akt pathway.
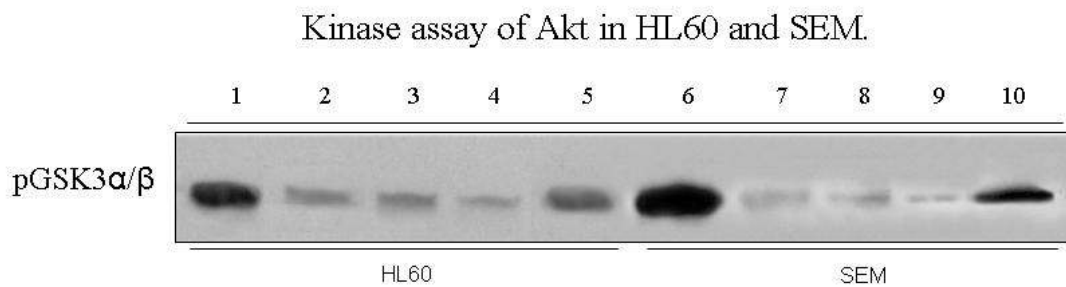

Figure 19. The Western blot shows the activity assay of Akt in HL60 and SEM cell lines in different condition: lane 1 HL60 untreated, lane 2 HL60 + Perifosine, lane 3 HL60 + ErPC, lane 4 HL60 + PI3K/Erk inhibitor, lane 5 HL60 + Doxorubicin, lane 6 SEM untreated, lane 7 SEM + Perifosine, lane 8 SEM + ErPC, lane 9 SEM + PI3K/Erk inhibitor, lane 10 SEM + Doxorubucin.

3.11 Phosphorylation state of some Akt direct substrates: GSK3, mTOR and p21 in AML and ALL cell lines after conventional and new drugs treatments.

The effects of the conventional and new drugs were studied also on the phosphorylation of Akt direct substrate. The HL60 and SEM cell lines were treated with the concentrations and the times defined. As we can observe the new drugs Perifosine, ErPC and PI3K/Erk inhibitor can inhibit the phopshorylation of the GSK3, mTOR and P21. This result can suggest that these drugs can inhibit different process of the cells, such as glucose metabolism, protein synthesis and cell cycle.

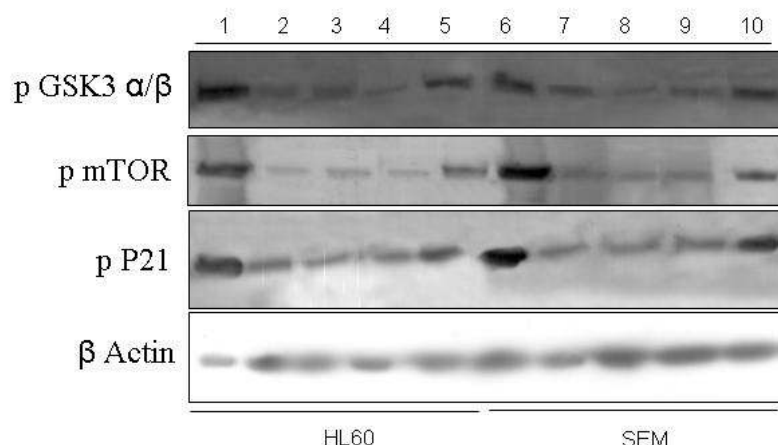Phosphorylation of Akt direct substrates after drugs treatment

Figure 20. Analysis of phosphorylation state of GSK3 α/β, mTOR and p21 in HL60 and SEM cell lines. Lane 1 HL60 untreated, lane 2 HL60 + Perifosine, lane 3 HL60 + ErPC, lane 4 HL60 + PI3K/Erk inhibitor, lane 5 HL60 + Doxorubicin, lane 6 SEM untreated, lane 7 SEM + Perifosine, lane 8 SEM + ErPC, lane 9 SEM + PI3K/Erk inhibitor, lane 10 SEM + Doxorubucin. As control for the correct loading of the protein in all the lanes, it was used β actin, as showed in the last panel.

3.12 EPO determines subcellular redistribution of phosphorylated p473Ser Akt in K562 cells.

Since previous investigations have highlighted that p-Akt may migrate to the nucleus [reviewed in Neri et al 2002], we sought to analyze the subcellular distribution of Akt after its serine 473 residue phosphorylation in K562 erythroleukemia cells, that were induced to differentiate by 96 h of EPO treatment. As shownin Fig. 21, either with Benzidine staining or with flow cytometric analysis with anti CD71, after 4 days of treatment more than 50% of cells were differentiated.
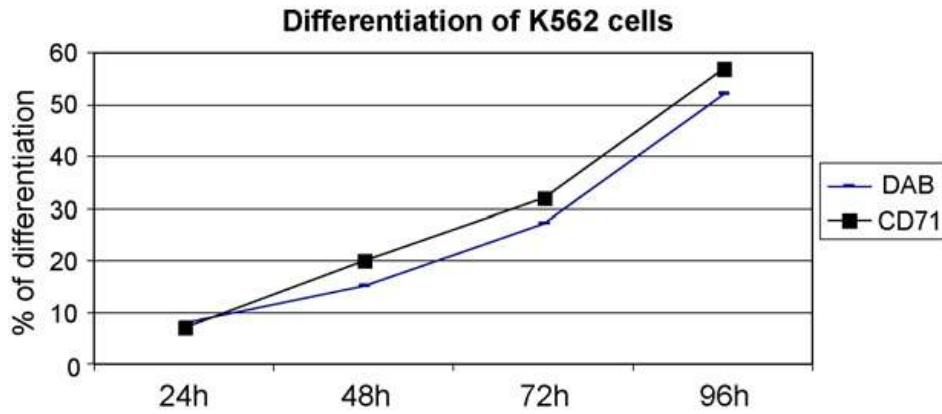
Figure 21. K562 differentiation after EPO exposure. Either with Benzidine staining or with anti CD71 flow cytometric analysis, after 96 h of EPO treatment K562 cells disclosed more than 50% of erythroid differentiation. Viability was always more than 90% and S.D. was less than 10%. Data are representative of three independent experiments.

No changes of total Akt amount were found in all the examined times of EPO treatment. However, using the anti p473Ser Akt antibody, we found that Akt activation was evident already at 5min, with a peak of Akt phosphorylation 10 min after EPO stimulation and decreased until 30 min, maintaining a phosphorylation level higher than the untreated control (Fig. 22).
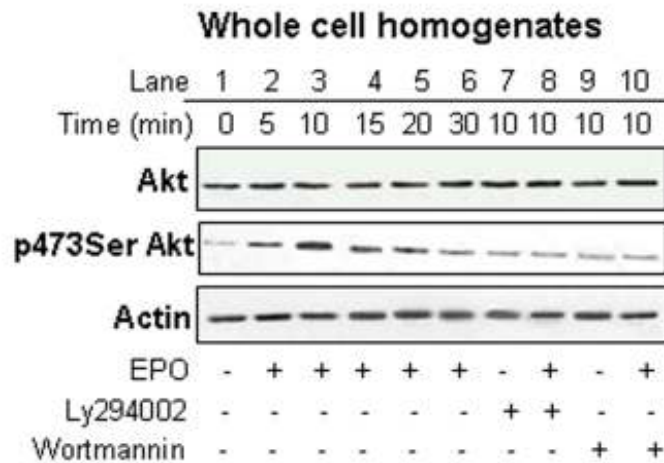

Figure 22. Akt expression and phosphorylation levels in K562 cells. Western blot on unstimulated K562 cells, cells exposed to EPO or cells exposed to EPO plus Ly294002 or Wortmannin. In each lane 20µg proteins were loaded, as confirmed by anti-actin antibody.

Pre-treatment of cells with 25μM Ly294002 or 100 nM Wortmannin alone did not modify Akt levels. Cells pre-treated with 25μM Ly294002 or 100 nM Wortmannin before EPO stimulation showed Akt expression and phosphorylation levels comparable, at each time point, to those of untreated cells. Western blotting performed on nuclei isolated from K562 EPO treated cells showed increased amount of total nuclear Akt in a range from 10 to 20min, with a peak at 15 min (Fig. 23A). The nuclear immunolabelling of p473Ser Akt started 10min after treatment, increased to a maximum intensity 15 min after stimulation in parallel with total Akt nuclear translocation and after 30min was slightly higher than the control sample. Cell number was not affected by pre-treatment with Ly294002 before EPO stimulation and no modifications of Akt distribution and phosphorylation state were observable. In Ly294002 or Wortmannin and EPO treated samples, Akt expression and phosphorylation levels were comparable to those of untreated cells. The human leukemia T-cell derived Jurkat cell line was used as positive control [Fukuda et al 2005, Seminario et al 2003]. Immunocytochemical analysis showed total Akt mainly in the cytoplasm of untreated K562 cells. Nevertheless, nuclei, identified by means of phase-contrast microscopy (data not shown), showed also in control cells the presence of only a small amount of fluorescence (Fig. 23B). Immunostaining intensity increased in the nucleus after 15min of EPO stimulation and greatly decreased, at the level of untreated cells, after 30min of treatment. Cells pre-treatment with 25μM Ly294002 prevented the intranuclear Akt translocation. Immunostaining using anti p473Ser Akt antibody in control K562 cells showed fluorescence predominantly located in the cytoplasm (Fig. 23B). After 15min of EPO stimulation, immunofluorescence increased and concentrated inside the nuclear compartment to strongly decrease after 30 min. Pre-treatment with Ly294002 prevented the p473Ser Akt translocation into the nucleus after 15 min of EPO treatment (Fig. 23B).
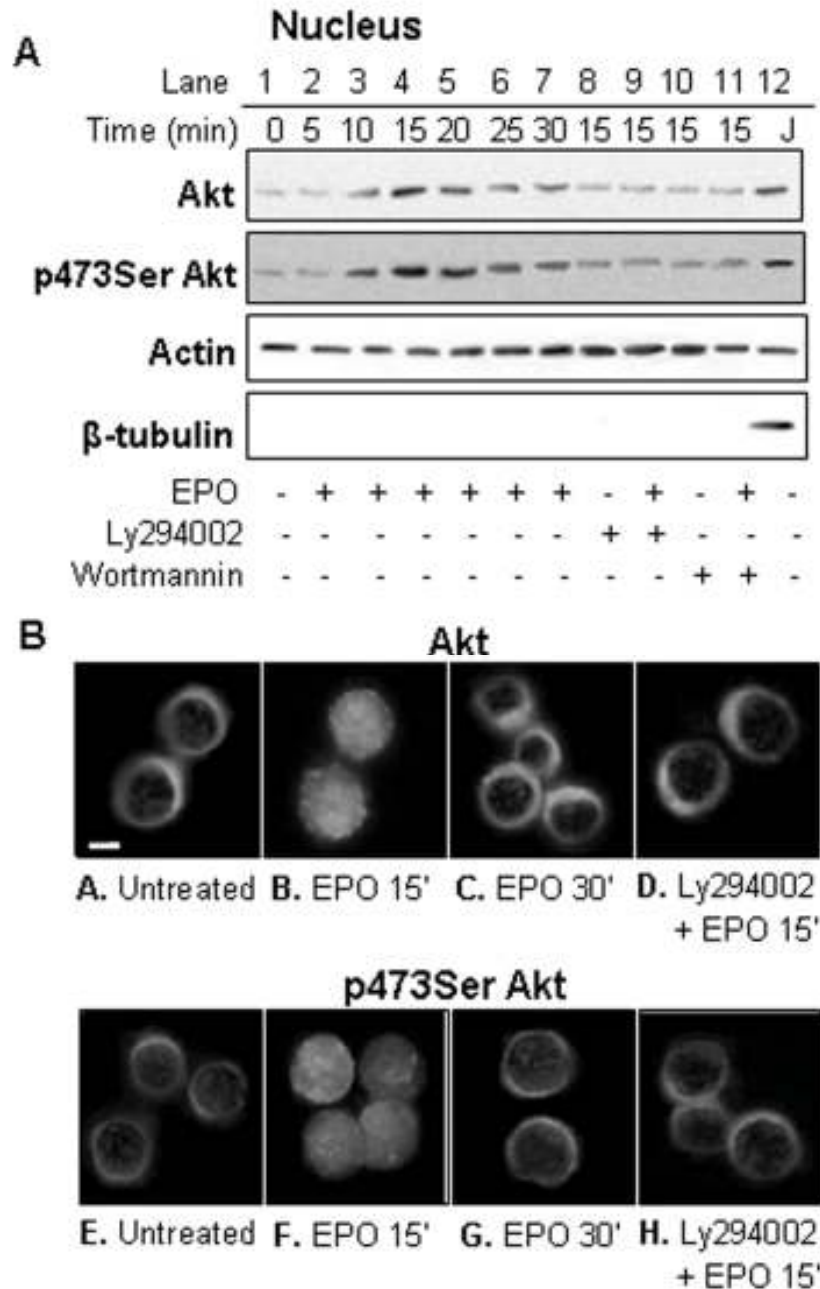
Figure 23. Akt expression and phosphorylation levels in nuclei of K562 cells. (A)Western blot on unstimulated K562 cells, cells exposed to EPO or cells exposed to EPO plus Ly294002 or Wortmannin. Total homogenates from Jurkat cells (J) were used as positive control for Akt MW. In each lane 20μg proteins were loaded, as confirmed by anti-actin antibody. β-Tubulin assessed absence of cytoplasmic contaminants in nuclear fractions. (B) Total and p473Ser Akt distribution in K562 cells treated with EPO studied by fluorescence microscopy. Scale bar: 5μm.

3.13 Exposure of K562 cells to EPO increases Akt kinase activity in whole cell homogenates and in nuclei.

K562 cells treated with EPO exhibited an increase of total immunoprecipitable Akt activity levels in kinase assay performed in vitro toward the exogenous substrate histone H2B. A rise of Akt activity was detected early after 5 min and peaked 10 min after EPO treatment in whole cell homogenates, to reduce, after 30min, to levels still higher than the control. The dependence on the PI3K/Akt pathway was further assessed, when cells, pre-treated with 25μM Ly294002 or with 100 nM Wortmannin, even if stimulated with EPO did not show any increase in Akt activity (Fig. 24A). Akt immunoprecipitated from nuclei showed after 10 min an increased enzymatic activity, that reached a peak 15 min after EPO stimulation, was still high after 20min and lasted even if at a lower intensity until 30 min. Cells pre-treated with 25μM Ly294002 or 100 nM Wortmannin exhibited Akt activity levels comparable to those of untreated cells (Fig. 24B). Similar results were obtained using anti-phosphorylated Akt substrate (PAS) antibody to reveal phosphorylation of recombinant histone H2B after in vitro Akt kinase assay. In whole cell homogenates the highest labeling was detectable after 10 min of EPO treatment (Fig. 24C) and in isolated nuclei an increase in phosphorylation of H2B PAS sites (i.e. serine/threonine phosphorylation) was observed maximal after 15min of EPO administration (Fig. 24D).
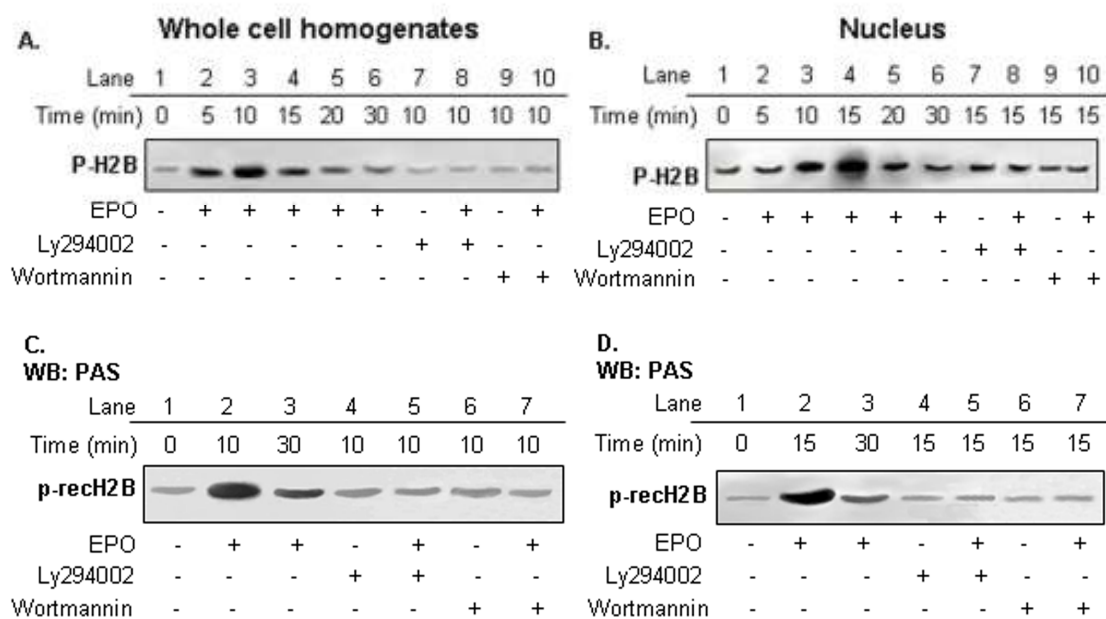

Figure 24. Total Akt kinase activity. (A) Autoradiogram of Akt activity on whole K562 cell homogenates, after EPO administration alone or plus Ly294002 or Wortmannin. The Akt kinase

assay was performed toward the exogenous substrate histone H2B by using radioactive isotope. (B) Autoradiogram of nuclear Akt activity after EPO exposure alone or plus Ly294002 or Wortmannin. The Akt kinase assay was performed toward the exogenous substrate histone H2B by using the radioactive isotope. (C) Non-radioactive analysis showing the results of total Akt activity on whole K562 cell homogenates, after EPO alone or plus Ly294002 or Wortmannin. The Akt kinase assay was performed toward the exogenous substrate recombinant histone H2B and revealed by Western blot with anti-PAS antibody. (D) Non-radioactive analysis showing the results of nuclear Akt activity, after EPO alone or plus Ly294002 or Wortmannin. The Akt kinase assay was performed toward the exogenous substrate recombinant histone H2B and revealed by anti-PAS antibody.

3.14 The majority of nuclear Akt is phosphorylated on serine 473 residue after EPO stimulation.

To exclude that nuclear Akt activity could be simply a consequence of its translocation to the nucleus, the amount of phosphorylated versus unphosphorylated Akt in nuclei of K562 cells treated with 3 U/ml EPO was compared by performing double round immunoprecipitation from nuclear fractions. A first immunoprecipitation round was performed using anti p473Ser Akt antibody and revealed by Western blot using anti Akt. Increased p473Ser Akt in nuclei was observed after 15min of EPO treatment (Fig. 25). A little amount of p473Ser Akt, appreciably more intense than control nuclei, was immunoprecipitable after 30min of EPO administration. The second round of immunoprecipitation using anti Akt antibody was carried out on the supernatants derived from first immunoprecipitation round. The remaining unphosphorylated Akt was revealed by anti Akt antibody. This analysis showed that unphosphorylated Akt was very faint or extremely low compared to p473Ser Akt level in both samples, thus showing that almost all nuclear Akt is phosphorylated on serine 473 residue (Fig. 25).
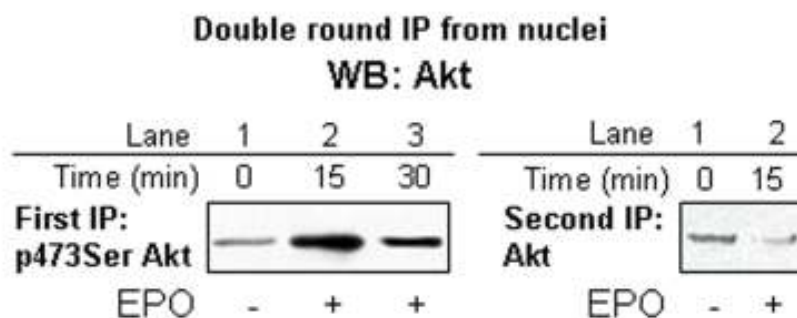
Figure 25. The majority of nuclear Akt is phosphorylated. Panel on the left: first round of IP carried out with anti-p473Ser Akt on nuclei isolated from K562 cells. Panel on the right: second round of IP with anti Akt antibody. Western blot detection was carried out with anti Akt antibody. Results were replicated in three independent experiments.

3.15 Different Akt inhibitors induce different levels of Akt phosphorylation on serine 473 residue.

In order to test whether the activation of nuclear Akt in EPO treated cells is required for differentiation to proceed or whether the increase is only a consequence of differentiation and to explore the relevance of this activation, we used a panel of Akt inhibitors interfering with the activation process (inhibitors VI, VII and VIII) or acting on its kinase activity after the activation (inhibitors IX). Inhibitor VI and VII interact with the PH domain, inhibit PtdIns(3,4,5)P3 binding to Akt and consequently membrane translocation and activation of Akt [Hiromura et al 2004]. Inhibitor VIII was found to be cell active and to block phosphorylation of Akt at threonine 308 and serine 473 thus reducing the levels of active Akt in cells [Barnett et al 2005]. At variance, inhibitor IX selectively inhibits Akt kinase activity without blocking phosphorylation of Akt itself at either threonine 308 or serine 473 [Jin et al 2004]. Akt total amount was unchanged, independently from EPO or inhibitor treatments. When cells treated with EPO were pre-treated with inhibitors blocking Akt activation, no detectable raise in phosphorylation on serine 473 was observed. On the contrary, the inhibitor acting on the kinase activity of Akt did not modify the increase of phosphorylation level on the 473 serine residue (Fig. 26A). We used Akt inhibitor VIII or IX as representative inhibitors of the two different mechanisms of Akt inhibition to detect total and p473Ser Akt localization by immunofluorescence during EPO stimulation and inhibitors treatment. In control cells both total or p473Ser phosphorylated Akt are distributed throughout the cytoplasm and their distribution is not affected by inhibitors (Fig. 26B and C, images A and D control, B and E). Treatment with Akt inhibitor VII did not modify the distribution of Akt in both native and phosphorylated form after EPO exposure (Fig. 26B and C, image C). On the contrary, in Akt inhibitor IX-treated samples, 15 min of EPO administration triggered nuclear translocation of total or p473Ser phosphorylated Akt (Fig. 26B and C, image F).
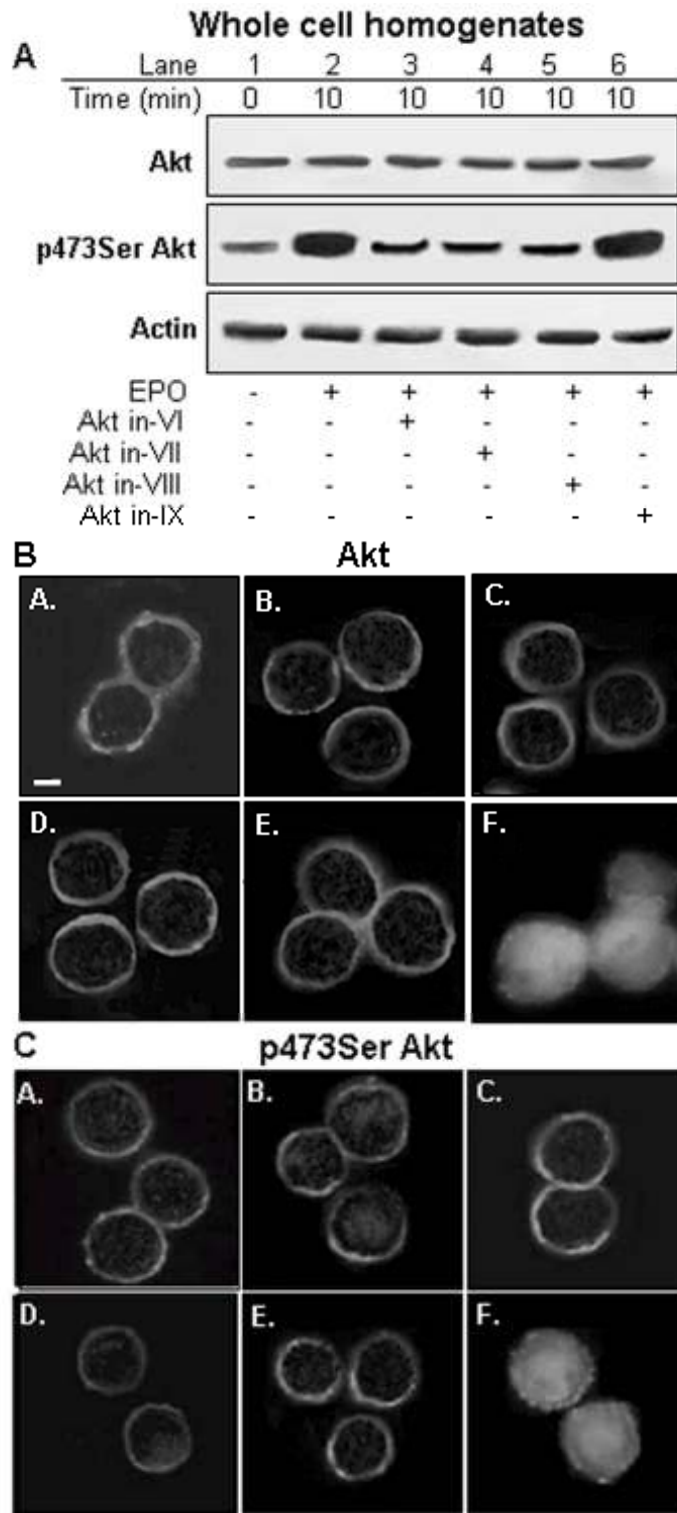
Figure 26. Akt expression and phosphorylation levels after specific inhibition. (A)Western blot showing Akt expression and phosphorylation levels in whole K562 cell homogenates from unstimulated cells, cells exposed to EPO or cells exposed to EPO after pre-treatment with a panel of different Akt inhibitors: Akt inhibitor VI (in-VI), Akt inhibitor VII (in-VII), Akt inhibitor VIII (in-VIII) and Akt inhibitor IX (in-IX) (B) Analysis of Akt distribution in K562 cells treated with different Akt inhibitors and EPO by fluorescence microscopy. (A) Control cells, (B) cells treated

with Akt in-VII, (C) cells pre-treated with Akt in-VII and stimulated for 15min with EPO, (D) control cells, (E) cells treated with Akt in-IX, (F) cells pre-treated with Akt in-IX and stimulated for 15min with EPO. Scale bar: 4μm. (C) Distribution of p473Ser Akt in K562 cells treated with different Akt inhibitors and EPO by fluorescence microscopy. (A) Control cells, (B) cells treated with Akt in-VII, (C) cells pre-treated with Akt in-VII and stimulated 15 min with EPO, (D) control cells, (E) cells treated with Akt in-IX and (F) cells pre-treated with Akt in-IX and stimulated 15 min with EPO.

3.16 The phosphorylation state of Akt determines its translocation to the nuclear compartment.

We performed subcellular fractionation experiments in order to determine whether or not Akt phosphorylation and activation could be related to its nuclear translocation. We first evaluated the activity of Akt kinase in the nuclear fractions of cells pretreated for 12 h with the different inhibitors followed by exposure to EPO. The increase of enzymatic activity induced by EPO after 15 min was abolished independently of the type of Akt inhibitor used (Fig. 27A). When cells were pre-treated with inhibitors blocking the activation, i.e. the phosphorylation on these activating residues of the enzyme, neither total Akt nor both its p308 and p473 phosphory lated forms translocates to the nuclear interior (Fig. 27A). On the contrary, when cells were treated with inhibitor IX, EPO treatment triggered the nuclear translocation of Akt phosphorylated on threonine 308 residue and on serine 473 residue (Fig. 27A). It is worthy to note that cell viability in all cases was at least 90% (Fig. 27A). As described above (Fig. 24), we investigated the amount of phosphorylated and unphosphorylated Akt in nuclei of K562, by a first immunoprecipitation round performed using anti p473Ser Akt antibody and a second round using anti Akt antibody was carried out on supernatant of first round of immunoprecipitated samples. In both rounds the product of immunoprecipitation was revealed by Western blot using anti Akt antibody. This analysis showed that the strong rise in p473Ser Akt labelling in nuclei from cells treated with EPO for 15min, or with EPO and inhibitor IX, became extremely low after the second round of immunoprecipitation thus showing that, even if its enzymatic activity is inhibited, almost all nuclear Akt, capable to translocate to the nucleus, is in its phosphorylated and active form on serine 473 residue (Fig. 27B). On the contrary, the faint band observable in samples pre-treated with inhibitor VI, VII and VIII, remained substantially unchanged after the second immunoprecipitation (Fig. 27B). The use of Akt inhibitors suggests therefore an essential role of Akt activation for its nuclear translocation.
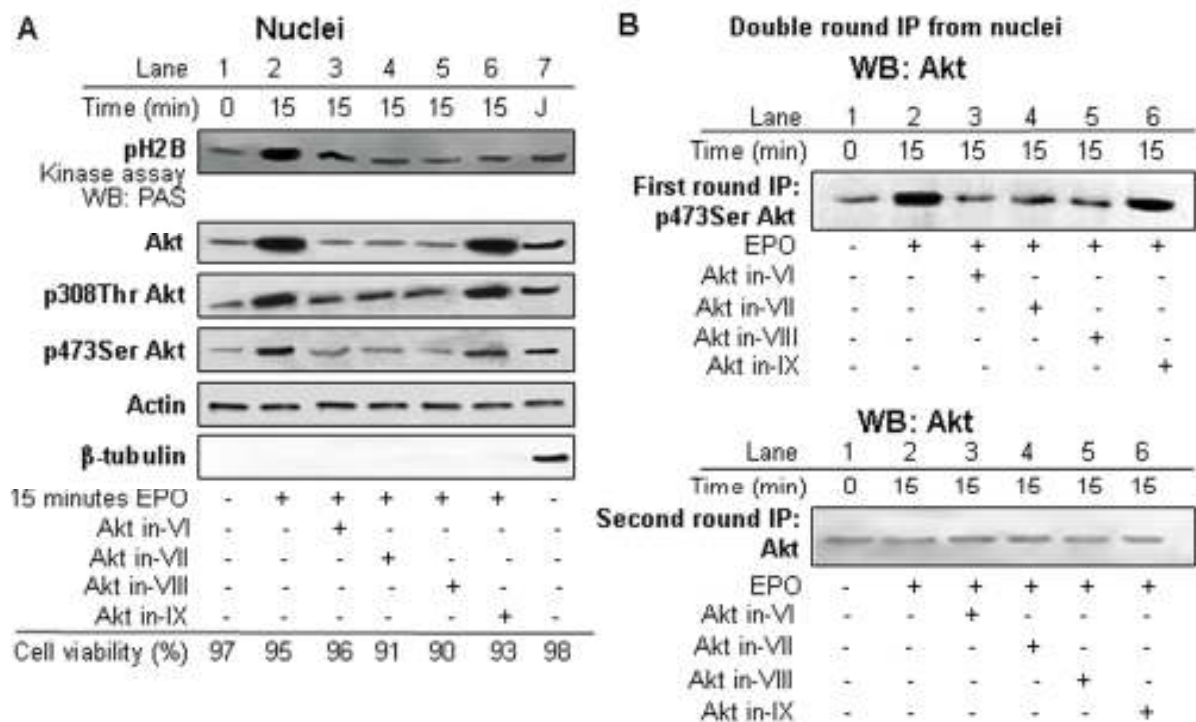
Figure 27. Effect of different Akt inhibitors on Akt translocation and activity. (A) In the first row is shown Akt kinase activity into nuclei of K562 cells after pre-treatment with different Akt inhibitors and EPO stimulation. In the following rows total Akt, p308Thr Akt and p473Ser Akt into nuclei were also analyzed by Western blotting. Viability of the cells was always more than 90%; data are representative of three different experiments. (B) Upper panel: first round of IP carried out on nuclei of K562 cells with p473Ser Akt. Lower panel: second round of IP with anti Akt antibody performed on first round supernatants. The detection was carried out with anti Akt antibody on control samples and on the samples exposed to EPO for 15min plus several types of Akt inhibitors.

3.17 Effects of down modulation or Akt inhibition on erythroid differentiation.

To give new insights into functional consequences for a developmental role of active Akt translocation into the nucleus, we have down-modulated the expression of Akt during EPO treatment of K562 cells. By means of siRNA sequences, a reduced amount of Akt protein was obtained in control cells as well as in differentiating cells, in which Akt expression was forcedly

silenced in the aim of counteracting the EPO induced nuclear translocation of Akt protein (Fig. 28A).

As reported in Fig. 28B, the reduced expression of Akt results in a large decrease of the erythoid differentiation level. Similarly when cells were pre-treated with specific Akt inhibitors the increase of EPO-induced hemoglobin synthesis in the cells was significantly suppressed and differentiation did not occur, thus indicating that nuclear Akt enzymatic activity plays a relevant role in K562 cells erythroid maturation. This set of data demonstrates that a reduced expression of Akt considerably impairs the differentiative potential of EPO and supports the contention that Akt is indispensable for the progression of tumoral erythroleukemia cells along the erythoid lineage.
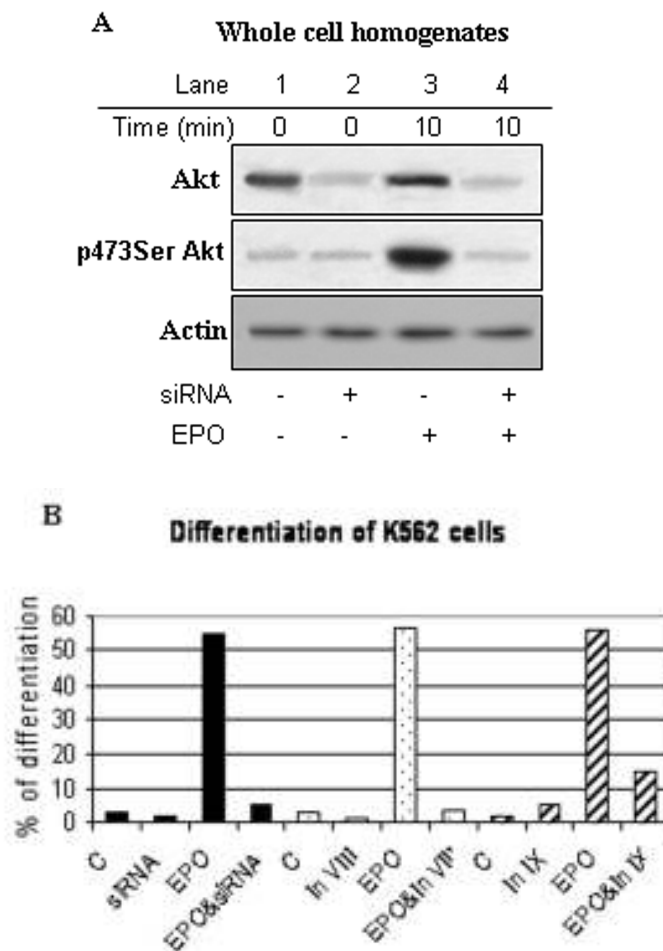
Figure 28. Down-modulation of Akt expression or biochemical inhibition impairs erythroid differentiation. K562 erythroleukemia cells, subjected to RNA interference assay in control (−) or differentiating conditions for 4 days (+), were subjected to immunochemical analysis to evaluate Akt expression (A) and to cytofluorimetric determination to estimate their differentiation level (B). In K562 cells EPO induces hemoglobin synthesis, which was hampered by siRNA sequences or

treatment with different Akt inhibitors. Values are percentage of CD71-positive cells. Viability was always more than 80% and S.D. less than 10%. The data are representative of three separate experiments performed in duplicate.

4 Discussion and Conclusions

In this project we have investigated the involvement of the PI3K/Akt network in acute leukemia. The obtained results on patients affected by acute leukemia suggested that PI3K/Akt network should be involved in this malignancy. The results showed that PI3K and Akt are up-regulated in patients affected by AML and ALL, and also in cell lines with acute leukemia, if compared with healthy donor controls. In particular, PI3K and Akt activity levels were enhanced in ALL cell lines and in ALL patients examined. The data observed in patients were paralleled and reinforced by data obtained in acute leukemia cell lines. The Akt expression levels were very high in acute leukemia cell lines, with very high activation levels, detected by the phosphorylation on the serine 473 residue, in ALL cells. The PI3K activity is higher in patients, compared to the cell lines, about 2 times more. Our data seem to indicate that, in the contest of a major activation of the PI3K/Akt axis in acute leukemias, powerful activation of Akt and some main downstream targets can be observed in ALL. Moreover, it is known that the bad prognosis of some patients depends on the activation of signaling pathways, and a bad prognosis is associated to patients that have higher activation of Akt [Kornblau et al 2006]. Preliminary studies, showed 50-70% of patients affected by AML have phosphorylated Akt on threonin 308 and serin 473 [Brandts et al 2005]. The constitutive activation of PI3K/Akt network may be necessary for AML cell survival and it may determine a bad prognosis [Min et al 2003].

These observations represented the hypothesis for testing in acute leukemia cell models different newly developed inhibitors which act on PI3K/Akt pathway, maybe inhibiting its up-regulation. The involvement of PI3K/Akt network in apoptosis and drug treatment resistance, it is been demonstrate. In particular in Neri et al 2003 it is showed the importance of the constitutive activation of PI3K/Akt pathway in drug resistance in HL60 AR (apoptosis resistant clone). The constitutive activation of PI3/Akt pathway, in particular the tyrosine phosphorylation of P85 seen in HL-60 AR cells was due to a possible autocrine production of IGF-I [Neri et al 2003]; an alteration on this molecule colud be responsible of the increased activity in ALL cell lines. The increased activity of PI3K/Akt pathway in acute leukemia, it may depends also on alteration or mutation of some phosphatases such as PTEN or SHIP, by negatively regulating the Akt pathway. Another mechanism to induce PI3K/Akt constitutive activation may depends on expression of double tandem internal duplication of FLT3, an upstream gene of PI3K [Kiyoi et al 1998, Xu et al 2003] or lost of function of PTEN and SHIP.

Our data confirmed that the administration of the new drugs developed as PI3K/Akt inhibitors was able to induce a strong reduction of proliferation and to induce apoptosis, at least for the drugs directly acting on PI3K and Akt (Perifosine, ErPC and PI3K/Erk inhibitors). These effects occurred via PI3K/Akt, as demonstrated by the weakened phosphorylation levels of the phosphorylation on serin 473 of Akt and of its downstream targets GSK3, mTOR and p21 involved in such critical cell processes, while treatment with Doxorubicin does not inhibits phosphorylation of downstream target and activity of Akt. The results we have obtained allow to conclude that the specific PI3K/Akt drugs used in this work can revert the proliferation and survival advantages of cancer cells, providing thus the basis for the identification of novel drugs to introduce in AML and ALL treatments, in order to ameliorate the current clinical protocols.

Sequencing analysis on the AML and ALL patients could contribute to identify the molecular alterations underlying the leukemogenesis. We found one recurrent (2 cell lines out of 6 and all 4 patients) intronic transition, the IVS4a+30g, that may be involved in mechanisms of splicing of Akt. Data on Akt alterations in acute leukemias are poor in literature; thus, more detailed analyses in a wider group of patients are necessary to confirm the importance of the identified change and its potential functional meaning and to detect other genetic alterations on the PI3K/Akt network useful to suggest novel molecular targets for specific therapy. Mutations on PI3K and Akt genes are frequency in different types of tumor [Bellacosa et al 2005].
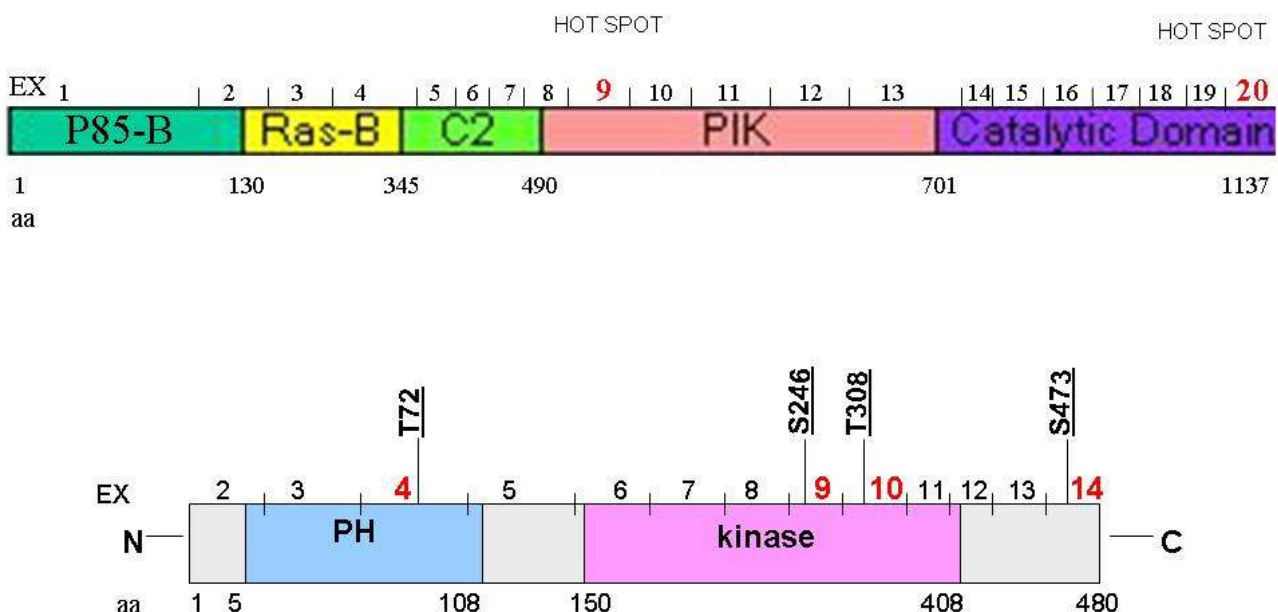

Figure 29. Schematic representation of Akt domains.

In this work we also addressed the study of the role of Akt during the initial steps of the human erythroid differentiation. We have shown, for the first time to our knowledge, that EPO treatment activates Akt in K562 erythroleukemia cells and induces the translocation of Akt active form in the nuclear compartment in a rapid and transient manner, suggesting that Akt plays a crucial role in EPO mediated erythroid differentiation.

This conclusion is based on several lines of evidence. In vivo Akt phosphorylation and in vitro Akt kinase activity were up-regulated by EPO administration to K562 cells. Both phosphorylation and activity of Akt were down-regulated by the pharmacological PI3K inhibitor, Ly294002, thus showing that Akt activation is in the PI3K/Akt signal transduction axis.

We have previously demonstrated in K562 cells the nuclear translocation of PI3K and the increase in intranuclear in vivo synthesis of PtdIns(3,4,5)P_3 in response to EPO [Neri et al 2002]. In the present study immunoprecipitable nuclear Akt activity was detectable after 10 minutes and showed an increase that reached a maximum after 15-20 minutes. This enhancement was similar but delayed of 5 to 10 minutes when compared to Akt activation in whole cells and may be explained by the time required by Akt for translocating into this cell fraction, where almost all Akt results phosphorylated in the sites of activation. The nuclear peak of enzymatic activity occurred later when compared with whole cells and the fact that at 5 minutes of stimulation nuclei did not show an increase in activity, ruled out the possibility that the increase we measured in nuclear Akt activity was due to a cytoplasmic contamination. These events, which occur after 15 minutes of EPO stimulation, are accompanied by a rise of the enzymatic activity of this kinase into nuclei of K562 cells.

The localization of p473Ser Akt mainly into the nucleus indicated that some putative Akt targets reside within this organelle. Known substrates are for example the forkhead transcription factors, that link the PI3K/Akt cascade to gene expression that regulates cell growth, survival, and metabolism [Kops et al 1999]. Akt phosphorylates the nuclear receptors RAR alpha (retinoic acid receptor alpha) and inhibits its transactivation [Srinivas et al 2006]. It has recently been reported that acinus, a nuclear factor required for apoptotic chromatin condensation, is a direct target of Akt [Hu et al 2005] and zyxin, a focal adhesion molecule, is phosphorylated in the nucleus on serine 142 by Akt, leading to its association with acinus [Chan et al 2007]. Therefore, by regulating zyxin/acinus complex formation in the nucleus, Akt influences apoptosis.

Although it is now clear that Akt migrates to the nucleus as the result of treatment with growth factors, the mechanism by which Akt translocates into the nucleus remains unclear. There are several reports indicating that Akt nuclear translocation may be attributable to an activation of

PI3K. In human mature T-cell leukemia the mechanism of Akt nuclear translocation involves PI3K, that has been suggested as a physiological regulator of Akt within the nucleus as well as the cytoplasm [Pekarsky et al 2000]. In osteoblast-like MC3T3-E1 cells exposed to proliferative growth factor, Akt nuclear translocation appears to be mediated by PI3K [Borgatti et al 2000]. It has been shown before that nuclear PtdIns(3,4,5)P$_3$ facilitates translocation of proteins with PH domain. For example both nuclear PI3K and PtdIns(3,4,5)P$_3$ are necessary for the nuclear translocation of PKC-ζ [Neri et al 1999] or for PIKE-L binding and residency into the nucleus that is compromised by mutations of its PH domain leading to a decreased activation of Akt [Hu et al 2005].

More recently a report by Xuan Nguyen et al 2006 showed that Akt phosphorylation is coupled to its nuclear translocation, which requires PI3K activity for the maintenance of Akt activation within the nucleus under conditions of NGF stimulation. Consistently with this report, we have found that there is a relevant p473 Serine residue phosphorylation of Akt recovered in the nucleus, with only a very limited amount of protein not phosphorylated on serine residues and treatment of cells with Ly294002 fully inhibited EPO-stimulated Akt translocation to the nucleus. Thus, Akt nuclear translocation may be attributable to an activation of PI3K. We demonstrated with a different biochemical method, as Western blot or immunoprecipitation, that Akt nuclear residence is regulated by its phosphorylation status, whereas its kinase activity appears to be not required. It is worthy to note that, when nuclear Akt kinase activity is inhibited in the nucleus either by activation plus translocation block and either by kinase activity inhibition, erythroid maturation is not taking place.

Although the enhanced Akt activity is usually associated with the proliferative and survival signals also in leukemia cells, a growing body of evidences indicates a relevant role also in erythroid differentiation. Our data gain relevance when compared with data obtained in human erythroid progenitors. EPO induced maturation of human CD34$^+$ erythroid progenitors is dependent on the PI3K/Akt signaling pathway [Myklebust et al 2002, Bouscary et al 2003]. In the context of EPO signaling it has been shown that Akt phosphorylates both GATA-1 and Foxo3a, transcription factors of crucial importance in erythropoiesis [Bouscary et al 2003, Kadri et al 2005]. Enforced expression of Myr-Akt, a constitutively active form of Akt, highly augmented EPO-induced erythropoiesis and induced cell hypersensitivity to EPO in the earliest committed cells of the erythroid lineage, the erythroid burst-forming unit (BFU-E), human erythroid progenitors CD34$^+$ CD71$^+$ CD45RA$^-$ GPA$^-$ [Sivertsen et al 2006]. These effects were completely counteracted by the PI3K inhibitor Ly294002.

Ghaffari and co-workers recently demonstrated that enforced expression of activated Akt in murine fetal liver progenitor cells overrode the need for EPO to induce erythroid differentiation [Ghaffari et

al 2006]. They concluded that the enhanced erythroid maturation of activated-Akt-transduced cells was not limited to its anti-apoptotic or proliferative effect as no increase in total cell number was observed. Increased activation of the PI3K/Akt signaling pathway may be particularly relevant to normal hematopoiesis and to patients with polycythemia vera (PV).

These results are strengthened by the identification of molecules downstream to Akt that are relevant in human erythropoiesis. Akt has been described as a mediator of EPO modulation of GATA-1 transcription factor that may coordinate EPO-R signaling and GATA-1 activities during hematopoietic cell differentiation [Zhao et al 2006]. K-ras has been identified as the major regulator for cytokine dependent Akt activation in erythroid differentiation in vivo [Zhang et al 2005].

To conclude, this work provided evidences for the up-regulation of the PI3K/Akt axis in acute leukemias, and in particular in ALL. Since alterations in the PI3K/Akt network play important roles in the etiology, maintenance and progression of acute leukemia, we investigated also the genetic pattern of PI3K and Akt, finding a potential lesion in Akt gene associated with acute leukemia. We demonstrated that agents inhibiting PI3K and Akt are able to reduce proliferation and apoptosis of leukemia cells, and thus to revert the advantage of cancer on normal cells. Also if this pathway has so far not been studied in detail in this disease, our preliminary data indicate a strong therapeutic potential for the PI3K and Akt inhibitors analyzed; it is likely that the PI3K/Akt inhibitor compounds will be effective in strategies involving their combination with drugs that inhibit other targets, and their inclusion into current leukemia therapeutic protocols may be of particular relevance for a better clinical management.

Taken together, our data indicate that Akt may play a major function during erythroid differentiation, by becoming active and translocating into the nucleus where it may activate several substrates involving transcription factors responsible for cell maturation and advancement of the erythroid differentiation. Important step in this field will be the identification of the molecular events produced by Akt activation during erythroleukemia cells differentiation, among the multiple effects exerted by this multifunctional kinase into the nucleus.

These findings may help identifying novel target into the nucleus essential for differentiation and thus useful for anticancer therapy of hematological malignancies.

5 References

- Abraham R.T. PI 3-kinase related kinases: 'big' players in stress-induced signaling pathways, DNA Repair. 2004; 3: 883–887.

- Actor B., et al. Comprehensive analysis of genomic alterations in gliosarcoma and its two tissue components, Genes Chromosomes Cancer. 2002; 34: 416–427.

- Adams, K. W. and Cooper, G. M. Rapid turnover of mcl-1 couples translation to cell survival and apoptosis. J. Biol. Chem. 2007; 282: 6192–6200.

- Alessi, D. R. and Cohen, P. Mechanism of activation and function of protein kinase B. Curr. Opin. Genet. Dev. 1998; 8: 55–62.

- Alessi, D. R., et al. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Bα. Curr. Biol. 1997; 7: 261–269.

- Ali K., et al. Essential role for the p110delta phosphoinositide 3-kinase in the allergic response, Nature. 2004; 431: 1007–1011.

- Alloatti G., et al. Phosphoinositide 3-kinase gamma controls autonomic regulation of the mouse heart through Gi-independent downregulation of cAMP level, FEBS Lett. 2005; 579: 133–140.

- Alsabti EA, et al. Clinical trials in vaccination with leukemia associated antigens in acute myelogenous leukemia. The Japanese Journal of Experimental Medicine. 1979; 49:157–68.

- Arbiser J.L., et al. Solenopsin, the alkaloidal component of the fire ant (Solenopsis invicta), is a naturally occurring inhibitor of phosphatidylinositol-3-kinase signaling and angiogenesis, Blood. 2007; 109: 560–565.

- Arden, K. C. and Biggs, 3rd, W. H. Regulation of the FoxO family of transcription factors by phosphatidylinositol-3 kinase-activated signaling. Arch. Biochem. Biophys. 2002; 403: 292–298.

- Arrigo, A. P. The cellular "networking" of mammalian Hsp27 and its functions in the control of protein folding, redox state and apoptosis. Adv. Exp. Med. Biol. 2007; 594: 14–26.

- Asano T, et al. The PI 3-kinase/Akt signaling pathway is activated due to aberrant Pten expression and targets transcription factors NFkappaB and c-Myc in pancreatic cancer cells. Oncogene. 2004; 23: 8571–8580.

- Autret A, et al. Early phosphatidylinositol 3-kinase/Akt pathway activation limits poliovirus-induced JNK-mediated cell death. J Virol. 2008; 82: 3796-3802.

- Avellino R, et al. Rapamycin stimulates apoptosis of childhood acute lymphoblastic leukemia cells. Blood. 2005; 106: 1400-1406.

- Bachman KE, et al. The PIK3CA gene is mutated with high frequency in human breast cancers. Cancer Biol. Ther. 2004; 3: 772–775.

- Backman S.A., et al. Early onset of neoplasia in the prostate and skin of mice with tissuespecific deletion of Pten, Proc. Natl. Acad. Sci. U. S. A. 2004; 101: 1725–1730.

- Bader A.G., et al. Oncogenic PI3K deregulates transcription and translation, Nat. Rev., Cancer. 2005; 5: 921–929.

- Bader, A. G. and Vogt, P. K. An essential role for protein synthesis in oncogenic cellular transformation. Oncogene. 2004; 23: 3145–3150.

- Baer MR, et al. Expression of the neural cell adhesion molecule CD56 is associated with short remission duration and survival in acute myeloid leukemia with t(8;21)(q22;q22). Blood. 1997; 90: 1643−1648.

- Baer MR, K et al. Phase 3 study of the multidrug resistance modulator PSC-833 in previously untreated patients 60 years of age and older with acute myeloid leukemia: Cancer and Leukemia Group B Study 9720. Blood. 2002; 100:1224–32.

- Baer R, et al. Transcription factors of the bHLH and LIM families: synergistic mediators of T cell acute leukemia? Curr Top Microbiol Immunol. 1997; 220: 55-65.

- Bandyopadhyay G.K., et al. Increased p85/55/50 expression and decreased phosphotidylinositol 3-kinase activity in insulin-resistant human skeletal muscle, Diabetes. 2005; 5: 2351–2359.

- Barata JT, et al. Activation of PI3K is indispensable for interleukin 7-mediated viability, proliferation, glucose use, and growth of T cell acute lymphoblastic leukemia cells. J Exp Med. 2004; 200: 659-669.

- Barata JT, et al. Common gamma chain-signaling cytokines promote proliferation of T-cell acute lymphoblastic leukemia. Haematologica. 2004; 89: 1459-1467.

- Barata JT, et al. IL-7-dependent human leukemia T-cell line as a valuable tool for drug discovery in T-ALL. Blood. 2004; 103: 1891-1900.

- Barata JT, et al. Interleukin-7 in T-cell acute lymphoblastic leukemia: an extrinsic factor supporting leukemogenesis? Leuk Lymphoma. 2005; 46: 483-495.

- Barata JT, et al. Interleukin-7 promotes survival and cell cycle progression of T-cell acute lymphoblastic leukemia cells by down-regulating the cyclin-dependent kinase inhibitor p27(kip1). Blood. 2001; 98: 1524-1531.

- Barata JT, et al. Molecular and functional evidence for activity of murine IL-7 on human lymphocytes. Exp Hematol. 2006; 34: 1133-1142.

- Barber D.F., et al. Class IB-phosphatidylinositol 3-kinase (PI3K) deficiency ameliorates IA-PI3K-induced systemic lupus but not T cell invasion, J. Immunol. 2006; 176: 589–593.

- Barnett S.Fet al. Malinowski, E.M. McAvoy, D.D. Nahas, R.G. Robinson, H.E. Huber, Identification and characterization of pleckstrin-homology-domain-dependent and isoenzyme-specific Akt inhibitors, Biochem. J. 2005; 385: 399–408.

- Barnett, et al. (2005). Identification and characterization of pleckstrin-homology-domain-dependent and isoenzyme-specific Akt inhibitors. Biochem J, 385, 399-408.

- Barnett, S.F., et al. (2005). Identification and characterization of pleckstrin-homology-domain-dependent and isoenzyme-specific Akt inhibitors. Biochem J, 385, 399-408.

- Barragan E, et al. Prognostic implications of Wilms' tumor gene (WT1) expression in patients with de novo acute myeloid leukemia. Haematologica. 2004; 89: 926−933.

- Barthwal MK, et al. Negative regulation of mixed lineage kinase 3 by protein kinase B/AKT leads to cell survival. J Biol Chem. 2003; 278: 3897-3902.

- Basu S, et al. Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis. Mol Cell. 2003 Jan;11(1):11-23.

- Baudard M, et al. Acute myelogenous leukaemia in the elderly: retrospective study of 235 consecutive patients. British Journal of Haematology. 1994; 86: 82–91.

- Bauer, A., et al. The NF-κB regulator Bcl-3 and the BH3-only proteins Bim and Puma control the death of activated T cells. Proc. Natl. Acad. Sci. U.S.A. 2006; 103: 10979–10984.

- Behzad, H., et al. Cytokine-mediated FOXO3a phosphorylation suppresses FasL expression in hemopoietic cell lines: investigations of the role of Fas in apoptosis due to cytokine starvation. Cytokine. 2007; 38: 74–83.

- Beinke, S. and Ley, S. C. Functions of NF-κB1 and NF-κB2 in immune cell biology. Biochem. J. 2004; 382: 393–409.

- Bellacosa A, et al. Activation of AKT kinases in cancer: implications for therapeutic targeting. Adv. Cancer Res. 2005; 94: 29-86.

- Bellacosa A,. A retroviral oncogene, akt, encoding a serine threonine kinase containing an SH2-like region. Science. 1991; 254: 274–277.

- Bellacosa A., et al. Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas, Int. J. Cancer. 1995; 64: 280–285.

- Berneman ZN, et al. Vaccination of Acute Myeloid Leukemia Patients with Dendritic Cells Electroporated with mRNA Encoding the Wilms' Tumor Protein WT1: A Phase I/II Trial. Blood. 2007; 110: 158.

- Bertelsen B.I., et al. Molecular analysis of the PI3K-AKT pathway in uterine cervical neoplasia: frequent PIK3CA amplification and AKT phosphorylation, Int. J. Cancer. 2006; 118: 1877–1883.

- Beuvink I., et al. The mTOR inhibitor RAD001 sensitizes tumor cells to DNA-damaged induced apoptosis through inhibition of p21 translation. Cell. 2005; 120: 747–759.

- Bharti, A. C. and Aggarwal, B. B. Nuclear factor-κB and cancer: its role in prevention and therapy. Biochem. Pharmacol. 2002; 64: 883–888.

- Bi L., et al. Early embryonic lethality in mice deficient in the p110beta catalytic subunit of PI 3-kinase. Mamm. Genome. 2002; 13: 169–172.

- Bi L., et al. Proliferative defect and embryonic lethality in mice homozygous for a deletion in the p110alpha subunit of phosphoinositide 3-kinase. J. Biol. Chem. 1999; 274: 10963–10968.

- Biwa T., et al. Granulocyte/macrophage colony-stimulating factor plays an essential role in oxidized low density lipoprotein-induced macrophage proliferation. J. Atheroscler. Thromb. 2000; 7: 14–20.

- Biwa T., et al. Sites of action of protein kinase C and phosphatidylinositol 3-kinase are distinct in oxidized low density lipoprotein-induced macrophage proliferation. J. Biol. Chem. 2000; 275: 5810–5816.

- Blanco-Aparicio,C. et al. Mice expressing myrAKT1 in the mammary gland develop carcinogen-induced ER positive mammary tumors that mimic human breast cancer. Carcinogenesis. 2007; 28: 584–594.

- Blume-Jensen P. and T. Hunter, Oncogenic kinase signalling. Nature. 2001; 411: 355–365.

- Blume-Jensen, P., et al. The kit receptor promotes cell survival via activation of PI 3-kinase and subsequent Akt-mediated phosphorylation of Bad on Ser136. Curr. Biol. 1998; 8: 779–782.

- Borgatti P, et al. Threonine 308 phosphorylated form of Akt translocates to the nucleus of PC12 cells under nerve growth factor stimulation and associates with the nuclear matrix protein nucleolin. J Cell Physiol. 2003; 196: 79-88.

- Borgatti, P., et al. (2000). Translocation of Akt/PKB to the nucleus of osteoblast-like MC3T3-E1 cells exposed to proliferative growth factors. FEBS Lett, 477, 27-32.

- Boudeau J., et al. LKB1, a protein kinase regulating cell proliferation and polarity. FEBS Lett. 2003; 546: 159–165.

- Bouscary, D., et al. (2003). Critical role for PI 3-kinase in the control of erythropoietin-induced erythroid progenitor proliferation. Blood, 101, 3436-3443.

- Bouscary, D., et al. (2003). Critical role for PI 3-kinase in the control of erythropoietin-induced erythroid progenitor proliferation. Blood, 101, 3436-3443.

- Bozulic, L., et al. PKBα/Akt1 acts downstream of DNA-PK in the DNA double-strand break response and promotes survival. Mol. Cell. 2008; 30: 203–213.

- Brach, M. A., et al. Prolongation of survival of human polymorphonuclear neutrophils by granulocyte-macrophage colony-stimulating factor is caused by inhibition of programmed cell death. Blood. 1992; 80: 2920–2924.

- Brandts CH, et al. Constitutive activation of Akt by Flt3 internal tandem duplications is necessary for increased survival, proliferation, and myeloid transformation. Cancer Res. 2005; 65: 9643-9650.

- Brazil,D.P and Hemmings BA. Ten years of protein kinase B signalling: a hard Akt to follow. Trends Biochem. Sci. 2001; 26 : 657–664.

- Brenner MK, et al. Gene-marking to trace origin of relapse after autologous bone-marrow transplantation. Lancet. 1993; 341: 85–6.

- Brock, C., et al. Roles of Gβγ in membrane recruitment and activation of p110γ /p101 phosphoinositide 3-kinase γ . J. Cell Biol. 2003; 160: 89–99.

- Broderick,D.K. et al. Mutations of PIK3CA in anaplastic oligodendrogliomas, high-grade astrocytomas, and medulloblastomas. Cancer Res. 2004; 64: 5048–5050.

- Brognard J., et al. Dennis Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation, Cancer Res. 2001; 61: 3986–3997.

- Budihardjo, I., et al. Biochemical pathways of caspase activation during apoptosis. Annu. Rev. Cell Dev. Biol. 1999; 15: 269–290.

- Bullinger L and Valk PJ. Gene expression profiling in acute myeloid leukemia. J Clin Oncol. 2005; 23: 6296-6305. Review.

- Bunn, H.F. (2007). New agents that stimulates erythropoiesis. Blood ,109, 868-873.

- Burgering B.M., P.J. Coffer, Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction, Nature. 1995; 376: 599–602.

- Burnett AK, et al. A comparison of low-dose cytarabine and hydroxyurea with or without alltrans retinoic acid for acute myeloid leukemia and highrisk myelodysplastic syndrome in patients not considered fit for intensive treatment. Cancer. 2007; 109: 1114–24.

- Burnett AK, et al. A phase 2 evaluation of single agent clofarabine as first line treatment for older patients with AML who are not considered fit for intensive chemotherapy. Blood. 2004; 104: 248.

- Byrd JC, et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: Results from Cancer and Leukemia Group B (CALGB 8461). Blood. 2002; 100: 4325−4336.

- Byun D.S., et al. Frequent monoallelic deletion of PTEN and its reciprocal association with PIK3CA amplification in gastric carcinoma, Int. J. Cancer. 2003; 104: 318–327.

- Cairns P., et al. Frequent inactivation of PTEN/MMAC1 in primary prostate cancer, Cancer Res. 1997; 57: 4997–5000.

- Calderwood, S. K. and Ciocca, D. R. Heat shock proteins: stress proteins with Janus-like properties in cancer. Int. J. Hyperthermia. 2008; 24: 31–39.

- Campbell I.G., et al. Mutation of the PIK3CA gene in ovarian and breast cancer, Cancer Res. 2004; 64: 7678–7681.

- Cantley L.C. and B.G. Neel, New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway, Proc. Natl. Acad. Sci. U. S. A. 1999; 96: 4240–4245.

- Cantley LC. The phosphoinositide 3-kinase pathway. Science. 2002; 296: 1655-1657.

- Cantley, L. C., et al. Oncogenes and signal transduction. Cell. 1991; 64: 281–302.

- Cardoso B.A., et al. Aberrant signaling in T-cell acute lymphoblastic leukemia: biological and therapeutic implications. Brazilian Journal of Medical and Biological Research. 2008; 41: 344-350.

- Carmeliet P and R.K. Jain, Angiogenesis in cancer and other diseases, Nature. 2000; 407: 249–257.

- Carnesecchi S., et al. Insulin-induced vascular endothelial growth factor expression is mediated by the NADPH oxidase NOX3, Exp. Cell Res. 2006; 312: 3413–3424.

- Carpten JD, et al. A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. Nature. 2007; 448: 439–444.

- Castillo S.S., et al. Preferential inhibition of Akt and killing of Akt-dependent cancer cells by rationally designed phosphatidylinositol ether lipid analogues, Cancer Res. 2004; 64: 2782–2792.

- Chan, C.B., et al. (2007). Akt phosphorylation of zyxin mediates its interaction with acinus-S and prevents acinus-triggered chromatin condensation. Cell Death Differ, in press.

- Chan, T. O. and Tsichlis, P. N. PDK2: a complex tail in one Akt. Science STKE. 2001; PE1

- Chang,C.J. et al. PTEN regulates Mdm2 expression through the P1 promoter. J. Biol. Chem. 2004; 279: 29841–29848.

- Chen Z, et al. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. Nature. 2005; 436 : 725–730.

- Chen, C., et al. The Rel/NF-κB family directly activates expression of the apoptosis inhibitor Bcl-xL. Mol. Cell. Biol. 2000; 20: 2687–2695.

- Chen,M.L. et al. The deficiency of Akt1 is sufficient to suppress tumor development in Pten+/- mice. Genes Dev. 2006; 20: 1569–1574.

- Cheng J.Q., et al. Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA, Proc. Natl. Acad. Sci. U. S. A. 1996; 93: 3636–3641.

- Cheson BD, et al. Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. Journal of Clinical Oncology. 2003; 21: 4642–9.

- Chipuk, J. E., et al. PUMA couples the nuclear and cytoplasmic proapoptotic function of p53. Science. 2005; 309: 1732–1735.

- Clayton E., et al. A crucial role for the p110delta subunit of phosphatidylinositol 3-kinase in B cell development and activation, J. Exp. Med. 2002; 196: 753–763.

- Coffer, P. J. and Woodgett, J. R. Molecular cloning and characterisation of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families. Eur. J. Biochem. 1991; 201: 475–481.

- Coffer, P. J et al. Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. Biochem. J. 1998; 335: 1–13.

- Cohen P.L. and R.A. Eisenberg, Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease, Annu. Rev. Immunol. 1991; 9: 243–269.

- Cong L.N., et al. Physiological role of Akt in insulin-stimulated translocation of GLUT4 in transfected rat adipose cells, Mol. Endocrinol. 1997; 11: 1881–1890.

- Cook, D., et al. Wingless inactivates glycogen synthase kinase-3 via an intracellular signalling pathway which involves a protein kinase C. EMBO J. 1996; 15: 4526–4536.

- Crackower M.A., et al. Regulation of myocardial contractility and cell size by distinct PI3K-PTEN signaling pathways. Cell. 2002; 110: 737–749.

- Cripe LD and Hinton S. Acute myeloid leukemia in adults. Curr Treat Options Oncol. 2000; 1: 9-17. Review.

- Cross, D. A., et al. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature. 1995; 378: 785–789.

- Crul M, et al. Phase I and pharmacological study of daily oral administration of perifosine (D-21266) in patients with advanced solid tumours. Eur J Cancer. 2002;38:1615-1621.

- Cully M., Het al. PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. Nat. Rev., Cancer. 2006; 6: 184–192.

- Dahia,P.L. PTEN, a unique tumor suppressor gene. Endocr. Relat. Cancer. 2000; 7: 115–129.

- Dann S.G., et al. mTOR Complex1-S6K1 signaling: at the crossroads of obesity, diabetes and cancer, Trends Mol. Med. 2007; 13: 252–259.

- Datta S.R., et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell. 1997; 91: 231–241.

- De Siervi A, et al. Transcriptional activation of p21waf1/cip1 by alkylphospholipids: role of the mitogen-activated protein kinase pathway in the transactivation of the human p21waf1/cip1 promoter by Sp1. Cancer Res. 2004;64:743-750.

- Defeo-Jones D., et al. Tumor cell sensitization to apoptotic stimuli by selective inhibition of specific Akt/PKB family members. Mol. Cancer Ther. 2005; 4: 271–279.

- del Peso L., et al. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science. 1997; 278: 687–689.

- del Peso, L., et al. Regulation of the forkhead transcription factor FKHR, but not the PAX3-FKHR fusion protein, by the serine/threonine kinase Akt. Oncogene. 1999; 18: 7328–7333.

- Del Prete A., et al. Defective dendritic cell migration and activation of adaptive immunity in PI3Kgamma-deficient mice, EMBO J. 2004; 23: 3505–3515.

- Del Re DP, et al. Focal adhesion kinase as a RhoA-activable signaling scaffold mediating Akt activation and cardiomyocyte protection. J Biol Chem. 2008 Dec 19; 283: 35622-35629.

- Delcommenne, M., et al. Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. Proc. Natl. Acad. Sci. U.S.A. 1998; 95: 11211–11216.

- Déléris P, et alM. SHIP-2 and PTEN are expressed and active in vascular smooth muscle cell nuclei, but only SHIP-2 is associated with nuclear speckles. Biol Chem. 2003; 278: 38884-38891.

- Dijkers, P. F., et al. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. Curr. Biol. 2000; 10: 1201–1204.

- Dijkers, P. F., et al. FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity. J. Cell Biol. 2002; 156: 531–542.

- Dijkers, P. F., et al. Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27KIP1. Mol. Cell. Biol. 2000; 20: 9138–9148.

- Ding, Q., et al. Degradation of Mcl-1 by β-TrCP mediates glycogen synthase kinase 3-induced tumor suppression and chemosensitization. Mol. Cell. Biol. 2007; 27: 4006–4017.

- Disperati P, et al. Acute promyelocytic leukemia in patients aged 70 years and over – A single center experience of unselected patients. Leukemia & Lymphoma. 2007; 48: 1654–8.

- Doble, B. W. and Woodgett, J. R. GSK-3: tricks of the trade for a multi-tasking kinase. J. Cell Sci. 2003; 116: 1175–1186.

- Döhner K, et al. Prognostic significance of partial tandem duplications of the MLL gene in adult patients 16 to 60 years old with acute myeloid leukemia and normal cytogenetics: A study of the Acute Myeloid Leukemia Study Group Ulm. J Clin Oncol. 2002; 20: 3254−3261.

- Donepudi M and Grütter MG. Structure and zymogen activation of caspases. Biophys Chem. 2002; 101-102: 145-153. Review.

- Dong, B., et al. Ca2+/calmodulin directly interacts with the pleckstrin homology domain of AKT1. J. Biol. Chem. 2007; 282: 25131–25140.

- Dong, L. Q. and Liu, F. PDK2: the missing piece in the receptor tyrosine kinase signaling pathway puzzle. Am. J. Physiol. Endocrinol. Metab. 2005; 289: 187–196.

- Douglas D.A., et al. Novel mutations of epidermal growth factor receptor in localized prostate cancer, Front. Biosci. 2006; 11: 2518–2525.

- Doukas J., et al. Isoform-selective PI3K inhibitors as novel therapeutics for the treatment of acute myocardial infarction, Biochem. Soc. Trans. 2007; 35: 204–206.

- Doukas J., et al. Phosphoinositide 3-kinase gamma/delta inhibition limits infarct size after myocardial ischemia/reperfusion injury, Proc. Natl. Acad. Sci. U. S. A. 2006; 103: 19866–19871.

- Downward J. How BAD phosphorylation is good for survival. Nat Cell Biol 1999; 1: E33-5.

- Downward, J. PI 3-kinase, Akt and cell survival. Semin. Cell Dev. Biol. 2004; 15: 177–182.

- Dreesen O and Brivanlou AH. Signaling pathways in cancer and embryonic stem cells. Stem Cell Rev. 2007; 3: 7-17.

- Druker BJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med. 2001; 344: 1031−1037.

- Dudek, H., et al. Regulation of neuronal survival by the serine–threonine protein kinase Akt. Science. 1997; 275: 661–665.

- Duronio V. The life of a cell: apoptosis regulation by the PI3K/PKB pathway. Biochem. J. 2008; 415: 333–344.

- Duronio V., et al. Downstream signalling events regulated by phosphatidylinositol 3-kinase activity. Cell. Signal. 1998; 10: 233–239.

- Dutta, J., et al. Current insights into the regulation of programmed cell death by NF-κB. Oncogene. 2006; 25: 6800–6816.

- Earnshaw, W. C., et al. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu. Rev. Biochem. 1999; 68: 383–424

- Eguchi-Ishimae M, et al. NOTCH1 mutation can be an early, prenatal genetic event in T-ALL. Blood. 2008; 111: 376-378.

- Ekert, P. G., et al. Cell death provoked by loss of interleukin-3 signaling is independent of Bad, Bim, and PI3 kinase, but depends in part on Puma. Blood. 2006; 108: 1461–1468.

- Ellisen LW, et al. TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell. 1991; 66: 649-661.

- Engelman J.A., et al. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat. Rev. Genet. 2006; 7: 606–619.

- Eom, T. Y., et al. Neural precursor cells are protected from apoptosis induced by trophic factor withdrawal or genotoxic stress by inhibitors of glycogen synthase kinase 3. J. Biol. Chem. 2007; 282: 22856–22864.

- Erdlenbruch B, et al. (1999). Cancer Chemother. Pharmacol., 44, 484–490.

- Ermoian R.P., et al. Dysregulation of PTEN and protein kinase B is associated with glioma histology and patient survival. Clin. Cancer Res. 2002, 8: 1100–1106.

- Erneux, C., et al. The diversity and possible functions of the inositol polyphosphate 5-phosphatases. Biochim. Biophys. Acta Mol. Cell Biol. Lipids. 1998, 1436: 185–199.

- Faderl S, et al. Clofarabine and cytarabine combination as induction therapy for acute myeloid leukemia (AML) in patients 50 years of age or older. Blood. 2006; 108: 45–51.

- Fang J., et al. PI3K/PTEN/AKT Signaling Regulates Prostate Tumor Angiogenesis. Cell. Signal. 2007; 19: 2487–2497.

- Feldman BJ, et al. A carboxy-terminal deletion mutant of Notch1 accelerates lymphoid oncogenesis in E2A-PBX1 transgenic mice. Blood. 2000; 96: 1906-1913.

- Fey MF. ESMO Minimum Clinical Recommendations for diagnosis, treatment and follow-up of acute myeloblastic leukaemia (AML) in adult patients. Annals of Oncology. 2003; 14: 1161–2.

- Fiedler W, et al. Vascular endothelial growth factor, a possible paracrine growth factor in human acute myeloid leukemia. Blood. 1997; 89: 1870–5.

- Fingar, D. C. and Blenis, J. Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. Oncogene. 2004; 23: 3151–3171.

- Fiorenza, M. T., et al. TCL1 promotes blastomere proliferation through nuclear transfer, but not direct phosphorylation, of AKT/PKB in early mouse embryos. Cell Death Differ. 2008; 15: 420–422.

- Firestein G.S., Evolving concepts of rheumatoid arthritis. Nature. 2003; 423: 356–361.

- Folkman J., Angiogenesis, Annu. Rev. Med. 2006; 57: 1–18.

- Franke, T. F., et al. Direct regulation of the Akt proto-oncogene product by phosphatidylinositol 3,4-bisphosphate. Science. 1997; 275: 665–668.

- Franke, T. F., et al. PI3K: downstream AKTion blocks apoptosis. Cell. 1997; 88: 435–437.

- Franke, T. F., et al. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell. 1995; 81: 727–736.

- Freeman,D.J. et al. PTEN tumor suppressor regulates p53 protein levels and activity through phosphatase-dependent and –independent mechanisms. Cancer Cell. 2003; 3: 117–130.

- Fukuda, R., et al. (2005). Alteration of phosphatidylinositol 3-kinase cascade in the multilobulated nuclear formation of adult T cell leukemia/lymphoma (ATLL). Proc Natl Acad Sci USA, 102, 15213-15218.

- Fukuda, R., et al. (2005). Alteration of phosphatidylinositol 3-kinase cascade in the multilobulated

- Galanis E., et al. Phase II trial of temsirolimus (CCI-779) in recurrent glioblastoma multiforme: a North Central Cancer Treatment Group Study. J. Clin. Oncol. 2005; 23: 5294–5304.

- Gallia G.L.,et al. PIK3CA gene mutations in pediatric and adult glioblastoma multiforme. Mol. Cancer Res. 2006; 4: 709–714.

- Gangloff Y.G., et al. Disruption of the mouse mTOR gene leads to early postimplantation lethality and prohibits embryonic stem cell development. Mol. Cell. Biol. 2004; 24: 9508–9516.

- Gao D.C. N., et al. G1 cell cycle progression and the expression of G1 cyclins are regulated by PI3K/AKT/mTOR/p70S6K1 signaling in human ovarian cancer cells. Am. J. Physiol. Cell Physiol. 2004; 287: 281–291.

- Gao N., et al. Role of PI3K/AKT/mTOR signaling in the cell cycle progression of human prostate cancer. Biochem. Biophys. Res. Commun. 2003; 310: 1124–1132.

- García-Rostán G, et al. Mutation of the PIK3CA gene in anaplastic thyroid cancer. Cancer Res. 2005; 65: 10199–10207.

- Gardai, S. J., et al. Phosphorylation of Bax Ser184 by Akt regulates its activity and apoptosis in neutrophils. J. Biol. Chem. 2004; 279: 21085–21095.

- Garrido, C., et al. HSP27 and HSP70: potentially oncogenic apoptosis inhibitors. Cell Cycle. 2003; 2: 579–584.

- Gerber HP, et al. VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism. Nature. 2002; 417: 954–8.

- Ghaffari, S., et al. (2006). AKT induces erythroid-cell maturation of JAK2-deficient fetal liver progenitor cells and is required for Epo regulation of erythroid-cell differentiation. Blood, 107, 1888-1891.

- Ginisty H, et al. Structure and functions of nucleolin. J Cell Sci. 1999; 112: 761-772.

- Glass C.K. and J.L. Witztum, Atherosclerosis. The road ahead. Cell. 2001; 104: 503–516.

- Goel A., et al. Frequent inactivation of PTEN by promoter hypermethylation in microsatellite instability-high sporadic colorectal cancers, Cancer Res. 2004; 64: 3014–3021.

- Gold, M. R., et al. The B cell antigen receptor activates the Akt (protein kinase B)/glycogen synthase kinase-3 signaling pathway via phosphatidylinositol 3-kinase. J. Immunol. 1999; 163: 1894–1905.

- Goldstone AH, et al. Attempts to improve treatment outcomes in acute myeloid leukemia (AML) in older patients: the results of the United Kingdome Medical Research Council AML11trial. Blood. 2001; 98: 1302–11.

- Gothert JR, et al. NOTCH1 pathway activation is an early hallmark of SCL T leukemogenesis. Blood. 2007; 110: 3753-3762.

- Gottlieb,T.M. et al. Cross-talk between Akt, p53 and Mdm2: possible implications for the regulation of apoptosis. Oncogene. 2002; 21: 1299–1303.

- Granville C.A., et al. Handicapping the race to develop inhibitors of the phosphoinositide 3-kinase/Akt/mammalian target of rapamycin pathway. Clin. Cancer Res. 2006; 12: 679–689.

- Greenberg PL, et al. Mitoxantrone, etoposide, and cytarabine with or without valspodar in patients with relapsed or refractory acute myeloid leukemia and high-risk myelodysplastic syndrome: a phase III trial (E2995). Journal of Clinical Oncology. 2004; 22: 1078–86.

- Grimwade D, et al. The predictive value of hierarchical cytogenetic classification in older adults with acute myeloid leukemia (AML): analysis of 1065 patients entered into the United Kingdom Medical Research Council AML11 trial. Blood. 2001; 98: 1312–20.

- Grimwade D, et al.; The Medical Research Council Adult and Children's Leukaemia Working Parties. The importance of diagnostic cytogenetics on outcome in AML: Analysis of 1,612 patients entered into the MRC AML 10 trial. Blood. 1998; 92: 2322−2333.

- Grimwade D, F et al. The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukemia Working Parties. Blood. 1998; 92: 2322–33.

- Gu,J. et al. Shc and FAK differentially regulate cell motility and directionality modulated by PTEN. J. Cell Biol. 1999; 146: 389–403.

- Gu,J. et al. Tumor suppressor PTEN inhibits integrin- and growth factor-mediated mitogen-activated protein (MAP) kinase signaling pathways. J. Cell Biol. 1998; 143: 1375–1383.

- Guba M., et al. Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. Nat. Med. 2002; 8: 128–135.

- Guertin D.A. and D.M. Sabatini, Defining the role of mTOR in cancer. Cancer Cell. 2007; 12: 9–22.

- Guo D, et al. Notch-1 regulates Akt signaling pathway and the expression of cell cycle regulatory proteins cyclin D1, CDK2 and p21 in T-ALL cell lines. Leuk Res. 2008 Dec 15. in press.

- Gupta, S., et al. Binding of ras to phosphoinositide 3-kinase p110α is required for ras-driven tumorigenesis in mice. Cell. 2007; 129: 957–968.

- Hammerman PS, et al. Beginnings of a signal-transduction pathway for bioenergetic control of cell survival. Trends Biochem Sci 2004; 29: 586-92. Review.

- Hammerman, P.S., et al. (2005). Pim and Akt oncogenes are independent regulators of hematopoietic cell growth and survival. Blood, 105, 4477-4483.

- Harima Y., et al.Mutation of the PTEN gene in advanced cervical cancer correlated with tumor progression and poor outcome after radiotherapy. Int. J. Oncol. 2001; 18: 493–497.

- Havasi, A., et al. Hsp27 inhibits Bax activation and apoptosis via a phosphatidylinositol 3-kinase-dependent mechanism. J. Biol. Chem. 2008; 283: 12305–12313.

- Hay N. and N. Sonenberg, Upstream and downstream of mTOR. Genes Dev. 2004; 18: 1926–1945.

- Hennessy B.Tet al. Exploiting the PI3K/AKT pathway for cancer drug discovery. Nat. Rev. Drug Discov. 2005; 4: 988–1004.

- Hideshima T, et al. Perifosine, an oral bioactive novel alkylphospholipid, inhibits Akt and induces in vitro and in vivo cytotoxicity in human multiple myeloma cells. Blood. 2006; 107: 4053-4062.

- Hinton, H. J. and Welham, M. J. Cytokine-induced protein kinase B activation and bad phosphorylation do not correlate with cell survival of hemopoietic cells. J. Immunol. 1999; 162: 7002–7009.

- Hiromura, M., et al. (2004). Inhibition of Akt kinase activity by a peptide spanning the betaA strand of the proto-oncogene TCL1. J Biol Chem, 279, 53407-53418.

- Hiromura, Met al. (2004). Inhibition of Akt kinase activity by a peptide spanning the betaA strand of the proto-oncogene TCL1. J Biol Chem, 279, 53407-53418.

- Hirsch, E., et al. Central role for G protein-coupled phosphoinositide 3-kinase γ in inflammation. Science. 2000; 287: 1049–1053.

- Hoemann CD, et al. Two distinct Notch1 mutant alleles are involved in the induction of T-cell leukemia in c-myc transgenic mice. Mol Cell Biol. 2000; 20: 3831-3842.

- Hollestelle A., et al. Phosphatidylinositol-3-OH kinase or RAS pathway mutations in human breast cancer cell lines, Mol. Cancer Res. 2007; 5: 195–201.

- Hong, S. Y., et al. Involvement of two NF-κB binding elements in tumor necrosis factor α-, CD40-, and Epstein–Barr virus latent membrane protein 1-mediated induction of the cellular inhibitor of apoptosis protein 2 gene. J. Biol. Chem. 2000; 275: 18022–18028.

- Hu Y, et al. Akt phosphorylates acinus and inhibits its proteolytic cleavage, preventing chromatin condensation. EMBO J. 2005; 24: 3543-3554.

- Hu, Y., et al. (2005). Akt phosphorylates acinus and inhibits its proteolytic cleavage, preventing chromatin condensation. EMBO J, 24, 3543-35454.

- Hu, Y., et al. (2005). Phosphoinositol lipids bind to phophatidylinositol 3 (PI3)-kinase enhancer GTPase and mediate its stimulatory effect on PI3-kinase and Akt signalings. PNAS, 102, 16853-16858.

- Huang B and Porter G. Expression of proline-rich Akt-substrate PRAS40 in cell survival pathway and carcinogenesis. Acta Pharmacol Sin. 2005; 26: 1253-1258.

- Huang ME, et al. Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. Blood. 1988; 72: 567−572.

- Huang, H. M., et al. Mcl-1 is a common target of stem cell factor and interleukin-5 for apoptosis prevention activity via MEK/MAPK and PI-3K/Akt pathways. Blood. 2000; 96: 1764–1771.

- Huddleston, H., et al. (2003). Functional p85alpha gene is required for normal murine fetal erythropoiesis. Blood, 102, 142-145.

- Hundal, R. S., et al. Oxidized low density lipoprotein inhibits macrophage apoptosis by blocking ceramide generation, thereby maintaining protein kinase B activation and Bcl-XL levels. J. Biol. Chem. 2003; 278: 24399–24408.

- Iantorno M, et al. Ghrelin has novel vascular actions that mimic PI 3-kinase-dependent actions of insulin to stimulate production of NO from endothelial cells. Am J Physiol Endocrinol Metab. 2007; 292: E756-64.

- Ihle N.T., et al. The phosphatidylinositol-3-kinase inhibitor PX-866 overcomes resistance to the epidermal growth factor receptor inhibitor gefitinib in A-549 human non-small cell lung cancer xenografts. Mol. Cancer Ther. 2005; 4: 1349–1357.

- Ikeda, S., et al. Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3β and β-catenin and promotes GSK-3β-dependent phosphorylation of β-catenin. EMBO J. 1998; 17: 1371–1384.

- Jabbour EJ, et al. Adult Acute Myeloid Leukemia. Mayo Clinic Proceedings. 2006; 81: 247-260.

- Jacinto, E. and Lorberg, A. TOR regulation of AGC kinases in yeast and mammals. Biochem. J. 2008; 410: 19–37.

- Jastrzebski, K., et al. Coordinate regulation of ribosome biogenesis and function by the ribosomal protein S6 kinase, a key mediator of mTOR function. Growth Factors. 2007; 25: 209–226.

- Jelkmann, W. (2007). Erythropoietin after a century of research: younger than ever. Eur J Haematol, 78, 183-205.

- Jendrossek V, et al. (1999). Int. J. Oncol., 14, 15–22.

- Jendrossek V, et al. (2001). Anticancer Res., 21, 3389–3396.

- Jiang B.H, et al. Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. J. Biol. Chem. 1996; 271: 17771–17778.

- Jiang B.H. and Ling-Zhi Liu. PI3K/PTEN signaling in tumorigenesis and angiogenesis. Biochimica et Biophysica Acta. 2008; 1784: 150–158.

- Jiang B.H., et al. Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells, Proc. Natl. Acad. Sci. U. S. A. 2000; 97: 1749–1753.

- Jin, X., et al. (2004). Inhibition of AKT survival pathway by a small molecule inhibitor in human endometrial cancer cells. Br J Cancer, 91, 1808-1812.

- Jin, X., et al. (2004). Inhibition of AKT survival pathway by a small molecule inhibitor in human endometrial cancer cells. Br J Cancer, 91, 1808-1812.

- Jones G.E., et al. Requirement for PI 3-kinase gamma in macrophage migration to MCP-1 and CSF-1, Exp. Cell Res. 2003; 290: 120–131.

- Jones, P. F., et al. Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily. Proc. Natl. Acad. Sci. U.S.A. 1991; 88: 4171–4175.

- Jones, R. G et al. Protein kinase B regulates T lymphocyte survival, nuclear factor κB activation, and Bcl-XL levels in vivo. J. Exp. Med. 2000; 191: 1721–1734.

- Jope RS and Johnson GV. The glamour and gloom of glycogen synthase kinase-3. Trends Biochem Sci. 2004; 29: 95-102. Review.

- Jou S.T., et al. Essential, nonredundant role for the phosphoinositide 3- kinase p110delta in signaling by the B-cell receptor complex, Mol. Cell. Biol. 2002; 22: 8580–8591.

- Kadri, Z., et al. (2005). Phosphatidylinositol 3-kinase/Akt induced by erythropoietin renders the erythroid differentiation factor GATA-1 competent for TIMP-1 gene transactivation. Mol Cell Biol, 25, 7412-7422.

- Kandel,E.S and Hay N The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. Exp. Cell Res. 1999; 253: 210–229.

- Kang S., et al. Vogt, Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic. Proc. Natl. Acad. Sci. U. S. A. 2005; 102: 802–807.

- Kang, S., et al. Mutated PI 3-kinases: cancer targets on a silver platter. Cell Cycle. 2005; 4: 578–581.

- Kantarjian H, et al. Phase 2 clinical and pharmacologic study of clofarabine in patients with refractory or relapsed acute leukemia. Blood. 2003; 102: 2379–86.

- Kantarjian HM, et al. Clofarabine: past, present, and future. Leukemia & Lymphoma. 2007; 48: 1922–30.

- Kaplan, D. R., et al. Phosphatidylinositol metabolism and polyoma-mediated transformation. Proc. Nat. Acad. Sci. U.S.A. 1986; 83: 3624–3628.

- Kappelmayer J, et al. Pgp and FLT3: identification and modulation of two proteins that lead to chemotherapy resistance in acute myeloid leukemia. Curr Med Chem. 2007; 14: 519-530. Review.

- Karathanasis NV, et al. Gene polymorphisms in childhood ALL. Pediatr Blood Cancer. 2009; 52: 318-323.

- Karin, M. and Ben-Neriah, Y. Phosphorylation meets ubiquitination: the control of NF-κB activity. Annu. Rev. Immunol. 2000; 18: 621–663.

- Karni, R., et al. The gene encoding the splicing factor SF2/ASF is a proto-oncogene. Nat. Struct. Mol. Biol. 2007; 14: 185–193.

- Kashii, Y., et al. (2000). A member of Forkhead family transcription factor, FKHRL1, is one of the downstream molecules of phosphatidylinositol 3-kinase-Akt activation pathway in erythropoietin signal transduction. Blood, 96, 941-949.

- Khan S., et al. PTEN promoter is methylated in a proportion of invasive breast cancers. Int. J. Cancer. 2004; 112: 407–410.

- Kim KT, et al. Constitutively activated FLT3 phosphorylates BAD partially through pim-1. Br J Haematol. 2006; 134: 500-509.

- King M. E. and Jacob M. Roweb Recent Developments in Acute Myelogenous Leukemia Therapy. TheOncologist. 2007; 12: 14–21.

- Kirkegaard T, et al. AKT activation predicts outcome in breast cancer patients treated with tamoxifen. J. Pathol. 2005; 207: 139–146.

- Kitanaka, A., et al. (1994). Antisense src expression inhibits proliferation and erythropoietin- induced erythroid differentiation of K562 human leukemia cells. Biochem Biophys Res Commun, 201, 1534-1540.

- Kitaura J., et al. IgE and IgE+Ag-mediated mast cell migration in an autocrine/paracrine fashion. Blood. 2005; 105: 3222–3229.

- Kiyoi H, et al. Internal tandem duplication of the FLT3 gene is a novel modality of elongation mutation which causes constitutive activation of the product. Leukemia 1998; 12: 1333-1337.

- Klingmuller, U., et al. (1997). Identification of a novel pathway important for proliferation and differentiation of primary erythroid progenitors. Proc Natl Acad Sci USA, 94, 3016-3021.

- Knapper S, et al. A phase 2 trial of the FLT3 inhibitor lestaurtinib (CEP701) as first-line treatment for older patients with acute myeloid leukemia not considered fit for intensive chemotherapy. Blood. 2006; 108: 3263–70.

- Kogoshi H, et al. Gammasecretase inhibitors suppress the growth of leukemia and lymphoma cells. Oncol Rep. 2007; 18: 77-80.

- Koh H, et al. Cloning and characterization of a nuclear S6 kinase, S6 kinase-related kinase (SRK); a novel nuclear target of Akt. Oncogene. 1999; 18: 5115-5119.

- Kohn AD, et al. Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. J Biol Chem. 1996; 271: 31372-31378.

- Kohn, A. D., et al. Insulin stimulates the kinase activity of RAC-PK, a pleckstrin homology domain containing Ser/Thr kinase. EMBO J. 1995; 14: 4288–4295.

- Kolb HJ, et al. Graft-versus-leukemia reactions in allogeneic chimeras. Blood. 2004; 103: 767–76.

- Kondapaka SB, et al. Perifosine, a novel alkylphospholipid, inhibits protein kinase B activation. Mol Cancer Ther. 2003; 2: 1093-1103.

- Konishi, T., et al. Overexpression of hRFI inhibits 5-fluorouracil-induced apoptosis in colorectal cancer cells via activation of NF-κB and upregulation of BCL-2 and BCL-XL. Oncogene. 2006; 25: 3160–3169.

- Konstantinov SM and Berger MR. (1999). Cancer Lett., 144, 153–160.

- Kops, G.J., et al. (1999). Direct control of the Forkhead transcription factor AFX by protein kinase B. Nature, 398, 630-634.

- Kordes U, et al. Transcription factor NF-kappaB is constitutively activated in acute lymphoblastic leukemia cells. Leukemia. 2000; 14: 399-402.

- Kornblau SM, et al. Studying the right cell in acute myelogenous leukemia: dynamic changes of apoptosis and signal transduction pathway protein expression in chemotherapy resistant ex-vivo selected "survivor cells". Cell Cycle. 2006; 5: 2769-2777.

- Kottaridis PD, et al. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: Analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. Blood. 2001; 98: 1752−1759.

- Kovacina KS, et al. Identification of a proline-rich Akt substrate as a 14-3-3 binding partner. J Biol Chem. 2003; 278: 10189-10194.

- Kreuz, S., et al. NF-κB inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. Mol. Cell. Biol. 2001; 21: 3964–3973.

- Ksiezak-Reding H, et al. Akt/PKB kinase phosphorylates separately Thr212 and Ser214 of tau protein in vitro. Biochim Biophys Acta. 2003; 1639: 159-68.

- Kubota Y, et al. Constitutive activation of PI3K is involved in the spontaneous proliferation of primary acute myeloid leukemia cells: direct evidence of PI3K activation. Leukemia. 2004; 18: 1438-1440.

- Kubota, Y., et al. (1996). Wortmannin, a specific inhibitor of phosphatidylinositol-3-kinase, inhibits erythropoietin-induced erythroid differentiation of K562 cells. Leukemia, 10, 720-726.

- Kubota, Y., et al. (2001). Src transduces erythropoietin-induced differentiation signals through phosphatidylinositol 3-kinase. EMBO J, 20, 5666-5677.

- Kumar, R., and Hung, M.C. Signaling intricacies take center stage in cancer cells.. Cancer Res. 2005; 65: 2511–2515.

- Kuwana, T., et al. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. Mol. Cell. 2005; 17: 525–535.

- Kyoung Pyo H, et al. Phosphorylation of tau at THR212 and SER214 in human neuronal and glial cultures: the role of AKT. Neuroscience. 2004; 127: 649-658.

- Laffargue M., et al.Phosphoinositide 3-kinase gamma is an essential amplifier of mast cell function, Immunity. 2002; 16: 441–451.

- Lee J.T. Jr., et al. Phosphatidylinositol 3′-kinase activation leads to multidrug resistance protein-1 expression and subsequent chemoresistance in advanced prostate cancer cells. Cancer Res. 2004; 64: 8397–8404.

- Lee J.W., et al. PIK3CA gene is frequently mutated in breast carcinomas and hepatocellular carcinomas. Oncogene. 2005; 24: 1477–1480.

- Leevers S.J., et al. The Drosophila phosphoinositide 3-kinase Dp110 promotes cell growth. EMBO J. 1996; 15: 6584–6594.

- Leith CP, et al. Acute myeloid leukemia in the elderly: assessment of multidrug resistance (MDR1) and cytogenetics distinguishes biologic subgroups with remarkably distinct responses to standard chemotherapy: a Southwest Oncology Group study. Blood. 1997; 89: 3323–9.

- Lemmon, M. A. and Ferguson, K. M. Signal-dependent membrane targeting by pleckstrin homology (PH) domains. Biochem. J. 2000; 350: 1–18.

- Lennon R, et al. Hemopexin induces nephrin-dependent reorganization of the actin cytoskeleton in podocytes. J Am Soc Nephrol. 2008; 19: 2140-2109.

- Leong KG and Karsan A. Recent insights into the role of Notch signaling in tumorigenesis. Blood. 2006; 107: 2223-2233.

- Levis M, et al. In vitro studies of a FLT-3 inhibitor combined with chemotherapy: sequence of administration is important to achieve synergistic cytotoxic effects. Blood. 2004; 104: 1145–50.

- Lewis HD, et al. Apoptosis in T cell acute lymphoblastic leukemia cells after cell cycle arrest induced by pharmacological inhibition of notch signaling. Chem Biol. 2007; 14: 209-219.

- Li G., et al. Conditional loss of PTEN leads to precocious development and neoplasia in the mammary gland, Development. 2002; 129: 4159–4170.

- Li J., et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science. 1997; 275: 1943–1947.

- Li X, et al. Autoposphorylation of AKT at threonine 72 and serine 246. A potential mechanism of regulation of AKT kinase activity. J. Biol. Chem. 2006; 281: 13837-13843.

- Li Zet al. Roles of PLCbeta2 and -beta3 and PI3Kgamma in chemoattractant-mediated signal transduction. Science. 2000; 287: 1046–1049.

- Lian,Z. et al. Class reunion: PTEN joins the nuclear crew. Oncogene. 2005; 24: 7394–7400.

- Liaw D., et al. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat. Genet. 1997; 16: 64–67.

- Lin HK, et al. Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. Proc Natl Acad Sci U S A. 2001; 98: 7200-7205.

- Lindsay,Y. et al. Localization of agonist-sensitive PtdIns(3,4,5)P3 reveals a nuclear pool that is insensitive to PTEN expression. J. Cell Sci. 2006; 119: 5160–5168.

- Link W, et al. Membrane localization of all class I PI 3-kinase isoforms suppresses c-Myc-induced apoptosis in Rat1 fibroblasts via Akt. J. Cell. Biochem. 2005; 95: 979–989.

- Linseman, D. A., et al. Glycogen synthase kinase-3β phosphorylates Bax and promotes its mitochondrial localization during neuronal apoptosis. J. Neurosci. 2004; 24: 9993–10002.

- Liu Y., et al. Polo-like kinases inhibited by wortmannin. Labeling site and downstream effects. J. Biol. Chem. 2007; 282: 2505–2511.

- Liu,Z. et al. Human tumor mutants in the p110alpha subunit of PI3K. Cell Cycle. 2006; 5: 675–677.

- Livasy C.A., et al. EGFR expression and HER2/neu overexpression/amplification in endometrial carcinosarcoma. Gynecol. Oncol. 2006; 100: 101–106.

- Lizcano, J. M., et al. Regulation of BAD by cAMP-dependent protein kinase is mediated via phosphorylation of a novel site, Ser155. Biochem. J. 2000; 349: 547–557.

- Lopez-Pajares, et al. Phosphorylation of MDMX mediated by Akt leads to stabilization and induces 14-3-3 binding. J. Biol. Chem. 2008; 283: 13707–13713.

- Lotem, J. and Sachs, L. Control of apoptosis in hematopoiesis and leukemia by cytokines, tumor suppressor and oncogenes. Leukemia. 1996; 10: 925–931.

- Lowenberg B, et al. Acute Myeloid Leukemia. New England Journal of Medicine. 1999; 341: 1051–62.

- Lowenberg B, et al. Mitoxantrone versus daunorubicin in in induction-consolidation chemotherapy—the value of low-dose cytarabine for maintenance of remission, and an assessment of prognostic factors in acute myeloid leukemia in the elderly: final report.

European Organization for the Research and Treatment of Cancer and the Dutch-Belgian hemato-Oncology Cooperative Hovon Group. Journal of Clinical Oncology. 1998; 16: 872–81.

- Lowenberg B, et al. On the value of intensive remission induction chemotherapy in elderly patients of 65+ years with acute myeloid leukemia: a randomized phase III study of the European Organization for Research and Treatment of Cancer Leukemia Group. Journal of Clinical Oncology. 1989; 7: 1268–74.

- Lu J, et al. p38-MAP kinase activation followed by BIM induction is essential for glucocorticoidinduced apoptosis in lymphoblastic leukemia cells. FEBS Lett. 2006; 580: 3539-3544.

- Lu Y, et al. JAK/STAT and PI3K/AKT pathways form a mutual transactivation loop and afford resistance to oxidative stress-induced apoptosis in cardiomyocytes. Cell Physiol Biochem. 2008;21(4):305-14.

- Luo JM, et al. Mutation analysis of SHIP gene in acute leukemia. Zhong-guo Shi Yan Xue Ye Xue Za Zhi. 2004; 12: 420-426.

- Lusis A.J., Atherosclerosis. Nature. 2000; 407: 233–241.

- Ma, K, et al. PI(3,4,5)P 3 and PI(3,4)P 2 levels correlate with PKB/Akt phosphorylation at Thr308 and Ser473, respectively; PI(3,4)P 2 levels determine PKB activity. Cell. Signalling. 2008; 20: 684–694.

- Maehama,T and Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5- trisphosphate. J. Biol. Chem. 1998; 273: 13375–13378.

- Malek K et al. Effects of the IMP-dehydrogenase inhibitor, Tiazofurin, in bcr-abl positive acute myelogenous leukemia. Part I. In vivo studies. Leuk Res. 2004; 28: 1125−1136.

- Manning, B.D., and Cantley, L.C. AKT/PKB signaling: navigating downstream. Cell. 2007; 129: 1261–1274.

- Marchisio, M., et al. (1998). Phosphatidylinositol 3-kinase in HL-60 nuclei is bound to the nuclear matrix and increases during granulocytic differentiation. Biochem Biophys Res Commun, 253, 346-351.

- Marcucci G., et al. Phase 1 and pharmacodynamic studies of G3139, a Bcl-2 antisense oligonucleotide, in combination with chemotherapy in refractory or relapsed acute leukemia. Blood. 2003; 101: 425−432.

- Marsh D.J., et al. PTEN mutation spectrum and genotypephenotype correlations in Bannayan-Riley-Ruvalcaba syndrome suggest a single entity with Cowden syndrome. Hum. Mol. Genet. 1999; 8: 1461–1472.

- Marshall S. Role of insulin, adipocyte hormones, and nutrient-sensing pathways in regulating fuel metabolism and energy homeostasis: a nutritional perspective of diabetes, obesity, and cancer. Sci. STKE. 2006 re7.

- Marte, B. M. and Downward, J. PKB/Akt: connecting phosphoinositide 3-kinase to cell survival and beyond. Trends Biochem. Sci. 1997; 22: 355–358.

- Martelli A.M., et al. A new selective AKT pharmacological inhibitor reduces resistance to chemotherapeutic drugs, TRAIL, all-transretinoic acid, and ionizing radiation of human leukemia cells. Leukemia. 2003; 17: 1794–1805.

- Marygold, S. J. and Leevers, S. J. Growth signaling: TSC takes its place. Curr. Biol. 2002; 12: 785–787.

- Maser R.S., et al. Chromosomally unstable mouse tumours have genomic alterations similar to diverse human cancers. Nature. 2007; 447: 966-971.

- Mathews V., et al. Single-agent arsenic trioxide in the treatment of newly diagnosed acute promyelocytic leukemia: durable remissions with minimal toxicity. Blood. 2006; 107: 2527–632.

- Matkovic, K., et al. (2006). The role of the nuclear Akt activation and Akt inhibitors in all-trans-retinoic acid-differentiated HL-60 cells. Leukemia, 20, 941-951.

- Maurer, U., et al. Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. Mol. Cell. 2006; 21: 749–760.

- Mayer R.J., et al. Intensive postremission chemotherapy in adults with acute myeloid leukemia. Cancer and Leukemia Group B. New England Journal of Medicine. 1994; 331: 896–903.

- Mayo LD,et al. Phosphorylation of human p53 at serine 46 determines promoter selection and whether apoptosis is attenuated or amplified. J Biol Chem. 2005; 280: 25953-25959.

- Mayo, L. D. and Donner, D. B. A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. Proc. Natl. Acad. Sci. U.S.A. 2001; 98: 11598–11603.

- Menghini R, et al. Phosphorylation of GATA2 by Akt increases adipose tissue differentiation and reduces adipose tissue-related inflammation: a novel pathway linking obesity to atherosclerosis. Circulation. 2005; 111: 1946-53.

- Menzin J., et al. The outcomes and costs of acute myeloid leukemia among the elderly. Archives of Internal Medicine. 2002; 162: 1597–603.

- Mercado-Gómez O, et al. Inhibition of Wnt and PI3K signaling modulates GSK-3beta activity and induces morphological changes in cortical neurons: role of tau phosphorylation. Neurochem Res. 2008; 33: 1599-1609.

- Meske V, et al. Coupling of mammalian target of rapamycin with phosphoinositide 3-kinase signaling pathway regulates protein phosphatase 2A- and glycogen synthase kinase-3 -dependent phosphorylation of Tau. Biol Chem. 2008; 283: 100-109.

- Miled N., et al.Mechanism of two classes of cancer mutations in the phosphoinositide 3-kinase catalytic subunit. Science. 2007; 317: 239–242.

- Milligan DW, et al. Guidelines on the management of acute myeloid leukaemia in adults. British Journal of Haematology. 2006; 135: 450–74.

- Min YH, et al. Constitutive phosphorylation of Akt/PKB protein in acute myeloid leukemia: its significance as a prognostic variable. Leukemia 2003, 17: 995-997.

- Minami Y, et al. Different antiapoptotic pathways between wild-type and mutated FLT3: insights into therapeutic targets in leukemia. Blood. 2003; 102: 2969-2975.

- Minshall, C., et al. Requirement for phosphatidylinositol 3'-kinase to protect hemopoietic progenitors against apoptosis depends upon the extracellular survival factor. J. Immunol. 1996; 156: 939–947.

- Mirmohammadsadegh A., et al. Epigenetic silencing of the PTEN gene in melanoma. Cancer Res. 2006; 66: 6546–6552.

- Mishra, R., et al. Glycogen synthase kinase-3β induces neuronal cell death via direct phosphorylation of mixed lineage kinase 3. J. Biol. Chem. 2007; 282: 30393–30405.

- Mizoguchi M., et al. Genetic alterations of phosphoinositide 3-kinase subunit genes in human glioblastomas. Brain Pathol. 2004; 14: 372–377.

- Moll, U. M., et al. Transcription-independent pro-apoptotic functions of p53. Curr. Opin. Cell Biol. 2005; 17: 631–636.

- Mora, A., et al. PDK1, the master regulator of AGC kinase signal transduction. Semin. Cell Dev. Biol. 2004; 15: 161–170.

- Moule, S. K., et al. Regulation of protein kinase B and glycogen synthase kinase-3 by insulin and β-adrenergic agonists in rat epididymal fat cells: activation of protein kinase B by wortmannin-sensitive and -insensitive mechanisms. J. Biol. Chem. 1997; 272: 7713–7719.

- Mungamuri SK, et al. Survival signaling by Notch1: mammalian target of rapamycin (mTOR)-dependent inhibition of p53. Cancer Res. 2006; 66: 4715-4724.

- Myers, M. P., et al. The lipid phosphatase activity of PTEN is critical for its tumor suppressor function. Proc. Natl. Acad. Sci. U.S.A. 1998; 95: 13513–13518.

- Myers,M.P. et al. P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. Proc. Natl Acad. Sci. USA. 1997; 94: 9052–9057.

- Myklebust, J.H., et al. (2002). Activation of phosphatidylinositol 3-kinase is important for erythropoietin-induced erythropoiesis from CD34(+) hematopoietic progenitor cells. Exp Hematol, 30, 990-1000.

- Nakatani K., et al. Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines, J. Biol. Chem. 1999; 274: 21528–21532.

- Nakayama K, et al. Sequence mutations and amplification of PIK3CA and AKT2 genes in purified ovarian serous neoplasms. Cancer Biol. Ther. 2006; 5: 779–785.

- Nanri T, et al. Mutations in the receptor tyrosine kinase pathway are associated with clinical outcome in patients with acute myeloblastic leukemia harboring t(8;21)(q22;q22). Leukemia. 2005; 19: 1361−1366.

- Naoe T and Kiyoi H. Normal and oncogenic FLT3. Cell Mol Life Sci. 2004; 61: 2932-2938.

- Nelen MR, et al. Germline mutations in the PTEN/MMAC1 gene in patients with Cowden disease. Hum. Mol. Genet. 1997; 6: 1383–1387.

- Neri LM, et al. The phosphoinositide 3-kinase/AKT1 pathway involvement in drug and all-trans-retinoic acid resistance of leukemia cells. Mol Cancer Res. 2003 Jan;1(3):234-46.

- Neri, L.M., et al. (1999). Monocytic differentiation of HL-60 cells is characterized by the nuclear translocation of phosphatidylinositol 3-kinase and of definite phosphatidylnositol-specific phospholipase C isoforms. Biochem Biophys Res Commun, 259, 314-320.

- Neri, L.M., et al. (1999). Monocytic differentiation of HL-60 cells is characterized by the nuclear translocation of phosphatidylinositol 3-kinase and of definite phosphatidylnositol-specific phospholipase C isoforms. Biochem Biophys Res Commun, 259, 314-320.

- Neri, L.M., et al. (2002). Erythropoietin-induced erythroid differentiation of K562 cells is accompanied by the nuclear translocation of phosphatidylinositol 3-kinase and intranuclear generation of phosphatidylinositol (3,4,5) triphosphate. Cell Signal, 14, 21-29.

- Neri, L.M., et al. (2002). The nuclear phosphoinositide 3-kinase/Akt pathway: a new second messenger system. Biochim Biophys Acta, 1584, 73-80.

- Nicholson KM and Anderson NG. The protein kinase B/Akt signalling pathway in human malignancy. Cell Signal. 2002; 14: 381-395. Review.

- Nishii K, et al. Characteristics of t(8;21) acute myeloid leukemia (AML) with additional chromosomal abnormality: Concomitant trisomy 4 may constitute a distinctive subtype of t(8;21) AML. Leukemia. 2003; 17: 731−737.

- Oda K., et al. High frequency of coexistent mutations of PIK3CA and PTEN genes in endometrial carcinoma, Cancer Res. 2005; 65: 10669–10673.

- Okano J, et al. Genetic alterations in esophageal cancer. Methods Mol. Biol. 2003; 222: 131–145.

- Okkenhaug K. and B. Vanhaesebroeck, PI3K in lymphocyte development, differentiation and activation. Nat. Rev. Immunol. 2003; 3: 317–330.

- Okkenhaug K., et al. Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. Science. 2002; 297: 1031–1034.

- Ollikainen M., et al. Patterns of PIK3CA alterations in familial colorectal and endometrial carcinoma. Int. J. Cancer. 2007; 121: 915–920.

- Oren,M. et al. Regulation of p53: intricate loops and delicate balances. Biochem. Pharmacol. 2002; 64: 865–871.

- Osaki, M., et al. PI3K–Akt pathway: its functions and alterations in human cancer. Apoptosis. 2004; 9: 667–676.

- Ozes, O. N., et al. NF-κB activation by tumour necrosis factor requires the Akt serine–threonine kinase. Nature. 1999; 401: 82–85.

- Ozpolat, B., et al. Programmed cell death-4 tumor suppressor protein contributes to retinoic acid-induced terminal granulocytic differentiation of human myeloid leukemia cells. Mol. Cancer Res. 2007; 5: 95–108.

- Pagano L, et al. The role of Gemtuzumab Ozogamicin in the treatment of acute myeloid leukemia patients. Oncogene, 2007; 26: 3679–90.

- Palamarchuk A, et al. Akt phosphorylates and regulates Pdcd4 tumor suppressor protein. Cancer Res. 2005; 65: 11282-11286.

- Palamarchuk A, et al. Akt phosphorylates Tal1 oncoprotein and inhibits its repressor activity. Cancer Res. 2005; 65: 4515-4519.

- Palomero T, et al. Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. Nat Med. 2007; 13: 1203-1210.

- Pan, D., et al. Tuberous sclerosis complex: from Drosophila to human disease. Trends Cell Biol. 2004; 14: 78–85.

- Pap, M. and Cooper, G. M. Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-kinase/Akt cell survival pathway. J. Biol. Chem. 1998; 273: 19929–19932.

- Park, J. R. Cytokine regulation of apoptosis in hematopoietic precursor cells. Curr. Opin. Hematol. 1996; 3: 191–196.

- Parmar S et al. Phase II trial of arsenic trioxide in relapsed and refractory acute myeloid leukemia, secondary leukemia and/or newly diagnosed patients at least 65 years old. Leukemia Research. 2004; 28: 909–19.

- Parsons DW, et al. Colorectal cancer: mutations in a signalling pathway. Nature. 2005; 436: 792.

- Patel V, et al. Perifosine, a novel alkylphospholipid, induces p21WAF1 expression in squamous carcinoma cells through a p53-independent pathway, leading to loss in cyclin-dependent kinase activity and cell cycle arrest. Cancer Res. 2002;62:1401-1409.

- Patrucco E., et al. PI3Kgamma modulates the cardiac response to chronic pressure overload by distinct kinase-dependent and -independent effects. Cell. 2004; 118: 375–387.

- Pautas C, et al. Randomized Comparison of Standard Induction with Daunorubicin (DNR) for 3 Days vs Idarubicin (IDA) for 3 or 4 Days in AML pts Aged 50 to 70 and of Maintenance with Interleukin 2. Final Analysis of the ALFA 9801 Study. Blood. 2007;.110: 162.

- Pear WS, et al. Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. J Exp Med. 1996; 183: 2283-2291.

- Pedrero J.M., et al.Frequent genetic and biochemical alterations of the PI 3-K/AKT/PTEN pathway in head and neck squamous cell carcinoma. Int. J. Cancer. 2005; 114: 242–248.

- Pekarsky, Y., et al. (2000). Tcl1 enhances Akt kinase activity and mediates its nuclear translocation. Proc Natl Acad Sci USA, 97, 3028-3033.

- Pekarsky, Y., et al. Tcl1 enhances Akt kinase activity and mediates its nuclear translocation. Proc Natl Acad Sci U S A. 2000; 97: 3028-3033.

- Philp A.J., et al. The phosphatidylinositol 3′-kinase p85alpha gene is an oncogene in human ovarian and colon tumors. Cancer Res. 2001; 61: 7426–7429.

- Piatkowska-Jakubas B, et al. Use of L-asparaginase in acute lymphoblastic leukemia: recommendations of the Polish Adult Leukemia Group. Pol Arch Med Wewn. 2008; 118: 664-669. Review.

- Plas DR and Thompson CB. Akt-dependent transformation: there is more to growth than just surviving. Oncogene. 2005; 24: 7435–7442.

- Pui CH, et al. Expression analysis of genes at 3q26-q27 involved in frequent amplification in squamous cell lung carcinoma. Eur. J. Cancer. 1999; 35: 641–646.

- Rahmani M, et al. Coadministration of histone deacetylase inhibitors and perifosine synergistically induces apoptosis in human leukemia cells through Akt and ERK1/2 inactivation and the generation of ceramide and reactive oxygen species. Cancer Res. 2005;65:2422-2432.

- Ravi, R. and Bedi, A. NF-κB in cancer: a friend turned foe. Drug Resist. 2004; 7: 53–67.

- Ries LG, et al. SEER Cancer Statistics Review, 1975-2004, National Cancer Institute. Bethesda, MD, http://seer.cancer.gov/csr/1975_2004/, based on November 2006 SEER data submission, posted to the SEER web site, 2007.

- Risau W., Mechanisms of angiogenesis. Nature. 1997; 386: 671–674.

- Rodriguez-Viciana, P., et al. Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature. 1994; 370: 527–532.

- Romashkova, J. A. and Makarov, S. S. NF-κB is a target of AKT in anti-apoptotic PDGF signalling. Nature. 1999; 401: 86–90.

- Roos, W. P. and Kaina, B. DNA damage-induced cell death by apoptosis. Trends Mol. Med. 2006; 12: 440–450.

- Rosner, M., et al. Akt regulates nuclear/cytoplasmic localization of tuberin. Oncogene. 2007; 26: 521–531.

- Rössig L, et al. Akt-dependent phosphorylation of p21(Cip1) regulates PCNA binding and proliferation of endothelial cells. Mol Cell Biol. 2001; 21: 5644-5657.

- Rowe JM, et al. A phase 3 study of three induction regimens and of priming with GM-CSF in older adults with acute myeloid leukemia: a trial by the Eastern Cooperative Oncology Group. Blood. 2004; 103: 479–85.

- Rowe JM. Preface: Significant advances in the biology and therapy of AML over the past four decades. Best Pract Res Clin Haematol. 2006; 19: 259–262.

- Ruiter GA, et al. Anti-cancer alkyl-lysophospholipids inhibit the phosphatidylinositol 3-kinase-Akt/PKB survival pathway. Anticancer Drugs. 2003; 14: 167-173.

- Samuels Y. and K. Ericson. Oncogenic PI3K and its role in cancer. Curr. Opin. Oncol. 2006; 18: 77–82.

- Samuels Y. and V.E. Velculescu., Oncogenic mutations of PIK3CA in human cancers. Cell Cycle. 2004; 3: 1221–1224.

- Samuels Y., et al. High frequency of mutations of the PIK3CA gene in human cancers. Science. 2004; 304: 554.

- Samuels Y., et al. Mutant PIK3CA promotes cell growth and invasion of human cancer cells. Cancer Cell. 2005; 7: 561–573.

- Sarbassov D.D., et al. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science. 2005; 307: 1098–1101.

- Sarbassov D.D., et al. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol. Cell. 2006; 22: 159–168.

- Sasaki T., et al. Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. Science. 2000; 287: 1040–1046.

- Scheid, M. P. and Duronio, V. Dissociation of cytokine-induced phosphorylation of Bad and activation of PKB/Akt: involvement of MEK upstream of Bad phosphorylation. Proc. Natl. Acad. Sci. U.S.A. 1998; 95: 7439–7444.

- Scheid, M. P. and Woodgett, J. R. PKB/AKT: functional insights from genetic models. Nat. Rev. Mol. Cell Biol. 2001; 2: 760–768.

- Scheid, M. P., et al. Phosphatidylinositol (3,4,5)P 3 is essential but not sufficient for protein kinase B (PKB) activation; phosphatidylinositol (3,4)P 2 is required for PKB phosphorylation at Ser-473: studies using cells from SH2-containing inositol-5-phosphatase knockout mice. J. Biol. Chem. 2002; 277: 9027–9035.

- Scheid, M. P., et al. Role of phosphatidylinositol 3-OH kinase activity in the inhibition of apoptosis in haemopoietic cells: phosphatidylinositol 3-OH-kinase inhibitors reveal a difference in signalling between interleukin-3 and granulocyte-macrophage colony stimulating factor. Biochem. J. 1995; 312: 159–162.

- Scheid, M. P.,et al. Phosphoinositide-dependent phosphorylation of PDK1 regulates nuclear translocation. Mol. Cell. Biol. 2005; 25: 2347–2363.

- Schetelig J, et al. Comparable Outcome after Allogeneic Hematopoietic Cell Transplantation from Matched Unrelated and Sibling Donors in Elderly Patients with Acute Myeloid Leukemia—Results of a Retrospective Analysis in 368 Patients. Blood. 2007; 110: 172.

- Schiller G and Lee M. Long-term outcome of high-dose cytarabine-based consolidation chemotherapy for older patients with acute myelogenous leukemia. Leukemia and Lymphoma. 1997; 25: 111–9.

- Schlondorff J. Nephrin. AKTs on actin: The slit diaphragm-actin cytoskeleton signaling network expands. Kidney Int. 2008; 73: 524-526.

- Schon M.P. and W.H. Boehncke, Psoriasis, N. Engl. J. Med. 2005; 352: 1899–1912.

- Schrager CA, et al. Clinical and pathological features of breast disease in Cowden's syndrome: an underrecognized syndrome with an increased risk of breast cancer. Hum. Pathol. 1998; 29: 47–53.

- Schubert, K. M. and Duronio, V. Distinct roles for extracellular-signal-regulated protein kinase (ERK) mitogen-activated protein kinases and phosphatidylinositol 3-kinase in the regulation of Mcl-1 synthesis. Biochem. J. 2001; 356: 473–480.

- Seminario, et al. (2003). PTEN expression in PTEN-null leukemic T cell lines leads to reduced proliferation via slowed cell cycle progression. Oncogene, 22, 8195-8204.

- Seminario, M.C., et al. (2003). PTEN expression in PTEN-null leukemic T cell lines leads to reduced proliferation via slowed cell cycle progression. Oncogene, 22, 8195-8204.

- Sevilla, L., et al. Transcriptional regulation of the bcl-x gene encoding the anti-apoptotic Bcl-xL protein by Ets, Rel/NFκB, STAT and AP1 transcription factor families. Histol. Histopathol. 2001; 16: 595–601.

- Shaw R.J. and L.C. Cantley, Ras, PI(3)K and mTOR signalling controls tumour cell growth. Nature. 2006; 441: 424–430.

- Shayesteh L., et al.PIK3CA is implicated as an oncogene in ovarian cancer. Nat. Genet. 1999; 21: 99–102.

- Shin I, et al. Arteaga CL. PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. Nat Med. 2002; 8: 1145-1152.

- Shioi T., et al.The conserved phosphoinositide 3-kinase pathway determines heart size in mice, EMBO J. 2000; 19: 2537–2548.

- Sivertsen, E.A., et al. (2006). PI3K/Akt-dependent Epo-induced signaling and target genes in human early erythroid progenitor cells. Br J Haematol, 135, 117-128.

- Skinner H.D., et al. Vascular endothelial growth factor transcriptional activation is mediated by hypoxiainducible factor 1alpha, HDM2, and p70S6K1 in response to phosphatidylinositol 3-kinase/AKT signaling. J. Biol. Chem. 2004; 279: 45643–45651.

- Slovak ML, et al. Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: A Southwest Oncology Group/Eastern Cooperative Oncology Group study. Blood. 2000; 96: 4075−4083.

- Smith TJ, et al. Update of Recommendations for the Use of White Blood Cell Growth Factors: An Evidence-Based Clinical Practice Guideline. Journal of Clinical Oncology. 2006; 24: 3187–205.

- Sonenshein, G. E. Rel/NF-κB transcription factors and the control of apoptosis. Semin. Cancer Biol. 1997; 8: 113–119.

- Srinivas, H., et al. (2006). Akt phosphorylates and suppresses the transactivation of retinoic acid receptor alpha. Biochem J, 395, 653-662.

- Staal S.P. Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma, Proc. Natl. Acad. Sci. U. S. A. 1987; 84: 5034–5037.

- Stahl J.M., et al. Cheng, M.W. Bosenberg, M. Kester, L. Sandirasegarane, G.P. Robertson, Deregulated Akt3 activity promotes development of malignant melanoma, Cancer Res. 2004; 64: 7002–7010.

- Stambolic V., et al. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell. 1998; 95: 29–39.

- Starkman BG, et al. IGF-I stimulation of proteoglycan synthesis by chondrocytes requires activation of the PI 3-kinase pathway but not ERK MAPK. Biochem J. 2005; 389: 723-9.

- Stephens L., et al. Phosphoinositide 3-kinases as drug targets in cancer, Curr. Opin. Pharmacol. 2005; 5: 357–365.

- Stephens L.R., et al.The G beta gamma sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. Cell. 1997; 89: 105–114.

- Stiles B, et al. Essential role of AKT-1/protein kinase B alpha in PTEN-controlled tumorigenesis. Mol. Cell. Biol. 2002; 22: 3842–3851.

- Stone RM and Mayer RJ. The approach to the elderly patient with acute myeloid leukemia. Hematology/Oncology Clinics of North America. 1993; 7: 65−79.

- Stone RM, et al. Acute myeloid leukemia. Hematology. 2004: 98–117.

- Stone RM, et al. Granulocyte-macrophage colony-stimulating factor after initial chemotherapy for elderly patients with primary acute myelogenous leukemia. New England Journal of Medicine. 1995; 332: 1671–7.

- Stone RM, et al. Postremission therapy in older patients with de novo acute myeloid leukemia: a randomized trial comparing mitoxantrone and intermediate-dose cytarabine with standard-dose cytarabine. Blood. 2001; 98: 548–53.

- Stone RM. Novel therapeutic agents in acute myeloid leukemia. Experimental Hematology. 2007; 35: 166–7.

- Stoyanov B., et al., Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase. Science. 1995; 269: 690–693.

- Suhara, T., et al. Suppression of Akt signaling induces Fas ligand expression: involvement of caspase and Jun kinase activation in Akt-mediated Fas ligand regulation. Mol. Cell. Biol. 2002; 22: 680–691

- Summers S.A., et al. The role of glycogen synthase kinase 3beta in insulinstimulated glucose metabolism, J. Biol. Chem. 274 (1999) 17934–17940.

- Sun,H. et al. (1999) PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. Proc. Natl Acad. Sci. USA, 96, 6199–6204.

- Suzuki A., et al. High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice, Curr. Biol. 8 (1998) 1169–1178.

- Tallman MS, et al. Drug therapy for acute myeloid leukemia. Blood 2005;106:1154−1163.

- Tamura,M. et al. (1999) Tumor suppressor PTEN inhibition of cell invasion, migration, and growth: differential involvement of focal adhesion kinase and p130Cas. Cancer Res., 59, 442–449.

- Tan, Y., et al. (1999) p90RSK blocks bad-mediated cell death via a protein kinase C-dependent pathway. J. Biol. Chem. 274, 34859–34867

- Tan, Y., et al. (2000) BAD Ser-155 phosphorylation regulates BAD/Bcl-XL interaction and cell survival. J. Biol. Chem. 275, 25865–25869

- Tang, E. D., et al. (1999) Negative regulation of the forkhead transcription factor FKHR by Akt. J. Biol. Chem. 274, 16741–16746

- Tazzari P.L., et al. Martelli, Multidrug resistance-associated protein 1 expression is under the control of the phosphoinositide 3 kinase/Akt signal transduction network in human acute myelogenous leukemia blasts, Leukemia 21 (2007) 427–438.

- Teitell, M. A. (2005) The TCL1 family of oncoproteins: co-activators of transformation. Nat. Rev. Cancer 5, 640–648

- Thomas, et al. (2002) High-resolution structure of the pleckstrin homology domain of protein kinase B/Akt bound to phosphatidylinositol (3,4,5)-trisphosphate. Curr. Biol. **12**, 1256–1262

- Tilly H, et al. Low-dose cytarabine versus intensive chemotherapy in the treatment of acute nonlymphocytic leukemia in the elderly. Journal of Clinical Oncology 1990;8:272–9.

- Tilly H, et al. Treatment of acute myeloblastic leukemia in elderly patients with low-dose cytosine arabonoside [abstract, article in French]. Presse Medicale 1985;14(15): 827–831.

- Toker A and Yoeli-Lerner M. (2006) Akt signaling and cancer: surviving but not moving on. Cancer Res., 66, 3963–3966.

- Tzortzatou-Stathopoulou F, et al. Low relapse rate in children with acute lymphoblastic leukemia after risk-directed therapy. J Pediatr Hematol Oncol. 2001; 23: 591-597. Review.

- Uddin S, et al. Inhibition of phosphatidylinositol 3'-kinase induces preferentially killing of PTEN-null T leukemias through AKT pathway. Biochem Biophys Res Commun 2004; 320: 932-938.

- Um S.H., et al. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity, Nature 431 (2004) 200–205.

- Urso B., et al. The alphaisoform of class II phosphoinositide 3-kinase is more effectively activated by insulin receptors than IGF receptors, and activation requires receptor NPEY motifs, FEBS Lett. 460 (1999) 423–426.

- Valentino L and Pierre J. JAK/STAT signal transduction: regulators and implication in hematological malignancies. Biochem Pharmacol 2006; 71: 713-721.

- Valk PJ, et al. Prognostically useful gene-expression profiles in acute myeloid leukemia. N Engl J Med. 2004; 350: 1617-1628.

- van Dam P.A., et al. Expression of c-erbB-2, c-myc, and c-ras oncoproteins, insulin-like growth factor receptor I, and epidermal growth factor receptor in ovarian carcinoma, J. Clin. Pathol. 47 (1994) 914–919.

- Van den Heuvel-Eibrink MM, et al. CD34-related coexpression of MDR1 and BCRP indicated a clinically resistant phenotype in patients with acute myeloid leukemia (AML) of older age. Annals of Hematology 2007;86(5):329–37.

- Van der Holt B, et al. The value of the MDR1 reversal agent PSC-833 in addition to daunorubicin and cytarabine in the treatment of elderly patients with previously untreated

acute myeloid leukemia (AML), in relation to MDR1 status at diagnosis. Blood 2005;106(8):2646–54.

- Van Leeuwen FE. Risk of acute myelogenous leukemia and myelodysplasia following cancer treatment. Bailliere's Clinical Haematology 1996;9(1):57–85.

- van Weeren, P. C., et al. (1998) Essential role for protein kinase B (PKB) in insulin-induced glycogen synthase kinase 3 inactivation: characterization of dominant-negative mutant of PKB. J. Biol. Chem. 273, 13150–13156

- Vanhaesebroeck, B. and Alessi, D. R. (2000) The PI3K–PDK1 connection: more than just a road to PKB. Biochem. J. 346, 561–576

- Vanhaesebroeck, B., et al. (2005) Signalling by PI3K isoforms: insights from gene-targeted mice. Trends Biochem. Sci. 30, 194–204.

- Varma S and Khandelwal RL. Effects of rapamycin on cell proliferation and phosphorylation of mTOR and p70(S6K) in HepG2 and HepG2 cells overexpressing constitutively active Akt/PKB. Biochim Biophys Acta. 2007; 1770: 71-78.

- Vecchione C., et al. Protection from angiotensin II-mediated vasculotoxic and hypertensive response in mice lacking PI3Kgamma, J. Exp. Med. 201 (2005) 1217–1228.

- Viglietto G, et al. Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer. Nat Med. 2002; 8: 1136-1144.

- Vivanco I. and C.L. Sawyers, The phosphatidylinositol 3-kinase AKT pathway in human cancer, Nat. Rev., Cancer 2 (2002) 489–501.

- Vogt et al. Cancer-specific mutations in phosphatidylinositol 3-kinase. Trends Biochem. Sci. 2007; 32: 342-349.

- Voigt, P., et al. (2005) Assigning functional domains within the p101 regulatory subunit of phosphoinositide 3-kinase γ. J. Biol. Chem. **280**, 5121–5127.

- Vousden KH. Activation of the p53 tumor suppressor protein. Biochim Biophys Acta. 2002; 1602: 47-59. Review.

- Wallen H, et al. Ablative allogenic hematopoietic cell transplantation in adults 60 years of age and older. Journal of Clinical Oncology 2005;23(15): 3439–46.

- Wang D, et al. Correlation between MDR1 genetic polymorphism and prognosis in acute myeloid leukemia [abstract, article in Chinese]. Zhonghua Yi Xue Za Zhi 2007;87(20): 1384-1388.

- Wang G.L., et al.Hypoxia-inducible factor 1 is a basic-helix–loop–helix–PAS heterodimer regulated by cellular O2 tension, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 5510–5514.

- Ward S.G. and P. Finan, Isoform-specific phosphoinositide 3 (2003) 426–434.

- Webb, P. R., et al. (2000) Regulation of neutrophil apoptosis: a role for protein kinase C and phosphatidylinositol-3-kinase. Apoptosis **5**, 451–458

- Weng AP, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science 2004; 306: 269-271.

- Weng AP, et al. c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. Genes Dev 2006; 20: 2096-2109.

- Weng,L. et al. (2001) PTEN induces apoptosis and cell cycle arrest through phosphoinositol-3-kinase/Akt-dependent and -independent pathways. Hum. Mol. Genet., 10, 237–242.

- Weng,L.P. et al. (2001) PTEN coordinates G(1) arrest by down-regulating cyclin D1 via its protein phosphatase activity and up-regulating p27 via its lipid phosphatase activity in a breast cancer model. Hum. Mol. Genet., 10, 599–604.

- White-Gilbertson, S., et al. (2008) Transformation, translation and TRAIL: an unexpected intersection. Cytokine Growth Factor Rev. **19**, 167–172

- Whitman SP, et al. The MLL partial tandem duplication: Evidence for recessive gain-of-function in acute myeloid leukemia identifies a novel patient subgroup for molecular-targeted therapy. Blood 2005;106:345−352.

- Whitman, M., et al. (1985) Association of phosphatidylinositol kinase activity with polyoma middle-T competent for transformation. Nature 315, 239–242.

- Whitman, M., et al. (1987) Evidence for two distinct nphosphatidylinositol kinases in fibroblasts: implications for cellular regulation. Biochem. J. 247, 165–174

- Whitman, M., et al. (1988) Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. Nature 332, 644–646

- Wick, M. J., et al. (2003) Mouse 3-phosphoinositide-dependent protein kinase-1 undergoes dimerization and trans-phosphorylation in the activation loop. J. Biol. Chem. **278**, 42913–42919

- Williams, M. R., et al. (2000) The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. Curr. Biol. **10**, 439–448

- Willis, S. N. and Adams, J. M. (2005) Life in the balance: how BH3-only proteins induce apoptosis. Curr. Opin. Cell Biol. **17**, 617–625

- Willner J., et al. Alternate molecular genetic pathways in ovarian carcinomas of common histological types, Hum. Pathol. 38 (2007) 607–613.

- Woods A., et al. LKB1 is the upstream kinase in the AMP-activated protein kinase cascade, Curr. Biol. 13 (2003) 2004–2008.

- Wu H., et al. PTEN signaling pathways in melanoma, Oncogene 22 (2003) 3113–3122.

- Wu, J. and Kaufman, R. J. (2006) From acute ER stress to physiological roles of the unfolded protein response. Cell Death Differ. **13**, 374–384

- Wu, X. and Deng, Y. (2002) Bax and BH3-domain-only proteins in p53-mediated apoptosis. Front. Biosci. **7**, d151–d156

- Wullschleger S., et al. TOR signaling in growth and metabolism, Cell 124 (2006) 471–484.

- Wymann M.P. and R. Marone, Phosphoinositide 3-kinase in disease: timing, location, and scaffolding, Curr. Opin. Cell Biol. 17 (2005) 141–149.

- Wymann M.P., et al. Phosphoinositide 3-kinase signaling—Which way to target? Trends Pharmacol. Sci. 24 (2003) 366–376.

- Xia C., et al. Regulation of angiogenesis and tumor growth by p110 alpha and AKT1 via VEGF expression, J. Cell. Physiol. 209 (2006) 56–66.

- Xin, M., et al. (2007) Protein kinase Cζ abrogates the proapoptotic function of Bax through phosphorylation. J. Biol. Chem. 282, 21268–21277

- Xing D and Orsulic S. (2005) A genetically defined mouse ovarian carcinoma model for the molecular characterization of pathway-targeted therapy and tumor resistance. Proc. Natl Acad. Sci. USA, 102, 6936–6941.

- Xu Q, et al. Survival of acute myeloid leukemia cells requires PI3 kinase activation. Blood 2003; 102: 972-980.

- Xuan Nguyen, T.L., et al. (2006). Akt phosphorylation is essential for nuclear translocation and retention in NGF-stimulated PC12 cells. Biochim Biophys Res Commun, 349, 789-798.

- Yamamoto S., et al. Prognostic significance of activated Akt expression in pancreatic ductal adenocarcinoma, Clin. Cancer Res. 10 (2004) 2846–2850.

- Yang, E., et al. (1995) Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell 80, 285–291

- Yang, H. Y., et al. (2007) Roles for negative cell regulator 14-3-3σ in control of MDM2 activities. Oncogene 26, 7355–7362

- Yang, J., et al. (2002) Molecular mechanism for the regulation of protein kinase B/Akt by hydrophobic motif phosphorylation. Mol. Cell **9**, 1227–1240

- Yao, R. and Cooper, G. M. (1995) Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science **267**, 2003–2006

- Yilmaz OH, et al. Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. Nature 2006; 441: 475-482.

- You, H., et al. (2006) FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal. J. Exp. Med. 203, 1657–1663

- Zha, J., et al. (1996) Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-XL. Cell 87, 619–628

- Zha, J., et al. (1997) BH3 domain of BAD is required for heterodimerization with BCL-XL and pro-apoptotic activity. J. Biol. Chem. 272, 24101–24104

- Zhang A., et al. Genetic alterations in cervical carcinomas: frequent low-level amplifications of oncogenes are associated with human papillomavirus infection, Int. J. Cancer 101 (2002) 427–433.

- Zhang J, et al. PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. Nature 2006; 441: 518-522.

- Zhang, J. and Lodish, H.F. (2005). Identification of K-ras as the major regulator for cytokine-dependent Akt activation in erythroid progenitors in vivo. Proc Natl Acad Sci USA, 102, 14605-14610.

- Zhao, W., et al. (2006). Erythropoietin stimulates phosphorylation and activation of GATA-1 via the PI3-kinase/AKT signaling pathway. Blood, 107, 907-915.

- Zhou J, et al. In vivo activity of ABT-869, a multi-target kinase inhibitor, against acute myeloid leukemia with wild-type FLT3 receptor. Leukemia Research 2007; (Article in press), doi:10.1016/j.leukres. 2007.11.025.

- Zhou QL, et al. Akt substrate TBC1D1 regulates GLUT1 expression through the mTOR pathway in 3T3-L1 adipocytes. Biochem J. 2008; 411: 647-655.

- Zhu J, et al. Nephrin mediates actin reorganization via phosphoinositide 3-kinase in podocytes. Kidney Int. 2008; 73: 556-566.

- Zunino SJ and Storms DH. Resveratrol-induced apoptosis is enhanced in acute lymphoblastic leukemia cells by modulation of the mitochondrial permeability transition pore. Cancer Lett 2006; 240: 123-134.

- Zweidler-McKay PA and Pear WS. Notch and T cell malignancy. Semin Cancer Biol 2004; 14 329-340.

Acknowledgement

Nuclear translocation of active AKT is required for erythroid differentiation in erythropoietin treated K562 erythroleukemia cells

Silvia Missiroli [a,1], Daniela Etro [a,1], Francesca Buontempo [a], Keqiang Ye [b], Silvano Capitani [a], Luca Maria Neri [a,*]

[a] *Dipartimento di Morfologia ed Embriologia, Sezione di Anatomia Umana, Signal Transduction Unit, Universita' di Ferrara, Ferrara, Italy*
[b] *Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322, USA*

ARTICLE INFO

ABSTRACT

Erythroid differentiation of human erythroleukemia cell line K562 induced by erythropoietin is a complex process that involves modifications at nuclear level, including nuclear translocation of phosphatidyl-inositol 3-kinase.

In this work we show that erythropoietin stimulation of K562 cells can induce nuclear translocation of active Akt, a downstream molecule of the phosphatidyl-inositol 3-kinase signaling pathway. Akt shows a peak of activity in whole cell homogenates at earlier stage when compared to the nucleus, which shows a peak delayed of 10 min. Akt increases its intranuclear amount and activity rapidly and transiently in response to EPO. Almost all Akt kinase that translocates to the nucleus shows a marked phosphorylation on serine 473. Nuclear enzyme translocation is blocked by the phosphatidyl-inositol 3-kinase inhibitor Ly294002 or Wortmannin. The specific Akt pharmacological inhibitor VI, VII and VIII that act as blocking enzyme activation inhibited translocation as well, whereas Akt inhibitor IX, that inhibits Akt activity, did not block Akt nuclear translocation.

When cells were treated by means of siRNA sequences or with the Akt inhibitors the differentiation process was arrested, thus showing the requirement of the nuclear translocation of the active enzyme to differentiate.

These findings strongly suggest that the intranuclear translocation of active Akt kinase represents an important step in the signaling pathway that mediates erythropoietin-induced erythroid differentiation.

© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

The phosphoinositide 3-kinase (PI3K)/Akt signaling network is crucial to widely divergent physiological processes that include cell cycle progression, differentiation, transcription, translation and apoptosis (Brazil et al., 2004). Membrane localization of PI3K by growth factors stimulates the generation of phosphatidylinositol (3,4,5) trisphosphate (PtdIns(3,4,5)P$_3$), activating a number of important downstream substrates (Hanada et al., 2004). The serine/threonine kinase Akt is a well-characterized downstream target of PI3K. In unstimulated cells, Akt resides within the cytoplasm in an inactive state, but binding of PtdIns(3,4,5)P$_3$ to its pleckstrin homology (PH) domain recruits inactive Akt to the plasma mem-

brane. To complete Akt activation, a first phosphorylation within its catalytic loop at threonine 308 by phosphoinositide-dependent protein-kinase 1 (PDK1) and a second phosphorylation, commonly believed to represent full activation of Akt, on serine 473 in its C-terminally hydrophobic motif by a kinase not yet conclusively identified are needed (Williams et al., 2000; Sarbassov et al., 2005). Activated Akt phosphorylates and modulates the activity of many proteins in cell growth, survival and apoptosis, including BAD and Caspase 9, apoptosis signal regulated kinase 1 (Ask1), FoxO family transcription factors, glycogen synthetase kinase 3β, and inhibitor kinase B (IkB) kinase (IKK) function (Chang et al., 2003; Steelman et al., 2004; Hennessy et al., 2005). Akt is involved in differentiation by activating nuclear transcription factors such as the forkhead transcription factor FKHR essential for differentiation and survival of hematopoietic and neuronal cells (Gotz et al., 2005).

Binding of Erythropoietin (EPO) to its receptor (EPO-R), essential for erythroid cell proliferation and differentiation (Bunn, 2007), triggers the phosphorylation and activation of EPO-R bound JAK2 tyrosine kinase, resulting in activation of several downstream signaling pathways that include PI3K/Akt pathway, MAP kinase, STAT5

---

and protein kinase C (Jelkmann, 2007). Several studies suggest that the PI3-kinase/AKT signaling pathway may have an important role in supporting erythropoiesis (Klingmuller et al., 1997; Huddleston et al., 2003; Hammerman et al., 2005) and AKT is rapidly phosphorylated and activated in response to EPO (Kashii et al., 2000; Bouscary et al., 2003).

EPO scarcely stimulates K562 human erythroleukemia cell proliferation, but induces erythroid differentiation (Kitanaka et al., 1994). Suppression of PI3K activity by the fungal metabolite Wortmannin reduces the expression of erythroid differentiation in K562 cells (Kubota et al., 1996) and antisense src RNA expression interferes with hemoglobin synthesis, lying src immediately upstream of PI3K in the signal transduction of erythroid differentiation induced by EPO (Kubota et al., 2001).

We have previously demonstrated that, in addition to its well-established role at the plasma membrane, PI3K translocates to the nucleus in response to EPO exposure of K562 cells, with increased PI3K activity and $PtdIns(3,4,5)P_3$ production that occurs early after treatment at the nuclear level (Neri et al., 2002a,b). Indeed evidence collected from other leukemic cell types points to a key role played by PI3K (Marchisio et al., 1998; Neri et al., 1999a) and by Akt (Matkovic et al., 2006) in the transduction of differentiating signals from cell periphery to the nucleus.

Since we observed that PI3K plays a role in erythroid differentiation at the nuclear level, we felt that K562 could constitute a useful model to investigate the relevance of the PI3K downstream molecule Akt in erythroid differentiation induced by EPO. Here we show that PI3K activation is a prerequisite for the nuclear translocation of Akt, that Akt activation by phosphorylation is also critical for its nuclear translocation and that increased Akt activity rapidly occurs in the nucleus in response to the cytokine. These events appear to be essential for EPO-induced erythroid differentiation that is blocked by Akt down modulation or Akt inhibitors, indicating that nuclear Akt may play a crucial role triggering the signal transduction cascade necessary for EPO's erythroid differentiating signal from cell periphery to the nucleus.

2. Materials and methods

2.1. Materials

RPMI-1640, fetal calf serum (FCS), normal goat serum (NGS), bovine serum albumin (BSA), peroxidase-conjugated IgG, antibody to β-tubulin and β-actin, ATP, acrylamide/bis-acrylamide 37:1 and 1,4-diazabicycle-[2.2.2]octane were from Sigma (St. Louis, MO). Human recombinant erythropoietin (EPO) and Lumi-Light chemo-luminescence detection kit were from Roche Molecular Biochemicals (Germany). Anti CD71 FITC was from BD Pharmingen, Milan, Italy. [γ-^{32}P] ATP and Cy2 secondary antibodies were from Amersham Pharmacia Biotech (Uppsala, Sweden). Antibody to lamin-B, Akt inhibitor VI H-AVTDHPDRLWAWEKF-OH (TCL110-24), VII H-YGRKKRRQRRR-AVTDHPDRLWAWEKF-OH (TAT-TCL110-24), VIII 1,3-Dihydro-1-(1-((4-(6-phenyl-1H-imidazo [4,5-g] quinoxalin-7-yl) phenyl) methyl)-4-piperidinyl)-2H-benzimidazol-2-one, IX 9-Methoxy-2-methylellipticinium acetate, Wortmannin and Ly294002 were from Merck-Calbiochem (La Jolla, CA). Small interfering RNA (siRNA) sequences targeting the human Akt1 gene (Gene accession number NM_001014431) were purchased from QIAGEN (QIAGEN S.p.A., Milan, Italy). Antibody to Akt was from Transduction Laboratories (Franklin Lakes, NJ) and anti p308Thr Akt, anti p473Ser Akt, anti-phosphorylated Akt substrate (PAS) antibodies from Cell Signaling Technology Inc. (Danvers, MA). Immunoprecipitation matrix was from Santa Cruz Biotechnology (Santa Cruz, CA); the kit for protein concentration detection was from Pierce (Rockford, IL). Histone

H2B was from Boehringer Mannheim and recH2B was from UBI (Lake Placid, NY).

2.2. Cell culture and hemoglobin detection

K562 human erythroleukemia cells were cultured in RPMI-1640 medium with 10% FCS. EPO stimulation was performed at 3 U/ml. LY294002 was used at 25 μM and Wortmannin was used at 100 nM, the PI3K inhibitors were administered 1 h prior EPO stimulation, Akt inhibitor VI and VII were used at 50 μM final concentration, VIII at 25 μM and IX at 20 μM; the Akt inhibitors were administered 12 h prior to EPO stimulation. The hemoglobin determination kit was from Vector Laboratories (Burlingame, CA). For hemoglobin determination, the benzidine staining solution was freshly prepared as described in the manufacturer's protocol. The expression of the specific erythroid antigen CD71 on K562 cell surface was determined by direct immunofluorescent staining with FITC conjugated mouse monoclonal antibodies against human CD71. Analysis was performed with FACScan from Becton Dickinson. FITC conjugated mouse IgG1 was used as negative control.

2.3. Preparation of cell and nuclear fractions

This was accomplished as reported by Bertagnolo et al. (1997).

2.4. Western blotting analysis

Proteins separated on 0.1% SDS-PAGE were transferred to nitro-cellulose sheets, stained by Ponceau S and saturated in TBS with 5% not-fat dry milk and 0.1% Tween-20 (T) 1 h at room temperature (RT), then incubated 2 h in blocking buffer containing primary antibody 1:1000 dilutions. After 4 washes in TBS with 0.1% T, samples were incubated 45 min at RT with secondary antibody and washed as previously described. Bands were visualized by chemo-luminescence method. Anti-actin antibody was used to ensure equal protein loading and anti β-tubulin antibody to assess purity of the nuclear fraction.

2.5. Immunoprecipitation (IP)

Total or nuclear homogenates from 10^7 or 10^8 cells respectively were resuspended in 50 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS plus phosphatases and proteases inhibitors. IP was performed according to the manufacturer's instruction using the IP matrix.

2.6. Akt kinase activity assay

Total Akt IP were resuspended in 20 mM HEPES pH 7.4, 10 mM MgCl$_2$, 10 mM MnCl$_2$. The reaction was started by mixing 10 μl IP, 100 μM ATP, 100 μCi/ml [γ-^{32}P] ATP or P-ATP, 4 μg H2B and incubated 40 min at RT. The reaction was stopped by adding 1 M Tris pH 6.8, 8% SDS, 40% glycerol, 20% β-mercapto-ethanol and samples were loaded in SDS-PAGE. After electrophoresis, gel was dried and H2B phosphorylation was revealed by auto-radiography or Western blot.

2.7. In situ immunofluorescence

Cells plated onto 0.1% poly-L-lysine-coated glass slides were fixed in ethanol 10 min at RT, incubated in PBS with 2% BSA and 3% NGS for 30 min and washed with 5 mM MgCl$_2$ PBS. Antibody to Akt, p473Ser Akt and Cy2 were diluted 1:100, 1:75 and 1:1000 respectively, in PBS with 2% BSA, 3% NGS, 5 mM MgCl$_2$. The coverslips were mounted in glycerol containing 1,4-diazabicyclo [2.2.2]

octane to retard fading; slides were observed using a Zeiss Axiophot epifluorescence microscope coupled with a Photometric Cool Snap CCD camera for image acquisition.

2.8. RNA interference assays

Small interfering RNA (siRNA) sequences targeting the human Akt1 gene (Gene accession number NM_001014431) were synthesized by QIAGEN (QIAGEN S.p.A., Milan, Italy). Four specific sequence were as follows: Hs_Akt1_6 sense r(CCA GGA CCA UGA GAA GCU U)dTdT, antisense r(AAG CUU CUC AUG GUC CUG G)dTdT; Hs_Akt1_5 sense r(UCA CAC CAC CUG ACC AAG A)dTdT, antisense r(UCU UGG UCA GGU GGU GUG A)dTdT; Ha_Akt1_10 sense r(CGC UUG GUC CCG AGG CCA A)dTdT, antisense r(UUG GCC UCG GGA CCA AGC G)dTdG; Hs_Akt1_11 sense r(CCA UGA GCG ACG UGG CUA U)dTdT, antisense r(AUA GCC ACG UCG CUC AUG G)dTdG. The lyophilized siRNA was dissolved in sterile Suspension Buffer (100 mM potassium acetate, 30 mM Hepes-KOH, 2 mM magnesium acetate, pH 7.4) to obtain a 20 μM solution. Then, oligonucleotides were heated to 90 °C for 1 min followed by 1 h at 37 °C. As a control, non-silencing fluorescein-labeled duplex siRNA, also purchased from QIAGEN, was used.

Exponentially growing K562 cells were transfected in contemporary with 6 μg of each siRNA duplexes using Lipofectamine2000 (Invitrogen), according to the manufacturer's protocols. In brief, 1 day before transfection, cells were seeded without antibiotics at 10^6 cells/well in six-well tissue culture plate. The cells were transfected with siRNA and were incubated with siRNA plus Lipofectamine2000 in Opti-MEM I or in Opti-MEM I with Lipofectamine2000 (control cells). After 5 h, cells transfected with fluorescein-labeled siRNA were subjected to cytofluorimetric analysis to determine transfection efficiency that resulted higher than 60%. After 48 h from transfection cells were treated with EPO for 10 min and then cells were harvested for analysis.

3. Results

3.1. EPO determines subcellular redistribution of phosphorylated p473Ser Akt in K562 cells

Since previous investigations have highlighted that p-Akt may migrate to the nucleus (reviewed in Neri et al., 2002b), we sought to analyze the subcellular distribution of Akt after its serine 473 residue phosphorylation in K562 erythroleukemia cells, that were induced to differentiate by 96 h of EPO treatment. As shown in Fig. 1, either with Benzidine staining or with flow cytometric analysis with anti CD71, after 4 days of treatment more than 50% of cells were differentiated.
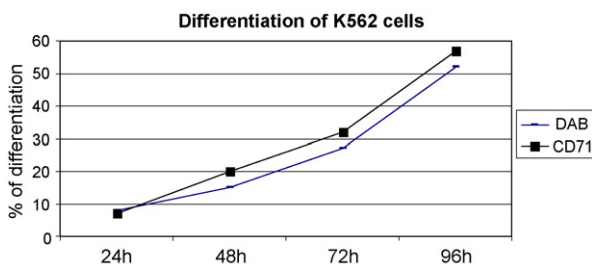

Fig. 1. K562 differentiation after EPO exposure. Either with Benzidine staining or with anti CD71 flow cytometric analysis, after 96 h of EPO treatment K562 cells disclosed more than 50% of erythroid differentiation. Viability was always more than 90% and S.D. was less than 10%. Data are representative of three independent experiments.
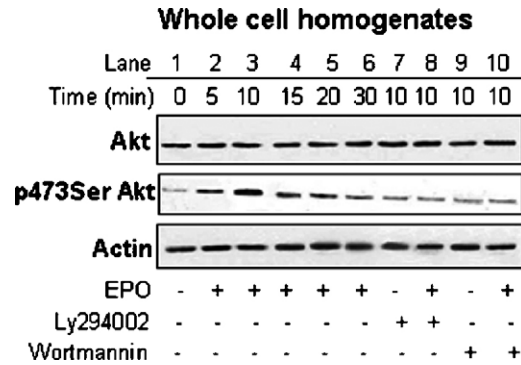

Fig. 2. Akt expression and phosphorylation levels in K562 cells. Western blot on unstimulated K562 cells, cells exposed to EPO or cells exposed to EPO plus Ly294002 or Wortmannin. In each lane 20 μg proteins were loaded, as confirmed by anti-actin antibody.

No changes of total Akt amount were found in all the examined times of EPO treatment. However, using the anti p473Ser Akt antibody, we found that Akt activation was evident already at 5 min, with a peak of Akt phosphorylation 10 min after EPO stimulation and decreased until 30 min, maintaining a phosphorylation level higher than the untreated control (Fig. 2). Pre-treatment of cells with 25 μM Ly294002 or 100 nM Wortmannin alone did not modify Akt levels. Cells pre-treated with 25 μM Ly294002 or 100 nM Wortmannin before EPO stimulation showed Akt expression and phosphorylation levels comparable, at each time point, to those of untreated cells.

Western blotting performed on nuclei isolated from K562 EPO-treated cells showed increased amount of total nuclear Akt in a range from 10 to 20 min, with a peak at 15 min (Fig. 3A). The nuclear immunolabelling of p473Ser Akt started 10 min after treatment, increased to a maximum intensity 15 min after stimulation in parallel with total Akt nuclear translocation and after 30 min was slightly higher than the control sample. Cell number was not affected by pre-treatment with Ly294002 before EPO stimulation and no modifications of Akt distribution and phosphorylation state were observable. In Ly294002 or Wortmannin and EPO treated samples, Akt expression and phosphorylation levels were comparable to those of untreated cells. The human leukemia T-cell derived Jurkat cell line was used as positive control (Fukuda et al., 2005; Seminario et al., 2003).

Immunocytochemical analysis showed total Akt mainly in the cytoplasm of untreated K562 cells. Nevertheless, nuclei, identified by means of phase-contrast microscopy (data not shown), showed also in control cells the presence of only a small amount of fluorescence (Fig. 3B). Immunostaining intensity increased in the nucleus after 15 min of EPO stimulation and greatly decreased, at the level of untreated cells, after 30 min of treatment. Cells pre-treatment with 25 μM Ly294002 prevented the intranuclear Akt translocation. Immunostaining using anti p473Ser Akt antibody in control K562 cells showed fluorescence predominantly located in the cytoplasm (Fig. 3B). After 15 min of EPO stimulation, immunofluorescence increased and concentrated inside the nuclear compartment to strongly decrease after 30 min. Pre-treatment with Ly294002 prevented the p473Ser Akt translocation into the nucleus after 15 min of EPO treatment (Fig. 3B).

3.2. Exposure of K562 cells to EPO increases Akt kinase activity in whole cell homogenates and in nuclei

K562 cells treated with EPO exhibited an increase of total immunoprecipitable Akt activity levels in kinase assay performed
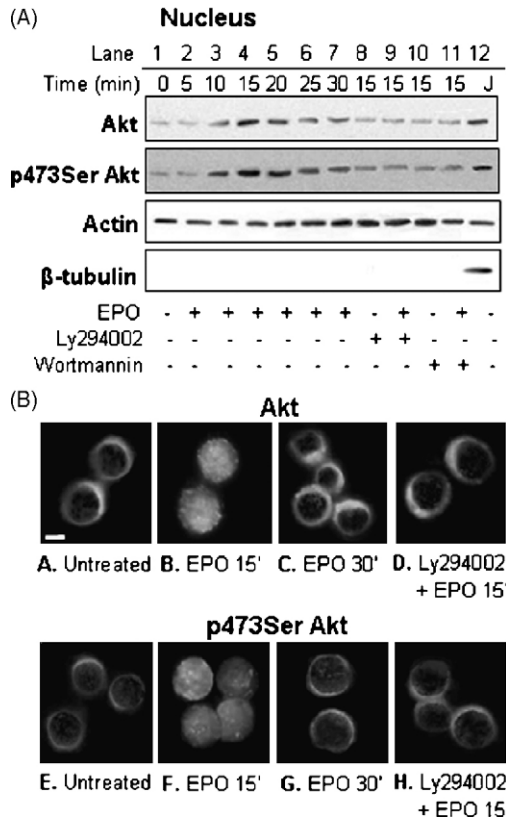
(A)

Fig. 3. Akt expression and phosphorylation levels in nuclei of K562 cells. (A) Western blot on unstimulated K562 cells, cells exposed to EPO or cells exposed to EPO plus Ly294002 or Wortmannin. Total homogenates from Jurkat cells (J) were used as positive control for Akt MW. In each lane 20 μg proteins were loaded, as confirmed by anti-actin antibody. β-Tubulin assessed absence of cytoplasmic contaminants in nuclear fractions. (B) Total and p473Ser Akt distribution in K562 cells treated with EPO studied by fluorescence microscopy. Scale bar: 5 μm.

in vitro toward the exogenous substrate histone H2B. A rise of Akt activity was detected early after 5 min and peaked 10 min after EPO treatment in whole cell homogenates, to reduce, after 30 min, to levels still higher than the control. The dependence on the PI3K/Akt pathway was further assessed, when cells, pre-treated with 25 μM Ly294002 or with 100 nM Wortmannin, even if stimulated with EPO did not show any increase in Akt activity (Fig. 4A). Akt immunoprecipitated from nuclei showed after 10 min an increased enzymatic activity, that reached a peak 15 min after EPO stimulation, was still high after 20 min and lasted even if at a lower intensity until 30 min.

Cells pre-treated with 25 μM Ly294002 or 100 nM Wortmannin exhibited Akt activity levels comparable to those of untreated cells (Fig. 4B).

Similar results were obtained using anti-phosphorylated Akt substrate (PAS) antibody to reveal phosphorylation of recombinant histone H2B after in vitro Akt kinase assay. In whole cell homogenates the highest labeling was detectable after 10 min of EPO treatment (Fig. 4C) and in isolated nuclei an increase in phosphorylation of H2B PAS sites (i.e. serine/threonine phosphorylation) was observed maximal after 15 min of EPO administration (Fig. 4D).

3.3. The majority of nuclear Akt is phosphorylated on serine 473 residue after EPO stimulation

To exclude that nuclear Akt activity could be simply a consequence of its translocation to the nucleus, the amount of phosphorylated versus unphosphorylated Akt in nuclei of K562 cells treated with 3 U/ml EPO was compared by performing double round immunoprecipitation from nuclear fractions. A first immunoprecipitation round was performed using anti p473Ser Akt antibody and revealed by Western blot using anti Akt. Increased p473Ser Akt in nuclei was observed after 15 min of EPO treatment (Fig. 5). A little amount of p473Ser Akt, appreciably more intense than control nuclei, was immunoprecipitable after 30 min of EPO administration.

The second round of immunoprecipitation using anti Akt antibody was carried out on the supernatants derived from first
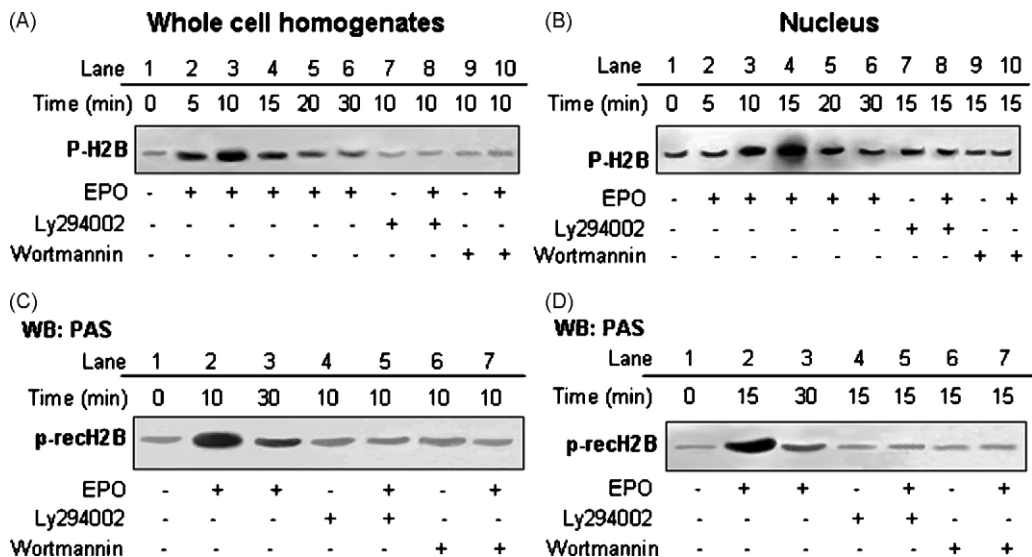

Fig. 4. Total Akt kinase activity. (A) Autoradiogram of Akt activity on whole K562 cell homogenates, after EPO administration alone or plus Ly294002 or Wortmannin. The Akt kinase assay was performed toward the exogenous substrate histone H2B by using radioactive isotope. (B) Autoradiogram of nuclear Akt activity after EPO exposure alone or plus Ly294002 or Wortmannin. The Akt kinase assay was performed toward the exogenous substrate histone H2B by using the radioactive isotope. (C) Non-radioactive analysis showing the results of total Akt activity on whole K562 cell homogenates, after EPO alone or plus Ly294002 or Wortmannin. The Akt kinase assay was performed toward the exogenous substrate recombinant histone H2B and revealed by Western blot with anti-PAS antibody. (D) Non-radioactive analysis showing the results of nuclear Akt activity, after EPO alone or plus Ly294002 or Wortmannin. The Akt kinase assay was performed toward the exogenous substrate recombinant histone H2B and revealed by anti-PAS antibody.
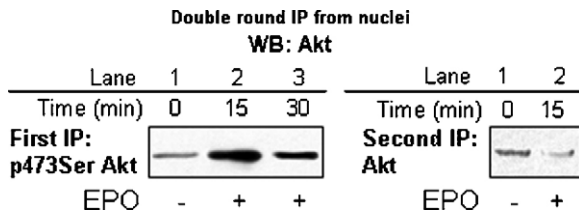
Fig. 5. The majority of nuclear Akt is phosphorylated. Panel on the left: first round of IP carried out with anti-p473Ser Akt on nuclei isolated from K562 cells. Panel on the right: second round of IP with anti Akt antibody. Western blot detection was carried out with anti Akt antibody. Results were replicated in three independent experiments.

immunoprecipitation round. The remaining unphosphorylated Akt was revealed by anti Akt antibody. This analysis showed that unphosphorylated Akt was very faint or extremely low compared to p473Ser Akt level in both samples, thus showing that almost all nuclear Akt is phosphorylated on serine 473 residue (Fig. 5).

3.4. Different Akt inhibitors induce different levels of Akt phosphorylation on serine 473 residue

In order to test whether the activation of nuclear Akt in EPO treated cells is required for differentiation to proceed or whether the increase is only a consequence of differentiation and to explore the relevance of this activation, we used a panel of Akt inhibitors interfering with the activation process (inhibitors VI, VII and VIII) or acting on its kinase activity after the activation (inhibitors IX). Inhibitor VI and VII interact with the PH domain, inhibit PtdIns(3,4,5)P$_3$ binding to Akt and consequently membrane translocation and activation of Akt (Hiromura et al., 2004). Inhibitor VIII was found to be cell active and to block phosphorylation of Akt at threonine 308 and serine 473 thus reducing the levels of active Akt in cells (Barnett et al., 2005). At variance, inhibitor IX selectively inhibits Akt kinase activity without blocking phosphorylation of Akt itself at either threonine 308 or serine 473 (Jin et al., 2004).

Akt total amount was unchanged, independently from EPO or inhibitor treatments. When cells treated with EPO were pre-treated with inhibitors blocking Akt activation, no detectable raise in phosphorylation on serine 473 was observed. On the contrary, the inhibitor acting on the kinase activity of Akt did not modify the increase of phosphorylation level on the 473 serine residue (Fig. 6A).

We used Akt inhibitor VIII or IX as representative inhibitors of the two different mechanisms of Akt inhibition to detect total and p473Ser Akt localization by immunofluorescence during EPO stimulation and inhibitors treatment. In control cells both total or p473Ser phosphorylated Akt are distributed throughout the cytoplasm and their distribution is not affected by inhibitors (Fig. 6B and C, images A and D control, B and E). Treatment with Akt inhibitor VII did not modify the distribution of Akt in both native and phosphorylated form after EPO exposure (Fig. 6B and C, image C). On the contrary, in Akt inhibitor IX-treated samples, 15 min of EPO administration triggered nuclear translocation of total or p473Ser phosphorylated Akt (Fig. 6B and C, image F).

3.5. The phosphorylation state of Akt determines its translocation to the nuclear compartment

We performed subcellular fractionation experiments in order to determine whether or not Akt phosphorylation and activation could be related to its nuclear translocation. We first evaluated the activity of Akt kinase in the nuclear fractions of cells pre-treated for 12 h with the different inhibitors followed by exposure to EPO. The increase of enzymatic activity induced by EPO after
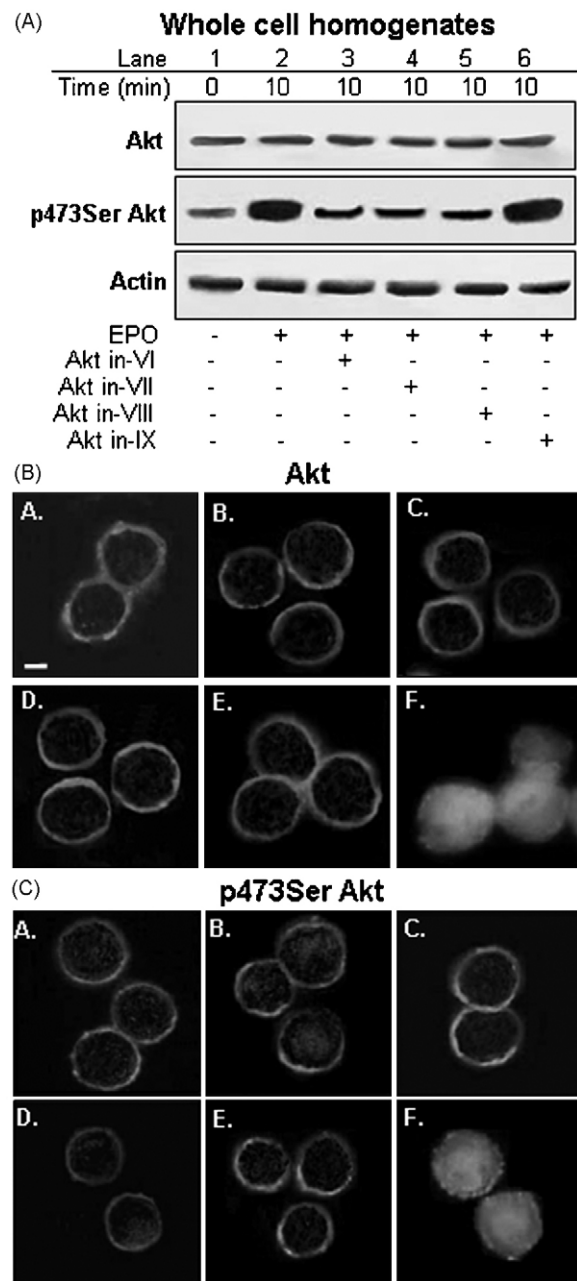

Fig. 6. Akt expression and phosphorylation levels after specific inhibition. (A) Western blot showing Akt expression and phosphorylation levels in whole K562 cell homogenates from unstimulated cells, cells exposed to EPO or cells exposed to EPO after pre-treatment with a panel of different Akt inhibitors: Akt inhibitor VI (in-VI), Akt inhibitor VII (in-VII), Akt inhibitor VIII (in-VIII) and Akt inhibitor IX (in-IX) (B) Analysis of Akt distribution in K562 cells treated with different Akt inhibitors and EPO by fluorescence microscopy. (A) Control cells, (B) cells treated with Akt in-VII, (C) cells pre-treated with Akt in-VII and stimulated for 15 min with EPO, (D) control cells, (E) cells treated with Akt in-IX and (F) cells pre-treated with Akt in-IX and stimulated for 15 min with EPO. Scale bar: 4 μm. (C) Distribution of p473Ser Akt in K562 cells treated with different Akt inhibitors and EPO by fluorescence microscopy. (A) Control cells, (B) cells treated with Akt in-VII, (C) cells pre-treated with Akt in-VII and stimulated 15 min with EPO, (D) control cells, (E) cells treated with Akt in-IX and (F) cells pre-treated with Akt in-IX and stimulated 15 min with EPO.

15 min was abolished independently of the type of Akt inhibitor used (Fig. 7A).

When cells were pre-treated with inhibitors blocking the activation, i.e. the phosphorylation on these activating residues of the enzyme, neither total Akt nor both its p308 and p473 phosphory-
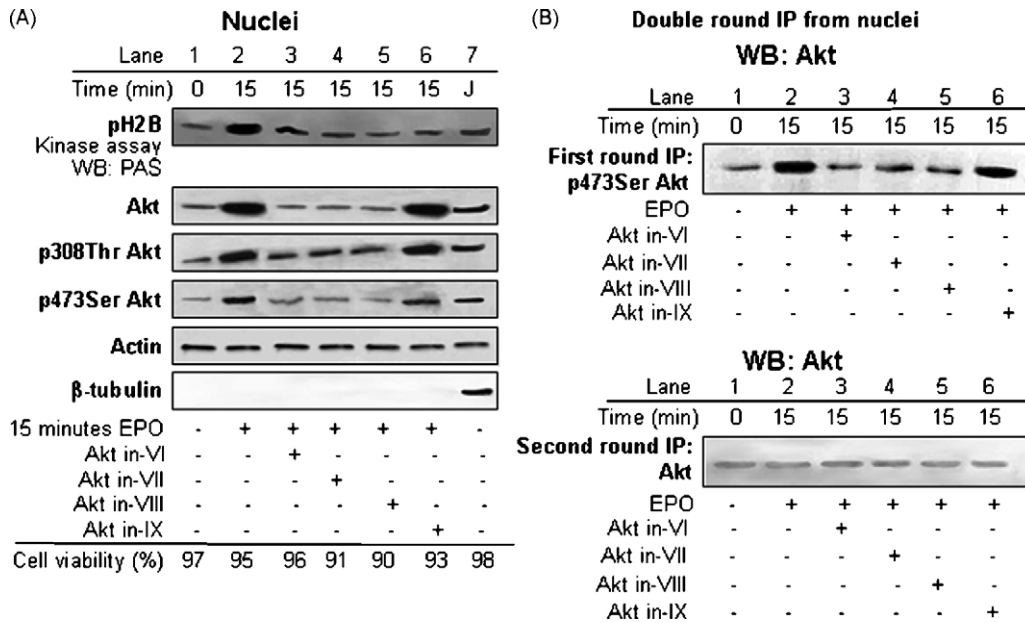
Fig. 7. Effect of different Akt inhibitors on Akt translocation and activity. (A) In the first row is shown Akt kinase activity into nuclei of K562 cells after pre-treatment with different Akt inhibitors and EPO stimulation. In the following rows total Akt, p308Thr Akt and p473Ser Akt into nuclei were also analyzed by Western blotting. Viability of the cells was always more than 90%; data are representative of three different experiments. (B) Upper panel: first round of IP carried out on nuclei of K562 cells with p473Ser Akt. Lower panel: second round of IP with anti Akt antibody performed on first round supernatants. The detection was carried out with anti Akt antibody on control samples and on the samples exposed to EPO for 15 min plus several types of Akt inhibitors.

lated forms translocates to the nuclear interior (Fig. 7A). On the contrary, when cells were treated with inhibitor IX, EPO treatment triggered the nuclear translocation of Akt phosphorylated on threonine 308 residue and on serine 473 residue (Fig. 7A). It is worthy to note that cell viability in all cases was at least 90% (Fig. 7A).

As described above (Fig. 4), we investigated the amount of phosphorylated and unphosphorylated Akt in nuclei of K562, by a first immunoprecipitation round performed using anti p473Ser Akt antibody and a second round using anti Akt antibody was carried out on supernatant of first round of immunoprecipitated samples. In both rounds the product of immunoprecipitation was revealed by Western blot using anti Akt antibody.

This analysis showed that the strong rise in p473Ser Akt labeling in nuclei from cells treated with EPO for 15 min, or with EPO and inhibitor IX, became extremely low after the second round of immunoprecipitation thus showing that, even if its enzymatic activity is inhibited, almost all nuclear Akt, capable to translocate to the nucleus, is in its phosphorylated and active form on serine 473 residue (Fig. 7B). On the contrary, the faint band observable in samples pre-treated with inhibitor VI, VII and VIII, remained substantially unchanged after the second immunoprecipitation (Fig. 7B). The use of Akt inhibitors suggests therefore an essential role of Akt activation for its nuclear translocation.

3.6. Effects of down modulation or Akt inhibition on erythroid differentiation

To give new insights into functional consequences for a developmental role of active Akt translocation into the nucleus, we have down-modulated the expression of Akt during EPO treatment of K562 cells. By means of siRNA sequences, a reduced amount of Akt protein was obtained in control cells as well as in differentiating cells, in which Akt expression was forcedly silenced in the aim of counteracting the EPO induced nuclear translocation of Akt protein (Fig. 8A).

As reported in Fig. 8B, the reduced expression of Akt results in a large decrease of the erythoid differentiation level. Similarly when
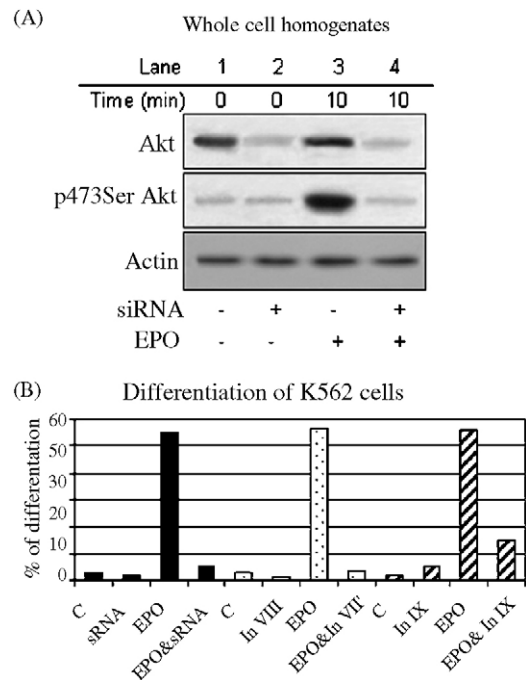

Fig. 8. Down-modulation of Akt expression or biochemical inhibition impairs erythroid differentiation. K562 erythroleukemia cells, subjected to RNA interference assay in control (−) or differentiating conditions for 4 days (+), were subjected to immunochemical analysis to evaluate Akt expression (A) and to cytofluorimetric determination to estimate their differentiation level (B). In K562 cells EPO induces hemoglobin synthesis, which was hampered by siRNA sequences or treatment with different Akt inhibitors. Values are percentage of CD71-positive cells. Viability was always more than 80% and S.D. less than 10%. The data are representative of three separate experiments performed in duplicate.

cells were pre-treated with specific Akt inhibitors the increase of EPO-induced hemoglobin synthesis in the cells was significantly suppressed and differentiation did not occur, thus indicating that nuclear Akt enzymatic activity plays a relevant role in K562 cells erythroid maturation.

This set of data demonstrates that a reduced expression of Akt considerably impairs the differentiative potential of EPO and supports the contention that Akt is indispensable for the progression of tumoral erythroleukemia cells along the erythoid lineage.

4. Discussion

In this paper we observed the role of Akt during the initial steps of the human erythroid differentiation and we have shown, for the first time to our knowledge, that EPO treatment activates Akt in K562 erythroleukemia cells and induces the translocation of Akt active form in the nuclear compartment in a rapid and transient manner, suggesting that Akt plays a crucial role in EPO mediated erythroid differentiation.

This conclusion is based on several lines of evidence. In vivo Akt phosphorylation and in vitro Akt kinase activity were up-regulated by EPO administration to K562 cells. Both phosphorylation and activity of Akt were down-regulated by the pharmacological PI3K inhibitors, Ly294002 or Wortmannin, thus showing that Akt activation is in the PI3K/Akt signal transduction axis.

We have previously demonstrated in K562 cells the nuclear translocation of PI3K and the increase in intranuclear in vivo synthesis of PtdIns(3,4,5)P$_3$ in response to EPO (Neri et al., 2002b). In the present study immunoprecipitable nuclear Akt activity showed an increase starting from 10 min that reached a maximum after 15–20 min. This enhancement was similar but delayed of 5–10 min when compared to Akt activation in whole cells and may be explained by the time required by Akt for translocating into this cell fraction, where almost all Akt results phosphorylated in the sites of activation. The nuclear peak of enzymatic activity delayed when compared with whole cells and the fact that at 5 min of stimulation nuclei did not show an increase in activity, demonstrate unlikely that the increase we measured in nuclear Akt activity was due to a cytoplasmic contamination. These events, which occur after 15 min of EPO stimulation, are accompanied by a rise of the enzymatic activity of this kinase into nuclei of K562 cells.

The localization of p473Ser Akt mainly into the nucleus indicated that some putative Akt targets reside within this organelle. Known substrates are, for example the forkhead transcription factors, that link the PI3K/Akt cascade to gene expression that regulates cell growth, survival, and metabolism (Kops et al., 1999). Akt phosphorylates the nuclear receptors RAR alpha (retinoic acid receptor alpha) and inhibits its transactivation (Srinivas et al., 2006). It has recently been reported that acinus, a nuclear factor required for apoptotic chromatin condensation, is a direct target of Akt (Hu et al., 2005b) and zyxin, a focal adhesion molecule, is phosphorylated in the nucleus on serine 142 by Akt, leading to its association with acinus (Chan et al., 2007). Therefore, by regulating zyxin/acinus complex formation in the nucleus, Akt influences apoptosis.

Although it is now clear that Akt migrates to the nucleus as the result of treatment with growth factors, the mechanism by which Akt translocates into the nucleus remains unclear. There are several reports indicating that Akt nuclear translocation may be attributable to an activation of PI3K. In human mature T-cell leukemia the mechanism of Akt nuclear translocation involves PI3K that has been suggested as a physiological regulator of Akt within the nucleus as well as the cytoplasm (Pekarsky et al., 2000). In osteoblast-like MC3T3-E1 cells exposed to proliferative growth factor, Akt nuclear translocation appears to be mediated by PI3K

(Borgatti et al., 2000). It has been shown before that nuclear PtdIns(3,4,5)P$_3$ facilitates translocation of proteins with PH domain. For example both nuclear PI3K and PtdIns(3,4,5)P$_3$ are necessary for the nuclear translocation of PKC-ζ (Neri et al., 1999b) or for PIKE-L binding and residency into the nucleus that is compromised by mutations of its PH domain leading to a decreased activation of Akt (Hu et al., 2005a).

More recently a report by Xuan Nguyen et al. (2006) showed that Akt phosphorylation is coupled to its nuclear translocation, which requires PI3K activity for the maintenance of Akt activation within the nucleus under conditions of NGF stimulation. Consistently with this report, we have found that there is a relevant p473 serine residue phosphorylation of Akt recovered in the nucleus, with only a very limited amount of protein not phosphorylated on serine residues and treatment of cells with Ly294002 fully inhibited EPO-stimulated Akt translocation to the nucleus. Thus Akt nuclear translocation may be attributable to an activation of PI3K. We demonstrated with a different biochemical method that Akt nuclear residence is regulated by its phosphorylation status, whereas its kinase activity appears to be not required. It is worthy to note that, when nuclear Akt kinase activity is inhibited in the nucleus either by activation plus translocation block and either by kinase activity inhibition, erythroid maturation is not taking place.

Our siRNA down modulation data address the issue of whether the increase of the amount of nuclear Akt we have observed in erythoid differentiation is merely designed to the function of Akt in mature cells or, more intriguingly, it is functionally relevant to the maturation mechanism.

Although the enhanced Akt activity is usually associated with the proliferative and survival signals also in leukemia cells, a growing body of evidences indicates a relevant role also in erythroid differentiation. Our data gain relevance when compared with data obtained in human erythroid progenitors. EPO induced maturation of human CD34$^+$ erythroid progenitors is dependent on the PI3K/Akt signaling pathway (Myklebust et al., 2002; Bouscary et al., 2003). In the context of EPO signaling it has been shown that Akt phosphorylates both GATA-1 and Foxo3a, transcription factors of crucial importance in erythropoiesis (Bouscary et al., 2003; Kadri et al., 2005). Enforced expression of Myr-Akt, a constitutively active form of Akt, highly augmented EPO-induced erythropoiesis and induced cell hypersensitivity to EPO in the earliest committed cells of the erythroid lineage, the erythroid burst-forming unit (BFU-E), human erythroid progenitors CD34$^+$ CD71$^+$ CD45RA$^-$ GPA$^-$ (Sivertsen et al., 2006). These effects were completely counteracted by the PI3K inhibitor Ly294002.

Interestingly, Ghaffari et al. (2006) recently demonstrated that enforced expression of activated Akt in murine fetal liver progenitor cells overrode the need for EPO to induce erythroid differentiation. They concluded that the enhanced erythroid maturation of activated-Akt-transfected cells was not limited to its anti-apoptotic or proliferative effect as no increase in total cell number was observed. Increased activation of the PI3K/Akt signaling pathway may be particularly relevant to normal hematopoiesis and to patients with polycythemia vera (PV).

These results are strengthened by the identification of molecules downstream to Akt that are relevant in human erythropoiesis. Akt has been described as a mediator of EPO modulation of GATA-1 transcription factor that may coordinate EPO-R signaling and GATA-1 activities during hematopoietic cell differentiation (Zhao et al., 2006). K-ras has been identified as the major regulator for cytokine dependent Akt activation in erythroid differentiation in vivo (Zhang and Lodish, 2005).

Taken together, these data indicate that Akt may play a major function during erythroid differentiation, by becoming active and translocating into the nucleus where it may activate several

substrates involving transcription factors responsible for cell maturation and advancement of the erythroid differentiation. In this latter event, as also demonstrated by down regulation by means of siRNA sequences, Akt could play an active role during EPO-driven differentiation of tumoral cells, thus constituting a molecular target for therapeutic intervention.

Important step in this field will be the identification of the molecular events produced by Akt activation during erythroleukemia cells differentiation, among the multiple effects exerted by this multifunctional kinase into the nucleus.

These findings may help identifying novel target into the nucleus essential for differentiation and thus useful for anticancer therapy of hematological malignancies.

Acknowledgements

References

Barnett SF, Defeo-Jones D, Fu S, Hancock PJ, Haskell KM, Jones RE, et al. Identification and characterization of pleckstrin-homology-domain-dependent and isoenzyme-specific Akt inhibitors. Biochem J 2005;385:399–408.

Bertagnolo V, Marchisio M, Capitani S, Neri LM. Intranuclear translocation of phospholipase C beta2 during HL-60 myeloid differentiation. Biochem Biophys Res Commun 1997;27(235):831–7.

Borgatti P, Martelli AM, Bellacosa A, Casto R, Massari L, Capitani S, et al. Translocation of Akt/PKB to the nucleus of osteoblast-like MC3T3-E1 cells exposed to proliferative growth factors. FEBS Lett 2000;477:27–32.

Bouscary D, Pene F, Claessens YE, Muller O, Chretien S, Fontenay-Roupie M, et al. Critical role for PI 3-kinase in the control of erythropoietin-induced erythroid progenitor proliferation. Blood 2003;101:3436–43.

Brazil DP, Yang ZZ, Hemmings BA. Advances in protein kinase B signaling: Akt ion on multiple fronts. Trends Biochem Sci 2004;29:233–42.

Bunn HF. New agents that stimulates erythropoiesis. Blood 2007;109:868–73.

Chan CB, Liu X, Tang X, Fu H, Ye K. Akt phosphorylation of zyxin mediates its interaction with acinus-S and prevents acinus-triggered chromatin condensation. Cell Death Differ 2007;14:1688–99.

Chang F, Lee JT, Navolanic PM, Steelman LS, Shelton JG, Blalock WL, et al. Involvement of PI3/Akt pathway in cell cycle progression, apoptosis and neoplastic transformation: a target for cancer chemotherapy. Leukemia 2003;17:590–603.

Fukuda R, Hayashi A, Utsunomiya A, Nukada Y, Fukui R, Itoh K, et al. Alteration of phosphatidylinositol 3-kinase cascade in the multilobulated nuclear formation of adult T cell leukemia/lymphoma (ATLL). Proc Natl Acad Sci USA 2005;102:15213–8.

Ghaffari S, Kitidis C, Zhao W, Marinkovic D, Fleming MD, Luo B, et al. AKT induces erythroid-cell maturation of JAK2-deficient fetal liver progenitor cells and is required for Epo regulation of erythroid-cell differentiation. Blood 2006;107:1888–91.

Gotz R, Wiese S, Takayama S, Camarero GC, Rossoll W, Schweizer U, et al. Bag1 is essential for differentiation and survival of hematopoietic and neuronal cells. Nat Neurosci 2005;8:1169–78.

Hammerman PS, Fox CJ, Birnbaum MJ, Thompson CB. Pim and Akt oncogenes are independent regulators of hematopoietic cell growth and survival. Blood 2005;105:4477–83.

Hanada M, Feng J, Hemmings BA. Structure, regulation and function of PKB/Akt—a major therapeutic target. Biochim Biophys Acta 2004;1697:3–16.

Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/Akt pathway for cancer drug discovery. Nat Rev Drug Discov 2005;4:988–1004.

Hiromura M, Okada F, Obata T, Auguin D, Shibata T, Roumestand C, et al. Inhibition of Akt kinase activity by a peptide spanning the betaA strand of the proto-oncogene TCL1. J Biol Chem 2004;279:53407–18.

Hu Y, Liu Z, Ye K. Phosphoinositol lipids bind to phophatidylinositol 3 (PI3)-kinase enhancer GTPase and mediate its stimulatory effect on PI3-kinase and Akt signalings. PNAS 2005a;102:16853–8.

Hu Y, Yao J, Liu Z, Liu X, Fu H, Ye K. Akt phosphorylates acinus and inhibits its proteolytic cleavage, preventing chromatin condensation. EMBO J 2005b;24:3543–54.

Huddleston H, Tan B, Yang FC, White H, Wenning MJ, Orazi A, et al. Functional p85alpha gene is required for normal murine fetal erythropoiesis. Blood 2003;102:142–5.

Jelkmann W. Erythropoietin after a century of research: younger than ever. Eur J Haematol 2007;78:183–205.

Jin X, Gossett DR, Wang S, Yang D, Cao Y, Chen J, et al. Inhibition of AKT survival pathway by a small molecule inhibitor in human endometrial cancer cells. Br J Cancer 2004;91:1808–12.

Kadri Z, Maouche-Chretien L, Rooke HM, Orkin SH, Romeo PH, Mayeux P, et al. Phosphatidylinositol 3-kinase/Akt induced by erythropoietin renders the erythroid differentiation factor GATA-1 competent for TIMP-1 gene transactivation. Mol Cell Biol 2005;25:7412–22.

Kashii Y, Uchida M, Kirito K, Tanaka M, Nishijima K, Toshima M, et al. A member of Forkhead family transcription factor, FKHRL1, is one of the downstream molecules of phosphatidylinositol 3-kinase-Akt activation pathway in erythropoietin signal transduction. Blood 2000;96:941–9.

Kitanaka A, Waki M, Kamano H, Tanaka T, Kubota Y, Ohnishi H, et al. Antisense src expression inhibits proliferation and erythropoietin-induced erythroid differentiation of K562 human leukemia cells. Biochem Biophys Res Commun 1994;201:1534–40.

Klingmuller U, Wu H, Hsiao JG, Toker A, Duckworth BC, Cantley LC, et al. Identification of a novel pathway important for proliferation and differentiation of primary erythroid progenitors. Proc Natl Acad Sci USA 1997;94:3016–21.

Kops GJ, de Ruiter ND, De Vries-Smits AM, Powell DR, Bos JL, Burgering BM. Direct control of the Forkhead transcription factor AFX by protein kinase B. Nature 1999;398:630–4.

Kubota Y, Tanaka T, Kitanaka A, Ohnishi H, Okutani Y, Waki M, et al. Src transduces erythropoietin-induced differentiation signals through phosphatidylinositol 3-kinase. EMBO J 2001;20:5666–77.

Kubota Y, Tanaka T, Yamaoka G, Yamaguchi M, Ohnishi H, Kawanishi K, et al. Wortmannin, a specific inhibitor of phosphatidylinositol-3-kinase, inhibits erythropoietin-induced erythroid differentiation of K562 cells. Leukemia 1996;10:720–6.

Marchisio M, Bertagnolo V, Colamussi ML, Capitani S, Neri LM. Phosphatidylinositol 3-kinase in HL-60 nuclei is bound to the nuclear matrix and increases during granulocytic differentiation. Biochem Biophys Res Commun 1998;253:346–51.

Matkovic K, Brugnoli F, Bertagnolo V, Banfic H, Visnjic D. The role of the nuclear Akt activation and Akt inhibitors in all-trans-retinoic acid-differentiated HL-60 cells. Leukemia 2006;20:941–51.

Myklebust JH, Blomhoff HK, Rusten LS, Stokke T, Smeland EB. Activation of phosphatidylinositol 3-kinase is important for erythropoietin-induced erythropoiesis from CD34(+) hematopoietic progenitor cells. Exp Hematol 2002;30:990–1000.

Neri LM, Borgatti P, Capitani S, Martelli AM. The nuclear phosphoinositide 3-kinase/Akt pathway: a new second messenger system. Biochim Biophys Acta 2002a;1584:73–80.

Neri LM, Bortul R, Tabellini G, Borgatti P, Baldini G, Celeghini C, et al. Erythropoietin-induced erythroid differentiation of K562 cells is accompanied by the nuclear translocation of phosphatidylinositol 3-kinase and intranuclear generation of phosphatidylinositol (3,4,5) triphosphate. Cell Signal 2002b;14:21–9.

Neri LM, Marchisio M, Colamussi ML, Bertagnolo V. Monocytic differentiation of HL-60 cells is characterized by the nuclear translocation of phosphatidylinositol 3-kinase and of definite phosphatidylinositol-specific phospholipase C isoforms. Biochem Biophys Res Commun 1999a;259:314–20.

Neri LM, Martelli AM, Borgatti P, Colamussi ML, Marchisio M, Capitani S. Increase in nuclear phosphatidylinositol 3-kinase activity and phosphatidylinositol (3,4,5) trisphosphate synthesis precede PKC-zeta translocation to the nucleus of NGF-treated PC12 cells. FASEB J 1999b;13:2299–310.

Pekarsky Y, Koval A, Hallas C, Bichi R, Tresini M, Malstrom S, et al. Tcl1 enhances Akt kinase activity and mediates its nuclear translocation. Proc Natl Acad Sci USA 2000;97:3028–33.

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorilation and regulation of Akt/PKB by the rictor–mTOR complex. Science 2005;307:1098–101.

Seminario MC, Precht P, Wersto RP, Gorospe M, Wange RL. PTEN expression in PTEN-null leukemic T cell lines leads to reduced proliferation via slowed cell cycle progression. Oncogene 2003;22:8195–204.

Sivertsen EA, Hystad ME, Gutzkow KB, Dosen G, Smeland EB, Blomhoff HK, et al. PI3K/Akt-dependent Epo-induced signaling and target genes in human early erythroid progenitor cells. Br J Haematol 2006;135:117–28.

Srinivas H, Xia D, Moore NL, Uray IP, Kim H, Ma L, et al. Akt phosphorylates and suppresses the transactivation of retinoic acid receptor alpha. Biochem J 2006;395:653–62.

Steelman LS, Pohnert SC, Shelton JG, Franklin RA, Bertrand FE, McCubrey JA. JAK/STAT, Raf/MEK/ERK. PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. Leukemia 2004;18:189–218.

Williams MR, Arthur JS, Balendran A, van deer Kaay J, Poli V, Cohen P, et al. The role of 3-phosphoinositide-dependent protein-kinase 1 in activating AGC kinases defined in embryonic stem cells. Curr Biol 2000;10:439–48.

Xuan Nguyen TL, Choi JW, Lee SB, Ye K, Woo SD, Lee KH, et al. Akt phosphorylation is essential for nuclear translocation and retention in NGF-stimulated PC12 cells. Biochim Biophys Res Commun 2006;349:789–98.

Zhang J, Lodish HF. Identification of K-ras as the major regulator for cytokine-dependent Akt activation in erythroid progenitors in vivo. Proc Natl Acad Sci USA 2005;102:14605–10.

Zhao W, Kitidis C, Fleming MD, Lodish HF, Ghaffari S. Erythropoietin stimulates phosphorylation and activation of GATA-1 via the PI3-kinase/AKT signaling pathway. Blood 2006;107:907–15.