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PI3K-Independent AKT Activation in Cancers: A Treasure Trove for Novel Therapeutics

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Abstract

AKT/PKB serine threonine kinase, a critical signaling molecule promoting cell growth and survival pathways, is frequently dysregulated in many cancers. Although phosphatidylinositol-3-OH kinase (PI3K), a lipid kinase, is well characterized as a major regulator of AKT activation in response to a variety of ligands, recent studies highlight a diverse group of tyrosine (Ack1/TNK2, Src, PTK6) and serine/threonine (TBK1, IKBKE, DNAPKcs) kinases that activate AKT directly to promote its pro-proliferative signaling functions. While some of these alternate AKT activating kinases respond to growth factors, others respond to inflammatory and genotoxic stimuli. A common theme emerging from these studies is that aberrant or hyperactivation of these alternate kinases is often associated with malignancy. Consequently, evaluating the use of small molecular inhibitors against these alternate AKT activating kinases at earlier stages of cancer therapy may overcome the pressing problem of drug resistance surfacing especially in patients treated with PI3K inhibitors.

Keywords

Ack1; TNK2; ACK; AKT; SRC; TBK1; IKBKE; DNAPKcs; PI3K; PTEN; Tyrosine phosphorylation; Cancer; EGFR; HER2; Kinase inhibitor

AKT/PKB kinase is an indispensable conduit for signal transduction pathways that regulate processes intrinsic to the survival and defense of the organism i.e. cell growth, proliferation, survival and immunity. Activation of AKT is tightly regulated in normal cells, and at the minimum, three events appear to be required for full activation of its enzymatic activity. The first event is the rapid translocation of AKT to the plasma membrane in response to specific stimuli, the subsequent two events are phosphorylations at Thr308 and Ser473, that transform it into a potent kinase (Burgering and Coffey, 1995; Dong and Liu, 2005; Franke et al., 1995; Sarbassov et al., 2005; Stephens et al., 1998; Stokoe et al., 1997). While phosphatidylinositol-3-OH kinase (PI3K), a lipid kinase is a major regulator of AKT activation in response to a variety of ligands, recent studies highlight diverse group of tyrosine (Ack1/TNK2, Src, PTK6) and serine/threonine (TBK1, IKBKE, DNAPKcs) kinases that also activate AKT directly to promote growth, proliferation and oncogenic transformation. In this review, we will highlight the molecular mechanisms by which Ser/Thr/Tyr kinases, but not lipid kinases directly activate AKT. Further, we will discuss how pathological activation of these alternate activators bypasses PI3K-mediated AKT activation to promote malignancy.

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Frontline AKT activators: PI3K and PTEN

Addition of ligands or growth factors such as insulin, epidermal growth factor (EGF), PDGF (Platelet Derived Growth Factor) or IGF (Insulin like Growth Factor) to serum deprived cells rapidly activate the cognate Receptor Tyrosine Kinases (RTKs) by tyrosine phosphorylation. Ligand bound RTKs dimerize, associate with the lipid kinase, PI3K, which in turn phosphorylates the phospholipid phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-P2) or (PIP2) at the 3' position to generate PtdIns-3,4,5-P3 or (PIP3) (Zhao and Roberts, 2006). Higher concentration of PIP3 in the inner leaflet of the plasma membrane attracts cytosolic proteins such as AKT. AKT binds to the phospholipid via its PH domain, undergoes conformational changes that facilitate phosphorylation by PDK1 at Thr308 and at Ser473 by PDK2 (mTORC2 complex) thereby activating the kinase (Manning and Cantley, 2007). Activated AKT phosphorylates multiple proteins including BAD, MDM2, GSK3 β in the cytosol and the proapoptotic FOXOs proteins in the nucleus to regulate growth, metabolism, survival and tumorigenesis (Greer and Brunet, 2005; Huang and Tindall, 2007; Manning and Cantley, 2007). In normal cells, the RTK/PI3K pathway functions as a frontline activator of AKT to activate intracellular signaling pathways (Brugge et al., 2007). This signaling cascade is exquisitely regulated and is turned off rapidly by the activation of lipid phosphatases such as phosphatase and tensin homolog (PTEN) which removes the 3' phosphate or the SHIP phosphatase which removes the 5' phosphate from PIP3 to generate PIP2 (Aman et al., 1998; Zhao and Roberts, 2006). Lowering of PIP3 levels result in rapid downregulation of AKT signaling primarily due to its inability to be recruited to the plasma membrane.

Cancer cells represent one pathological scenario where this regulation has gone astray. Deregulation of AKT can occur either due to (i) amplification, mutations, deletions and substitutions in RTK genes, which constitutively activate RTKs or (ii) due to activating mutations in PI3K (PI3KCA) or (iii) deletion of the negative regulator and tumor suppressor phosphatase, PTEN (Burke et al., 2011). These modes of aberrant activation relay chronic signals that are pro-growth, proliferative and pro-survival. Not surprisingly, chemical compounds that can inhibit RTKs/PI3K pathway are potent suppressors of AKT signaling and several drugs are in clinical trials that efficiently target this pathway and inhibit growth of various tumors (Burke et al., 2011).

Although, a significant fraction of the cancer patients respond favorably to treatment with PI3K inhibitors, disease recurrence is a frequent outcome (Norman, 2011). The pro-survival cellular mechanisms alleviating the requirement of PI3K/PTEN mediated AKT activation is becoming increasingly apparent. Here we have focused on recent discoveries of PI3K-independent AKT activators that reveal new players contributing significantly to its oncogenic signaling functions. Uncovering of these alternate AKT activators not only deepens the complexity of AKT signaling, but it also provides a treasure trove of novel therapeutic targets that in combination with PI3K inhibitors can be used to abrogate AKT dependent and AKT-independent cancer cell survival.

Alternate AKT activators

From a number of recent studies it is becoming apparent that there exist another group of responders i.e. the secondary responders or the alternate responders that also pick up the signals from RTKs to activate AKT or can activate it when constitutively activated by autoactivating mutations (Guo et al., 2011a; Guo et al., 2011b; Joung et al., 2011; Mahajan et al., 2010b; Xie et al., 2011). These alternate responders belong to a diverse group of serine/threonine and tyrosine kinases that can interact with AKT to promote cell transformation, importantly without requiring participation of the PI3K pathway. These

alternate AKT activating kinases, their mode of action and conditions where their signaling acquire significance are discussed below.

Ack1 or TNK2

Ack1, a ubiquitously expressed non-receptor tyrosine kinase (NRTK), integrates signals from a multiple ligand-activated RTKs to modulate activity of intracellular tumor suppressor proteins and steroid receptors (Galisteo et al., 2006; Mahajan et al., 2010b; Mahajan and Mahajan, 2010; Mahajan et al., 2007; Mahajan et al., 2005). Recent studies have uncovered AKT as a key Ack1-interacting protein. Ack1 forms robust complex with all the three isoforms of AKT and directly regulates AKT's plasma membrane recruitment, 'shunting' the PI3K pathway (Mahajan et al., 2010b; Mahajan and Mahajan, 2010). This is mediated by a novel phosphorylation event in AKT, Tyrosine 176 that is invariant from yeast to mammals. Tyr176-phosphorylated AKT preferentially binds another membrane phospholipid, phosphatidic acid (PA) (Mahajan et al., 2010b), as opposed to PI3K activated AKT which specifically binds PIP3, facilitating its translocation to plasma membrane even in the presence of the PI3K inhibitor, LY294002 or PI3K siRNA.

While in normal cells Ack1 mediated AKT activation is regulated by growth factors, growth factor independent AKT activation can be mediated by the somatic mutants of Ack1 kinase. One such autoactivating somatic mutation, E346K, has been identified in the Ack1 kinase domain in ovarian cancer (Mahajan et al., 2010b; Mahajan and Mahajan, 2010). The Ack1-E346K mutant co-localizes with AKT and cause AKT Tyr176 phosphorylation followed by Ser473/Thr308-phosphorylation in serum starved conditions indicating that the somatic autoactivating mutations in Ack1 are sufficient to activate both Ack1 and AKT (Mahajan et al., 2010b). AKT2 expression is reported to be significantly increased in high grade ovarian tumors (Cheng et al., 1992; Saad et al., 2010). Thus, presence of high levels of AKT2 in a subset of ovarian cancers harboring Ack1 autoactivating mutations may increase the risk of tumor development.

Src

One of the first tyrosine kinases shown to directly phosphorylate AKT/PKB at highly conserved trysoine residues, Tyr315 and Tyr326, is Src (Chen et al., 2001). As seen with Ack1, Src mediated AKT Tyr-phosphorylation is not inhibited by LY294002, the PI3K inhibitor. Interestingly the constitutive kinase activity of myristoylated AKT (myr-Akt) was shown to be further enhanced by constitutively active Src mutant, Src-Y527F. Interaction between the two kinases involves the SH3 domain of Src and proline-rich motif (PXXP) in the C-terminal regulatory region of AKT (Jiang and Qiu, 2003). Notably, although AKT mutated at both Tyr315 and Tyr326 residues retained Thr308-phosphorylation, it lost ability to phosphorylate substrates upon EGF stimulation. The effect of these mutations on AKT Ser473 phosphorylation is not known. Nevertheless, cells expressing the myristoylated doubly mutated AKT (Y315F/Y326F) show detachment induced apoptosis and lack the ability to inhibit the activity of Forkhead transcription factors. The physiological significance of Src mediated AKT activation is further strengthened from the observation that the tumor necrosis family (TNF) family member TRANCE and its cognate receptor TRANCE-R, require both c-src and PI3K to activate PKB in dendritic cells and osteoclasts, with TRAF6 acting as a mediator between c-src and TRANCE-R, to promote survival. The cytokines IL α and LPS, which function through Toll like receptors also appear to activate AKT via Src family of tyrosine kinases (Wong et al., 1999).

PTK6

Protein tyrosine kinase 6 (PTK6), is another NRTK that has recently been shown to directly phosphorylate AKT and promote its activation in response to EGF (Zheng et al., 2010). Although, mass spectrometric analysis revealed PTK6 mediated AKT phosphorylation at Tyr215 and Tyr326 residues, mutational analysis revealed that Tyr215 is not the primary site of phosphorylation. In contrast, Y315F/Y326F double mutant displayed significant loss of AKT Tyr-phosphorylation indicating that these are likely to be the major sites targeted by PTK6. Interestingly, activation of AKT by PTK6 is most evident in cells exposed to low levels of EGF (0.1ng/ml versus 1ng/ml of EGF) suggesting that under higher concentrations of EGF, the RTK/PI3K pathway may be robustly activated, masking the contribution of PTK6 mediated AKT activation. It also opens up the possibility that phosphorylation at Y315/326 is in itself insufficient to activate AKT and cells require additional growth factor stimulation.

AKT tyrosine phosphorylation at 474 by an unknown kinase

High levels of basal AKT activity were observed in serum starved ovarian cancer cell line SKOV3 that displayed AKT phosphorylation at Tyr474 (Conus et al., 2002). Tyr474 site is located adjacent to Ser473 in the C-terminal regulatory region. Mutation at Tyr474 reduced Thr-308 phosphorylation and AKT activation, however, the identity of tyrosine kinase needed for AKT Tyr474 phosphorylation is not known.

Overall, multiple tyrosine kinases apparently activate AKT in PI3K-independent manner by phosphorylating AKT at multiple tyrosine residues e.g. Tyr176, Tyr215, Tyr315, Tyr326 and Tyr474 residues (Figure 1). Majority of these tyrosine phosphorylation events (Tyr176, Tyr215, Tyr315, Tyr326) occur in the kinase domain, phosphorylation of which is likely to induce conformational changes in AKT. For instance, molecular modeling revealed that Tyr176 and Ser473 in AKT are located in regions with increased conformational flexibility and phosphorylation at Tyr176 is likely to induce substantial conformational change and thus affect the loop harboring Ser473 (Mahajan et al., 2010b). Similarly, AKT modified at other tyrosine residues may adopt a completely different conformation. Therefore, inhibitors that target one modified form of AKT may be ineffective against other modified types of AKT. Guided molecular modeling approaches combined with high throughput synthetic chemistry for selective screening of small molecule inhibitors against specifically modified may be able to prevent AKT activation in cancers. Apart from tyrosine kinases, certain serine/threonine kinases also activate AKT in a PI3K independent manner, some of which are discussed below.

IKBKE

IKBKE or IKK ϵ (I-kappa-B kinase epsilon,) is a Ser/Thr kinase that plays a critical role in interferon mediated anti-viral immunity, exemplified in the IKBKE knockout mice which are hypersusceptible to viral infection due to defects in the IFN signaling pathway (Tenoever et al., 2007). It is one of the five distinct members of the I κ B kinase (IKK, inhibitor of nuclear factor κ B kinase) family (Boehm et al., 2007). While earlier studies revealed that IKK ϵ /IKBKE kinase directly phosphorylates STAT1 (S708A) (Tenoever et al., 2007), more recent studies have reported its ability to activate AKT directly (Guo et al., 2011a; Xie et al., 2011). Mass spectrometric analysis revealed three sites, Ser137, Thr308 and Ser473, at which AKT is phosphorylated (Guo et al., 2011). Of these, Ser137 phosphorylation site is present only in AKT1/3 and appears not to be essential for IKBKE mediated AKT activation *in vitro*. However, it may be essential in specific cellular context or in response to certain ligands. In cells expressing constitutively active IKK ϵ /IKBKE, AKT activation was unaffected by several well known pharmacological PI3K inhibitors e.g. LY294002,

Wortmannin, or compounds such as perifosine and triciribine that target PH domain of AKT. These findings suggest that the fraction of AKT complexed with IKBKE is refractory to PI3K inhibition due to direct activation. Further. It indicates that under certain conditions cells can dispense with regulatory steps to quickly activate AKT.

TBK1

TANK-binding kinase 1 (TBK1), also called as NAK (NF- κ B-activated kinase), or T2K (TRAF2-associated kinase) is a Ser/Thr kinase and a nonconforming member of the IKK-related kinase family, like IKBKE (Pomerantz and Baltimore, 1999). While TBK1 is demonstrated to be critical for the phosphorylation and activation of interferon response factors, IRF3 and IRF7, that play key roles in the induction of type I interferon (Hacker and Karin, 2006), recent studies indicate that TBK1 can also directly activate AKT by phosphorylation (Joung et al., 2011; Ou et al., 2011; Xie et al., 2011). It interacts with AKT following activation of Toll like receptor in macrophages by agonists, poly(I:C) and LPS, to facilitate activation of IRF3. AKT was found to be phosphorylated at three residues, Thr195, Ser378 and Ser473 by TBK1 in a PI3K-independent manner (Joung et al., 2011). Pharmacological inhibition of PI3K kinase had no affect on TBK1 mediated AKT activation (Joung et al., 2011). A complementary genetic study also concluded that TBK1 phosphorylated AKT at Ser473 when stably expressed in *Ric*^{-/-} cells (which lack mTORC2/Ser473 kinase activity) (Xie et al., 2011) and knockdown of TBK1 by shRNA reduced the residual AKT activation seen in *Ric*^{-/-} cells. Conversely, increased expression of TBK1 observed in the mTOR knockout cardiac muscle supports the hypothesis for mTORC2-independent role for TBK1 in AKT activation. However, in contrast to other studies, activation of AKT in this system partly required PI3K function, as this dependency could be for membrane recruitment of AKT via PH domain wherein it can be phosphorylated by membrane-associated IKK ϵ /TBK1 (Xie et al., 2011). MEFs derived from the IKBKE/TBK1 double knockout cells exhibited delayed, but did not exhibit complete loss of AKT phosphorylation, suggesting that other kinases may be recruited for AKT activation. Phosphorylation of AKT substrates, FoxO and GSK3 β , was also modestly reduced in growth factor stimulated IKBKE/TBK1 double knockout cells, consistent with the notion that these are backup pathways.

Ou et al reported that glucose but not insulin induced AKT activation in TBK1 null MEFs and EGF mediated activation was slightly impaired (Ou et al., 2011). TBK1 interacts with a fraction of AKT that is constitutively present in the subcellular compartment, exocyst. A pathological outcome of TBK1 mediated AKT activation is the oncogenic transformation of cells that could be blocked by a selective TBK1 small molecule inhibitor. Collectively, these studies reinforce the notion that although these alternate AKT activators function in backup pathways in normal cells, untimely or aberrant activation of these pathways in cancerous cells can lead to sustained AKT signaling to promote malignancy.

DNA-PK, MRE11/ATM

DNA double strand breaks or DSBs are induced by ionizing radiation, by radiomimetic agents and also during V(D)J recombination (Gellert, 2002). Repair of DSBs is not only essential for the preservation of genomic integrity but also for cell survival. Cells exposed to ionizing radiation display increased AKT phosphorylation (Bozulic et al., 2008). One of the kinase that is significantly activated by DSBs is DNA PKcs which is a core factor of the non homologous endjoining repair machinery. DNAPKcs activated by DSBs is autophosphorylated and phosphorylates AKT directly at Ser473 (Bozulic et al., 2008). AKT/PKB α ^{-/-} mice are hypersensitive to DNA damage indicating a functional requirement of DNAPK mediated activation. Although AKT activation by DNAPKcs is modest (~10-fold

enhancement of kinase activity) it is apparently sufficient to promote cell survival, as this activation is suggested to be essential for p21 transcriptional regulation and for prevention of DNA damage induced apoptosis (Feng et al., 2004).

Recent papers indicate that other DNA damage induced pathways such as the Mre11/ATM activate AKT under genotoxic stress to promote survival (Fraser et al., 2011; Surucu et al., 2008). MRE11/ATM dependent Ser473-phosphorylated AKT is detected at DSBs in response to ionizing radiation that is also PI3K-independent (Fraser et al., 2011).

Somatic mutations in AKT

The pathological role of AKT in cell transformation is further underscored by the finding of a rare oncogenic mutation, E17K, in AKT PH domain which facilitates its recruitment to the plasma membrane and subsequent activation in the absence of RTK/PI3K signaling, likely due to altered phosphoinositide binding (Carpten et al., 2007). This activating mutation has mostly been detected in solid tumors e.g. breast (2-4%), endometrial (2-4%), NSCLC (1%) and in squamous cell carcinoma (Cohen et al., 2010; Pao and Girard, 2011). Strikingly, introduction of the E17K mutation into a breast epithelial cell line did not affect EGF independent growth or acinar morphogenesis, nor resembled a PI3KCA mutated phenotype (Lauring et al., 2010). Somatic mutations in other members of AKT family have also been identified in endometrial cancer patients e.g. D399N and R368C mutations in AKT2 and E438D mutation in AKT3 (Dutt et al., 2009). The functional significance of these mutations remains to be determined.

Pathological features underlying alternate AKT activation

AKT activation is a frequent phenomenon in many solid tumors and hematological malignancies. The RTK/PI3K/mTORC2/AKT signaling cascade can be attributed as being the underlying cause of dysregulated AKT activation in a high proportion of these cancers. However, a significant percentage of the cancers do not show aberrations in any of the RTK/PI3K pathway members, but have the characteristic activated AKT expression. Among these are a third of the breast, prostate, majority of the pancreatic tumors and hematological malignancies that do not contain activating PI3K mutations (PI3KCA) and/or display normal PI3K activity (Bose et al., 2006; Panigrahi et al., 2004; Sun et al., 2001). Likewise, only 10% of the oral squamous cell carcinoma (OSCC) cases contain missense mutations in PI3KCA (Cohen et al., 2011). Moreover, PTEN expression is often unchanged in breast and ovarian tumors and mutations are rare in acute myeloid leukaemia (AML) and OSCC (Sun et al., 2001). Notable is the constitutively active E17K mutant of AKT that is also found not to be mutated in AML, gliomas and in OSCC (Cohen et al., 2011; Schuller et al., 2008; Tibes et al., 2008). The question remains as to how cancer cells continue to exhibit significant activation of AKT which is apparently critical for their survival.

Key clues for hyperactivated status of AKT comes from studies of primary cancers and cell lines which have uncovered activated AKT irrespective of PI3K functional status (Chen et al., 2001; Joung et al., 2011; Mahajan et al., 2010b; Ou et al., 2011; Xie et al., 2011). These cancers harbor genetic alterations such as gene amplifications or somatic autoactivating mutations in Ack1, TBK1 or IKBKE genes that increase expression and/or activation of the kinases in a dysregulated fashion. Constitutive or facultative association and interaction of AKT with aforementioned kinases appear to obviate the dependency on PIP3 (Ack1) or PDK1/PDK2 (TBK1 and IKBKE). E346K-Ack1, identified as a somatic mutation in ovarian cancer, causes significant AKT activation in the absence of growth factors (Mahajan et al., 2010b). Expression levels of Tyr284-phosphorylated-Ack1 and Tyr176-phosphorylated-AKT were also positively correlated with the severity of disease progression, and inversely correlated with the survival of breast, prostate and pancreatic cancer patients suggesting a

significant contribution of Ack1 to PI3K-independent AKT activation (Mahajan et al., 2010a; Mahajan et al., 2010b; Mahajan K., 2012). Notable are Ack1 transgenic mice that display activated Ack1 and triple phosphorylated AKT, these mice develop prostatic intraepithelial neoplasia or PINs (Mahajan et al., 2010b).

IKBKE, another direct AKT activating kinase, has also been reported to be overexpressed in human primary breast, ovarian, gliomas and prostate cancer due to copy number gain or chromosome amplification resulting in increased mRNA and protein expression (Boehm et al., 2007; Guan et al., 2011). Its oncogenic function had been attributed to its ability to induce nuclear translocation of NF- κ B component, C-Rel (Agami, 2007; Harris et al., 2006). Elevated expression of IKBKE in glioma cells increased their resistance to apoptotic inducers such as adriamycin or UV irradiation by inducing Bcl2 expression (Guan et al., 2011). A significant percentage of breast tumors display elevated levels of IKBKE and Ser473-phosphorylated AKT, underscoring its role as a human breast cancer oncogene (Guo et al 2011). Consistent with the above, IKBKE can substitute for myristoylated or activated AKT, in cooperating with activated MEK to induce transformation and for growth of NIH3T3 xenograft tumors in nude mice (Boehm et al., 2007; Guo et al., 2011a).

The other non-canonical member of the IKK family, TBK1 is also highly expressed in many cancers including lung, breast and colon cancers (Barbie et al., 2009; Boehm et al., 2007; Kan et al., 2010). TBK1 is known to induce proliferation of endothelial cells and has a proangiogenic activity (Korherr et al., 2006). RNA interference experiments revealed loss of TBK1 was synthetic lethal in cells containing activated KRAS and induces apoptosis in cells driven by oncogenic *KRAS*. TBK1 was found to regulate expression of Bcl-XL and c-Rel in K-ras mutant cell lines (Barbie et al., 2009; Shen and Hahn, 2011). A somatic mutation, P675L, was recently identified in lung adenocarcinoma (Kan et al., 2010). Overexpression of Her2/neu and PTK6 has been observed in ductal invasive breast carcinomas and expression of PTK6 has been shown to be prognostic of long term survival of breast cancer patients (Aubele et al., 2007; Born et al., 2005).

Translational implications: Alternate AKT activators as drug targets

Due to aberrant signaling in cancers, PI3K/AKT/mTOR pathway has been targeted for inhibition to block tumor growth and metastasis. Several PI3K inhibitors are currently under clinical trials (Crunkhorn, 2010). In all likelihood as seen with cancers having BRAFV600E mutation, inhibition of the original oncogene is not sufficient and cells develop drug resistance by activating other pathways such as RTK or N-Ras (Nazarian et al., 2010). Not surprisingly, a recent study demonstrated that NOTCH/cMYC pathway activation conferred resistance to the dual PI3K-mTOR inhibitor BEZ-235 as well as other PI3K inhibitors (Muellner et al., 2011). Using yeast based assay, mutations in p110 alpha subunit of PI3K (I800M or I800L mutations) have been identified that confer resistance to PI3K inhibitors and were further validated in human cell lines (Vogt, 2008; Zunder et al., 2008). Breast cancer cell lines that are resistant to Her2/RTK inhibitor, lapatinib, also recover from initial suppression of AKT phosphorylation. These lapatinib resistant cell lines display increased AKT Ser473 phosphorylation and tyrosine phosphorylation of several tyrosine kinases such as Src, Yes, Fyn, Lck, Frk and Erbb2. Similarly primary tumors isolated from lapatinib treated Her2 positive breast cancer patients exhibited increased RNA expression of some of the SRC family members (Rexer et al., 2011). It indicates that activation of the SRC family tyrosine kinases could be a significant mechanism underlying lapatinib resistance. Moreover, treatment with both lapatinib and broad SFK family inhibitor, AZD0530 were required for tumor regression of Her2 positive xenografts (Green et al., 2009; Hennequin et al., 2006; Rexer et al., 2011).

With the molecular characterization of these alternate AKT kinases as significant players in cancer development and progression, development of selective and specific inhibitors of these kinases becomes paramount. Some of the small molecular inhibitors that inhibit the activation of alternate AKT kinases have recently emerged. AIM-100 (4-amino-5,6-biaryl-furo[2,3-d]pyrimidine derivatives), a selective Ack1 inhibitor, has recently been shown to inhibit not only Ack1 activity but also suppress AKT kinase activity suppressing proliferation of wide variety of cancer cell lines including prostate, breast, lung, melanoma and lung cancer (Mahajan et al., 2010a; Mahajan K., 2012). AIM-100 treatment arrested cells in G1 phase which resulted in significant decrease in proliferation of cancer cells and induced apoptosis. (Mahajan et al., 2010a; Mahajan K., 2012). These data collectively indicate that Ack1 mediated AKT Tyr176-phosphorylation is prognostic of progression of tumors and inhibitors of Ack1/AKT signaling could be novel therapeutic agents to treat cancer patients that exhibit AKT activation but have normal PI3K and PTEN levels.

Although PLX4032 or Verumfarenib is currently being used for the treatment of melanoma patients with BRAFV600E mutation (IC₅₀ of 31nM at 100uM ATP), it has also been found to be a potent inhibitor of Ack1 activity *in vitro* (Ic₅₀ of 19nm at 10uM ATP) (Bollag et al., 2010). Dasatinib, a broad tyrosine kinase inhibitor also inhibits Ack1 kinase and has been approved by FDA and is currently in trial for treatment of various cancers (Li et al., 2010). Thus, small molecule inhibitors e.g. PLX4032 or Dasatinib could be potentially used in patients whose tumors exhibit Ack1 activation and have normal PI3K levels.

A number of pharmaceutical companies and academic groups are currently developing selective TBK1/IKKBKE inhibitors. While the initial goal was to develop small molecule inhibitors that mitigate the pro-inflammatory cytokine responses in inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus (SLE) and psoriasis, more recent attention is directed towards preclinical characterization of these inhibitors in suppressing cancer (Xie et al., 2011). These TBK1/IKKBKE inhibitors display high selectivity with many of them working in the nanomolar range such as 6-aminopyrazolopyrimidine derivative (IC₅₀=13nM) and MPI-0485520 (IC₅₀=0.5-1)(Ou et al., 2011). BX795 an aminopyrimidine compound, although developed as a PDK1 inhibitor inhibits the catalytic activity of TBK1 and IKK ϵ (IC₅₀=10nm-1uM) (Clark et al., 2009). Similarly, several small molecule inhibitors of DNA-PK e.g. NU7026 and IC486241 (ICC) are available and have shown be to effective in killing a number of cancer cell lines such as breast and colon when combined with DNA damaging agents (Davidson et al., 2011). Mre11 inhibitor, mirrin is another recently developed small molecule inhibitor that induces G2 arrest in response to DNA damage (Dupre et al., 2008; Garner et al., 2009). Thus, these compounds hold significant potential especially in those cancers wherein overexpression or activation of the alternate kinase and AKT is observed (Table 1). Alternate kinase and AKT activation could be determined by immunohistochemical staining of tumor biopsies or second generations sequencing which reveal somatic autoactivating mutations and amplification. These patients especially in case of haemotological malignancies where mutations in the PI3K/PTEN are rare or non existent, could respond well to small molecule inhibitors directed towards alternate kinases. This personalized medicine approach would be highly advantageous and far more efficient.

Conclusion

From all these studies it is apparent that cancer cells utilize the enzymatic activity of disparate group of kinases to retain AKT activity in cells. Notably, the expression of many of the alternate kinases in human primary tumors may not be mutually exclusive and may underlie genetic or cellular heterogeneity apparent in many cancers. Accordingly, cancer derived from an individual patient may have to be screened by immunohistochemical

staining with specific antibodies on a single platform to determine the activation status of PI3K and the alternate kinases. Second generation DNA sequencing of Ack1, TBK1, IKBKE, PTK6 and SRC may have to be performed to uncover one or more somatic autoactivating mutations in the kinase genes in each cancer sample. Mouse models that are generated usually have one representative mutation for targeting and thus are unlikely to address the problem of drug resistance. Complex mice xenograft models would be needed that have multiple activated kinases overexpressed in the same tumor and the effect of combination therapy is needed to be evaluated. Treatment of recurrent tumors or non responsive tumors with multi-drug approach has to be considered to completely inhibit cancer growth. Overall, identification and characterization of these alternate AKT activating kinases has opened a treasure trove for development and pre-clinical evaluation of novel therapeutics for treatment of some of the most aggressive type of cancers.

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Figure. 1.
Schematic representation of multiple phosphorylation sites in AKT targeted by various kinases. The E17K mutation site is also shown.

Table 1

Inhibitors of alternate AKT activating kinases

Kinase	Phosphorylation site	PI3K	Inhibitors (IC50)	Cancer/ Gain or Loss of function
Ack1/TNK2	Tyr 176	Independent	AIM-100 (21nM); Dasatinib	Gain-Breast, prostate, lung, pancreas, brain, Erwing's sarcoma
PTK6	Tyr315, Tyr326	Not known		Breast
Src	Tyr315, Tyr326	Independent	Dasatinib	
IKBKE	Thr308, Ser473,	Independent Or dependent	6-aminopyrazolopyrimidine derivative(59nM),MPI-0485520 (Myrexix) *	Breast, ovarian, gliomas, prostate
TBK1	Thr308, Ser473	Independent Or dependent	6-aminopyrazolopyrimidine derivative (13nM), MPI-0485520 (Myrexix) *	Breast, lung, colon
DNA-PK	Ser473	Independent	NU7026, IC486241	Loss-lymphomas
Mre11/ ATM mediated (kinase unknown)	Ser473	Independent	mirrin	Loss-lymphomas

* Richards B et al. Cellular and In Vivo Properties of MPI-0485520, a Novel and Potent Small Molecule Inhibitor of IKK ϵ . FASEB 2010 Annual Meeting.