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Review

Involvement of the phosphoinositide 3-kinase/Akt signaling pathway in the resistance to therapeutic treatments of human leukemias

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Summary. A major factor undermining successful cancer treatment is the occurrence of resistance to conventional treatments such as chemotherapy and ionizing radiation. Evidence accumulated over the recent years has indicated the phosphoinositide 3-kinase/Akt signal transduction pathway as one of the major factors implicated in cancer resistance to conventional therapies. Indeed, the phosphoinositide 3-kinase/Akt axis regulates the expression and/or function of many anti-apoptotic proteins which strongly contributes to cancer cell survival. As a result, small molecules designed to specifically target key components of this signaling network are now being developed for clinical use as single therapeutic agents and/or in combination with other forms of therapy to overcome resistance. Initially, the phosphoinositide 3-kinase/Akt signal transduction pathway has been mainly investigated in solid tumors. Recently, however, this network has also been recognized as an important therapeutic target in human leukemias. Specific inhibition of this signalling pathway may be a valid approach to treat these diseases and increase the efficacy of standard types of therapy.

Key words: PI3K/Akt, Signal transduction, Phosphorylation, Resistance, Apoptosis

Introduction

The phosphoinositide 3-kinase (PI3K)/Akt signaling pathway has emerged as a key regulator of widely divergent physiological cellular processes which include apoptosis, proliferation, differentiation, motility, and metabolism (Brazil and Hemmings, 2001; Cantrell, 2001). However, disruption of normal PI3K/Akt signaling has now been documented to frequently occur in several types of human cancer and also to play an important role in their malignant progression (Roymans and Sleger, 2001; Nicholson and Anderson, 2002). Furthermore, it is now clear that activation of the PI3K/Akt axis may be one of the major factors undermining successful anticancer treatment (West et al., 2002). Therefore, this pathway is an attractive target for therapeutic strategies in patients suffering from cancer of various types, including hematopoietic malignancies. The purpose of this review is to discuss potential antineoplastic strategies targeting this signaling cascade in human leukemias. Nevertheless, we shall begin with an outline of the mechanisms which govern PI3K/Akt activation and of the responses generated along this pathway with a particular emphasis placed on human leukemias.

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The PI3K family of isozymes and their activation

There are multiple isoforms of PI3K in mammalian cells, and these are presently subdivided into three classes, referred to as I, II, and III (Cantrell et al., 2001; Vanhaesebroeck et al., 2001). Class I PI3Ks are the most studied because they are generally coupled to extracellular stimuli such as polypeptide growth factors and for this reason they will be the only isoforms considered relevant to this review. Their preferred in vivo substrate is phosphatidylinositol 4,5 bisphosphate ($PtdIns(4,5)P_2$) which is phosphorylated to phosphatidylinositol 3,4,5 trisphosphate ($PtdIns(3,4,5)P_3$). They are further subdivided into Class IA and IB PI3Ks. Class IA PI3Ks consist of a p110 catalytic subunit (α, ß, and δ) and an adaptor/regulatory subunit. There are at least

seven adaptor proteins that are generated by expression and alternative splicing of three different genes (p85α, p85ß, and p55γ). The regulatory subunits function as adaptors and act to localize PI3K to the plasma membrane by the interaction of their SH2 (Src homology) domains with phosphotyrosine residues in activated receptors. They also serve to stabilize p110 and to limit its activity (Hiles et al., 1992).

On the other hand, class IB PI3K or PI3Kγ, is made of a p110γ catalytic subunit and a p101 regulatory subunit, unrelated to p85. Indeed, p110γ cannot be activated in vitro by recombinant p85, in contrast to Class IA PI3Ks (Stoyanov et al., 1995). However, p110γ can also be activated by the ßγ subunits of heterotrimeric G proteins (Vanhaesebroeck et al., 2001). PI3Kγ up-regulation is a hallmark of inflammation (Wymann et al., 2003).

Akt isoforms and their activation

Akt, a 57-kDa serine/threonine protein kinase also known as protein kinase B (PKB), was initially described as the cellular homolog of the transforming viral oncogene v-Akt that caused murine T-cell lymphoma (Franke et al., 2003). There are three mammalian Akt isoforms: Akt1/α, Akt2/ß, and Akt3/γ, all of which share a high degree of homology at the amino acid level (Brazil and Hemmings, 2001). Akt1 and Akt2 are expressed in all tissues, but the highest expression is in brain, thymus, heart and lung. Akt3 expression is high in brain and testes (Steelman et al., 2004). Akt contains an amino-terminal pleckstrin homology (PH) domain, which binds the phosphorylated lipid products of PI3K (PtdIns(3,4,5)P_3, for example) which are generated at the plasma membrane. Once bound to the plasma membrane Akt is phosphorylated at Thr 308 by phosphoinositide- dependent protein kinase-1 (PDK-1, which also requires 3-phosphorylated inositol lipids for activation and plasma membrane translocation) and at Ser 473 by a kinase whose identity remains highly controversial (Brazil and Hemmings, 2001; Hill and Hemmings, 2002). The corresponding phosphorylation sites of Akt2 are Thr 309 and Ser 474, while those of Akt3 are Thr 305 and Ser 472 (Nicholson and Anderson, 2002). Moreover, recent findings seem to indicate that for a full activation, Akt needs to be also phosphorylated on Tyr 474 (Conus et al., 2002). Although Akt activation is predominantly dependent on the PI3K lipid products, PI3K-independent mechanisms have also been described. Protein kinase A and ß-adrenergic receptor agonists, as well as changes in intracellular Ca^{2+} levels have been reported to increase Akt activity in a manner which was insensitive to PI3K pharmacological inhibitors. However, some of these results have been questioned (Vanhaesebroeck and Alessi, 2000) so that further experiments will be needed to clarify this fundamental issue.

It should be emphasized here that Akt can be activated by many forms of cellular stress including heat shock, ultraviolet light, ischemia, hypoxia, hypoglycemia, and oxidative stress (West et al., 2002, and references therein). Stress-induced Akt up-regulation is likely to be viewed as a compensatory protective mechanism which cells activate for escaping death. This is very relevant to the topic of this review, because cells are likely to perceive chemotherapy as a cellular insult, and many types of chemotherapy exert their cytotoxic effects through the generation of reactive oxygen species.

Negative regulation of the PI3K/Akt pathway

Since 3-phosphorylated inositides are not hydrolyzed by any known phospholipase C, a counter-regulation by phosphatases has emerged as a crucial process to control PI3K-dependent signaling. PTEN (Phosphatase and TENsin homolog deleted on chromosome 10) is a dual specificity lipid and protein phosphatase that preferentially removes the 3-phosphate mainly from PtdIns (3,4,5)P_3 but is also active on phosphatidylinositol 3,4 bisphosphate (PtdIns (3,4,)P_2), thus antagonizing PI3K signaling pathways (Leslie and Downes, 2002; Sulis and Parsons, 2003). PTEN is often mutated and inactive in human cancer and this results in Akt activation. Therefore, PTEN is considered to be a tumor-suppressor gene. Two other phosphatases, SHIP-1 and SHIP-2 (for Src Homology domain-containing Inositol Phosphatases), are capable of removing the 5-phosphate from (3,4,5)P_3 to yield PtdIns (3,4,)P_2 (Backers et al., 2003). While SHIP-1 is predominantly expressed in hematopoietic cells, SHIP-2 is more ubiquitous (March and Ravichandran, 2002). Moreover, protein phosphatase 1 (PP1), and protein phosphatase 2A (PP2A) are capable of directly dephosphorylating Akt, thus down-regulating its function (Borgatti et al., 2003; Liu et al., 2003; Li et al., 2004).

The oncogenic role of the PI3K/Akt pathway

PI3K was discovered because of its association with the viral oncoproteins v-Src and polyoma middle-T antigen (Cantley et al., 1991). Since this initial finding, many other observations have provided compelling evidence that especially the class IA PI3K-mediated signaling pathway is involved in neoplastic transformation. Numerous oncogenes activate class IA PI3K and several components of the PI3K/Akt pathway are not appropriately regulated in a wide range of human cancers, including breast, colon, ovarian, pancreatic, lymphoid, and prostate cancers (Vivanco and Sawyers, 2002). For example, the p110α subunit of PI3K is amplified in ovarian (Shayesteh et al., 1999) and cervical cancer (Ma et al., 2000), while amplification of Akt1 has been reported in human gastric cancer (Staal, 1987) and that of Akt2 has been found in pancreatic (Ruggeri et al., 1998), breast, and ovarian carcinomas (Bellacosa et al., 1995). Remarkably, active Akt in human tumors has been linked with poorer clinical outcome (Ermoian et al.,

2002; Perez-Tenorio and Stal, 2002; Min et al., 2003). Moreover, elevated levels of phosphorylated Akt are detected in tumor cells which lack, or have a reduced level of PTEN (Whang et al., 1998; Li et al., 2002).

Up-regulation of the PI3K/Akt axis can also be caused by mutations affecting the single components of the signaling pathway. Indeed, the frequency of the genetic alterations affecting the PI3K/Akt cascade equals or exceeds that of any other pathway in cancer with the possible exception of the p53 pathway. For example, activating mutations in the PI3K p85 regulatory subunit have been described in ovarian and colon cancers (Vivanco and Sawyer, 2002), as well as in a Hodgkin's lymphoma-derived cell line (Jucker et al., 2002). Genetic alterations of both alleles of PTEN occur in nearly all types of human cancers examined, with the highest frequency of inactivation in glioblastoma and endometrial cancer (Li et al., 1997; Tashiro et al., 1997). The PI3K/Akt axis delivers survival signals by phosphorylating key apoptotic regulators, as described in the following section. However, it should not be overlooked the fact that this signaling pathway is strongly implicated in cell proliferation (Nicholson and Anderson, 2002), as well as in promoting anchorage-independent survival, tumor invasiveness, and neoangiogenesis (Garrouste et al., 2002; Suzuki et al., 2004; Tan et al., 2004). Conceivably, also these functions strongly contribute to the oncogenetic potential

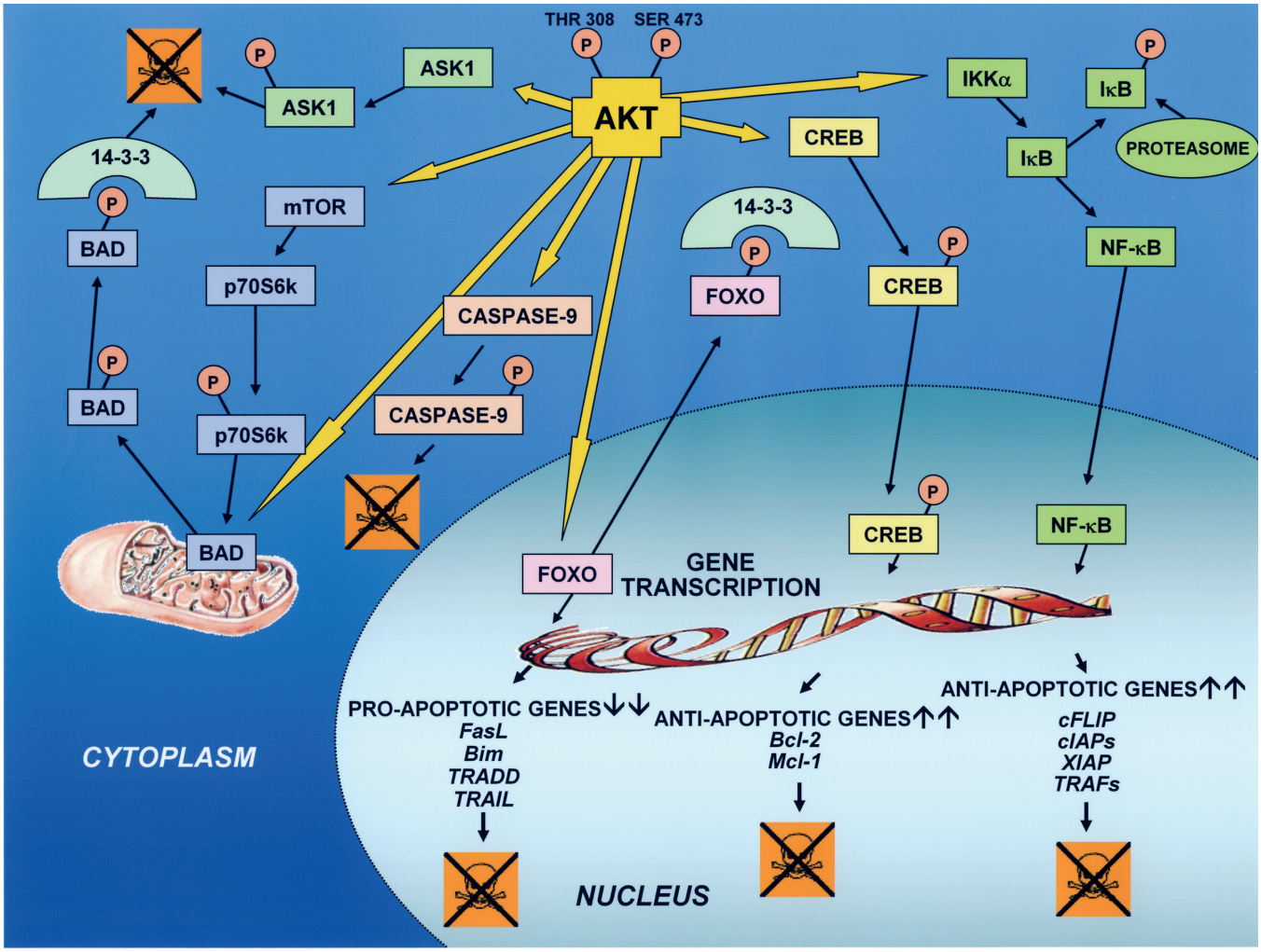

Fig. 1. Anti-apoptotic Akt signaling. Activated Akt promotes cell survival by multiple mechanisms: (1) by decreasing the transcription of pro-apoptotic genes by phosphorylating FOXO transcription factors which promotes their sequestration by 14-3-3 in the cytoplasm; (2) by increasing the transcription of anti-apoptotic genes by activating NF-κB and CREB transcription factors; (3) by phosphorylating and inactivating the pro-apoptotic proteins BAD, ASK1, and caspase-9; and (4) by activating mTOR which in turn phosphorylates and activates the pro-survival p70S6K. Note that, although substrates are placed in particular subcellular sites, the exact location of the phosphorylating events effected by Akt is still uncertain.

of the PI3K/Akt signaling.

Anti-apoptotic targets of PI3K/Akt pathway

Both chemotherapy and radiotherapy kill cancer cells mainly by inducing apoptosis. Because Akt is the prototypic kinase which promotes cellular survival to apoptotic insults, survival by Akt is the process controlled that has been most intensely investigated.

Numerous studies have highlighted that Akt enhances survival by directly phosphorylating key regulators of the apoptotic cascades. Akt phospharylates BAD, a member of the Bcl-2 family, at Ser 136. This modification promotes the sequestration of BAD by 14-3-3 proteins in the cytosol, thus preventing BAD from interacting with Bcl-2 or Bcl-XL at the mitochondrial membrane (Datta et al., 1997). Akt-dependent Bad phosphorylation at Ser 136 may also occur indirectly through the action of p70S6 kinase (p70S6K), another down-stream target of the PI3K/Akt pathway (Fig. 1) (Harada et al., 2001). However, this survival mechanism cannot operate universally because BAD is not expressed in all cell types (Kitada et al., 1998). Treatment with LY294002 (a pharmacological inhibitor selective for the PI3K/Akt pathway) reduced Ser 136 BAD phosphorylation and induced apoptosis in acute myeloid leukemia (AML) blasts with constitutively active Akt (Zhao et al., 2004). This finding may be an indication of the important role played by phosphorylated BAD to prevent apoptosis of leukemic cells.

Stress-activated protein kinases (SAPKs) are important mediators of apoptosis in cells exposed to stimuli such as heat shock, ionizing radiation, and osmotic stress (Benhar et al., 2002). Akt may interfere with SAPK signaling and thereby inhibit apoptosis because it phosphorylates and inactivates ASK1, a protein kinase which transduces signals to JNK and p38 MAP kinase pathways (Yoon et al., 2002; Yuan et al., 2003). Interestingly, it was shown that overexpression of Akt2 (which is often detected in ovarian carcinomas, as stated above) renders cisplatin-sensitive A2780S ovarian cancer cells resistant to cisplatin, a drug which has been widely used for treatment of human cancer. This finding emphasizes the important role played by Akt2 in chemoresistance (Yuan et al., 2003).

The aforementioned Akt targets regulate apoptosis prior to the release of cytochrome c from mitochondria and activation of the caspase-9 cascade which characterizes the execution phase of apoptosis. Nevertheless, there are data suggesting that Akt may also influence postmitochondrial events. Indeed, procaspase-9, the caspase initiator in the intrinsic apoptotic pathway, has been shown to be an Akt substrate. Akt-dependent procaspase-9 phosphorylation inhibited its intrinsic protease activity (Cardone et al., 1998). However, this finding has been somehow questioned, as it turned out that Akt most likely inhibits procaspase-9 activation through the action of an unknown cytosolic factor (Zhou et al., 2000). Another means by which Akt may promote cell survival is by phosphorylating transcription factors which control the expression of pro- and anti-apoptotic genes (Fig. 1). In fact, Akt either negatively affects factors that promote death gene expression or positively regulates factors inducing survival genes. An example of the former is the FOXO family of transcription factors, previously referred to as forkhead transcription factors (Arden and Biggs, 2002; Burgering and Medema, 2003). Phosphorylation of FOXO factors by Akt alters their intracellular localization: in the absence of Akt activation, FOXO proteins are predominantly localized in the nucleus where they are able to promote transcription of pro-apoptotic target genes such as *Fas-L* and *Bim* (Brunet et al., 1999; Dijkers et al., 2000). Activation of the PI3K/Akt pathway leads to the export of these factors from the nucleus and their accumulation and sequestration by 14-3-3 proteins in the cytoplasm (Biggs et al., 1999; Brunet et al. 1999; Cappellini et al., 2003). Thus, phosphorylation of FOXO proteins requires translocation of active Akt in the nucleus and there are numerous example of this subcellular localization of Akt (reviewed in Neri et al., 2002).

In addition to down-regulating FOXO activity, Akt is capable of positively regulating at least two other transcription factors. NF-kB is deeply involved in the regulation of critical cell functions such as proliferation, apoptosis, and survival (Li and Stark, 2002; Arlt and Schafer, 2002). The survival promoting activity of NF-κB is mediated by its ability to induce prosurvival genes such as *cIAP-1* and *-2*, *XIAP*, *c-FLIP*, and *TRAFs* (Li and Stark, 2002; Notarbartolo et al., 2004). NF-κB function is regulated through its association with the inhibitory cofactor I-κB, which sequesters NF-κB in the cytoplasm. Phosphorylation of I-κB by up-stream kinases, referred to as IKKs, promotes its degradation. This, in turn, allows NF-κB nuclear translocation and up-regulation of target genes (Fig. 1) (Greten and Karin, 2004). Akt phosphorylates directly and activates IKK· and, more importantly, it is believed to be essential for IKK-mediated destruction of I-κB (Sizemore et al., 2002).

Finally, the PI3K/Akt pathway has been shown to increase expression of the anti-apoptotic genes *Bcl-2* and *Mcl-1* in some cell types (Wang et al., 1999; Pugazhenthi et al., 2000; Yang et al., 2003). Akt-dependent enhanced expression of these two genes involves the cyclic AMP-response element binding protein (CREB) transcription factor. CREB is yet another direct target for phosphorylation by Akt and this phosphorylation occurs on a site (Ser 133) that increases CREB binding to accessory proteins necessary for gene induction (Du and Montminy, 1998). Mcl-1 protein seems to be very interesting for the scopes of this review, because its expression levels are related with resistance of human leukemias to a variety of chemotherapeutic agents (Kaufmann et al., 1998; van Stijn et al., 2003). Involvement of PI3K/Akt signaling in the regulation of

Mcl-1 expression in human leukemia cells has been shown by several groups (e.g. Ringshausen et al., 2002; Yu et al., 2003).

The PI3K/Akt axis and human leukemias

Activation of the PI3K/Akt survival pathway is a common feature to both acute and chronic human leukemias. As far as AML is concerned, a constitutive phosphorylation of Akt on both Ser 473 and Thr 308 has been detected in the majority of patients (70-80%) suffering from this type of neoplasm (Min et al., 2003; Tazzari et al., 2004) and Akt activation seems to be dependent on PI3K activity (Xu et al., 2003).

The phosphorylation of Akt was found to be significantly associated with phospho-FOXO1 factor (previously referred to as FKHR). Patients with phospho-FOXO1 had a significantly shorter overall survival than those without. Therefore, the detection of phospho-FOXO1 might provide a new tool for identifying AML patients with an unfavorable outcome (Cheong et al., 2003a).

Another critical target of Akt in AML blasts has very recently shown to be p27Kip1, a direct inhibitor of cyclin-dependent kinase (cdk) 2, one of the cdks responsible for the activation of E2F1 transcription factors that promote DNA replication (Sherr and Roberts, 1999). When phosphorylated by Akt, p27Kip1 mainly localizes to the cytoplasm where it cannot exert its inhibitory effect (Cappellini et al., 2003). Cytoplasmic localization of p27Kip1 in AML blasts with up-regulated Akt function was significantly associated with shorter disease-free survival and overall survival (Min et al., 2004a,b).

Activation of Akt in AML is also associated with BAD phosphorylation (Zhao et al., 2004) and enhanced NF-κB activity (Birkenkamp et al., 2004). Another down-stream target of PI3K signalling in AML blasts is XIAP. However, up-regulation of this anti-apoptotic protein in AML cells did not seem to require Akt activation (Carter et al., 2003).

The importance of the PI3K/Akt pathway in determining survival of AML blasts is emphasized by the finding that a selective pharmacological inhibitor of this network (LY294002) enhanced the apoptosis rate of AML blasts (Xu et al., 2003; Zhao et al., 2004).

The mechanisms which activate PI3K/Akt signalling in AML cells remain unclear even if, in about 30% of the cases, *N-Ras* gene mutations have been detected. These mutations resulted in constitutive Ras GTP-ase activation with a consequent effect on the PI3K/Akt pathway (Birkenkamp et al., 2004). Indeed, it is well established that Ras can activate the PI3K/Akt axis either by itself or through the Raf/MEK/ERK pathway (Blalock et al., 2003; Chang et al., 2003a,b). However, a recent study has implicated a dominant-negative mutation in the SHIP-1 phosphatase as a possible cause of Akt activation in AML (Luo et al., 2003). Also PTEN might be one of the factors involved in Akt up-regulation seen in AML, because in a recent study PTEN phosphorylation was detected in approximately 75% of patients suffering from AML. PTEN phosphorylation was significantly associated with Akt phosphorylation and with shorter overall survival (Cheong et al., 2003b). It has been reported that phosphorylation at the C-terminal regulatory domain stabilizes PTEN but makes it less active towards its substrate, PtdIns(3,4,5)P_3 (Vazquez et al., 2000, 2001). Others have reported that PTEN expression was low or absent from AML blasts (Xu et al., 2003). However, the level of PTEN expression did not correlate with the degree of Akt phosphorylation. As far as PTEN mutations are concerned, they do not seem to occur very frequently in AML (Dahia et al., 1999; Aggerholm et al., 2000). An absence of PTEN expression characterizes T-acute lymphoblastic leukemia cell lines such as CEM, Jurkat and MOLT-4, which have elevated levels of phosphorylated Akt (Uddin et al., 2004).

In some AML cases (approximately 25% of patients) there is an internal tandem duplication of the juxtamembrain domain of FLT3, which is a member of the class III receptor tyrosine kinase family. FLT3 plays an important role in regulating the proliferation, differentiation, and survival of hematopoietic cells (Stirewalt and Radich, 2003). This mutation is associated with a poor prognosis and results in Akt activation (Minami et al., 2003). Another possible mechanism of activation of the PI3K/Akt cascade in acute leukemia cells has been recently proposed. Vascular endothelial growth factor (VEGF) is a potent angiogenic molecule and pivotal regulator of neovascular response in hematological malignancies (Scavelli et al., 2004). It is intriguing that AML blasts produce and secrete VEGF and have demonstrable VEGF receptors, i.e. VEGFR-1 and VEGFR-2 (Bellamy et al., 2001). Using KG1 and HL60 human leukemic cell lines as experimental models, it has been demonstrated that VEGF stimulated rapid and sustained Akt phosphorylation through a mechanism which was dependent on PI3K since it could be inhibited by wortmannin (List et al., 2004). Therefore, at least in some AML cases, up-regulation of the PI3K/Akt axis may be due to an autocrine production of VEGF. A very recent study demonstrated that Akt up-regulation in AML blasts may be dependent on, or independent from, PI3K activation (Kubota et al., 2004).

As to chronic myelogenous leukemia (CML), the 210-kDa Bcr/abl tyrosine kinase, which is the primary molecular abnormality responsible for initiating and maintaining the leukemic phenotype of Philadelphia chromosome-positive cells (Goldman, 2004), associates with p85α of PI3K with a consequent activation of the PI3K/Akt pathway which is required for trasformation and growth of leukemic progenitors (Skorski et al., 1995, 1997).

The Bcr/abl tyrosine kinase (either 210-kDa or 185-kDa) fusion protein is also constitutively activated in some types of acute lymphoblastic leukemia (ALL)

(Brady, 2003). Hence, activation of PI3K pathway may be observed in ALL (Sattler et al., 1996).

Regarding chronic lymphocytic leukemia (CLL), there are several clues indicating an important role played by the PI3K/Akt/NF-κB axis in determining the survival of neoplastic B-cells (e.g. Barragan et al., 2002; Ringshausen et al., 2002; Cuní et al., 2004). Which may be the mechanism which promotes PI3K/Akt activation in B-CLL? Recent results point to the likelihood that serum albumin is the activating factor of this anti-apoptotic signalling pathway in B-CLL lymphocytes (Jones et al., 2003). However, full elucidation of the receptor and signal transduction mechanism through which albumin mediates up-regulation of PI3K/Akt axis is lacking.

Moreover, in human T-cell chronic lymphocytic/pro-lymphocytic leukemia (T-CLL/T-PLL), Akt is activated by the TCL1 oncogene (Pekarsky et al., 2000). TCL1 gene on chromosome 14q32.1 is constitutively activated by chromosome inversions and translocations observed in T-cell lymphoid malignancies (Lock, 2003). Evidence suggests that TCL1 oligomerizes with the PH domain of Akt at the plasma membrane, and that this facilitates Akt phosphorylation by PDK-1 (Auguin et al., 2004). While TCL1 binding to Akt is independent of Akt phosphorylation, the ability of TCL1 to stimulate Akt activity appears to be dependent on PI3K. Therefore, Akt phosphorylation and TCL1 binding may synergize to activate Akt, possibly due to the recruitment of the TCL1/Akt complex to the plasma membrane (Lock, 2003). TCL1 also facilitates Akt transport to the nucleus where it exerts some of its anti-apoptotic functions (Pekarski et al., 2000). Furthermore, the pathogenesis of T-CLL/T-PLL may also involve Nur77, a T-cell transcription factor required for T-cell receptor-mediated apoptosis. Akt phosphorylates Nur77, thereby blocking its DNA-binding ability and rendering the transcription factor inactive (Pekarski et al., 2003).

However, it should not been overlooked that a functioning PI3K signalling is required for normal hematopoiesis, since erythropoietin activates Akt in CD34+ hematopoietic progenitor cells (Myklebust et al., 2002), while inhibition of this pathway suppresses myeloid progenitor cell differentiation (Lewis et al., 2004).

PI3K/Akt activation and resistance to therapeutic treatments in human leukemias

The largely acknowledged critical role played by PI3K/Akt in cellular survival has led to an intense investigation into contribution of the signaling pathway to tumor cell survival in response to various types of therapeutic treatment.

It should be emphasized that administration of chemotherapeutic agents results in the modulation of the PI3K/Akt axis. Overall, the ability of chemotherapy to inhibit PI3K/Akt signaling correlates well with the cytotoxic effects of the drugs (West et al., 2002). Nevertheless, there is also evidence that chemotherapeutic agents can increase PI3K/Akt activity. For example, it has been reported that daunorubicin rapidly activates the PI3K/Akt pathway in U937 human leukemia cells (Plo et al., 1999). The mechanism directly responsible for such an activation is unclear, even though it is thought that the stimulus which produces apoptosis may also initiate an antagonistic anti-apoptotic program (Johnstone et al., 2002).

However, PI3K/Akt signalling is deeply involved in resistance to classical anti-neoplastic chemotherapeutic agents such as etoposide, anthracyclins, cisplatin (reviewed in West et al., 2002) as well as to ionizing radiation (e.g. Liang et al., 2003; Zhan and Han, 2004). Regarding human leukemias, an involvement of PI3K/Akt in causing resistance to drugs commonly used in treatment of AML, was first demonstrated by O'Gorman et al. (2000, 2001). These authors employed drug-resistant HL60 human leukemia cells to show that treatment with either wortmannin or LY294002 (two pharmacological inhibitors of PI3K) increased sensitivity to etoposide or doxorubicin. In contrast, an inhibitor of the Raf/MEK/ERK pathway (PD98059) did not sensitize HL60 cells to drug-induced apoptosis, indicating a lack of involvement of this pathway in chemoresistance.

More recently, our group has confirmed these data taking advantage of a HL60 cell clone, referred to as HL60AR (Apoptosis Resistant cells) which, when compared with parental (PT) HL60 cells, displayed a constitutively active PI3K/Akt axis (Neri et al., 2003). Interestingly, in HL60AR cells there was an autocrine secretion of insulin-like growth factor-1 which was responsible for PI3K/Akt activation. HL60AR cells are much more resistant than HL60PT cells to a wide variety of chemotherapeutic drugs as well as to all-trans retinoic acid (ATRA), a powerful differentiating agent for HL60 cells that is successfully employed also for the treatment of acute promyelocyitc leukemia (APL) (see Ohno et al., 2003 for an updated review). HL60AR resistance to drugs and ATRA could be lowered by overexpression of dominant negative PI3K or Akt, whereas forced expression of constitutively active Akt rendered HL60PT cells less sensitive to chemotherapeutic drugs or ATRA (Neri et al., 2003). Moreover, we have demonstrated that HL60AR cells are less sensitive to ionizing radiation than HL60PT cells. Treatment of HL60 cells with the PI3K inhibitors, wortmannin and LY294002, lowered radioresistance (Martelli et al., 2003). Involvement of the PI3K/Akt pathway in chemo- and radioresistance has also been shown in K562 and U937 human leukemia cells (Cataldi et al., 2003; Martelli et al., 2003).

The PI3K/Akt pathway has also been implicated in resistance of K562 human leukemia cells to STI571 (Kirschner and Baltensperger, 2003), even if these findings have been questioned (Marley et al., 2004). STI571 (imatinib, Gleevec, Glivec, CGP 57148) is an ATP-competitive selective inhibitor of Bcr-Abl

oncogene (Roskoski, 2003). STI571 has shown unprecedented efficacy for the treatment of CML (Hehlmann, 2003) and its effects on CML cells are reproduced by PI3K inhibitors such as wortmannin and LY294002 (Komatsu et al., 2003; Marley et al., 2004). STI571 activates the p38 MAP kinase-signaling pathway which plays an important role in the generation of the effects of STI571 on BCR-ABL-expressing cells, because its inhibition by means of the selective pharmacological inhibitor SB203580 blocked the STI571-induced multi-lineage differentiation of K562 cells as well as the growth inhibitory effects of STI571 on primary leukemic colony-forming unit granulocyte/macrophage progenitors from patients with CML (Kohmura et al., 2004; Parmar et al., 2004). As discussed earlier Akt activation hinders up-regulation of the p38 MAP kinase signaling pathway. Hence, inhibition of the PI3K/Akt axis is of benefit to the action of STI571. Furthermore, one of the STI571 targets is a FOXO transcription factor (Komatsu et al., 2003). FOXO transcription factor function is regulated through PI3K/Akt-dependent signal transduction. Thus, PI3K inhibitors such as wortmannin and LY294002 potentiate STI571 effects.

It has also been suggested that the PI3K/Akt axis is important for resistance to chlorambucil- and ionizing radiation-induced apoptosis of B-CLL lymphocytes, but surprisingly this hypothesis has never been tested by means of selective pharmacological inhibitors (Wickremasinghe et al., 2001).

Our group has recently shown that an up-regulated PI3K/Akt/NF-κB signalling pathway may be one of the factors implicated in resistance of human leukemic cell lines to arsenic trioxide (As_2O_3) (Tabellini et al., 2004a,b). As_2O_3 has been demonstrated to be effective especially for the treatment of APL. The mechanisms of action of As_20_3 are thought to involve growth inhibition and apoptosis induction (Ohno et al., 2003). Up-regulation of the PI3K/Akt/NF-κB pathway resulted in the enhanced expression of several anti-apoptotic factors which include XIAP, cIAPs, TRAFs (Tabellini et al., 2004a). As a consequence, As_20_3-elicited apoptosis was severely impaired in leukemic cells with an overactive PI3K/Akt signaling.

Finally, our laboratory has shown that the PI3K/Akt/NF-κB axis is also responsible for human leukemia cell resistance to Tumor necrosis factor-Related Apoptosis Inducing Ligand (TRAIL) (Bortul et al., 2003). TRAIL is one of the members of the TNF superfamily known to induce apoptosis in a wide variety of cancer, but not normal, cells (Wang and El-Deiry, 2003; Thorburn, 2004). AML blasts express TRAIL receptors (Min et al., 2004c) and the use of TRAIL as a therapeutic agent for AML has been proposed (Plasilova et al., 2002). Our results have clearly established that PI3K/Akt/NF-κB up-regulation results in enhanced expression of the FLICE inhibitory protein, cFLIP(L), in TRAIL-resistant HL60AR cells. cFLIP(L) is an inhibitor of caspase-8, the apical caspase of the death signalling cascade elicited by TRAIL (LeBlanc and Ashkenazi, 2003). Involvement of the PI3K/Akt pathway in TRAIL resistance has also been demonstrated by us in K562 and U937 leukemia cells (Martelli et al., 2003).

From the findings outlined above, it is clear that pharmacological inhibitors of the PI3K/Akt network should represent valuable tools to overcome resistance to therapeutic treatments which are currently used (or that might be used in the near future) for the treatment of human leukemias.

Inhibition of the PI3K/Akt pathway to overcome therapeutic resistance in human leukemias

Since activation of the PI3K/Akt axis confers therapeutic resistance, compounds which inhibit this pathway by targeting key regulatory proteins such as PI3K, Akt, mTOR, and Ras have potential for new effective therapies. Whether used alone or in combination with existing therapies, inhibitors of the PI3K/Akt pathway may exploit activation of the PI3K/Akt axis within cancer cells and/or enhance the efficacy of other forms of therapy.

Classical PI3K inhibitors

Two classical PI3K inhibitors, wortmannin and LY294002 have been widely used for in vitro studies on cancer cell lines in which they induce apoptosis and/or increase sensitivity to chemotherapeutic drugs, ionizing radiation, and TRAIL (West et al., 2002; Cataldi et al., 2003; Miyashita et al., 2003). Wortmannin is a fungal metabolite whereas LY294002 is a synthetic flavonoid derivative. Wortmannin irreversibly inhibits PI3K by interacting with the ATP-binding site of the catalytic subunit. In contrast, LY294002 is a reversible inhibitor of the ATP-binding site of PI3K (West et al., 2002).

However, neither wortmannin nor LY294002 are entirely specific for the PI3K/Akt pathway, as wortmannin also inhibits phospholipases C, D and A2, while LY294002 down-regulates casein kinase 2 activity (West et al., 2002).

There are numerous studies in which wortmannin and/or LY294002 have been employed to down-regulate in vitro the PI3K/Akt axis of human AML cells. As a consequence of the treatment, cells underwent apoptosis and/or became more sensitive to chemotherapeutic drugs, ionizing radiation, and TRAIL (O'Gorman et al., 2000; Bortul et al., 2003; Neri et al., 2003; Xu et al., 2003; Birkenkamp et al., 2004; Zhao et al., 2004). An interesting finding emerging from some of these investigations is that normal hematopoietic progenitors were less affected by PI3K inhibitors, suggesting a preferential targeting of leukemia cells (Xu et al., 2003; Zhao et al., 2004). This hypothesis was also strengthened by the results of a study in which the combination of STI571 and wortmannin was given to bone marrow cells isolated from healthy volunteers or patients suffering from CML: the combined treatment

inhibited cell growth and induced apoptosis in the cells derived from the leukemia patients, whereas normal bone marrow cells were unaffected (Klejman et al., 2002). When given singly, STI571 or wortmannin inhibited the phosphorylation of Akt and p70S6K and induced apoptosis in CML blasts. The combination STI571 plus wortmannin greatly potentiated apoptosis, which was associated with an increase in caspase-3 activation compared to either compound alone.

LY294002 induced apoptosis in B-CLL cells while sparing peripheral blood B-cells from healthy donors (Ringshausen et al., 2002), and increased dexamethasone- and fludarabine-induced apoptosis of B-CLL lymphocytes (Barragan et al., 2002).

Thus, the combination of a PI3K inhibitor such as wortmannin or LY294002 with conventional therapeutic agents may provide a better treatment for various types of acute or chronic leukemias which have become resistant to standard therapies.

Although the aforementioned studies suggest that blocking the PI3K/Akt pathway by means of wortmannin or LY294002 may be a valuable approach to treat human leukemias, there are some intrinsic disadvantages with these drugs. Wortmannin is soluble in organic solvents which may severely limits its use in clinical trails. As to LY294002, relatively few in vivo studies have been conducted to demonstrate its efficacy on the inhibition of growth of cancer xenogratfs (reviewed in West et al., 2002), but some side effects, such as dry and scaly skin, appeared in treated mice (West et al., 2002).

Novel selective Akt inhibitors

The traditional approach in developing kinase inhibitors has been to screen large compound libraries against the targeted purified kinase in vitro. This typically results in the identification of compounds which interact with the kinase's ATP-binding site. Because ATP-binding sites are similar across most kinases, inhibitors identified through this method usually display only a modest specificity. In an effort to develop effective Akt inhibitors and improve specificity, investigators have sought to interfere with the binding of PtdIns(3,4,5)P_3 to the PH domain of Akt. This effort has led to the synthesis of phosphatidylinositol ether analogues (PIAs), which were designed to inhibit this interaction (e.g. Hu et al., 2000). These inhibitors were synthesized based on the fact that they cannot be phosphorylated by PI3K on the 3-position of the myo-inositol ring. Indeed, they act as competitors for Akt activation at the plasma membrane and, thus, behave as downstream inhibitors of PI3K (West et al., 2002). A conceptual difference in the development of PIAs to inhibit Akt is that targeting the PH domain may minimize the lack of specificity observed with compounds that target the ATP-binding domain of Akt. Treatment of multiple cancer cell lines with these novel Akt inhibitors has demonstrated that several PIAs inhibited cancer cell growth (Castillo et al., 2004).

Using the experimental system consisting of HL60AR cells, we have demonstrated that one of these PIAs (1L-6-hydroxymethyl-chiro-inositol 2(R)-2-O-methyl-3-O-octadecylcarbonate) was able to restore sensitivity to chemotherapeutic drugs, ionizing radiation, ATRA, and TRAIL (Martelli et al., 2003). More recently, we have tested two novel PIAs, D-3-deoxy-2-O-methyl-*myo*-inositol 1-[(R)-2-methoxy-3-(octadecyloxy)propyl hydrogen phosphate and D-2,3-dideoxy-*myo*-inositol 1-[(R)-2-methoxy-3-(octadecyloxy)propyl hydrogen phosphate (see compounds PIA5 and PIA6 in Castillo et al., 2004) on HL60AR cells. These PIAs display improved metabolic stability and anticancer potential (Kozikowski et al., 2003). They were able to markedly increase sensitivity of HL60AR cells to etoposide or cytarabine at a concentration (5 μM) which was not toxic to human cord blood CD34+ hematopoietic precursor cells (Tabellini et al., 2004b). What may be the advantages given by these selective Akt inhibitors over PI3K inhibitors such as wortmannin or LY294002? The problem of systemic effects of PI3K inhibitors has not been investigated so far, but may be substantial, since the PI3K/Akt pathway regulates many physiological cell functions. By using selective Akt inhibitors side effects might be less marked. Indeed, our results have shown that 1L-6-hydroxymethyl-chiro-inositol 2(R)-2-O-methyl-3-O-octadecylcarbonate does not inhibit to the same extent as LY294002 the activation of PI3K down-stream targets which are independent from Akt, such as PKC-ζ (Martelli et al., 2003).

mTOR inhibitors

An alternative target to PI3K or Akt is represented by kinases located down-stream of Akt, such as the mammalian Target Of Rapamycin (mTOR or FRAP). mTOR, an evolutionary conserved kinase, functions as a molecular sensor which regulates protein synthesis upon the availability of nutrients such as amino acids (Fingar and Blenis, 2004). Both mTOR and PI3K, through independent and parallel pathways, modulate the activity of two kinases which control protein synthesis: p70S6K, a ribosomal kinase, and 4E-BP1, which binds and inhibits the eukaryotic initiation factor-4E (Fingar and Blenis, 2004). By controlling protein synthesis, p70S6K and 4E-BP1 also control cell growth and hypertrophy, which are important processes for neoplastic growth. Therefore, even more distal steps in the PI3K/Akt pathway may have the potential to be exploited to treat cancer (Bjornsti and Houghton, 2004). mTOR inhibitors include: rapamycin, CCI-779, and RAD001 (Huang and Houghton, 2003). Rapamycin is a macrocyclic lactone antibiotic which potently inhibits the growth of cancer cell lines and induces apoptosis (Panwalkar et al., 2004). Rapamycin has been approved by FDA as an immunosuppressant. It has, however, two disadvantages: poor solubility and chemical stability (Huang and Houghton, 2001). For this reasons, ester analogues of

rapamycin with improved aqueous stability and solubility have been synthesized. CCI-779 has been designed for intravenous injection and RAD001 for oral administration (Huang and Houghton, 2001). CCI-779 is already in Phase II clinical trial.

However, rapamycin failed to reverse drug resistance in HL60 cells (O'Gorman et al., 2000; Neri et al., 2003). In contrast, it has been reported that RAD001 was capable of sensitizing U937 leukemia cells to cytarabine (Xu et al., 2003). The reason for these conflicting findings is unclear.

Ras inhibitors

Earlier in this article, it was discussed that PI3K is a known down-stream substrate of the Ras GTPase in some cases of AML. Moreover, Bcr/abl-positive leukemias, including CML, frequently have overactivity of the Ras signaling pathway. Therefore, disruption of Ras activity may be effective for inhibition of the PI3K/Akt pathway. Ras becomes active only after post-translational addition of farnesyl or geranylgeranyl isoprenoid moieties to its carboxy terminus (Sebti, 2003). Without this modification, Ras stays in the cytosol where it cannot activate PI3K. Enzymes catalyzing transfer of these lipid moieties to Ras include farnesyltransferase, geranylgeranyltransferase type I, and geranylgeranyltransferase type II. Therefore, membrane localization of Ras could be inhibited by farnesyltransferase inhibitors (FTIs). Consistently, the FTI L-744832 has been shown to negatively affect both Akt phosphorylation and NF-κB activity of AML blasts with an overactive Ras (Birkenkamp et al., 2004).

Two FTIs (R115777 and SCH66336) are now undergoing Phase I or II clinical trials for hematological malignancies, including AML, CML, and ALL (Lancet and Karp, 2003).

Concluding remarks and future directions

There is no doubt that the activation of the PI3K/Akt pathway confers resistance to therapeutic treatments of various types of malignancies in vivo and in vitro, including those of hematopoietic origin. This finding is driving the furious development of compounds directed against components in the pathway. Nevertheless, a fundamental issue that still awaits answering is: will inhibition of this signaling network alter human diseases without deleterious side effects, such as perturbations of glucose homeostasis? In other words, can a therapeutic index be achieved when such an ubiquitous and fundamental pathway is targeted ? Perhaps a basis for a potential therapeutic index can be attributed to increased reliance on pathways promoting cellular survival by cancer cells exposed to forms of stress (chemotherapy for example) that are known for activating Akt. In addition, tumor cells might be more sensitive than normal cells to inhibition of this pathway because they often grow in hostile environments and would have therefore a higher reliance on survival signaling pathways. Therefore, even a partial inhibition of this pathway might be sufficient to negatively affect neoplastic growth while sparing normal cells.

Moreover, which component is the best target in such a heavily branched signaling network?

Would it be preferable to target single components of the branches further downstream of PI3K/Akt, such as mTOR, BAD, and the FOXO proteins that are more exclusively involved in cell growth, proliferation, and survival ? Or would "cocktails" of drugs affecting multiple steps of the pathway to be an even more effective form of therapy?

Additional work will be needed to address these issues to determine if the potential of PI3K/Akt inhibitors may be fully realized in cancer treatment. However, we may be entering a new era for cancer treatment using signal transduction modulators which could convert malignant hematological neoplasias into chronic and manageable diseases.

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