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ADAPTIVE CAPABILITIES OF THE PI3K/AKT/MTOR PATHWAY IN ACUTE MYELOID LEUKEMIA REVEALED BY THE USE OF SELECTIVE INHIBITORS

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1.ABSTRACT

The objective of the study was to investigate the sensitivity of primary blasts from AML patients to PI3K/Akt/mTor inhibitors through reverse-phase protein microarray. Reverse-phase microarray assays using phosphospecific antibodies (RPPA) can directly measure levels of phosphorylated protein isoforms. Mapping of deregulated kinases and protein signaling networks within tumors can provide a means to stratify patients with shared biological characteristics to the most optimal treatment, and identify drug targets. In particular, the PI3K/AKT/mTOR signaling pathways are frequently activated in blast cells from patients with acute myelogenous leukemia (AML), a neoplastic disorder characterized by the accumulation of genetically altered myelogenous cells displaying deregulated intracellular signalling pathways and aggressive clinical behavior with poor prognosis. By RPPA, we have analyzed the phosphorylome of 55 fresh peripheral blood and bone marrow specimens with newly diagnosed AML. Patients are diagnosed according to blast content, FAB classification and cytogenetic analysis. Samples are enriched for leukemic cells by performing Ficoll separation to yield a mononuclear fraction, followed by lymphocyte depletion. Only samples with > 80% blast cells were subjected to RPPA analysis. Collectively, our results indicate that: i) In good agreement with previous reports, by unsupervised hierarchical clustering our data validate a strong phosphorylation/activity of most members of the PI3K/Akt/mTOR pathway in >70% of samples from AML patients. This confirms that this pathway might indeed represent a pharmacological target in many patients. ii) In addition, our data indicate that the Akt pathway is hyper-activated in M4, M5 patients, compared to M0, M2 patients. iii) Furthermore, on the above basis, blast samples with high phosphoAkt were grown for 16 h either untreated or treated with the PI3K/Akt inhibitors Perifosine (phase II), Akt Inhibitor VIII (phase I), Triciribine (phase I), all at nanomolar-low micromolar dose. Akt allosteric inhibitors were used, so that Akt phosphorylation might be used as a read-out of inhibitor efficacy. Then cells were centrifuged and proteins extracted with a buffer suitable for both RPPA and western blotting analysis. Treatment with the above inhibitors had no effect on the phosphorylation of other selected targets, demonstrating the specificity of the above results (more than one different inhibitor was used to avoid off-target effects). Remarkably, the drugs were very effective in inducing apoptosis in all samples, though to a different degree. Unexpectedly, we observed that more than 50%

samples were characterized by paradoxical Akt phosphorylation upon drug administration.

iv) We therefore addressed the question why Akt inhibitors do not block Akt phosphorylation in those samples, and how do they trigger apoptosis short-circuiting Akt. First, we repeated the experiments at shorter time-points and observed that all drugs targeting Akt were able to abrogate phosphorylation at 2 and 4 hours treatment. However, this effect was followed by complete recovery and hyper-phosphorylation of Akt, far above basal level, after 20 hours. v) Next we asked whether initial Akt inhibition might trigger feedback signaling through its downstream effector mTORC1. To explore this possibility, blast samples with high phosphoAkt were grown for 2, 4 and 20 h either untreated or treated with the mTORC1 inhibitor rapamycin or with the mTOR dual kinase inhibitor Torin1, alone or in combination with the Akt inhibitor Perifosine. Although combined inhibition of both Akt and mTOR was not sufficient to reduce Akt phosphorylation below basal level after 20 hours, we observed that apoptosis was further increased. Thus, we conclude that there are Akt-regulated feedback pathways that are not sensitive to mTOR inhibition. In particular, we observed that catalytic inhibition of mTOR kinase leads to a new steady state, with Akt characterized by phosphorylation at T308 even in the absence of phosphorylation at S473.vi) We considered that Akt is the main effector of PI3K, which in turn is activated by most RTKs. Consequently we asked whether Akt inhibition unleashes Akt-dependent inhibition of RTKs expression. Expression of RTKs such as IR and IRS-1, was monitored accordingly. We found that in samples non responsive to the above drugs Akt phosphorylation is paralleled by high IRS-1. It is known that phosphorylation of the Akt direct target FOXO3a, leading to its degradation, down-regulates FOXO-dependent genes, such as IRS-1 and possibly other RTKs. Importantly, our results indicate that abrogation of Akt-dependent phosphorylation and degradation of FOXO3a leads to increased expression of IRS-1, hence reactivating PI3K and Akt signaling. vii) Based on the abovementioned result, samples were then treated with Perifosine in combination with the broad RTK inhibitor Sunitinib, known to block both IGF-1R and other RTKs upstream of PI3K, such as Flt-3, often activated in AML blasts. Strikingly, this drug combination blunted Akt phosphorylation after 20 hours also in those samples that were not sensitive to previous treatments, and triggered apoptosis extensively. Collectively, our results demonstrate that activation of PI3K/Akt in

leukemia is modulated by negative feedback mechanisms, including mTOR and RTKs-mediated signaling. We show that indeed, in more than 50% primary blasts from AML patients, blocking of Akt activity triggers release of Akt-dependent inhibition of RTKs expression, such as IR or IRS-1. Combined treatment with drugs targeting both Akt and mTOR is not sufficient to by-pass feedback mechanisms, although RTKs induction in these conditions is considerably weaker than that triggered by Akt inhibition alone. We further highlight that reduction of RTKs-dependent signaling by Sunitinib, in combination with Perifosine and Torin 1, very effectively blunts PI3K/Akt signaling, and trigger apoptosis. In conclusion, by means of selective inhibitors we have revealed that in primary leukemia blasts the PI3K/Akt/mTOR pathway possesses adaptive capabilities that disqualify administration of inhibitors of the above pathway as monotherapy for AML, but suggest that they could be very effective if administered in combination with RTKs inhibitors such as Sunitinib.

2. INTRODUCTION

2.1 ACUTE MYELOID LEUKEMIA

Acute Myeoloid Leukemia is an hematological disease originated by a block in differentiation of hematopoietic stem cells resulting in growth of a clonal population of neoplastic cells or blasts. (Fig 1) The overproduction of immature white cells interferes with the normal hematopoiesis.

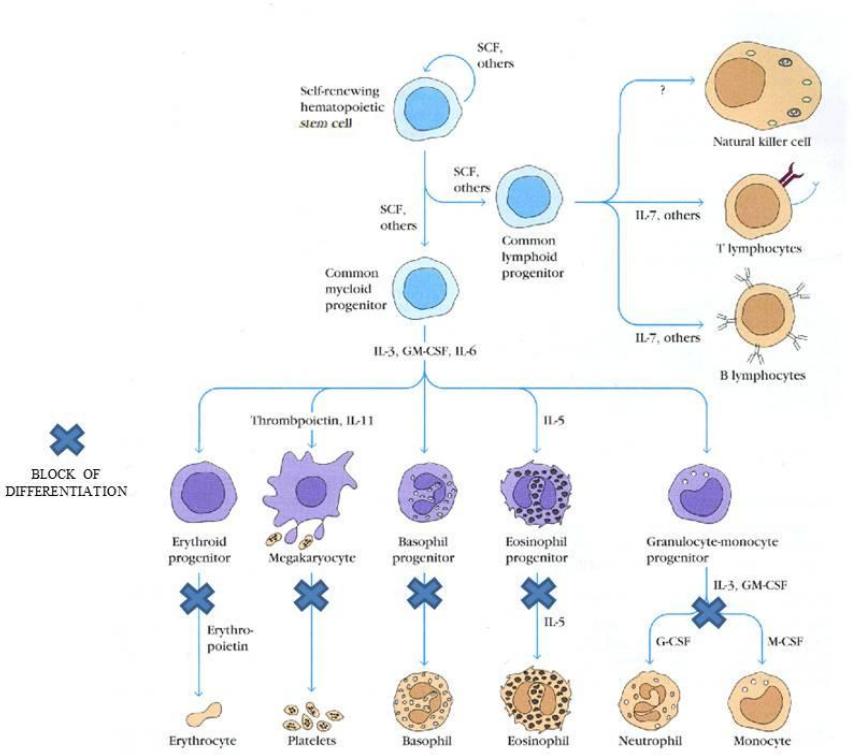


Fig 1

This disorder affects adults and its incidence increases with age. Remission rate with standard chemotherapy range from 50% to 80%. Nevertheless in most cases the patients have a relapse and die within 2 years from remission. (1) There are several subtypes of AML which are identified based on characteristics of the leukemia cells. Two systems have been used to classify AML into subtype the French-American-British (FAB) classification and the newer World Health Organization (WHO) classification. The different AML subtypes are classified according to French American British system into subtypes, M0 through M7, based on the type of cell from which the leukemia developed and how mature the cells are. This was based largely on how the leukemia cells looked under the microscope after staining. It is of note that acute promyelocytic leukemia is one of the most curable forms of AML. More than 70% of people with acute promyelocytic leukemia are sensible to ATRA therapy. The different FAB subtype are listed below:

Table 1

FAB subtype	Name	% of adult AML patients	Prognosis compared to average for AML
M0	Undifferentiated acute myeloblastic leukemia	5%	Worse
M1	Acute myeloblastic leukemia with minimal maturation	15%	Average
M2	Acute myeloblastic leukemia with maturation	25%	Better
M3	Acute promyelocytic leukemia (APL)	10%	Best
M4	Acute myelomonocytic leukemia	20%	Average
M4 eos	Acute myelomonocytic leukemia with eosinophilia	5%	Better
M5	Acute monocytic leukemia	10%	Average
M6	Acute erythroid leukemia	5%	Worse
M7	Acute megakaryoblastic leukemia	5%	Worse

The World Health Organization (WHO) classification of acute myeloid leukemia attempts to be more clinically useful and to produce more meaningful prognostic information than the FAB criteria. Each of the WHO categories contains numerous descriptive sub-categories of interest to the hematopathologist and oncologist; however, most of the

clinically significant information in the WHO schema is communicated via categorization into one of the subtypes listed below..

The WHO subtypes of AML are listed in table 2.

Table 2

AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
AML with inv(16)(p13,1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
AML with t(15;17)(q22;q12); *PML-RARA*
AML with t(9;11)(p22;q23); *MLLT3-MLL*
AML with t(6;9)(p23;q34); *DEK-NUP214*
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*
Provisional entity: AML with mutated *NPM1*
Provisional entity: AML with mutated *CEBPA*
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, not otherwise specified
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemias
Pure erythroid leukemia
Erythroleukemia, erythroid/myeloid
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma

WHO = World Health Organization

Swerdlow SH, Campo E, Harris NL, et al (eds): WHO classification of tumours of haematopoietic and lymphoid tissues. Lyon, France: IARC Press; 109–138, 2009.

Vardiman JW, Thiele J, Arber DA, et al: The 2008 revision of the World Health Organization classification of myeloid neoplasms and acute leukemia: Rationale and important changes. Blood 114:937–951, 2009

2.2 PROGNOSIS AND GENETICS

Acute Myueloid leukemia remission rate and survival percentage depend on a number of features, including age of the patient, cytogenetics, previous bone morrow disease (e.g myelodisplasia [MDS] or myeloproliferative disease). Prognosis and chromosomal aberration are linked very tightly. The classification of AML based on cytogenetics analysis divided patients in three main groups, those with favorable, intermediate, poor prognosis. People who have the poorest prognosis are those older than 60, those who have certain subtypes of AML, and those who develop AML after undergoing chemotherapy or radiation therapy for other cancers. About 40% to 50% of patients with AML have a normal karyotype and represent the largest subset of AML. (2). This subset has many difficulties in prognosis interpretation since not all patients have the same response to treatment. This is a result of the variability in gene mutations and gene expression in this population. Core-bonding factor (CBF) AML is a frequent subtype of AML with approximately favorable prognosis. This mutation t(8;21) results from translocations involving either AML1 or CBF, and is associated with FAB M2 subtype. In inv (16) mutation CBF on chromosome 16 is fused to MYH11 gene on chromosome 16 and is associated with FAB M4 subtype.(3,4,5) Mutations in the nucleophosmin 1(NPM1) gene and the CCAAT enhancer binding protein gene seems to confer a better prognosis, whereas FMS-like tyrosine kinase 3 related mutations are considered to have a very poor prognosis. Of note, the presence of *c-KIT* mutations in patients with otherwise favorable cytogenetic markers (eg, t(8:21), inv(16)) confers a higher risk of relapse and would place an otherwise better-risk patient into the intermediate-risk category.(5)- Other molecular markers, such as isocitrate dehydrogenase 1 and 2 *(IDH1, IDH2),* and methyltransferase gene, *DNMT3A* have been suggested to be predictive of risk and response to treatment, but the relationship between these markers and risk of relapse/death has not been fully elucidated.(6)

2.3 DEREGULATED SIGNAL TRANSDUCTION PATHWAYS IN ACUTE MYELOID LEUKEMIA

Most of patients treated with chemotherapy get to complete remission.

Nevertheless the relapse percentage is very high and the 5 year survival rate for Acute Myeloid leukemia is only at 20%. (7)

There is therefore urgent need to develop a more potent and effective drugs able to completely eliminate the aberrant growth of leukemic cells.

This uncontrolled blast growth is associated to a deregulated signal transduction pathways . Recent papers highlighted the importance of Receptor Tyrosine Kinase signaling pathways in leukemogenesis process (8)

The complex signaling networks downstream from RTKs and how alterations in these networks are translated into cellular responses especially when the cells are subjected to treatment are now under deep investigation. In this context it has been demonstrated that mutations in FLT3, cKit and RAS are frequent in Acute Myeloid Leukemia.

The activation status of growth and survival pathways including PI3K/Akt/mTor has also been found to be increased in AML blast cells.

2.4 PI3K/AKT/MTOR SIGNAL TRANSDUCTION PATHWAY IN ACUTE MYELOID LEUKEMIA

Constitutive activation of PI3K/Akt/mTor pathway is detectable in 50-80% of Acute Myeloid Leukemia patients (9 10) This aberrant activation is related to a *very poor prognosis with a low complete remission rate.* (11,12,13)

Moreover this unfavorable outcome caused by deregulation of PI3K/Akt/mTOR pathway is associated with the expression on leukemia cells surface of ATP binding cassette transporter MRP-1, involved in multi drug resistance (14,15).

A more recent report suggested that Akt constitutive activation could be associated to a favorable outcome. The authors in this paper explain that the myeloid precursor of blast cells population are led by this pathway to a S phase where are more susceptible to chemotherapy (16).

Constitutive PI3K/Akt/mTor activation is the results of several molecular factor such as activating mutations occurred to different Tyrosine Kinase receptor such as FLT3, C-kit and N-K- Ras (17,18,19,) or overexpression of the catalytic subunity of PI3K (20,21) , low level of PP2A and autocrine production of growth factor as IGF-1 and VEGF (22,23, 24,).

In 66 AML patients PDK1 expression was been analyzed and in 45% of patients it was found overexpressed and this upregulation was correlated with PKC activation, whereas Akt phosphorylation at T308 it was not analyzed. (25)

Furthermore interaction between stromal fibronectin and B1 integrin can activate PI3K/Akt/mTor pathway possibly through ILK1 upregulation (25,27) ILK1 seems to be correlated with phosphorylation of Akt at S473 throughout a direct interaction between mTorc2 and ILK1 in Acute Myeloid Leukemia cells. (28) Activating mutations at P110 PI3K or at PH domain of Akt were not detectable in Acute Myeloid Leukemia (29,30)

Despite PTEN deletions are very frequent in solid tumors and also in several cases of Acute Lymphoblastic Leukemia , in AML patients these genetic alterations are very rare. However PTEN activation can be mediated also by phosphorylation at its c-terminal domain.

This modification is able to stabilize PTEN and activate Akt (31). The complex mTORC2 is activated in primary AML cells and might control S473 Akt phosphorylation. Long term treatments with rapamycin and its derivative can disrupt the mTORC2 complex leading a decrease in Akt activity in leukemic cells.(32). It is noteworthy that PI3K/mTOR/Akt signaling pathway is central to a plethora of cellular mechanisms in a wide variety of cells including leukocyte Proteins within the PI3K/mTOR/Akt pathway therefore represent attractive targets for therapeutic intervention and drug development.

2.2 PHOSPHOINOSITIDE 3-KINASE

Phosphoinositides 3–kinases are a family of enzymes that play a pivotal role in important cellular regulatory mechanisms.

PI3K's are capable of phosphorylating the 3-OH position of phosphoinositide lipids (PIs) generating lipid second messangers. (32). Their function has been linked to the regulation of numerous biological processes including cell growth, differentiation, survival proliferation and migration. On the basis of structural similarities and substrates specificity, the PI3K family is divided into three classes termed I, II and III. All human class I members are heterodimers consisting of a catalytic subunit and a non catalytic subunit. They are known to phosphorylate PI, PIP, PIP_2 in vitro but have a strong preference for PIP_2 in vivo. Class I members are further subdivided into class IA and IB PI3Ks. Class IA consists of three isoforms (p110α, p110β and p110δ) whereas the only class IB member is termed p110γ

Class IA PI3Ks are commonly activated by tyrosine kinases, which generate docking sites for the p85/p55 adaptor subunit by phosphorylating tyrosines within p85/p55 consensus binding motifs on al large number of proteins. Effectors of class I PI3Ks are pleckstrin homology domain containing proteins such as Akt/PKB, BTK, TEC, ITK, BAM32 and small GTPase.

The action of PI3K's is antagonized by the PIP_3 phosphatases SHIP and PTEN

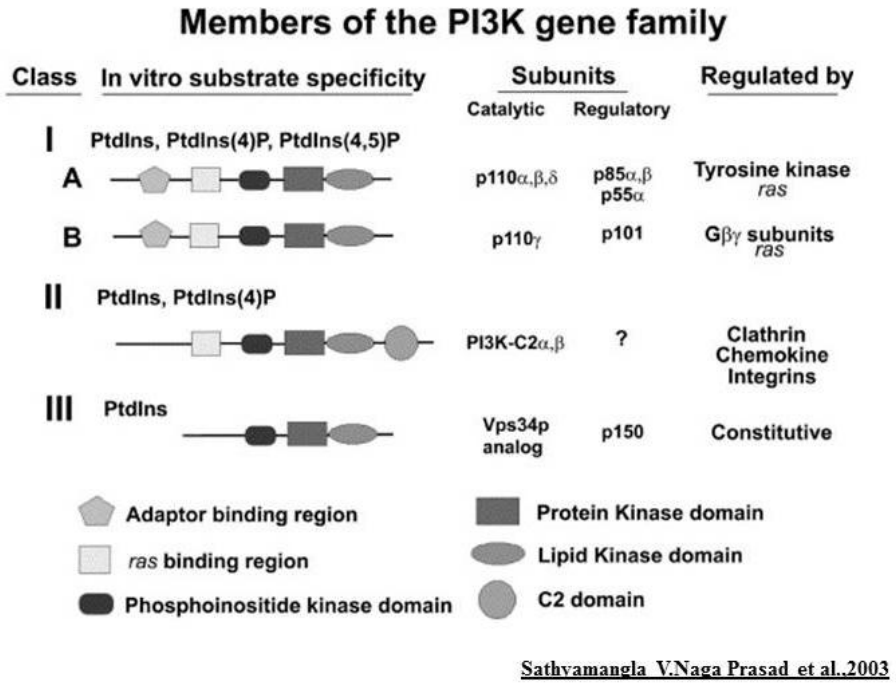
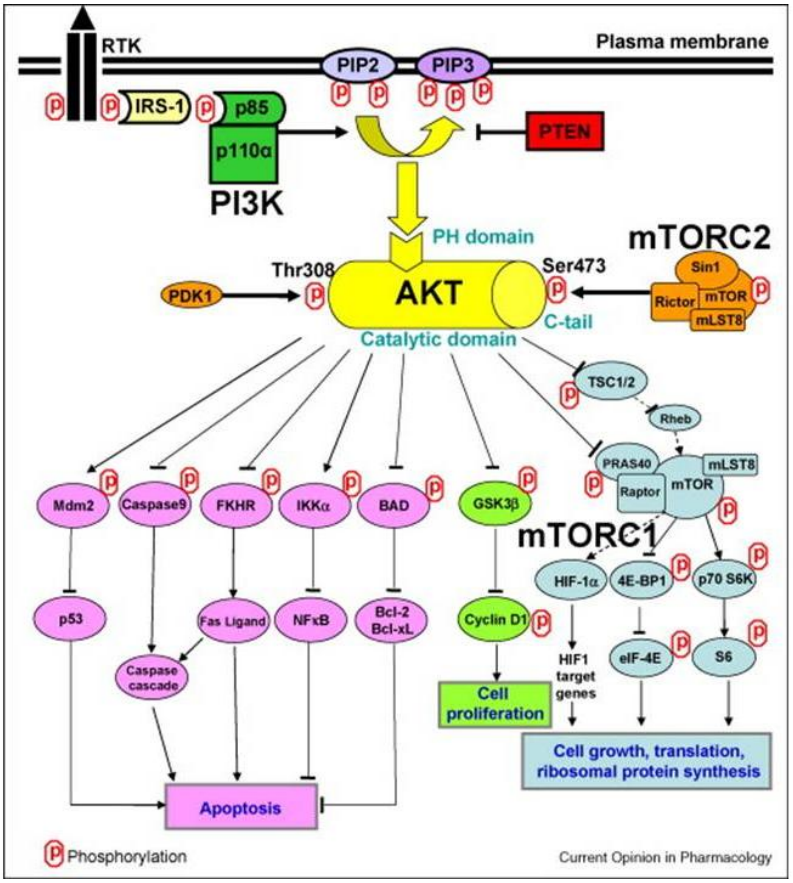


Fig 2 Schematic representation of the phosphoinositide 3-kinases (PI3Ks).

2.2.1 Akt/PKB

Akt is a 57-kDa serine/threonine protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration. The Akt family comprises three highly conserved isoforms : Akt1/a Akt2/b and Akt 3/g, which display a high degree of sequence homology (34). However functional differences exist between Akt isoforms, Akt1 is involved in cellular survival pathways , by inhibiting apoptotic processes(35), Akt2 is an important signaling molecule in the Insulin signaling pathway (36) and the role of Akt3 is less clear, though it appears to be predominantly expressed in the brain. Akt possesses a protein domain known as a PH domain, or Pleckstrin Homology domain. This domain binds to phosphoinositides with high affinity.

In the case of the PH domain of Akt, it binds either PIP_3 (phosphatidylinositol (3,4,5)-trisphosphate, PtdIns(3,4,5)P_3) or PIP_2 (phosphatidylinositol (3,4)-bisphosphate, PtdIns(3,4)P_2).(37) Once correctly positioned at the membrane via binding of PIP3, Akt can then be phosphorylated by its activating kinases, phosphoinositide dependent kinase 1 (PDK1) at threonine 308 and mTORC2 at serine 473 (38) Activated Akt can then go on to activate or deactivate its myriad substrates (e.g. mTOR) via its kinase activity. So far, over 100 Akt substrates have been identified (39) Each of these substrates has a key role in the regulation of cell survival and proliferation, either directly or through an intermediary (40).



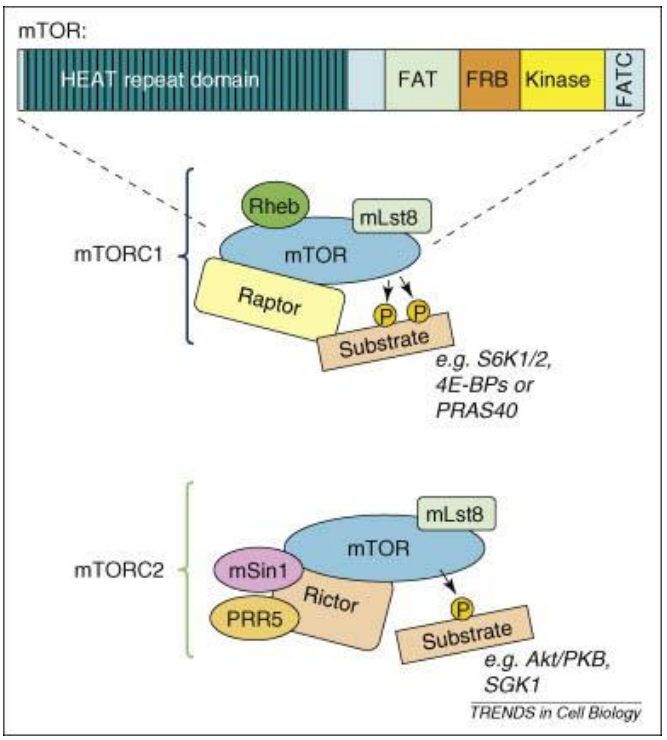
Yap TA et al.,Curr Opin Pharmacol. 2008

Fig 3 Schematic representation of PI3K–AKT pathway substrates and associated cellular functions.

2.2.2 mTOR

The target of rapamycin (TOR) was originally discovered in the budding yeast Saccharomyces cerevisiae, as a target of the macrolide fungicide rapamycin, through mutants that showed growth resistance to rapamycin (41). The structurally and functionally conserved mammalian counterpart (Mtor) was subsequently discovered biochemically based on its rapamycin inhibitory properties.

mTOR (the mammalian target of rapamycin) is a 289 kDa serine/threonine kinase that belongs to the PI3K-related protein kinase (PIKKs) family, since its c-terminus shares strong homology to the catalytic domain of PI3K. mTOR exists as two complexes referred to as MTORC1 and MTORC2. (Fig.4)



Xuemin Wang, **et al., Trends in Cell Biology, 2009**

Fig 4 Domain structure of mTOR. The N-terminus of mTOR contains two tandem repeated HEAT motifs (protein interaction domains found in Huntington,Elongation factor 3, PR65/A and TOR), followed by a FAT (domain shared by FRAP,Ataxia telangiectasia mutated, and TRRAP, all of which are PIKK family members) domain , a FRB (FKBP12-rapamycin-binding site,found in all eukaryotic TOR orthologs) domain, a PtdIns 3-kinase related catalytic domain , an auto inhibitory (repressor domain or RD domain) and a FATC (FAT C terminus) that is located at the C-terminus of the protein. The FRB domain forms a deep hydrophobic cleft that serves as the high affinity binding site for the inhibitory complex FKBP12-rapamycin

mTORC1 consists of mTOR, raptor, mlst8, and two negative regulators, PRAS40 and DEPTOR. This complex is characterized by the classic features of mTOR by functioning as a nutrient/energy/redox sensor and controlling protein synthesis. The activity of this complex is stimulated by insulin, growth factors, serum, phosphatidic acid, amino acids (particularly leucine), and oxidative stress.

mTORC1 is activated by the PI3K/AKT pathway (Fig 5) through the TSC1/TSC2 complex; Akt inhibits tuberous sclerosis 2 (TSC2 or hamartin) function through direct phosphorylation.

TSC2 is a GTPase-activating protein (GAP) that functions in association with the putative tuberous sclerosis1 (TSC1 or tuberin) to inactivate the small G protein Rheb

TSC2 phosphorylation by Akt represses GAP activity of the TSC1/TSC2 complex, allowing Rheb to accumulate in a GTP-bound state. Rheb-GTP then activates, through a mechanism not yet fully elucidated, the protein kinase mTOR.

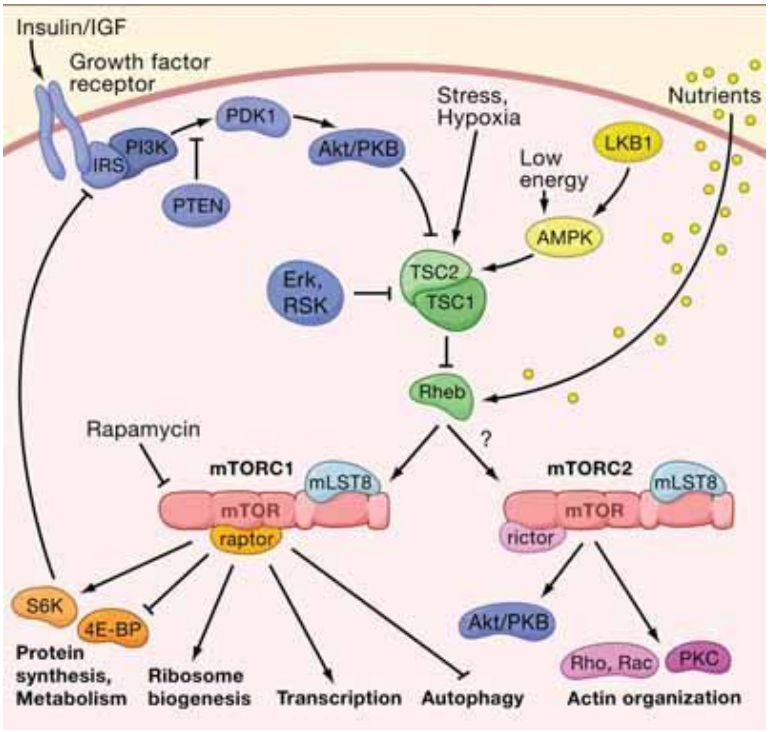
Akt also phosphorylates proline-rich Akt-substrate-40 (PRAS40), an inhibitor of mTORC1, and by doing so, it prevents the ability of PRAS40 to suppress mTORC1 signalling. Thus, this could be yet another mechanism by which Akt activates mTORC1. Moreover, PRAS40 is a substrate of mTORC1 itself, and it has been demonstrated that mTORC1-mediated phosphorylation of PRAS40 facilitates the removal of its inhibition on downstream signaling of mTORC1.

mTORC1 is a major regulator of ribosomal biogenesis and protein synthesis through the phosphorylation and activation of S6K and the phosphorylation and inactivation of the repressor of mRNA translation 4EBP1. Since they are the best characterized downstream targets of mTOR, the phosphorylation status of S6K and 4EBP1 are commonly used to evaluated mTorc1 activity in vivo. Activated mTORC1 phosphorylates S6K1, which phosphorylates S6 (40S ribosomal protein S6), enhancing the translation of mRNAs with a 5'-terminal oligopoly pyrimidine (5'-TOP). The targets of S6K1 include ribosomal proteins, elongation factors (eEF), and insulin growth factor II (IGF-II).

4EBP1 phosphorylation by mTORC1 on several amino acidic residues results in the release of the eukaryotic initiation factor 4E (eIF4E), that promote the translation of several proteins , such as c-Myc, cyclin D1, Cdk2, Rb protein, p27Kip1, VEGF and STAT3.(42,43,44) mTORC2 contains mTOR, rictor, Mlst8, Msin1, and the newly identified components Protor, Hsp70 and DEPTOR. Rictor is an mTOR-associated protein that is exclusive from mTORC2.

mTORC2 is activated by grow factors, phosphorylates PKC-α, AKT (ser473) and paxillin (focal adhesion-associated adaptor protein), and regulates the activity of the small GTPase Rac and Rho related to cell survival, migration, and regulation of the actin cytoskeleton. Hence mTORC1 and mTORC2 have different physiological functions. The complexes differ in their sensitivity to rapamycin: mTORC1 is sensitive and mTORC2 is resistant.

Several studies suggest the existence of a negative feedback loop from the mTOR-S6K1 pathway to the upstream IRS pathway (45) Activation of mTORC1 and S6K1 regulates IRS-1 both at the transcriptional level and through direct phosphorylation on specific residues which prevent its recruitment and binding to RTKs, leading to a negative feedback regulation of PI3K .This negative regulation of Akt activity by mTORC1 is a consequence of P70S6K mediated phosphorylation of insulin receptor substrates (IRS) 1 adapter protein, downstream of insulin receptor and/or Insulin-like Growth Factor-1 Receptor (IGF-1R). Indeed IRS-1 phosphorylation by p70S6K targets the adapter protein to proteasomal degradation. (46). Therefore at least in principle, inhibition of mTORC1 activity by rapamycin/rapalogs could results in hyperactivation of Akt and its substrates.



David Secko , The scientist, December 1, 2006

Fig 5 PI3K/Akt/ mTOR pathway mTOR exists in association with two different complexes, mTORC1 and mTORC2. mTORC1 consists of mTOR and regulatory associated protein of mTOR (Raptor), while mTORC2 consists of mTOR and rapamycin-independent companion of mTOR (Rictor). In the mTORC1 pathway, PI3K converts PIP_2 into PIP_3, which localizes Akt to the membrane. The TSC1 (hamartin) and TSC2 (tuberin) complex is inactivated by Akt-dependent phosphorylation. Inactivation of TSC2 results in activation of mTOR via the GTPase, Rheb. mTOR phosphorylates both p70 S6 kinase (p70S6K) and 4E-BP1 via independent pathways that promote cell proliferation. In the mTORC2 pathway, there is downstream signaling to the AGC kinases Akt, PKCα, and SGK1. Phosphorylation of Akt at Serine 473 by mTORC2 primes Akt for further phosphorylation at Threonine 308.

2.2.3 NEGATIVE REGULATION OFPI3K/AKT/mTOR PATHWAY

Negative regulation of the PI3K pathway is primarily accomplished through the action of the phosphatase and tensin homologue deleted on chromosome ten (PTEN) tumor suppressor proteins. PTEN encodes a lipid and protein phosphatase whose primary lipid substrate is PtdIns[3,4,5]P_3 (47). PTEN protein acts as a phosphatase to dephosphorylate phosphatidylinositol (3,4,5)-trisphosphate (PtdIns (3,4,5)P_3 or PIP_3). PTEN specifically catalyses the dephosporylation of the 3` phosphate of the inositol ring in PIP_3, resulting in the biphosphate product PIP_2 (PtdIns(4,5)P2). This dephosphorylation is important because it results in inhibition of the AKT signaling pathway. (48). Another negative regulator of the PI3K pathway is the PH domain leucine-rich repeat protein phosphatase (PHLPP). The phosphatases in the PHLPP family, PHLPP1 and PHLPP2 have been shown to directly dephosphorylate, and therefore inactivate, distinct Akt isoforms, at one of the two critical phosphorylation sites required for activation: S473. PHLPP2 dephosphorylates AKT1 and AKT3, whereas PHLPP1 is specific for AKT2 and AKT3. Lack of PHLPP appears to have effects on growth factor-induced Akt phosphorylation. When both PHLPP1 and PHLPP2 are knocked down using siRNA and cells are stimulated using epidermal growth factor, Akt phosphorylation at both S473 and T308 is increased dramatically. (49) Two other phosphatases, SH2 domain-containing inositol 5'phosphatase (SHIP)-1 and SHIP-2, remove the 5-phosphate from PtdIns[3,4,5]P_3 to produce PtdIns[3,4]P_2 and inactivate Akt (50). Mutations in these phosphatases, which eliminate their activity, can lead to tumor progression.

2.2.4 PI3K/AKT/mTOR PATHWAY AND SURVIVAL

This pathway regulate the activity of many proteins involved in apoptosis. Many of the effects of the /PI3K/Akt/mTOR pathway on apoptosis are mediated by Akt phosphorylation of key apoptotic effector molecules (*e.g*., Bcl-2, Mcl-1, Bad, Bim, CREB, Foxo, Caspase-9 and many others) (51). Akt can directly phosphorylate BAD on S136, causing its inactivation preventing it from interacting with anti-apoptotic

members of the Bcl-2 family of proteins (Bcl-2, Bcl-X_L) . Activated Akt can inhibit the release of cytochrome c from the mitochondria, which is a potent activator of the apoptotic caspases cascade. The Akt target, Foxo-3 is capable of upregulating Fas ligand (Fas-L) and Bim, two very important molecules that are potent inducers of apoptosis; however, when inactivated by Akt, Foxo-3 is localized to the cytosol where it is unable to increase expression of these genes (52). Akt can also phosphorylate Bim which inhibits its proapoptotic activity (53) p53 plays a key role in DNA damage-induced apoptosis. Recent studies have reported that the PI3K/AKT pathway inhibits p53-mediated transcription and apoptosis through the degradation of p53. Mdm2, a ubiquitin ligase for p53, plays a central role in regulation of the stability of p53 and serves as a good substrate for Akt. Akt phosphorylates Mdm2 at Ser^{186} and increased Mdm2 ubiquitination of p53. (54). Akt and mTor also control the activation of the NF-κB transcription factor. NF-κB is broadly associated with oncogenesis through its ability to control cell proliferation and to suppress apoptosis. It is shown that mTOR downstream from Akt controls NF-κB activity in PTEN-null/inactive prostate cancer cells via interaction with and stimulation of IKK. The mTOR-associated protein Raptor is required for the ability of Akt to induce NF-κB activity. Correspondingly, the mTOR inhibitor rapamycin is shown to suppress IKK activity in PTEN-deficient prostate cancer cells through a mechanism that may involve dissociation of Raptor from mTOR.(55). Moreover mTOR has been described as a key signaling regulator of autophagy. Autophagy is a highly conserved eukaryotic intracellular homeostatic process carrying out degradation of cytoplasm components, including damaged or superfluous organelles, toxic protein aggregates, and intracellular pathogens in lysosome.(56). Autophagy can be upregulated during metabolic, genotoxic or hypoxic stress conditions in order to ensure cell survival. Inhibition of mTOR kinase by specific inhibitors, rapamycin or nutrient deprivation, induces activation of autophagy. The role of mTOR in autophagy is conserved from yeast to mammals, and regulates the induction of autophagy process. In mammals the process may be mediated in part through mTOR-dependent phosphorylation of

eEF2K (eukaryotic translation elongation factor 2 kinase), where mTOR inhibition leads to activation of eEF2K and induction of autophagy.(57) The release of amino acids from autophagic degradation leads to the reactivation of mTORC1 and to the restoration of the cellular lysosomal population. In concert, these events caused by Akt/mTOR activation affect the survival status of the cell.

2.2.5 PI3K/AKT/MTOR PATHWAY AND CELL CYCLE

Once activated Akt/mTOR pathway has the ability to control the access to the cell cycle check point. Recent studies have investigated the mechanisms underlying the tumor-promoting effects of this pathway. Akt triggers a network that positively regulates G1/S cell cycle progression through direct phosphorylation and consequently inactivation of GSK3-beta, leading to increased cyclin D1. Cyclin D initiates the phosphorylation of pRB which facilitates subsequent pRB phosphorylation by Cyclin E. Moreover Akt inhibits the Forkhead family of transcription factors and the tumor suppressor tuberin (TSC2), leading to reduction of p27Kip1. High level of $P27^{KIP1}$ are required to maintain many cell types in quiescence. P27 translation and its protein stability decrease in response to mitogenic signaling. P27 degradation may be regulated through a Akt mediated upregulation of SKP2, a key component of the SCF ^{SKP2} ubiquitin ligase complex that mediates ubiquitination and degradation of p27. (58) Akt can also direct phosphorylates the cyclin dependent kinase inhibitors $p21^{cip1/waf1}$. This phosphorylation caused cytoplasmic accumulation of p21, preventing its access to nuclear Cyclin dependent kinases targets. The identification of p21Waf1/Cip1 and p27Kip1 as novel substrates of Akt provided new insights into mechanisms whereby hyperactivation of this lipid signaling pathway may lead to cell cycle deregulation in human cancers.(59). MTORC complexes integrates nutrient and mitogen signals to regulate cell growth and cell division. Rapamycin inhibits cell cycle progression via inhibition of mTOR. The activities of both the S6K1 and 4E-BP1/eIF4E pathways are required for mTOR

dependent G1-phase progression. Overexpression of constitutively active mutants of S6K1 or wild type eIF4E accelerates serum stimulated G1-phase progression and stable expression of wild type S6K1 confers a proliferative advantages in low-serum-containing media, suggesting that the activity of each of these pathways is limiting for cell proliferation. (60)

2.2.6 PI3K/AKT/MTOR PATHWAY AND METABOLISM

Among its many roles, Akt appears to be common to signaling pathways that mediate the metabolic effects of insulin in several physiologically important target tissues**.** The regulation of glucose homeostasis is one of the best-characterized Akt-mediated processes with strong isoform specificity. Insulin regulates whole body glucose homeostasis by inducing the uptake of glucose into muscle and fat cells and by inhibiting hepatic glucose output, both of which are under the regulation of Akt signaling. To regulate glucose disposal insulin-induces the redistribution of the GLUT4 glucose transporter from intracellular compartments to the plasma membrane of fat and muscle cells (61) . The increase in plasma membrane of GLUT4 promotes increased flux of glucose into those cells in a concentration dependent manner. Akt2 is the isoforms mainly involved in this mechanism. Deletion of Akt2 in mice, but not Akt1 or Akt3, results in fasting hyperglycemia, hyperinsulinemia, glucose intolerance and impaired glucose uptake by fat and muscle cells (61). The best characterized downstream effector of Akt required for regulation of GLUT4 trafficking to the plasma membrane is the RabGAP AS160. Phosphorylation by Akt leads to inhibition of the AS160 GAP activity towards Rab proteins, including Rab8, Rab10, and Rab14 (62). The subsequent activation of these Rab proteins facilitates the translocation of GLUT4 to the plasma membrane of adipocytes and muscle cells. Akt has been shown to regulate also the localization of the glucose transporter GLUT1 through the GSK-3 activity .Inhibition of basal GSK-3 activity (8–24 h) in several cell types, resulted in an approximately twofold increase in glucose uptake due to a similar increase in

protein expression of the facilitative glucose transporter 1 (GLUT1).(63) In addiction, Akt may indirectly activate the glycolysis rate-controlling enzyme phosphofructokinase-1 (PFK1) by directly phosphorylating phosphofructokinase 2 (PFK2), which produces the product, fructose-2.6-bisphosphate (Fru-1,6-P2), which is the most potent allosteric activator of PFK1(64). Hyperactive Akt activates MTORC1, which promotes HIF1a accumulation under normoxic conditions and increases GLUT1, HKII (hexokinase II) and lactate dehydrogenase (LDH) abundance. Moreover, insulin triggers the phosphorylation of 4E-BP1 and S6K1 in a rapamycin sensitive way. This provides evidence for a link between mTOR and glucose modulation. Activation of mTOR by glucose needs support from amino acids, and glucose increase DNA synthesis, a process that can be blocked by rapamycin. Phosphorylation of mTOR downstream targets triggered by insulin may be mediated by metabolites or energy generated from glucose and not glucose per se. mTOR controls the translation of Hypoxia-inducible transcription factor1 α mRNA.

HIF-1a upregulation leads to increased expression of angiogenic factors such as vascular endhotelial growth factor (VEGF) and platelet-derived growth factor.(PDGF) Moreover HIF-1a regulates the glycolytic pathway by controlling the expression of glucose-sensing molecules such as glucose transporter GLUT1 and GLUT3 (65). The impact of fatty acid on mTOR signaling is proposed to be tissue dependent. Free fatty acid (FFA)- modulated phosphorylation of mTOR downstream targets S6K1 and 4E-BP1 appears in tissues that depend on oxidative metabolism, but not in the ones that rely on glycolytic metabolism. Phosphatidic acid which harbors fatty acid chains, has been implicated in mitogenic activation of mTOR

mRNA translation and ribosomal biogenesis, two processes that are strongly affected by mTOR, consume high levels of cellular energy. This raises the possibility that mTOR activity is linked to cellular energy status. The ability of insulin to activate mTOR is impaired upon a reduction in cellular ATP levels by reduced glucose availability or the inhibition of mitochondrial respiration, suggesting that cellular

energy impacts mTOR activity . The effect of intracellular ATP levels on mTOR activity has been attributed to high dissociation constant of mTOR for ATP (66). The 5′AMP-activated protein kinase (AMPK) is regulated by even moderate changes in ATP levels and can sense the cellular AMP/ATP ratio. AMPK activity increases upon decline of the intracellular ATP. AMPK activation leads to a decrease in mTOR activity as measured by S6K1 phosphorylation (67). Also TSC2 contains multiple AMPK consensus phosphorylation sites, and two of these sites are phosphorylated by AMPK, both in vitro and in vivo. mTOR activity in TSC2 null cells is more refractive to energy deprivation compared with wild-type cells, and expression of a TSC2 mutant in which AMPK-targeted residues are substituted by alanine renders the phosphorylation of S6K1 more resistant to glucose deprivation. These results suggest that AMPK activates TSC2 (68) and these results imply that energy metabolism and protein synthesis are tightly coupled. This coupling is mediated by AMPK via activation of TSC2.

2.3. PI3K/AKT/mTOR INHIBITION

The PI3K/Akt/mTOR cascades are often activated by genetic alterations in upstream signaling molecules such as receptor tyrosine kinases (RTK). Some of the principal components of this pathways, are also activated/inactivated by mutations. This pathways have profound effects on proliferative, apoptotic and differentiation pathways. Dysregulation of this pathway can contribute to chemotherapeutic drug resistance and proliferation of cancer initiating cells There are many agents available that affect the PI3K pathway include monoclonal antibodies and tyrosine kinase inhibitors, as well as PI3K inhibitors, Akt inhibitors, rapamycin analogs, and mammalian target of rapamycin (mTOR) catalytic inhibitors. compounds that block both PI3K and mTOR (dual inhibitors).

2.3.1 PI3K INHIBITORS

LY294002 is a PI3K inhibitor for **p110α**, **p110δ** and **p110β** with **IC50** of 0.5 μM, 0.57 μM and 0.97 μM, respectively. LY294002 has been used extensively to study the role of PI3K/Akt pathway in normal and transformed cells. Inactivation of PI3K using LY294002 has been demonstrated to lead to the dephosphorylation of Akt at both T308 and S473, inducing specific G1 arrest in cell growth and finally to cell apoptosis . LY294002 also have antitumor activity in vitro and in vivo in a variety of tumor types. LY294002 is currently in Phase I clinical trials in patients with cancers (69)

2.3.2 AKT INHIBITORS

Perifosine is a synthetic novel alkylphospholipid (ALP), a new class of antitumor agents which targets cell membranes of active proliferating cells and inhibits PH domain mediated AKT membrane recruitment and activation. Importantly, Perifosine does not directly affect either activity of PI3K or phosphoinositide-dependent kinase 1 (PDK1). Perifosine has displayed significant anti-proliferative activity *in vitro* and *in vivo* in several human tumour model systems and is currently being tested in different clinical trials.(70) Perifosine exerts Akt-dependent and Akt-independent effects, indeed recent papers have documented that Perifosine targets both MTORC1 and MTORC2 by down regulating mTOR. (71) Perifosine reduced cell proliferation and induced apoptosis in several tumors type, including hematological disease as AML.

Triciribine

A cell-permeable and reversible tricyclic nucleoside that selectively inhibits the cellular phosphorylation/activation of Akt1/2/3. It does not inhibit known upstream activators of Akt i.e. PI3K or PDK. Exhibits little effect towards cellular signaling pathways mediated by PKC, PKA, SGK, Stat3, p38, ERK1/2, or JNK. It is shown to preferentially induce apoptosis and growth arrest in cancer cells with aberrant Akt activity both *in vitro* . and *in vivo*. Triciribine potently inhibits Akt signaling in human tumor cells with aberrant Akt, leading to inhibition of cell growth and induction of apoptosis. In a xenograft nude mice model, Triciribine significantly inhibits tumor growth in Akt-overexpressing cells but not in the tumors with low levels of Akt. (72)

VIII

Akt Inhibitor VIII is a cell-permeable quinoxaline compound that has been shown to potently, selectively, allosterically, and reversibly inhibit Akt1 and Akt2 isoforms. The inhibition appears to be pleckstrin homology (PH) domain-dependent and the Akt1/2 kinase inhibitor has no inhibitory effect against PH domain-lacking Akt, or other closely related AGC family kinases, PKA, PKC, and SGK, even at concentrations as high as 50 μM. Akt 1 / 2 is a isoform specific inhibitor that forms a PH domain-dependent inactive conformation with Akt1 and Akt2.(73)

2.3.3 mTOR INHIBITORS

Rapamycin

Sirolimus, also known as **rapamycin**, was originally developed as an antifungal agent. This use was abandoned when it was discovered to have potent immunosuppressive and antiproliferative properties. Rapamycin and analogue have

shown remarkable efficacies in preclinical and clinical trials treating organ transplant rejection, autoimmune diseases as well as tumors. The mode of action of rapamycin is to bind the cytosolic protein *FK-binding protein 12* (FKBP12) The coupled RPM-FKBP12 complex then targets the FKBP12-rapamycin binding region (FRB) in the C-terminus of target of rapamycin (TOR) protein and thereby decreases their activities and inhibits the downstream signaling events.

Rapamycin and a number of analogs are currently in Phase II AML trials, alone or with others chemotherapeutics. As a monotherapy rapamycin induced significant clinical responses in 4 out of 9 patients with either refractory or relapsed AML.(74) However, the effects of rapalogs, as previously reported, are limited and only cytostatic in vitro. More efficient anti leukemic activity in vitro and in vivo will probably be achieved with second generation mTOR inhibitors, now designated as TORC inhibitors .

Torin 1

Torin 1 is a potent and selective ATP-competitive mammalian target of rapamycin inhibitor. Torin1 inhibits phosphorylation of mTORC1 and mTORC2 substrates in cells at concentrations of 2 and 10 nM, respectively Torin1 causes cell cycle arrest through a rapamycin-resistant mechanism that is also independent of mTORC2. Torin1 disrupts mTORC1-dependent phenotypes more completely than rapamycin. Rapamycin-resistant functions of mTORC1 are required for cap-dependent translation. (75)

2.4 TYROSINE KINASE RECEPTORS

Tyrosine kinase receptors are a family of receptors with a similar structure. They each have a tyrosine kinase domain (which phosphorylates proteins on tyrosine residues), a hormone binding domain, and a carboxyl terminal segment with multiple tyrosines for auto phosphorylation. When hormone binds to the extracellular domain the receptors aggregate and the tyrosine kinase domains phosphorylate the C terminal tyrosine residues. The phosphorylation of specific tyrosine residues within the activated receptor creates binding sites for Src homology 2 (SH2) domain- and phosphotyrosine binding (PTB) domain-containing proteins Specific proteins containing these domains include Src and phospholipase Cγ. Phosphorylation and activation of these two proteins on receptor binding lead to the initiation of signal transduction pathways. Other proteins that interact with the activated receptor act as adaptor proteins and have no intrinsic enzymatic activity of their own. These adaptor proteins link RTK activation to downstream signal transduction pathways.

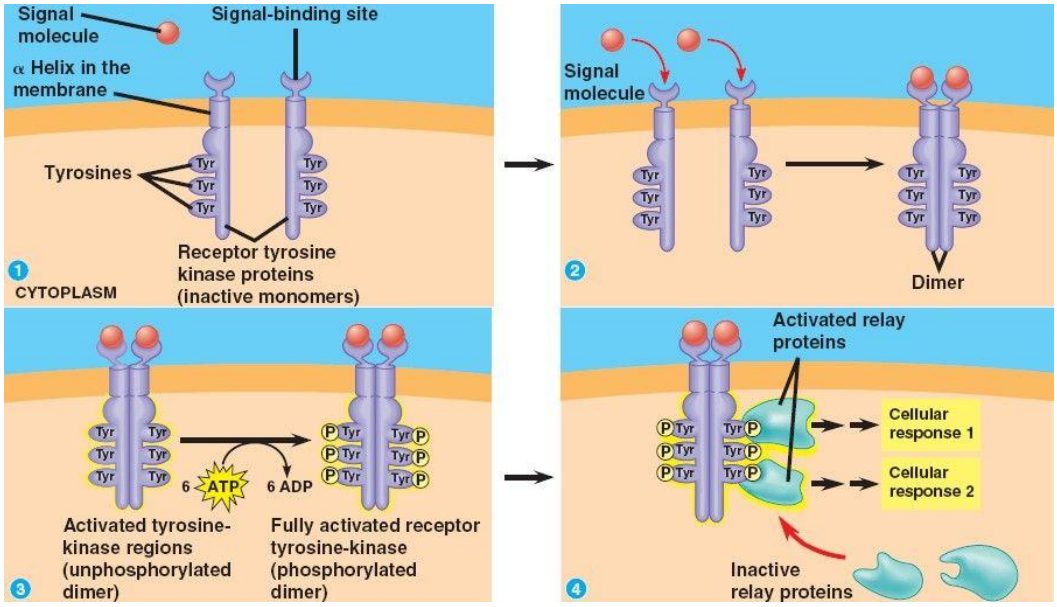


Fig 6 Receptor Tyrosine Kinases contain multiple tyrosine residues and are inactive in monomer state. Binding of signal molecules such as insulin causes 2 monomers to form a dimer. ATP donate a phosphate to each of the tyrosines. Relay proteins bind to the phosphorylated tyrosines and trigger different cellular responses

2.4.1 RTK's in Acute Myeloid Leukemia

In every receptor with tyrosine kinase activity there is an oncogenic potential. Structural modifications can lead to constitutive activation of RTKs and alterations in signal transduction. Deletions within the extracellular ligand-binding domain alter ligand responsiveness, and also point mutations are able to induce overall-ligand-independent conformational alterations and activate RTKs.

In Acute Myeloid Leukemia are extremely common mutations at RTKs or their downstream effector.

FLT3: the FMS-like tyrosine kinase 3 (FLT3) is the most frequently mutated gene in AML. FLT3 is a transmembrane receptor that has crucial role in normal hematopoiesis and in the control of cell proliferation. About one-third of all patients show either internal tandem duplications (ITDs) within the juxtamembrane domain of FLT3, or mutations within the activation loop.

FMS: FMS is a cell surface RTK and specific point mutations in this receptor have been implicated in neoplastic transformation by inducing ligand independence and constitutive activation of the tyrosine kinase activity. Patients with myeloplastic syndrome harboring FMS mutations were shown to have a significantly increased frequency of transformation to AML.

C-KIT: C-kit encodes a transmembrane receptor that is activated by stem cell factor (SCF). Activation of c-Kit correlates with the rate of proliferation of myeloid leukemia cells and with the excessive proliferation and aberrant differentiation of these cells.

IGF-1R . Insulin like growth factors has been described to be important for AML cell growth and autocrine IGF-1 production has been suggested to influence drug resistance .Activation of PI3K/Akt signaling pathway has been detected in blast cells

with IGF-1 mutations, and this contributes to survival and proliferation of leukemia cells.

3. AIMS

Signaling network maps and phosphoprotein profiles in cancer cells are considered powerful tools not only to understand precise molecular mechanisms, but also to determine molecular signatures of kinase activation, very helpful to identify new drug targets and to sort patients that do not respond to conventional treatments towards tailored therapy. Indeed, a major drawback of conventional therapies is drug resistance. Therefore, the definition of deregulated, functionally important, molecular network associated to defined subpopulation of patients is a potential solution to this problem.

Therefore, our broad objective is to investigate, by the use of Reverse Phase Protein Array, the responsiveness of AML patients to drugs targeting the PI3K/Akt/mTOR pathway, frequently hyperactivated in leukemia.

The specific aims are

a) to describe the phosphorylome (90 epitopes) of a large cohort (80) of AML patients and describe deregulated signaling patterns and aberrant protein phosphorylation, by means of reverse phase protein array and western blotting analysis;

b) to investigate the sensitivity of primary blasts from leukemia patients to PI3K/Akt/mTOR inhibitors, namely LY294002, Perifosine, Triciribine, Akt1/2 Inhibitor VIII, Rapamycin, Torin 1;

c) to explore the key regulatory events mediating the efficacy of the drugs.

4. MATERIALS AND METHODS

4.1 PATIENTS DEMOGRAPHICS AND CLINICAL CHARACTERISTICS

We generated a Reverse Phase Protein Array (RPPA) using protein derived from the leukemia cells enriched fraction from 80 AML patients at diagnosis. Diagnosis was made according to standard cytomorphology, cytochemistry and immunophenotyping criteria. (82) Samples were collected at the Oncohematology Laboratory (Modena, Italy, Director Prof. Mario Luppi), between 2008-2011 and stored in a BioBank in liquid nitrogen. The percentage of peripheral blood blasts was between 70% and 98%. Clinical details are resumed in

Table S1.

No of cases	**80**
Median	**63,8**
Minimum	**21**
Maximum	**86**
FAB	
M0	**5%**
M1	**15%**
M2	**15%**
M4	**30%**
M5	**25%**
RAEB	**5%**
AML sec	**5%**
CYTOGENETICS	
Favorable	**36%**
Intermediate	**44%**
Unfavorable	**20%**

Samples were collected from bone marrow and peripheral blood. Same day bone marrow and peripheral blood samples were available for 8 patients and were used to

compare proteins expression between peripheral blood and bone marrow. Immediately after collection the samples were purified by centrifugation on a *Ficoll-Hypaque* density gradient. Samples with less than 70% blasts were depleted of B and T lymphocytes using magnetic sorting with antibody conjugated anti CD3+ and anti CD19+. The samples were frozen in a cryoprotective solution containing 10% *DMSO* + 20% RPMI + 70% FBS and stored in liquid nitrogen.

4.2 CELL CULTURE AND DRUG TREATMENT

Human leukemia cell lines THP1, HL60, K562, Jurkat, CCRF CEM, CCRF CEM/ADR (Multi Drug Resistance phenotype obtained by continuous exposure to doxorubicin as described by Cenni et al(83)), were used as positive controls for correct staining, background and loading variation across the slides. Cells were cultured in RPMI 1640 (Euroclone LTD, UK) with 10% FCS, penicillin (100U/ml) (SIGMA ALDRICH) and streptomycin (100 µg/ml) (SIGMA ALDRICH), and maintained at 37°C in a humidified atmosphere with 5% CO_2.Where indicated primary AML blast cells were treated with kinase inhibitors are listed below:

DRUG	CONCENTRATION	TIME (hours)
LY294002	1mM	20
PERIFOSINE	20mM	2 4 20
Akt Inhibitor VIII	20mM	20
TRICIRIBINE	1mM	20
RAPAMYCIN	1mM	20
TORIN	0,1mM	20
SUNITINIB	0,05mM	20

4.3 **ARRAY ASSEMBLY AND PRINTING.**

Reverse-phase protein arrays. Primary blast cells were thawed and cultured in RPMI additioned with 20% fetal bovine serum for 20 hours. Cells were washed with ice–cold PBS 1X and lysed on ice for 20minutes in an appropriate lysis buffer: TPER Reagent (Pierce, Rockford, IL), 300 mM NaCl, 1 mM Na orthovanadate, 200 mM PEFABLOC (AEBSF) (Roche, Basel, Switzerland), 1ug/mL Aprotinin (Sigma, St. Louis, MO), 5 mg/mL Pepstatin A (Sigma), 1 mg/mL Leupeptin (Sigma). All samples were diluted to a final concentration of 0.5 mg/ml and then 30 μl of each sample, arrayed in a series of 6-fold dilutions, was printed in duplicate on slides. Commercial lysates derived from A431+EGF, Hela+Pervanadate and Jurkat Apoptotic cell lysates (BD Biosciences, Franklin Lakes, NJ). were also printed on each slide as low and high phosphorylation controls, respectively. The slides were then subjected to immunostaining with a panel of 90 commercially available antibodies primarily directed against specific phosphorylated or cleaved proteins, including PI-3K/Akt, ERK/MAPK, PKCs and caspase-dependent apoptosis. Each of these antibodies had previously undergone extensive validation for both phosphorylation and protein specificity using single band detection at the appropriate MW by Western blotting. To estimate the total protein amount, selected arrays were stained with Sypro Ruby protein blot stain (Molecular Probes, Eugene, OR, USA) and visualized on a Fluorchemk imaging system (Alpha Innotech, San Leandro, CA, USA). Slides were stained on an automated slide stainer (Dako, Carpinteria, CA, USA) using a biotin-linked peroxidase catalyzed signal amplification. Finally, the primary antibodies at concentrations ranging from 1:50 to 1:1000 were applied for 30 min followed by the secondary link antibody for 30 min (concentration 1:10 for anti-mouse antibodies and 1:5000 for anti-rabbit antibodies). For the complete list of the 92 stained antibodies with RPPA, please see **Table S2**. Each antibody was previously subjected to extensive validation for single band specificity by Western Blot (WB). For phospho-specific antibodies, each antibody was checked for specificity using cell extracts with and without appropriate ligand induction. The 92

antibodies used in this study were carefully selected based on both their extensive validation for specificity as well as detecting key signalling molecules known for their involvement in motility, invasion, pro-survival, and growth factor signaling. To allow normalization of total protein on printed arrays, one to two slides in each print run were stained with Sypro Ruby protein blot stain (Invitrogen) and the value of these stained arrays used for normalization of all end-point values. The intensity value for each end point was determined by identifying spots for each duplicate dilution curve for each sample that were within the linear dynamic range of the staining after background subtraction with each spot (within slide local background and also against a slide stained with secondary antibody only). Single intensity values were obtained by multiplying each spot in the linear range by its dilution factor and averaging candidate linear points. Finally, each value was normalized relative to the total protein intensity value for that sample derived from the Sypro Ruby-stained slide.

Table S2

1. AKT	cell signalling	1:50
2. EGFR	BioSource	1:100
3. ERK	cell signalling	1:100
4. ClCasp3 (D175)	cell signalling	1:50
5. phospho4EBP1 S65	cell signalling	1:100
6. phosphoAcetylCoA S79	cell signalling	1:100
7. phosphoAkt S473	cell signalling	1:100
8. phosphoCREB S133	cell signalling	1:100
9. phosphoERK T202-Y204	cell signalling	1:100
10. phosphoFKHR S256	cell signalling	1:100
11. phosphoFox01-03 T24-32	cell signalling	1:100
12. phosphoFKHRL1 S253	cell signalling	1:100
13. phosphoGSK3aB S21-9	cell signalling	1:100

69. phosphoNFkB S536	cell signalling	1:100
70. phosphoPKC delta T505	cell signalling	1:100
71. phosphoPKC theta T538	cell signalling	1:100
72. phosphoPKC zeta lambdaT410-403	cell signalling	1:100
73. phosphoPRAS40 T246	BioSource	1:1000
74. phosphoPTEN S380	cell signalling	1:500
75. phospho A RAF S299	cell signalling	1:100
76. phospho B RAF S445	cell signalling	1:100
77. phospho C RAF S338	cell signalling	1:200
78. phospho RAS GRF1 S916	cell signalling	1:100
79. phospho RSK3 T356-360	cell signalling	1:100
80. phospho S6rb S235-236	cell signalling	1:200
81. phospho SAPK-JNK T183Y185	cell signalling	1:50
82. phospho SHC Y317	cell signalling	1:100
83. phospho SMAD2 S465-467	cell signalling	1:250
84. phospho STAT5 Y694	cell signalling	1:50
85. phospho IKBa S32-36	BD	1:50
86. AKT2	cell signalling	1:100
87. CREB	cell signalling	1:100
88. eNOS	cell signalling	1:100
89. NFkB	cell signalling	1:50
90. PI3-K	cell signalling	1:100
91. PTEN	cell signalling	1:100

14. phosphoIRS1 S612	cell signalling	1:200
15. phosphomTOR S2448	cell signalling	1:100
16. phosphomTOR S2481	cell signalling	1:100
17. phosphoPDGFRB Y716	cell signalling	1:100
18. phosphoSTAT1 Y701	cell signalling	1:100
19. phosphoSTAT3 S727	cell signalling	1:50
20. phosphoSTAT3 Y705	cell signalling	1:50
21. phosphocABL T735	cell signalling	1:50
22. phosphocABL Y245	cell signalling	1:100
23. phosphoAKT-PKB S473	cell signalling	1:100
24. phosphoAKT T308	cell signalling	1:100
25. phosphoAMPKA S485	cell signalling	1:100
26. phosphoAMPKB S108	cell signalling	1:100
27. phosphoARRESTINB1 S412	cell signalling	1:100
28. phosphoASK1 S612	cell signalling	1:50
29. phosphoBAD S112	cell signalling	1:200
30. phosphoBAD S136	cell signalling	1:200
31. phosphoBcl2 S70	cell signalling	1:100
32. phosphoBcl2 T56	cell signalling	1:100
33. ClCasp7 D198	cell signalling	1:50
34. CleavedCasp9 D330	cell signalling	1:50
35. phosphoCateninB S33-35-T41	cell signalling	1:100
36. phosphoCofilin S3	cell signalling	1:100
37. phosphoEGFR Y992	cell signalling	1:50
38. phosphoEGFR Y1045	cell signalling	1:100
39. phosphoEGFR Y1068	cell signalling	1:100
40. phosphoEGFR Y1148	cell signalling	1:100
41. phosphoEGFR Y1173	cell signalling	1:100
42. phosphoEIF4E S209	cell signalling	1:100
43. phosphoEIF4G S1108	cell signalling	1:100
44. phosphoENOS S113	cell signalling	1:100
45. phosphoENOS S1177	cell signalling	1:100
46. ENOSNOS S116	Upstate	1:100
47. phosphoERB2HER2 Y1248	Upstate	1:100
48. phosphoERB3HER3 Y1289	Upstate	1:100
49. phosphoETK Y40	cell signalling	1:100
50. phosphoFADD S194	cell signalling	1:50
51. phosphoFAK Y576 577	BD	1:100
52. phosphoGSK3aB Y2790216	BioSource	1:100
53. phosphoHISTONEH3 S10	Upstate	1:100
54. phosphoIGF1 YY	cell signalling	1:100
55. phosphoIGF1 YYYY	cell signalling	1:100
56. phosphoLKB1 S334	cell signalling	1:100
57. phosphoLKB1 S428	cell signalling	1:100
58. phosphoMARCKS S152 156	cell signalling	1:100
59. phosphoMEK1 S298	cell signalling	1:100
60. phosphoMEK1-2 S217 221	cell signalling	1:100
61. phosphoPKCpan	cell signalling	1:100
62. phosphoP38	cell signalling	1:100
63. phosphoP70 6SK	cell signalling	1:100
64. phosphoP90RSK	cell signalling	1:100
65. phosphoPDK1 S241	cell signalling	1:200
66. phosphoPKA T197	cell signalling	1:100
67. phosphoPKCaS257	cell signalling	1:100
68. phosphoPKCab T638-641	cell signalling	1:100

5. RESULTS

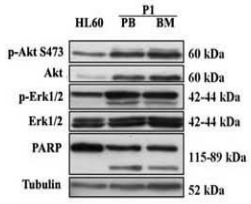
5.1 PHOSPHOPROTEIN ANALYSIS OF AML BLAST CELLS REVEALS DIFFERENT PROTEINS SIGNATURES CORRELATED WITH FAB CLASSIFICATION AND PROGNOSIS

A broad survey of multiple signaling pathways was initially performed on a population of 53 newly diagnosed AML patients, through RPPA analysis of 90 native and phosphorylated key endpoints related to cell growth, proliferation, survival and metabolism (Table 2, see MATERIALS AND METHODS). Blasts cells were obtained from bone marrow and/or pheripheral blood, at diagnosis (30 samples of pheripheral blood cells and 23 samples from bone marrow). 8 same patients blood and bone marrow specimens were available, and were therefore analyzed and compared, showing equivalent expression and phosphorylation of sampled proteins. Therefore in our analysis bone marrow and peripheral blood specimens were utilized interchangeably (Fig.1a,1b). Furthermore, 35 samples were fresh, collected and treated immediately after blood draw, whereas 18 were DMSO cryopreserved, obtained from a biobank at Policlinico di Modena. Thus protein profiles of fresh *vs* cryopreserved samples were compared by Western Blot and RPPA analysis. Overlapping profiles were observed in fresh samples and samples subjected to one freeze/thaw cycle. However, cleaved PARP and Caspase 3 were detectable in samples after repeated freeze/thaw cycles. (Fig. 1c,1d). Thus, in our study sets frozen samples were thawed only once. All samples were lysed in a buffer suitable to both RPPA and western blotting, then protein extracts were printed on nitrocellulose covered glass slides and proteins/phosphoproteins were detected with previously validated antibodies. By Microvigene software analysis a heatmap was generated, graphically representing the 90 endpoints scaled so that green represents low expression and red represents high expression (Fig. 2).

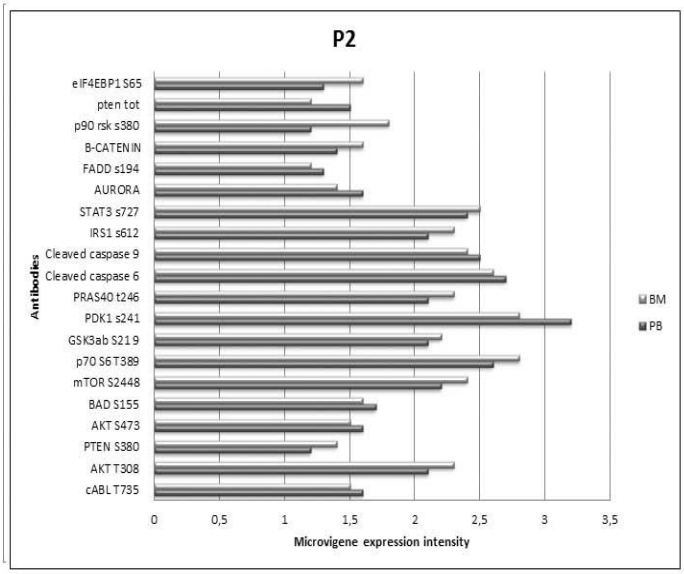
Because the FAB classification distinguish leukemia based on the degree of maturation, it is conceivable to expect different expression signatures for different FAB subtypes. FAB subtype comparison by unsupervised hierachical clustering analysis showed two groups of proteins tracking similarly. The first cluster by early myeloid M01-M2 patients was characterized by low levels of phosphorylated proteins involved in prosurvial pathways as pAkt-pPKC-pmTOR-pP70-pERK1/2, whereas signals related to proteins involved in proapoptotic functions are very high.

The second cluster is enriched for apoptosis (pBAD, pBCL2, pFADD, pFOXO). These proteins have significantly higher expression/phosphorylation in myeloid M4-M5 subtype.(Fig.3a,3b,3c) This difference might help to explain the poor prognosis associated to M4-M5 patients as well as the better outcome of M1-M2 patients.

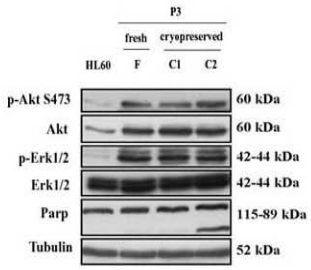
a)



b)



c)



d)

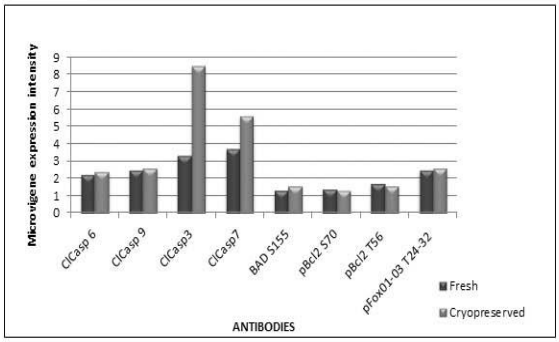


Fig. 1 **a)** Western blot shows expression of some of the proteins measured in one representative samples with matched blood (PB) and marrow (BM). HL60 cell lines were used as positive controls. **b)** Blast cells lysates from one sample, P2 representative of the 8 samples available, were printed onto RPPA arrays and assayed with 20 antibodies. The signal strength for each protein was similar regardless of source (blood or marrow). **c)** Western Blotting analysis shows that phosphorylation of Akt and Erk is not modified upon one freeze/thaw cycle (lane C1). However, repeated freeze/thaw cycles cause increase of cleaved PARP (lane C2). **d**) Blast cells lysates from one fresh sample and one sample subjected to repeated freeze/thaw cycles were analyzed through RPPA for apoptosis related proteins

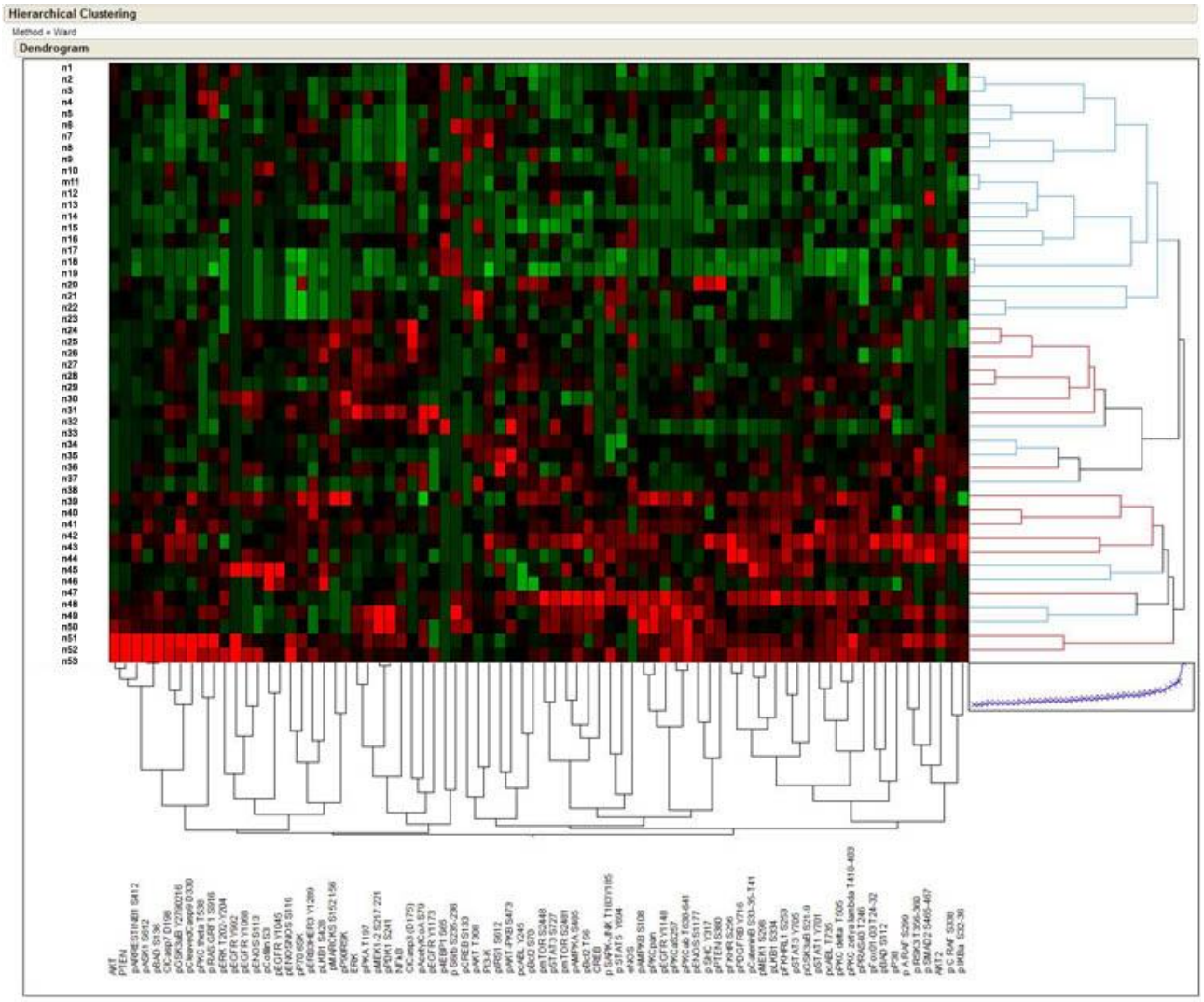
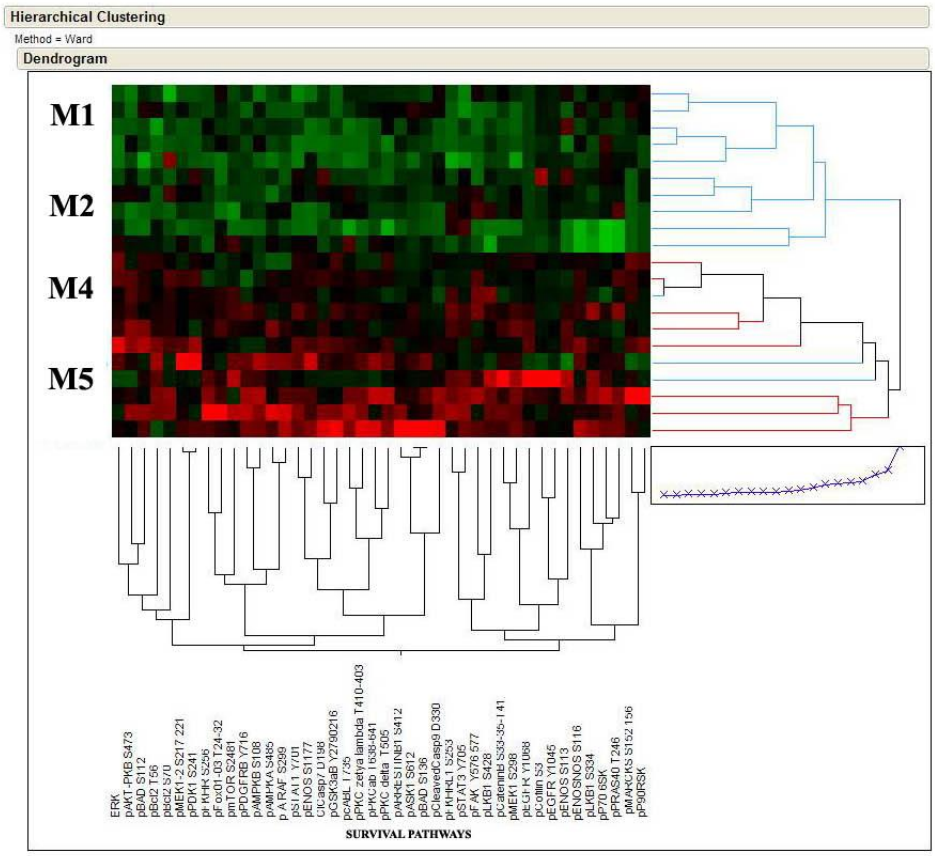


Fig. 2

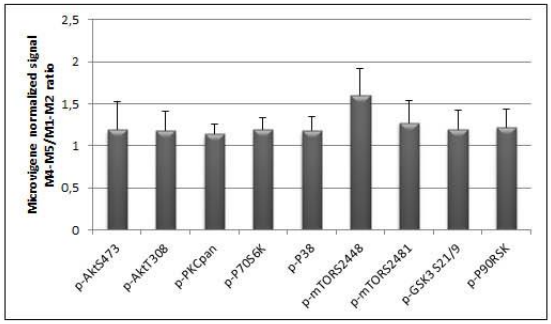
AML phosphoproteome

Through RPPA technology we analyzed about 90 endpoints involved in different signaling pathways: survival, apoptosis, oxidative-stress and metabolism. A heatmap generetad from Microvigene software is generated, graphically representing the 90 endpoints scaled so that green represents low expression and red represents high expression

a)



b)



c)

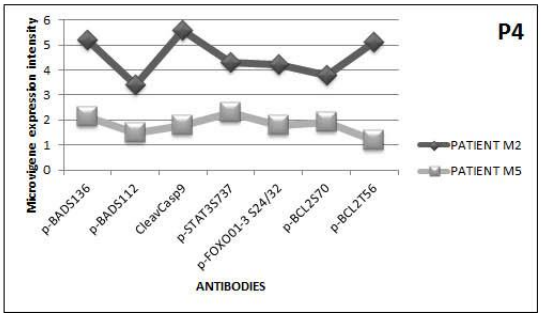


Fig.3 **a)** supervised Heat map based on FAM classification **b)** the reported signal intensities for each endpoint are expressed as a ratio between a mean value calculated in M4-M5patients and a mean value calculated in M1-M2 patients.Vertical bars indicate standard deviations **c)** Analysis of apoptosis related proteins. The signal intensity of one patient representative of the M2 and one patient representative of the M5 FAB subtype are shown.

5.2 AKT INHIBITION TRIGGERS PARADOXICAL AKT S473 PHOSPHORYLATION AS WELL AS SUSTAINED APOPTOSIS

Furthermore, the abovementioned unsupervised clustering of the 90 endpoints revealed two major classes of samples: one cluster with high Akt/mTOR pathway activation/phosphorylation and the other with a comparatively low level of signaling (Fig. 2). This result was not unexpected, as it is known that Akt phosphorylation at Ser473 can be detected in 50-60% of AML patients (30,78), and Akt/mTOR inhibitors are being developed as potential therapeutic (79). To get more insight into this important aspect, 10 samples with constitutive activation of PI3K/Akt were selected, and the effectiveness of PI3K/Akt inhibitors to diminish Akt phosphorylation and trigger apoptosis was studied. First, blast cells were grown for 20 hours in a medium additioned with either DMSO 0,01% (vehicle) or with the powerful ATP competitor of PI3K-p110 α,δ and β isoforms, LY294002 (1μM). Although cytotoxicity and low solubility prevented clinical development, LY294002 remains very useful in biochemical and pharmacological analysis. Samples were analyzed by western blotting and RPPA. Unexpectedly, PI3K inhibition had almost no effect on the phosphorylation of Akt S473 and its substrates GSK3, PRAS40 and FoxO 01/03. Phosphorylation at T308, as well as that of PDK1 S241, declined only slightly in most samples. Consequently the Akt kinase was not blocked by LY294002 (Fig 4a,4b) Remarkably however the drug was able to trigger cell death, as monitored by the increase of apoptosis related proteins such as cleaved caspase 9 and 6 (Fig. 4a). Hence, in a new study we explored whether this paradoxical Akt activity is a consequence of inhibitor binding to the ATP binding site of PI3K. We tested the following Akt inhibitors, already in clinical trials: Perifosine (phase II), Akt inhibitor VIII (phase I) and Triciribine (phase I).More than one inhibitor was used to avoid off targets effects. After 20 hours of drug administration, all the inhibitors were very effective in inducing apoptosis, though to a different degree (Fig. 5a). However, similarly to LY294002, these drugs caused paradoxical hyperphosphorylation of Akt

at its two regulatory sites Thr308 and Ser473, as well as high phosphorylation of its substrates pGSK3 (S21/9) pPRAS40 T246 in more than 70% samples (Fig. 5b). Interestingly, investigating this pathway by means of selective inhibitors allowed us to stratify blasts from AML patients in a group of responders, in which phosphorylation of Akt is blunted and cells undergo apoptosis, and one of not responders, characterized by persistent Akt activity above basal level. However it is important to mention that also the second group of samples undergo cell death in response to Akt inhibitors. Next, we investigated whether paradoxical Akt S473 phosphorylation in the non responder cluster might be due to the inactivation of the drug in water medium after 20 hours, or whether more complicated feedback mechanisms sustained Akt activity. To confirm this hypotesis, we repeated treatments at shorter times. Blast samples were cultured for 2 or 4 hours in a medium containing 20μM Perifosine. Figure 6 clearly shows that these short time treatments blunted Akt phosphorylation both at S473 and T308. In contrast, after 20 hours Akt was again hyperphosphorylated. The transient Akt inhibition suggests that feedback mechanisms promote reinduction of Akt activity.

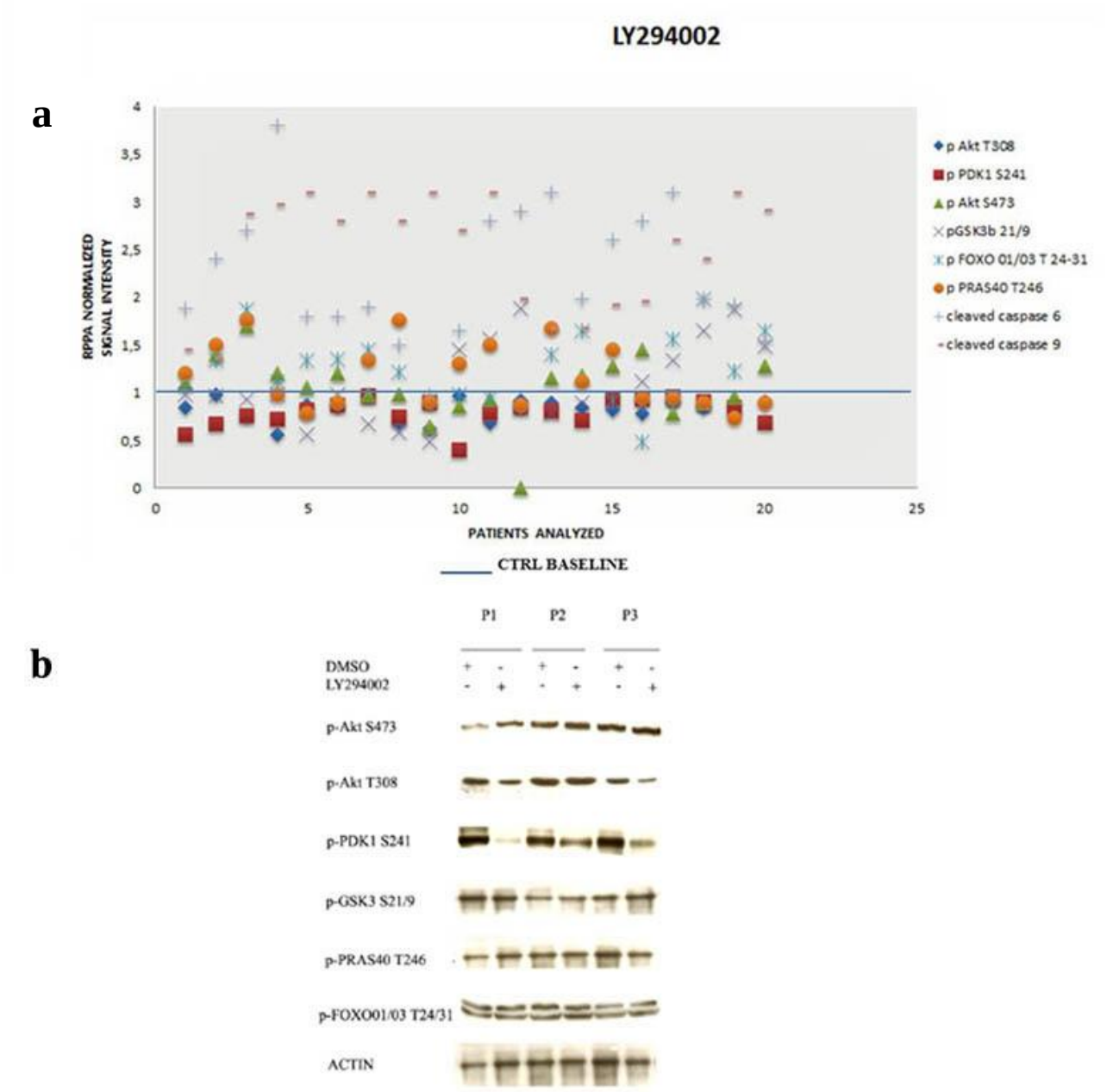
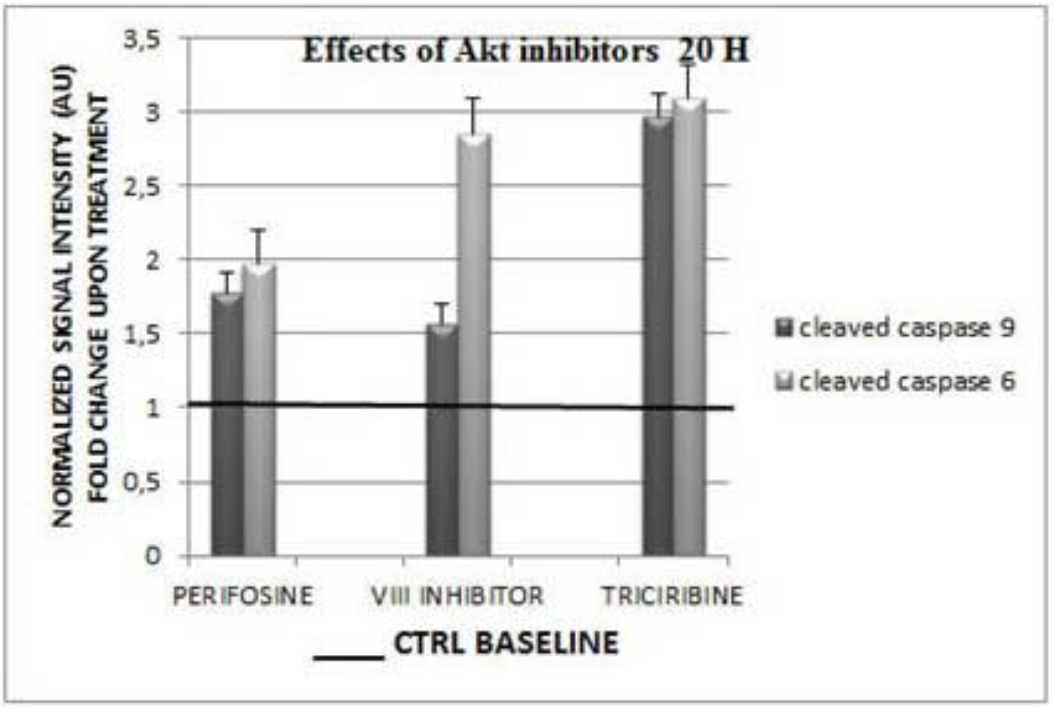


Fig.4

a)The signal intensity values shown in the graphic are normalized against the untreated samples. The dotted line indicates CTRL baseline of 1.
b) Western Blotting analysis shows expression of phosphoproteins involved in Akt signaling pathway in 3 representative samples treated with LY294002

a)



b)

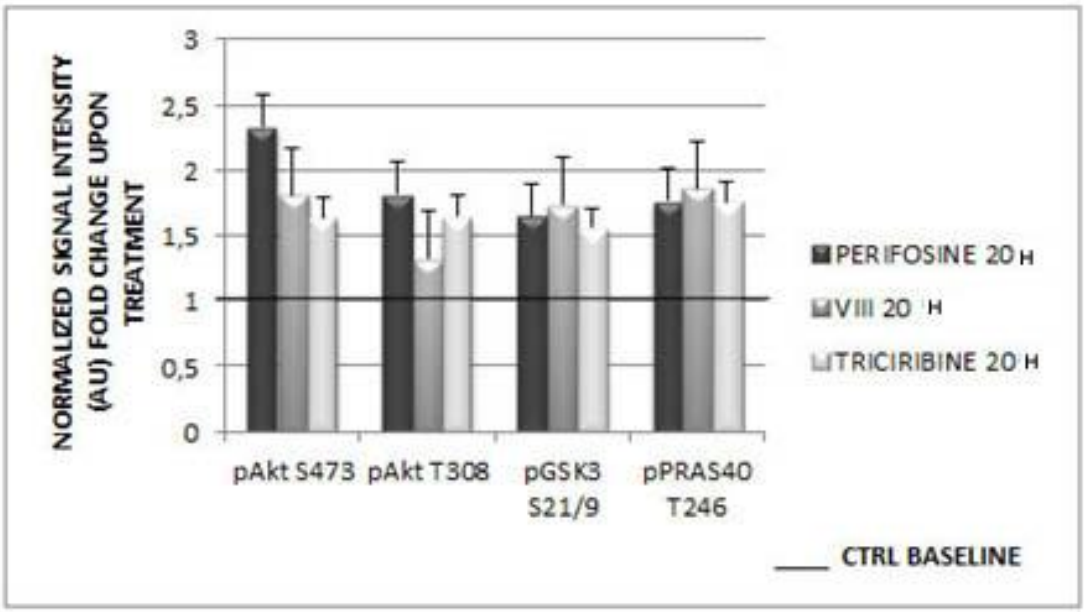


Fig.5 a) b) y-axis of the histograms represents *signal intensity* values obtained by Microvigene software. Y-axis, FOLD CHANGE VARIATION is calculated as a means value of ratio treatment versus vehicle(DMSO) .

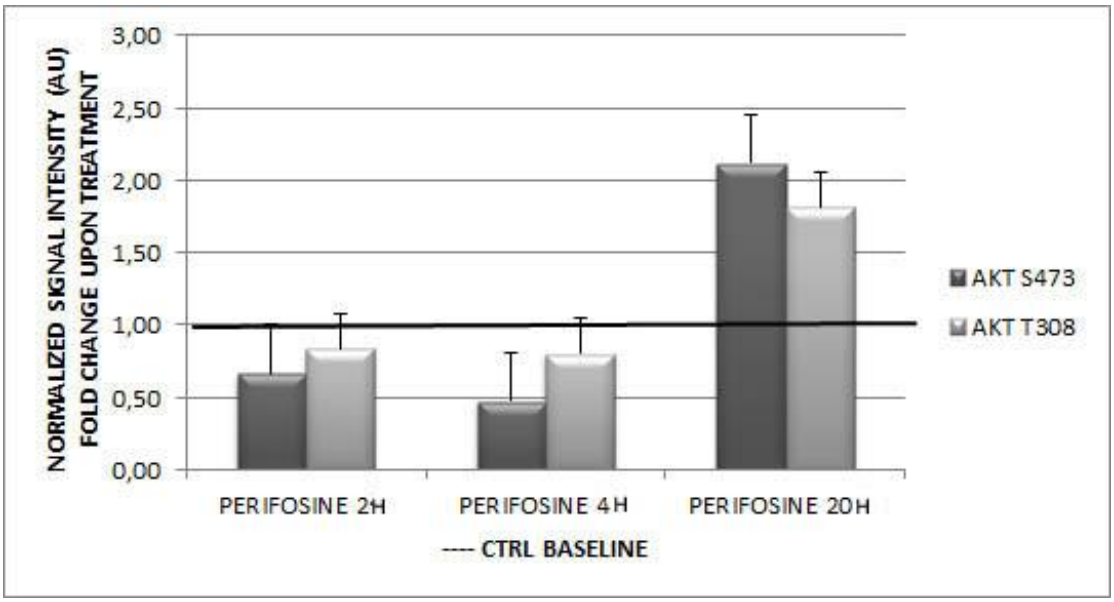


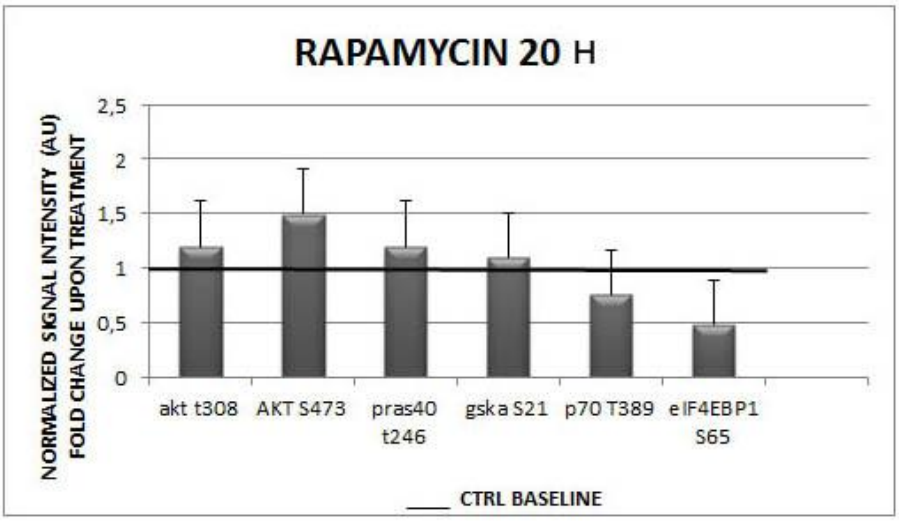
Fig. 6

5.3 COMBINED INHIBITION OF MTOR AND AKT ENHANCES CELL DEATH BUT DOES NOT COMPLETELY BLUNTS AKT ACTIVATION.

The mTOR complexes mTORC1 and mTORC2 function both upstream and downstream of Akt. mTORC1 is sensitive to rapamycin and inhibits Akt via a negative feedback loop which involves its substrate p70S6K. mTORC2 is rapamycin insensitive and directly phosphorylates Akt at S473. To explore the involvement of mTORC1/2 complexes in the rapid reinduction of Akt activity, we used the mTORC1 inhibitor rapamycin and the mTOR kinase inhibitor Torin 1. on 10 primary blast samples were tested. Sustained Akt S473 phosphorylation was observed in 60% samples after addition of Rapamycin for 20 hours (Fig 7). However, Rapamycin efficiently inhibited mTORC1, as from the low phosphorylation of its direct targets p70S6K and eIF4EBP1. Therefore, inactivation of p70S6K relieves feedback inhibition on IRS expression, which in turn might sustain Akt phosphorylation, as described recently by Tamburini et al., (75) and Shi Y et al. (76). Next, we reasoned that ATP competitive inhibitors of mTOR kinase, able to block both mTORC1 and

mTORC2, might be useful. Torin1 is an ATP-competitive mTOR inhibitor able to induce a significant decrease in the phosphorylation level of direct substrates of mTORC1 such as p70S6K and 4EBP1, and it also blocks the phosphorylation of Akt at S473 mediated by mTORC2. Therefore Torin 1 was used alone or in combination with Perifosine for 20 hours and protein extracts were analyzed by RPPA on 10 patients samples. Although reduction was only slightly below basal level, Torin1 inhibited Akt pS473 and abrogated the parodoxical phosphorylation observed above upon treatment with Perifosine alone. Of note, the combination triggered apoptosis very effectively (Fig 8). On the other hand, residual phosphorylation of Akt can be observed, sensitive neither to Akt inhibitors nor to mTOR inhibitors, indicating that alternative mechanisms exist that sustain Akt activity and maintain cell survival despite the block of a major survival pathway.

a)


RAPAMYCIN 20 H
NORMALIZED SIGNAL INTENSITY (AU)
FOLD CHANGE UPON TREATMENT
2,5
2
1,5
1
0,5
0
akt t308
AKT S473
pras40 t246
gska S21
p70 T389
eIF4EBP1 S65
CTRL BASELINE

b)

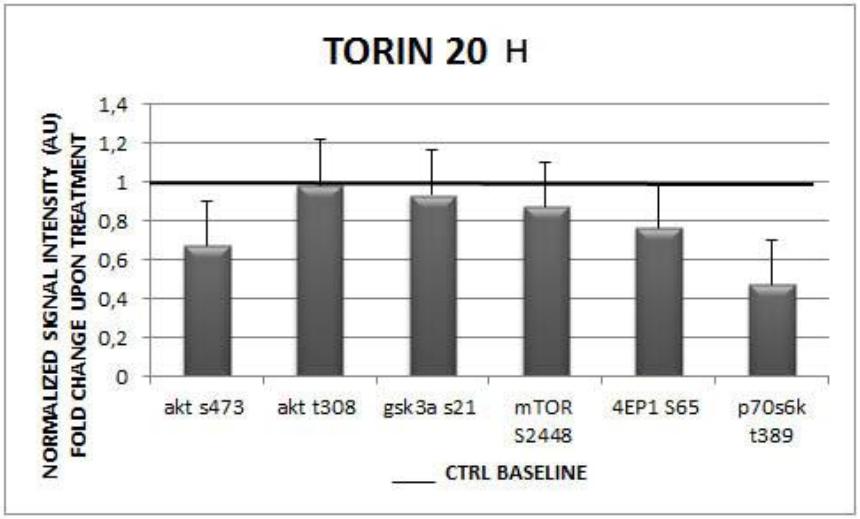

TORIN 20 H
NORMALIZED SIGNAL INTENSITY (AU)
FOLD CHANGE UPON TREATMENT
1,4
1,2
1
0,8
0,6
0,4
0,2
0
akt s473
akt t308
gsk3a s21
mTOR S2448
4EP1 S65
p70s6k t389
CTRL BASELINE

Fig. 7 a) b)

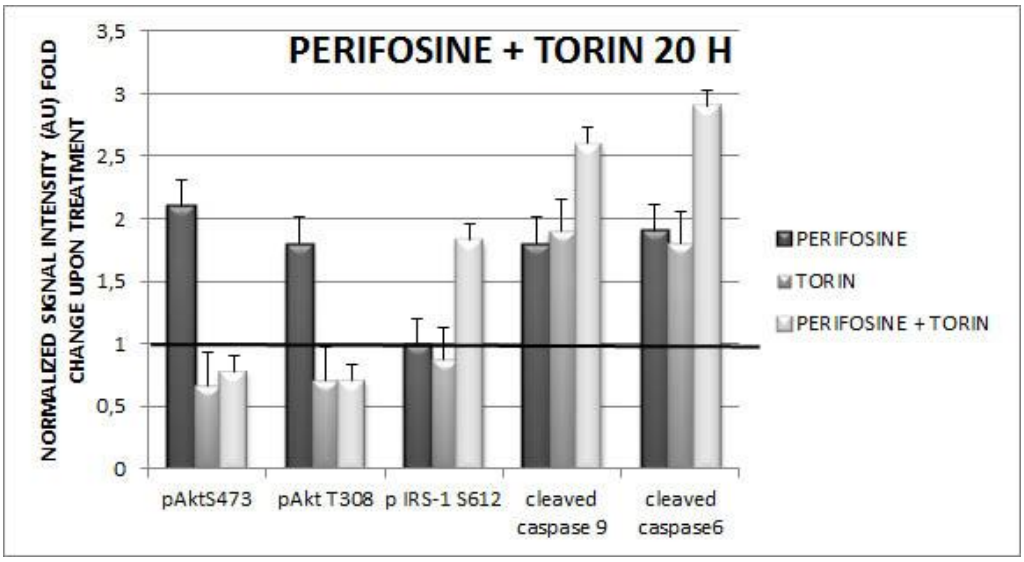


Fig. 8

5.4 RTK'S INHIBITION BLUNTS THE NEW STEADY STATE OF AKT ACTIVITY ORIGINATED FROM DRUGS TARGETING AKT/MTOR.

It was recently reported that mTORC1 inhibition can lead to activation of upstream receptor tyrosine kinase (RTK) signaling (80). In particular the Authors showed that mTOR and Akt inhibition induces expression and activation of multiple RTKs (80,81). We considered therefore that Akt is the main effector of PI3K,which in turn is activated by most RTK's.Thus, based on these reports, and on the results described above, we asked whether the inhibitor induced hyperactivation of Akt signaling in primary AML blast cells can be mediated by activation of growth factor receptor. Indeed, from previous analysis we knew that phosphorylation of the IRS-1 increased after Akt/mTOR inhibition (Fig. 8), in parallel with phosphorylation of Akt, therefore it is conceivable that the two events are finely balanced (Fig. 10). Tyrosine phosphorylation of the insulin receptors (IR) or IGF-1 receptors (IGF-1R) upon extracellular ligand binding induces the cytoplasmic binding of IRS-1 to these receptors. IRS-1 is an adapter molecules that provide docking sites for different SH2-

domain-containing proteins such as the phosphatidylinositol (PI) 3-kinase prompting it to a rapid activation. The protein levels of IRS-1 are regulated by the p70S6K, which targets IRS-1 for ubiquitin mediated degradation by the proteasome. We reasoned that the increased phosphorylation of IRS-1 observed in samples treated with Akt/mTOR inhibitors might result from the block of p70-mediated degradation of IRS-1 protein (76). Therefore, to confirm the existence of an alternative mechanism, dependent on RTKs such as IR/IRS-1, a further analysis of RPPA was carried out on 10 primary AML blasts specimens exposed to the broad RTKs inhibitor Sunitinib. Inibition of RTKs by Sunitinib at low concentration in combination with Perifosine or Torin1 very potently suppresses Akt signaling also in long time treatments (20 hours) (Fig. 9). We conclude that in cells where mTOR is inhibited, Akt signaling is dependent on the activation of upstream RTKs. In particular, it is known that phosphorylation of the Akt direct target Foxo03, leading to its degradation, down-regulates FOXO-dependent genes, such as IRS-1, IR and IGF-1 and possibly other RTKs. Conversely inhibition of Akt should abrogate FoxO degradation, resulting in up-regulation of Foxo-dependent RTKs. All together, we conclude that inhibition of Akt, accumulating FoxO3a into the nucleus, leads to increased expression of IRS-1, which in turn reactivates PI3K and Akt signaling.

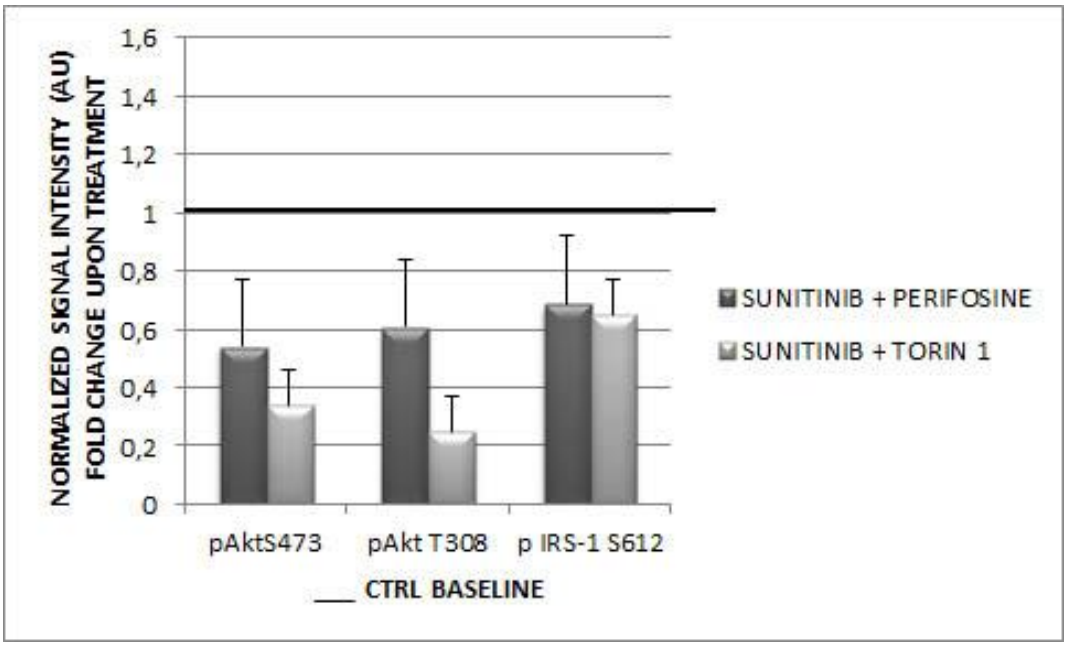
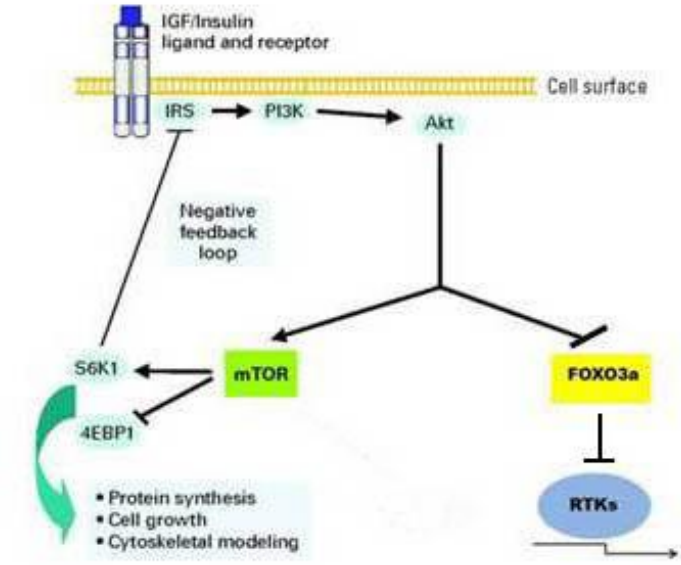

NORMALIZED SIGNAL INTENSITY (AU)
FOLD CHANGE UPON TREATMENT
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
pAktS473
pAkt T308
p IRS-1 S612
CTRL BASELINE
SUNITINIB + PERIFOSINE
SUNITINIB + TORIN 1

Fig 9

a)



b)

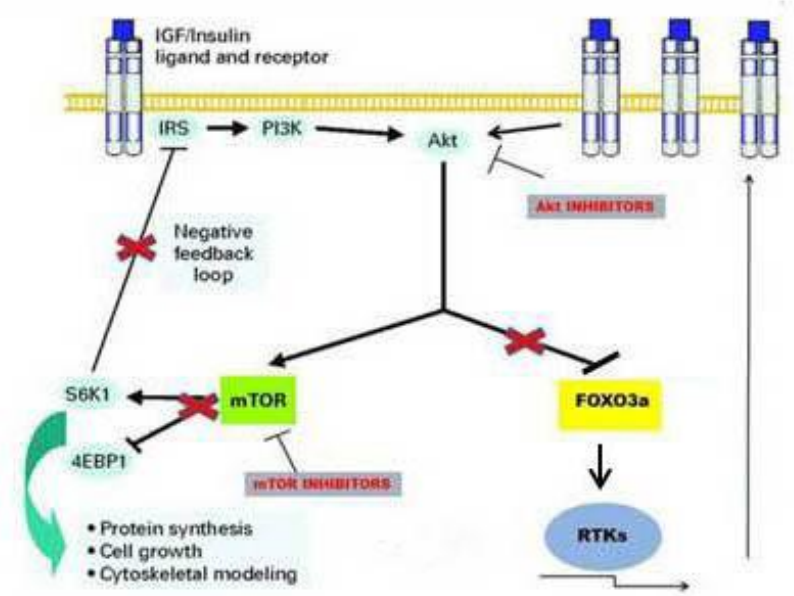


Fig 10 a) Feedback mechanism elicited by Akt and mTOR **b**) Akt/mTOR inhibition blocks the feedback mechanism and induces up-regulation of FoxO3a target genes.

6.DISCUSSION

The PI3K/Akt/mTOR pathway regulates several normal cellular functions that are also critical for tumorigenesis, including cellular proliferation, growth, survival and mobility. Components of this pathway are frequently abnormal in a variety of tumors, making them an attractive target for anti-cancer therapy. We show here that in primary Acute Myeloid Leukemia cells selective allosteric Akt inhibitors induced massive apoptosis. Paradoxically, however, this was not paralleled by decreased Akt phosphorylation. On the contrary, after 20 hours of drug addition to the medium, Akt S473 was hyperphosphorylated. We speculated that feedback signaling reactivates or maintains Akt activity by unknown mechanisms. This is likely to occur also in vivo in patients and can decrease the therapeutic effect of the drug. It is increasingly clear from recent reports that dysregulation of signaling by sustained activation/expression of receptor tyrosine kinases in cancer cells drives feedback inhibition of signaling network. Anticancer drugs inhibiting RTKs can relieve feedback mechanisms and reactivate signaling (81). The PI3K/Akt pathway is a key downstream physiological effector of growth factor receptors such as the Insulin receptor, IR, or the IGF-IR. Mutation or overexpression of other RTKs such as Flt3 or c-Kit may lead to dysregulation not only of PI3K/Akt but also of closely interconnected pathways, such as the mTOR pathway (80). Availability of specific drugs targeting these pathways may allow feedback mechanisms dissection. We show here experiments that reveal the extent and clinical implications of feedback mechanisms that allow leukemia cells to adapt to drugs targeting the Akt pathway over time. Experiments with Rapamycin in primary AML blasts indicate that one such feedback may be represented by mTOR. Indeed, the mTORC1 inhibitor Rapamycin potently reduced phosphorylation of its direct substrates p70S6K and 4EBP1, but elicited paradoxical Akt phosphorylation, most probably mediated by mTORC2. Moreover, prolonged inhibition of mTORC1 turns off p70S6K-mediated degradation of IRS-1, thus reactivating PI3K/Akt signaling.

We reasoned that the mTOR kinase inhibitor Torin 1, that blocks both mTORC1 and mTORC2 and therefore cannot cause the abovementioned feedback effects, should be more effective. However, Torin-1 reduced Akt phosphorylation only slightly below basal level. These results make it conceivable that other feedback mechanisms contribute to sustain Akt activity. Next we considered that inhibition of Akt kinase abrogates phosphorylation, and subsequent degradation, of its direct target Foxo. The transcription factor thus can accumulate in the nucleus and transactivate expression of its target gene, such as IR, IRS-1, IGF-IR as well as other RTKs. IRS-1 is an adaptor molecule that binds IGF-1 or Insulin receptors, upon extracellular ligand binding, through its SH2 domains. This binding allows IRS-1 to activate PI3K signaling. In our analysis, we observed that phosphorylation of IRS-1 increased in samples treated with Akt and mTOR inhibitors, consistent with the idea of an RTKs mediated feedback mechanism underlying persistent Akt activity. We used the broad tyrosine kinase inhibitor Sunitinib to confirm whether Akt feedback signaling involved RTKs. Remarkably, the inhibitor blocked phosphorylation of Akt deeply, and rescued the ability of Perifosine to flatten Akt activity. In conclusion, these findings demonstrate for the first time that Akt and mTOR inhibitors can trigger paradoxical Akt phosphorylation in leukemia blasts. The reason why this observation was missed by previous studies possibly resides in the use of either leukemic cell lines or few primary cells. It should be remembered, indeed, that around 40% samples do respond to Akt or mTOR inhibitors by decreasing Akt phosphorylation. In this study, by means of drugs selectively targeting either Akt or mTOR pathways, we have been able to relieve different aspects of the PI3K-dependent feedback and to demonstrate the existence of adaptative capabilities of leukemic cells, enabling them to escape Akt inhibition. This is an important finding, of consequence both for the biology of leukemia cells and for therapeutical strategy, as it demonstrates that Akt or mTOR inhibitors should not be proposed as monotherapy for the cure of AML. Conversely, in combination with RTKs inhibitors they very potently block Akt and avoid feedback signaling, while triggering massive apoptosis.

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