Targeting Akt in cancer therapy

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In an effort to improve therapeutic options in cancer, many investigational drugs are being developed to inhibit signaling pathways that promote the survival of cancer cells. The prototypic pathway that promotes cellular survival is the phosphoinositide 3′-kinase/Akt/mammalian target of rapamycin pathway, which is constitutively activated in many types of cancers. Mechanisms for activation of the serine/threonine kinase, Akt, include loss of tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) function, amplification or mutation of phosphoinositide 3′-kinase, amplification of Akt, activation of growth factor receptors and exposure to carcinogens. Activation of Akt promotes cellular survival as well as resistance to treatment with chemotherapy and/or radiation therapy. Immunohistochemical analyses have shown that Akt is activated in many types of cancers and preneoplastic lesions, and Akt activation is a poor prognostic factor in various cancers. Taken together, these data demonstrate that Akt is a valid target for inhibition. This review will focus on published data using different approaches to inhibit Akt. We will also consider how the complex regulation of the phosphoinositide 3′-kinase/Akt/mammalian target of rapamycin pathway poses practical issues concerning the design of clinical trials, potential toxicities and the likelihood of finding a therapeutic index when targeting such a critical cellular pathway. *Anti-Cancer Drugs* 18:861–874 © 2007 Lippincott Williams & Wilkins.

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The phosphoinositide 3′-kinase/Akt pathway

Initial activation of the phosphoinositide 3′-kinase (PI3K)/Akt pathway occurs at the cell membrane, where the signal for pathway activation is propagated through class IA PI3K (Fig. 1). Activation of PI3K can occur through tyrosine kinase growth factor receptors such as epidermal growth factor receptor (EGFR) and insulin-like growth factor-1 receptor (IGF-1R), cell adhesion molecules such as integrins, G-protein-coupled receptors, and oncogenes such as Ras. Once PI3K has been activated, PI3K catalyzes phosphorylation of the D3 position on phosphoinositides to generate the biologically active moieties phosphatidylinositol-3,4,5-triphosphate [$PI(3,4,5)P_3$] and phosphatidylinositol-3,4-bisphosphate [$PI(3,4)P_2$]. Upon generation, $PI(3,4,5)P_3$ binds to the pleckstrin homology (PH) domains of 3′-phosphoinositide-dependent kinase 1 (PDK-1) and the serine/threonine kinase Akt, causing both proteins to be translocated to the cell membrane where they are subsequently activated. The tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) antagonizes PI3K by dephosphorylating $PI(3,4,5)P_3$, thereby preventing translocation and activation of Akt and PDK-1.

Akt exists as three structurally similar isoforms, Akt1, 2 and 3, which are expressed in most tissues [1]. Activation of Akt1 occurs through two crucial phosphorylation events, the first of which occurs at T308 in the catalytic domain by PDK-1 [2,3]. Full activation requires a subsequent phosphorylation at S473 in the hydrophobic motif, which can be mediated by several kinases such as PDK-1 [4], integrin-linked kinase [5,6], Akt itself [7], DNA-dependent protein kinase [8,9] or mammalian target of rapamycin (mTOR) [10]. Phosphorylation of homologous residues in Akt2 and 3 occurs by the same mechanism. In addition to phosphorylation at T308 and S473, Akt can be regulated by phosphorylation at other sites or by binding to other proteins [11]. For example, an isoform of protein kinase C (PKC-ς) mediates an inhibitory phosphorylation of Akt at T34 in the PH domain [12]. Full activation of Akt also requires a tyrosine (Y) phosphorylation at Y474 [13]. Finally, Akt interacting proteins such as hsp90 [14], Tcl-1 [15], the C-terminal modulator protein [16], JNK-interaction protein [17] and TRB3 [18] can also affect Akt activity. The relative importance of these mechanisms and their role in cancer biology is unknown. Inhibitors of the chaperone protein hsp90, however, have recently shown promising anti-cancer activity owing to their inhibition of several client proteins, including Akt [14,19–22]. The fact that there are multiple mechanisms responsible for modulating Akt activity suggests that there are cell-specific and context-specific modes of regulation, which may have implications for developing inhibitors of Akt.

Akt recognizes and phosphorylates the consensus sequence RXRXX(S/T) when surrounded by hydrophobic residues. As this sequence is present in many proteins,

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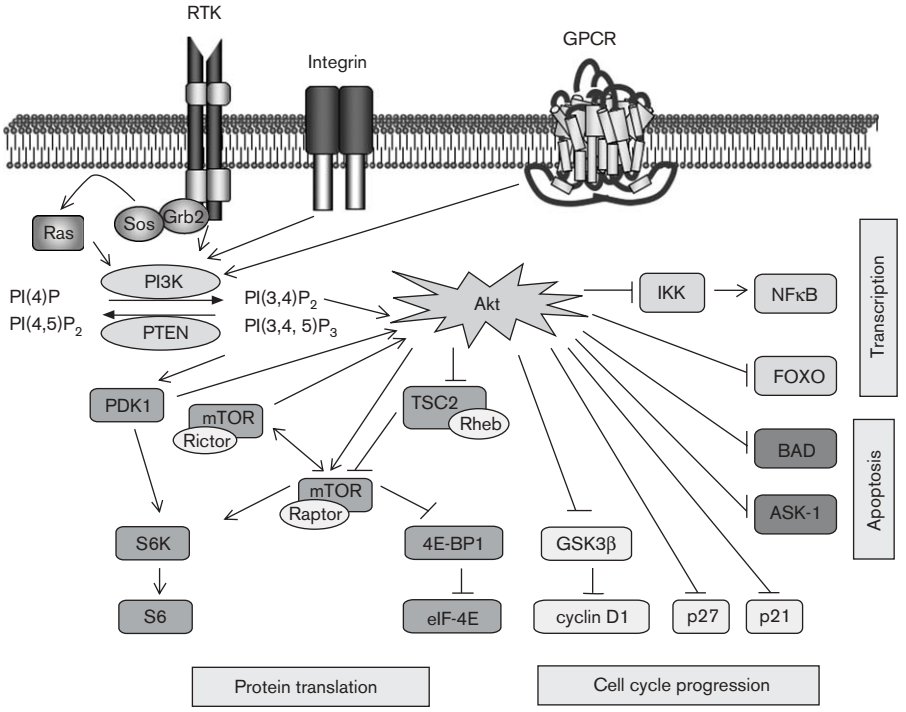
Fig. 1



The PI3K/Akt/mTOR pathway. Receptor tyrosine kinases such as IGF-IR and EGFR, integrins, and G-protein-coupled receptors can all stimulate PI3K. PI3K phosphorylates, PI(4)P and $PI(4,5)P_2$, at the 3′-position to generate $PI(3,4)P_2$ and $PI(3,4,5)P_3$, respectively. PTEN opposes the function of PI3K by removing 3′-phosphate groups. Generation of 3′-phosphoinositides activates both Akt and PDK-1, which phosphorylates Akt at T308. Akt propagates its signal to affect transcription, apoptosis, and cell cycle progression. Akt can activate mTOR directly by phosphorylation at S2448 or indirectly, by phosphorylation and inactivation of TSC2. When TSC2 is inactivated, the GTPase Rheb is maintained in its GTP-bound state, allowing for increased activation of mTOR. mTOR is present in two complexes: TORC1 (mTOR bound to Raptor) and TORC2 (mTOR bound to Rictor). TORC1 is responsible for activating S6 kinase 1, which activates ribosomal protein S6 and leads to increased protein translation. TORC1 also phosphorylates 4EBP-1, causing it to dissociate from eIF4E and freeing eIF4E to participate in the formation of the translation initiation complex. TORC2 can phosphorylate Akt at S473. ASK1, apoptosis signal-regulating kinase-1; EGFR, epidermal growth factor receptor; GPCR, G-protein-coupled receptor; IGF-IR, insulin-like growth factor-1 receptor; IKK, IκB kinase; NFκB, nuclear factor κB; mTOR, mammalian target of rapamycin; PDK1, 3′-phosphoinositide-dependent kinase 1; PI3K, phosphoinositide 3′-kinase; $PI(3,4)P_2$, phosphatidylinositol-3,4-bisphosphate; $PI(3,4,5)P_3$, phosphatidylinositol-3,4,5-triphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10; Rheb, Ras homolog enriched in brain; TSC2, tuberous sclerosis complex 2.

numerous Akt substrates have been identified and validated [23]. These substrates control key cellular processes such as apoptosis, cell cycle progression, transcription, and translation. For instance, Akt phosphorylates the FoxO subfamily of forkhead family transcription factors, which inhibits transcription of several proapoptotic genes, e.g. *Fas-L*, *IGFBP1* and *Bim* [24,25]. Additionally, Akt can directly regulate apoptosis by phosphorylating and inactivating proapoptotic proteins such as Bad, which controls release of cytochrome *c* from mitochondria, and apoptosis signal-regulating kinase-1, a mitogen-activated protein kinase kinase involved in stress-induced and cytokine-induced cell death [24,26,27]. In contrast, Akt can phosphorylate IκB kinase, which indirectly increases the activity of nuclear factor κB and stimulates the transcription of prosurvival genes [28–30]. Cell cycle progression can also be affected by Akt through its inhibitory phosphorylation of the cyclin-dependent kinase inhibitors, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ [31–33], and inhibition of glycogen synthase kinase (GSK)-3β by Akt stimulates cell cycle progression by stabilizing cyclin D1 expression [34]. Thus, Akt inhibition might have pleiotropic effects on cancer cells that could contribute to an antitumor response.

The best-studied downstream substrate of Akt is the serine/threonine kinase mTOR. Akt can directly phosphorylate and activate mTOR, as well as cause indirect activation of mTOR by phosphorylating and inactivating TSC2 (tuberous sclerosis complex 2, also called tuberin), which normally inhibits mTOR through the GTP-binding protein Rheb (Ras homolog enriched in brain). When TSC2 is inactivated by phosphorylation, the GTPase Rheb is maintained in its GTP-bound state, allowing for increased activation of mTOR. mTOR exists in two complexes: the TORC1 complex, where mTOR is bound to Raptor, and the TORC2 complex, where mTOR is bound to Rictor. In the TORC1 complex, mTOR

Table 1 Akt inhibitors

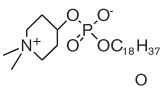
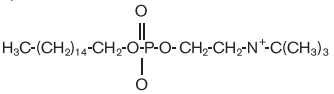
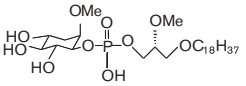
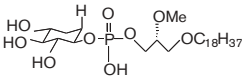
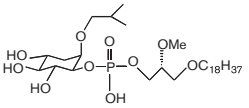
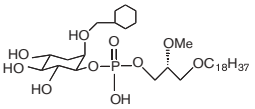
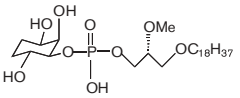
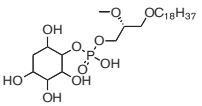
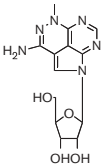
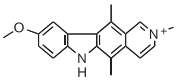
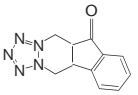
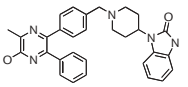
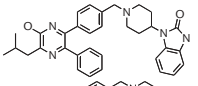
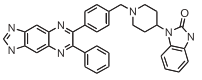
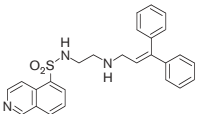
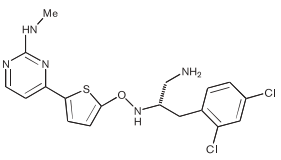
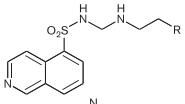
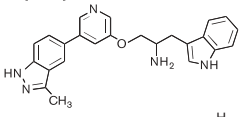
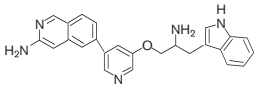
Agent	Structure	Mechanism of action	References
Lipid-based			
Perifosine		Bind PH domain; inhibit membrane translocation	[63–77]
Miltefosine			[78–87]
PIA5			[88–90]
PIA6			
PIA23			
PIA24			
PIA25			
PX-316			[91,92]
Small molecules			
API-2		Unknown	[93–96]
API-59CJ-OMe			[97,98]
KP-372			[99–103]
Canthine alkaloid analogs			
Akti1		Bind Akt at site other than PH domain; inhibition is PH domain dependent; some isoform specificity	[104–106]
Akti2			
Akti1,2			
ATP-competitive			
NL-71-101		Competitive inhibitor of ATP-binding domain	[107,108]
2-Pyrimidyl-5-amidothiophenes			[109]

Table 1 (continued)

Agent	Structure	Mechanism of action	References
Isoquinoline-5-sulfonamides			[110–112]
A-443654			[113–114]
A-423795			[115–117]
Peptide-based			
Akt-in	AVTDHPDRLWAWEKFH	Peptide mimetic; prevents membrane translocation	[118]
Pseudosubstrate	Ac-VELDPEFEPRARERTYAFGH		[119]
Akt ScFv		Single-chain antibody	[120]

API-2, Akt/protein kinase B signaling inhibitor-2; API-59CJ-OMe, 9-methoxy-2-methylellipticinium acetate; PH, pleckstrin homology PIA, phosphatidylinositol ether lipid analogs; PX-316, D-3-deoxy-phosphatidyl-myoinositol-1-(*R*)-2-methoxy-3-octadecyloxypropyl hydrogen phosphate.

phosphorylates its downstream effectors S6 kinase (S6K1) and 4EBP-1. S6K1 can then phosphorylate its substrate, a ribosomal protein called S6. 4EBP-1, when phosphorylated cannot bind effectively to its binding partner, eIF4E. The cumulative effect of each is to increase protein translation, especially of highly structured, capped mRNA species. Although mTOR is generally considered a downstream substrate of Akt, mTOR complexed with Rictor can also phosphorylate Akt at S473, thereby providing a level of positive feedback on the pathway [35]. Finally, S6K1 can also regulate the pathway by catalyzing an inhibitory phosphorylation on insulin receptor substrate proteins. This prevents insulin receptor substrate proteins from activating PI3K, which indirectly lowers activation of Akt [36,37].

Akt as a therapeutic target

Akt is a valid therapeutic target in cancer and many clinical observations support targeting Akt. First, immunohistochemical studies using antibodies that recognize Akt when phosphorylated at S473 have shown that activated Akt is detectable in cancers such as multiple myeloma, lung cancer, head and neck cancer, breast cancer, brain cancer, gastric cancer, acute myelogenous leukemia, endometrial cancer, melanoma, renal cell carcinoma, colon cancer, ovarian cancer, and prostate cancer [18,38–56]. Immunohistochemical analysis has also been used to demonstrate prognostic significance of Akt activation. Phosphorylation of Akt at S473 has been associated with poor prognosis in cancers of the skin [48], pancreas [54,57], liver [58], prostate [50], breast [53], endometrium [18], stomach [52], brain [49] and blood [51]. Tsurutani *et al*. [59] recently extended these studies by using antibodies against two sites of Akt phosphorylation, S473 and T308, to show that Akt activation is selective for nonsmall cell lung cancer (NSCLC) tumors versus normal tissue and is a better predictor of poor prognosis in NSCLC tumors than S473 alone. Collectively, these studies demonstrate that Akt activation is common to many cancers and correlates with poor prognosis.

Compelling evidence for directly targeting Akt in cancer comes from preclinical studies that employed biochemical or genetic approaches to inhibit Akt in cancer cells. In these experiments, inhibition of Akt induces apoptosis and/or cell cycle arrest, and increases responsiveness to chemotherapy or radiation *in vitro* and *in vivo* [60,61]. Inhibition of the Akt pathway is also observed with the administration of several standard chemotherapeutic and chemopreventive agents *in vitro*, and in some cases the cytotoxicity of these agents is a direct consequence of inhibition of Akt [62]. Furthermore, inhibiting Akt directly has advantages over targeting more distal components of the pathway. Because all substrates of Akt are not yet completely identified and the 'critical substrates' can be cell type-specific, inhibition of individual downstream components may miss key substrates that are responsible for Akt-regulated survival or proliferation. Therefore, directly targeting Akt may be more effective, though potentially more toxic. In addition, direct inhibition of Akt might circumvent feedback activation of Akt that has been observed with mTOR inhibitors. Approaches that directly target Akt are discussed below.

Akt inhibitors

The development of Akt inhibitors is a major effort within academia, industry and government. Of the various approaches to inhibit Akt, lipid-based compounds such as perifosine are the best developed and have demonstrated efficacy and tolerability in early clinical trials. Other approaches being developed to inhibit Akt, however, are present (Table 1) [63–120]. These approaches include the screening of small molecules that target the

ATP-binding domain or PH domain of Akt, and modeling the interaction of Akt with inhibitory binding proteins to aid in the design of peptides that inhibit Akt.

Lipid-based inhibitors

Lipid-based Akt inhibitors are the most mature in their development, and include the alkylphospholipids (ALPs) (perifosine, miltefosine, and edelfosine), phosphatidylinositol ether lipid analogs (PIAs), and D-3-deoxy-phosphatidyl-myoinositol-1-(*R*)-2-methoxy-3-octadecyloxyropyl hydrogen phosphate (PX-316).

Alkylphospholipids

Synthetic alkylphospholipids are membrane-permeable ether lipids with a single long alkyl chain, which facilitates their insertion into the outer leaflet of the plasma membrane. As they are relatively impervious to degradation by phospholipases, ALPs accumulate in lipid rafts of the plasma membrane and their accumulation disturbs the synthesis of de-novo phospholipids at the membrane, as well as the membrane translocation of Akt [63–65]. Three ALPs have been developed, perifosine, miltefosine and edelfosine, all of which have been shown to inhibit Akt signaling *in vitro* [66–68].

To date, the best-characterized and most developed inhibitor of Akt is perifosine. *In vitro*, perifosine inhibits translocation of Akt to the cell membrane, and inhibits the growth of melanoma, lung, prostate, colon and breast cancer cells in association with inhibition of Akt activity [67,68]. Perifosine has also been found to sensitize cancer cells to apoptosis and cell cycle arrest induced by radiation and other targeted and traditional chemotherapeutic agents *in vitro* [69–72]. Perifosine is the ALP most extensively tested *in vivo* and results from several clinical trials have been reported. In an initial phase I trial, the dose-limiting toxicity of oral administration of perifosine was gastrointestinal toxicity [68]. More recently, another phase I trial with perifosine was conducted in patients with incurable solid malignancies [73]. Using an increased loading dose and lower maintenance dosing, toxicities were minimized. Although nausea, diarrhea, dehydration and fatigue were seen during the loading phase, these toxicities were improved with use of prophylactic medications [73]. In this study, perifosine had clinical activity in a patient with uterine sarcoma (partial response) and a patient with renal cell carcinoma (stable disease). In neither study, however, was modulation of Akt assessed.

Phase II data with perifosine have also been reported. A phase II study of perifosine as a single agent in patients with previously untreated metastatic or locally advanced soft tissue sarcoma was completed in Canada in early 2006 [74]. Patients were given oral perifosine on an intermittent dosing schedule. Toxicities included diarrhea, vomiting, anorexia, and fatigue, and mild hematological side effects. No objective responses were seen and 12 out of 16 patients withdrew from the study owing to disease progression. Patients, however, were not selected for the trial based on activation of Akt in tumor specimens, and modulation of Akt was not monitored throughout the study. Moreover, the patients in this study had tumors of mixed histologic subtypes of soft tissue sarcoma and patients with chondrosarcoma, a tumor type that responded in earlier phase I trials, were excluded from this study.

Additional phase II studies investigating perifosine as a single agent in patients with other metastatic or refractory solid tumors have been reported. One study examined the effects of oral perifosine on previously untreated metastatic melanoma with similar results [75]. No response was seen in any of the evaluable patients. Three out of 14 patients had stable disease, while 11 progressed. Another study of oral perifosine in refractory androgen-independent prostate cancer did not demonstrate clinical activity [76]. Sixteen out of 19 patients had disease progression accompanied by mild toxicities while on perifosine and the study was ultimately discontinued after initial stages of accrual. A weakness of these phase II trials is that no patient with melanoma or prostate cancer responded in the phase I trials. In addition, Akt activation was not assessed before or during these phase II studies as part of the inclusion criteria. The reasons for the limited clinical activity are unclear, but it is possible that certain histologic subtypes and/or those with activation of one or more components of the PI3K/Akt/mTOR pathway might be the most likely to respond to perifosine. Moreover, in the latter study, attainable free drug levels were at least 10-fold less than those shown to be effective *in vitro*.

The most recent phase II study with perifosine was performed by Argiris *et al.* [77] in patients with incurable, recurrent or metastatic squamous cell carcinoma of the head and neck. Of 19 patients enrolled, 18 patients progressed after 8 weeks and one patient with stable disease was the only partial response observed. Levels of total and phospho-Akt, however, were analyzed by immunohistochemistry. High levels of total Akt in baseline tumor tissue correlated with better survival. Owing to the small sample size and lack of pretreatment biopsy tissues, correlation with levels of phosphorylated Akt was not of particular value in this study, although the attempt to analyze target inhibition in tumor tissue before and after treatment is laudable. Phase I studies combining perifosine with radiation, gemcitabine, docetaxel and paclitaxel are ongoing, as are additional phase II trials assessing the efficacy perifosine in refractory cancers of the breast, pancreas, prostate, head and neck, and lung.

Clinical studies with miltefosine and edelfosine are more limited. Originally considered as inhibitors of

phosphatidylcholine metabolism over 25 years ago, these alkylphospholipids were later shown to have broad antiproliferative effects in multiple types of cancer cells [64,78]. Phase I and II studies were conducted with oral miltefosine in the early 1990s, but did not reveal significant clinical activity [79–82]. Miltefosine, however, has recently been approved in Europe as a topical application for patients with cutaneous metastases, and was shown to be effective in treating some cutaneous lymphomas and breast cancer skin metastases, with little to no systemic side effects [83–87]. Akt activation has not been a consideration in the design or analysis of trials with miltefosine.

Phosphatidylinositol ether lipid analogs

Another group of lipid-based Akt inhibitors are the PIAs. PIAs were designed to interact with the PH domain of Akt and are structurally similar to $PI(3,4)P_2$ and $PI(3,4,5)P_3$. They are composed of an inositol ring, a linker (phosphate or carbonate) and an ether lipid side chain. Following a cell-based screening of 24 PIAs in breast and lung cancer cell lines that had high levels of active Akt, Castillo *et al.* [88] identified five PIAs that rapidly inhibited Akt activation as well as the phosphorylation of multiple downstream substrates, without affecting kinases upstream of Akt. These active PIAs showed structural characteristics such as a monomeric structure with a phosphate linker, and an inositol ring that bore two substitutions at either the 2′ and 3′ or the 4′ and 5′ positions. Subsequently, computer modeling and docking studies predicted that PIAs would bind to the PH domain of Akt with the inositol ring and lipid side chain in an altered position as compared to its endogenous substrates $PI(3,4)P_2$ and $PI(3,4,5)P_3$. This suggested that PIAs might inhibit the conformational changes caused by binding of the products of PI3K, and therefore might interfere with the translocation of Akt. Indeed, PIAs inhibited IGF-1-induced translocation of a fluorescent Akt-PH domain construct from the cytoplasm to the plasma membrane. PIAs also selectively induced apoptosis in breast cancer and NSCLC cell lines that have high levels of constitutively active Akt. Moreover, PIAs increase the efficacy of standard therapeutic agents or radiation [89]. Recently, Gills *et al.* [90] have identified molecular correlates of response to PIAs and confirmed that sensitivity to PIAs indeed correlates with levels of active but not total Akt. Other molecular correlates, however, were also identified that more closely correlated to response than active Akt [90]. Ongoing studies will confirm whether these markers can serve as predictive factors for response. The PIAs are currently being evaluated for in-vivo efficacy.

D-3-Deoxy-phosphatidyl-myoinositol-1-(*R*)-2-methoxy-3-octadecyloxyropyl hydrogen phosphate

PX-316 is another lipid-based inhibitor of Akt that also targets the PH domain and prevents membrane localization [91]. Preclinical studies were done with PX-316, which showed that daily intraperitoneal administration slowed the growth of breast and colorectal cancer xenografts (MCF-7 and HT-29 cells, respectively), and inhibited Akt *in vivo* [92]. Pharmacokinetic properties and the specificity of PX-316 have not been defined.

Small-molecule inhibitors

Using high-throughput screening of chemical libraries, several small-molecule inhibitors of Akt kinase have been identified. These inhibitors include Akt/protein kinase B signaling inhibitor-2 (API-2), 9-methoxy-2-methylellipticinium acetate (API-59CJ-OMe), KP372 and isoform-specific canthine alkaloid analogs.

Akt/protein kinase B signaling inhibitor-2

API-2 was identified as an Akt inhibitor after a screen of the National Cancer Institute (NCI)'s diversity set. API-2 inhibits Akt kinase activity, and stimulates apoptosis of xenografts of human breast, prostate, ovarian and pancreatic cancer cells exhibiting high Akt activity [93]. API-2 is a tricyclic nucleoside that demonstrated antitumor activity in phase I and II trials conducted over 20 years ago. Drug development, however, was discontinued owing to several drug-related toxicities including hepatotoxicity, hyperglycemia, thrombocytopenia and hypertriglyceridemia [94–96]. The recent identification of Akt inhibition as a mechanism underlying API-2 activity has provided new interest in studying this drug, and raises the possibility that lower doses may inhibit Akt and induce tumor cell apoptosis without the previously associated side effects. Clinical trials with API-2 are planned.

9-Methoxy-2-methylellipticinium acetate

Another small-molecule Akt inhibitor that was identified in an analysis of the NCI's anti-cancer drug screening database is API-59CJ-OMe. API-59CJ-OMe inhibited Akt kinase activity *in vitro* in endometrial cancer cell lines exhibiting high levels of Akt activity and preferentially induced apoptosis in these cell lines [97]. Similar results were observed in ovarian cancer cell lines, where API-59CJ-OMe was able to induce apoptosis in cell lines with constitutively active Akt, but had only minimal activity against those without elevated levels of Akt [98]. In both studies, however, induction of apoptosis and inhibition of Akt were assessed after 48 or 72 h of treatment, making it difficult to determine whether Akt inhibition was a direct consequence of drug treatment or whether it occurred as a secondary event.

KP372

KP372-1 is a nonisoform-specific inhibitor of Akt that was identified in a high-throughput screen of approximately 70 000 compounds using recombinant Akt kinase and a specific peptide substrate. In thyroid carcinoma cells with

high levels of active Akt, KP372 inhibited proliferation and induced apoptosis that was marked by the induction of caspase cleavage and sub-G_1 DNA content [99]. Cytotoxicity occurred in a dose-dependent manner, with IC_{50} values in the nanomolar range. At these relative IC_{50} concentrations, KP372 inhibited phosphorylation of Akt at S473 as well as Akt kinase activity. Similar results were observed *in vitro* in glioblastoma, acute myelogenous leukemia, Hodgkin's lymphoma, and squamous cell carcinoma of the head and neck cell lines, in which KP372 was able to downregulate phosphorylation of Akt at S473 and downstream substrates, as well as inhibit cell proliferation via induction of apoptosis and G_1 cell cycle arrest at doses less than 1 μmol/l [100–103]. Although in-vitro data indicate that KP372 is specific for the inhibition of Akt kinase, the compound inhibits additional kinases such as cyclin-dependent kinase 1, DNA-dependent protein kinase, extracellular signal-regulated kinase, mitogen-activated protein kinase kinase, GSK3β, and S6K at concentrations beyond the nanomolar range. To date, no in-vivo studies have been published with KP372.

Akti1, 2 and 3

Isoform-specific Akt inhibitors have also been identified in a high-throughput kinase activity screen. Canthine alkaloid analogs that specifically inhibit Akt1 and/or 2 with IC_{50}s in the micromolar range were synthesized from diphenylquinoxaline lead compounds [104,105]. Treatment with Akti1 or 2 sensitized cancer cells to chemotherapy-induced cell death, but maximum sensitization was seen with the dual Akt1/2 inhibitor, Akti1, 2 [106]. These studies are notable because this is the only published data on isoform-specific small-molecule Akt inhibitors. As Akt1 and 2 have different roles in development, this approach has the potential to mitigate toxicities in normal tissues. Further studies to test the efficacy of these compounds and the consequences of inhibiting both Akt1 and 2 *in vivo* are needed, but the concept of isoform-specific Akt inhibitors has possible advantages.

ATP-competitive inhibitors

NL-71-101 and doxazosin

Putative Akt inhibitors have been generated by chemically modifying existing compounds such as the protein kinase A (PKA) inhibitor, H89, and the α_1-adrenoreceptor antagonist, doxazosin. NL-71-101 is an analog of the PKA inhibitor, H89, which was modified to lose its activity against PKA and retain Akt inhibition. It exhibits modest specificity for Akt over PKA (2.4-fold), but its preclinical characterization is minimal [107]. Structural modification of the α_1-adrenoreceptor antagonist, doxazosin, has yielded compounds that both inhibit Akt and induce apoptosis in PC-3 cells *in vitro* [108]. Because Akt inhibition by these compounds, is only observed after several hours, however, it is unclear whether this is a direct or indirect effect. NL-71-101 and doxazosin analogs have only been tested in a few cell lines and have not been tested in animal models; therefore, their potential as therapeutics is unclear.

2-Pyrimidyl-5-amidothiophenes

Another series of compounds, 2-pyrimidyl-5-amidothiophenes were synthesized and evaluated for the ability to inhibit Akt *in vitro* [109]. After high-throughput screening of this series, a lead compound was identified as slightly selective for Akt3. This ATP-competitive compound exhibited an IC_{50} of 3 μmol/l against Akt3, and was further modified to yield a new series of Akt inhibitors. Of this series, one compound was particularly potent, and inhibited both proliferation and the phosphorylation of downstream substrates of Akt1, 2 and 3, in the low nanomolar range in DOV13 ovarian cancer cells. This compound, however, was 30 times more potent in inhibiting PKA. This relative selectivity for PKA and other AGC family kinases was confirmed in the melanoma cell line, UACC903, and represents a significant hurdle in the development of these compounds as Akt inhibitors.

Sulfonamide inhibitors

While generating synthetic calmodulin inhibitors, Ono-Saito *et al.* [110] discovered several analogs with shorter alkyl chains that showed nonspecific ATP-dependent inhibition of serine/threonine kinase activity. On the basis of this structure template, isoquinoline-5-sulfonamides were designed and assessed for their ability to inhibit Akt selectively. The structures of these compounds are derived from balanol, a natural fungal product that inhibits a broad spectrum of serine/threonine protein kinases including Akt, AMP-dependent protein kinase and protein kinase C [111]. The structure of Akt was used to virtually screen many sulfonamides and identify selective inhibitors of Akt. Eventually, several ATP-competitive inhibitors were developed with selectivity for Akt at in-vitro concentrations below the micromolar range [112]. These compounds have not been tested *in vivo*.

A-443654 and A-423795

The indazole-pyridine A-443654 was synthesized from a lead compound following a high-throughput chemical library screen, and was shown to inhibit the growth of PC-3, MiaPaCa-2 and 3T3-Akt1 xenografts [113]. At doses that inhibited tumor growth, inhibition of signaling downstream of Akt was observed in tumors, yet increased phosphorylation of Akt itself at S473 was observed. These paradoxical results raise questions about this compound's mechanism of action and the utility of measuring S473 alone to assess Akt activity. A recent study has shown synergy between A-443564 and the standard chemotherapy agents doxorubicin, camptothecin and paclitaxel in NSCLC cells. A-443654 was also able to sensitize prostate cancer PC3 xenografts to paclitaxel-induced

inhibition of tumor growth [114]. Unfortunately, Akt phosphorylation status was not assessed in the xenografts, but the synergistic effects and the observed tolerability of an Akt inhibitor combined with a standard chemotherapeutic agent *in vivo* are promising.

Subsequently, lead compounds generated from the indazole pyridine group were modified to produce a series of *trans*-3,4′-bispyridinylethylenes, some of which were capable of inhibiting Akt at nanomolar concentrations in human MiaPaCa-2 cells and murine prolymphocytic cells that overexpress Akt1 [115]. Following a high-throughput screen, the lead compound, A-423795, was found to inhibit Akt1 and phospho-GSK3β, a downstream substrate, with the lowest IC_{50} in these same cell lines [116]. A-423795 was then tested in a human MiaPaCa-2 pancreatic cancer xenograft model at its maximally tolerated dose in mice [117]. Although the compound had a significant effect on tumor growth, toxicities such as lethargy, weight loss and skin irritation at the site of injection developed, and ultimately caused for discontinuation of the study. Akt inhibition was not measured *in vivo*. In addition, compounds in this series have a short half-life in the plasma of mice, rats and monkeys, and are not isoform specific [115]. The observed toxicities indicate that A-423795 given with this schedule has a narrow margin of safety and requires subsequent studies to optimize a therapeutic index.

Peptide-based inhibitors

Akt-in

Peptide-based inhibitors of Akt are also being developed. Akt-in is a peptide composed of 15 amino acids designed to mimic the interaction between Akt and a coactivator, TCL1. The sequence of Akt-in encompasses a portion of the TCL1 protein that binds Akt in its PH domain, and inhibits Akt kinase activity and membrane translocation in lymphoid-derived cell lines. It also inhibits cellular proliferation in preclinical studies with transformed kidney epithelial cells and in fibrosarcoma xenografts but has poor oral bioavailability and cellular penetration [118]. Still, the mechanism of action of Akt-in raises the possibility that mimicking the interaction of Akt with its inhibitory binding proteins could be exploited for therapeutic value. This approach is understudied, but would most likely be successful through the identification or development of small molecules that mimic the interaction of Akt with binding proteins, rather than through the use of peptides.

Akt pseudosubstrates

Luo *et al.* [119] used the consensus sequence preferred by Akt to develop pseudopeptide substrates that have been shown to inhibit Akt and the growth of cancer cells *in vitro*. In the nanomolar range, these peptides inhibited growth and the ability of Akt to phosphorylate GSK3β, one of its downstream substrates, in HeLa and MiaPaCa cells. Two of these pseudosubstrates showed selective inhibition of Akt versus another closely related kinase, glucocorticoid-regulated protein kinase, which often overlaps with Akt inhibition.

scFv

Another peptide-based approach is the development of a single-chain antibody (scFv) against Akt. This antibody is the first genetically engineered scFv against Akt with inherent cell membrane-translocation activity and retention of Akt inhibitory function associated with induction of apoptosis *in vivo* [120]. Although the concept of peptide-based inhibitors is exciting, their development is currently hampered by their low bioavailability and stability *in vivo*.

Adenoviruses

Adenoviruses expressing a mutant inhibitory form of Akt have been developed. These adenoviruses selectively caused apoptosis in cancer cell lines expressing high levels of endogenous Akt activation [121]. Direct intratumoral injection of this construct inhibited the growth of ZR75-1 human breast cancer cells grown as xenografts. Such a genetic approach is limited by two factors, the concern over patient safety and the unlikely bioavailability after oral administration, which has become an unofficial benchmark for development set by other kinase inhibitors. Overall, the application of these genetic and peptide-based approaches is more limited than small molecule inhibitors.

Considerations in developing Akt inhibitors

The success of targeted kinase inhibitors, such as erlotinib and imatinib, and the strong rationale to target the PI3K/Akt pathway has fed optimism that Akt inhibitors might be clinically useful for cancer patients. As these drugs are predicted to modulate a target and not cause direct DNA damage like most standard chemotherapies, the traditional paradigm for clinical development may not be sufficient. Preclinical development should not only establish traditional endpoints such as efficacy, but should also strive to identify positive predictive factors for response, as well as 'off-target' effects. Clinical investigation should begin by focusing on patients who are most likely to be sensitive to the inhibitor, and evaluate target inhibition in tumor and surrogate tissues. As promising as inhibiting the PI3K/Akt pathway might be for cancer therapy, normal tissues also rely on pathway activation, which raises the question as to whether a therapeutic index can be achieved by targeting Akt.

Trial design

As Akt inhibitors are not traditional cytotoxic chemotherapies, special considerations should be given to

clinical trial design. For example, Akt inhibitors are likely to be most effective in patients whose tumors bear activation of the PI3K/Akt pathway. Therefore, phase I protocols with pathway inhibitors should determine the biologic effective dose, as well as the maximum tolerated dose, and should determine the relationship between these doses. Enrollment in clinical phase II or III trials should be initially limited to patients whose tumors bear activation of the pathway. Granted, this approach may result in the exclusion of patients who might obtain clinical benefit through unknown mechanisms, but *a priori*, Akt inhibition should be correlated with clinical efficacy. If efficacy against Akt is demonstrated in initial trials, then consideration should be given to other trials that do not exclude patients who lack pathway activation. By combining evaluation of changes in Akt activation status with traditional clinical responses in phase II trials, unambiguous outcomes and interpretations about a given Akt inhibitor could be derived (Table 3) [68,73–77,83–86]. This could be best observed when Akt pathway inhibition and clinical benefits are linked. For example, if Akt inhibition is accompanied by clinical benefit, then a targeted, effective drug has been identified. Conversely, if no inhibition of Akt and no clinical benefit are observed, then the dosing regimen should be carefully examined to ensure that the drug is likely reaching Akt in tumor tissue.

The conclusions are less obvious if there is disconnect between Akt inhibition and clinical benefit. For example, inhibition of Akt that is not accompanied by a clinical response could imply that the drug worked as expected, but that Akt is not important for tumor growth. While disappointing from an oncologist's perspective, the fact that an Akt inhibitor hits its target *in vivo* is important and this drug could be tested in other tumor types driven by the activation of the Akt pathway. The other ambiguous outcome that could occur is when a clinical response is not accompanied by pathway inhibition. From a practical perspective, clinical benefit is most important. It is also important to understand whether the effect on the pathway was truly negative. Assessment of other components in the pathway could be performed to confirm the lack of Akt inhibition. In addition, efforts should be made to understand 'off target', Akt-independent effects that might account for the inhibition of tumor growth. Recognizing these outcomes could assist in the development of Akt inhibitors.

Combining Akt inhibitors and other chemotherapies

Many studies show that Akt inhibitors not only have activity as single agents, but also are effective in combination with other types of chemotherapies. Preclinical data combining inhibitors of the PI3K/Akt/mTOR pathway with traditional chemotherapies have shown that this is a useful approach in many types of cancer cells [62]. If perifosine as well as other Akt inhibitors continue to have limited clinical activity as single agents, combining Akt inhibitors with traditional chemotherapies might be a better approach. On the basis of the observed feedback activation of Akt by mTOR inhibitors, however, it is possible that Akt inhibitors might be most effective when combined with other inhibitors whose targets are proximal to Akt. These targets include IGF-IR and PI3K, as well as erbB family members such as erbB2 and EGFR. For example, combining rapamycin, an mTOR inhibitor, with an inhibitor of IGF-1R abrogated feedback activation of Akt and enhanced cytotoxicity of rapamycin in glioma cells [122]. Similarly, synergistic effects between rapamycin and LY294002, an upstream inhibitor of PI3K, have been observed *in vitro* [123–125].

Another potentially useful combination is proximal inhibition of erbB family members and distal inhibition of Akt. Inhibition of Akt phosphorylation has been demonstrated to be a requirement for the anti-proliferative effects of the Her-2/*neu* antagonist, trastuzumab (Herceptin; Genentech, San Francisco, California, USA), and trastuzumab-resistant cells exhibit sustained

Table 2 Summary of clinical trial data with perifosine and miltefosine

	Tumor type	Response	Toxicities (CTC grade 3–4)	References
Phase I perifosine (oral)	Mixed solid tumors	One PR (leiomyosarcoma), two SD (renal cell carcinoma)	Nausea (3%), vomiting (3%), fatigue (11%)	[68]
		Two SD (liver cell carcinoma and melanoma of the eye)	Nausea (5%), diarrhea (5%)	[73]
Phase II perifosine (oral)	Metastatic or locally advanced soft tissue sarcoma	No responses, disease progression (75%)	Nausea (13%), vomiting (13%), diarrhea (13%), fatigue (6%)	[74]
	Metastatic melanoma	No responses, disease progression (79%)	Nausea (6%), diarrhea	[75]
	Androgen-independent prostate cancer	No responses, disease progression (84%)	Nausea (11%), diarrhea (22%), anorexia (6%)	[76]
	Recurrent, incurable or metastatic squamous cell carcinoma of the head and neck	One SD, disease progression (95%)	Nausea, vomiting, constipation, fatigue	[77]
Miltefosine (topical)	Breast cancer skin metastases	PR (22%), SD (39%)	Adverse skin reactions	[83]
		CR (4%), PR (36%), SD (44%)		[84]
		CR (23%), PR (20%), SD (33%)		[85]
	Cutaneous lymphoma	CR (17%), PR (33%), SD (8%)		[86]

CR, complete response; PR, partial response; SD, stable disease; CTC, Common Toxicity Criteria.

activation of the PI3K/Akt pathway [126–128]. Additionally, resistance to EGFR inhibitors has been observed to correlate with activation of the PI3K/Akt pathway. For example, cancer cell lines with mutant PTEN that have high levels of Akt are resistant to EGFR antagonists such as gefitinib, but sensitivity can be restored by pharmacologic inhibition of the PI3K/Akt pathway [129,130]. Similarly, EGFR inhibition can be effectively combined with PTEN restoration. She *et al.* [131] showed that this approach inhibited growth of breast cancer xenografts, which was not seen with either EGFR inhibition or PTEN induction alone. Similar results have been observed in NSCLC, prostate and leukemia cell lines, linking PTEN status, and thus Akt activity, with sensitivity to EGFR inhibition [71,132–135]. Interestingly, Sordella *et al.* [136] found that NSCLC cells transfected with gefitinib-sensitizing EGFR mutations had increased levels of activated Akt and these cells were more sensitive than their wild-type counterparts not only to gefitinib, but also to inhibitors of the PI3K/Akt pathway. This phenomenon has also been observed in gefitinib-treated patients with advanced NSCLC, where EGFR mutations correlated with Akt activation, as measured by immunohistochemical analysis of tumor tissue specimens [137].

Collectively, these data suggest that the use of EGFR antagonists with Akt inhibitors may be especially beneficial in patients whose tumors harbor mutations in EGFR and/or PTEN, as well as patients who have developed resistance to EGFR inhibition. Proximal inhibition of receptor tyrosine kinases combined with inhibition of distal pathway components, such as Akt and mTOR, may abrogate feedback activation that results from distal inhibition alone, and thus represent a possible mechanism to overcome resistance to Akt inhibition.

Potential problems associated with Akt inhibition

Despite the potential of achieving clinical benefit with Akt inhibition, problems could arise. Undesirable effects of Akt inhibition could include the possible promotion of metastasis and the induction of profound toxicities. Inhibition of Akt1 might increase metastatic potential. Recently, Yoeli-Lerner *et al.* [138] found that overexpression of Akt1 resulted in a decrease of breast cancer cell motility and invasion in three different breast cancer cell types. This occurred through degradation of the transcription factor nuclear factor of activated T cells, which had been previously shown to promote the invasion of carcinoma cells *in vitro*. Similarly, Liu *et al.* [139] showed that overexpression of constitutively active Akt1 in breast cancer cells results in decreased metastasis through phosphorylation of the tumor suppressor TSC2, but not nuclear factor of activated T cells. On the basis of these studies, inhibition of Akt1 might promote metastasis. To date, however, no clinical observations to support or refute this hypothesis exist.

As the PI3K/Akt pathway plays a central role in many normal physiologic functions and is activated in many normal tissues, Akt inhibition may cause serious side effects. Akt1 knockout mice ($Akt1^{-/-}$) are small in size and the males are infertile [140], whereas Akt2 knockout mice ($Akt2^{-/-}$) develop a diabetic phenotype [141]. Although these would be serious side effects to occur in a patient receiving drugs that target the pathway, complications from insulin resistance could likely be managed medically and would have to be weighed against the benefits of receiving a potentially effective cancer therapy. Furthermore, $Akt1^{-/-}$ mice do not develop a diabetic phenotype and loss of fertility is less likely to be of importance after childbearing years, when most cancer occurs. On the basis of studies showing that Akt inhibition can exacerbate preclinical models of glaucoma, Alzheimer's disease, Parkinson's disease and schizophrenia, neurotoxicity could also be significant [142–144]. Although these toxicities are important to consider, they may not occur with short-term therapy and may reverse following withdrawal of Akt inhibition.

It is undetermined whether an isoform-specific approach to inhibit Akt would maximize efficacy and/or minimize toxicities. The different phenotypes of isoform-specific knockout mice suggest that selective inhibition of certain isoforms might mitigate toxicity. In light of recent data linking Akt1 with suppression of metastasis, it would seem that inhibition of Akt2 and 3, but not Akt1, would be desirable. The data supporting Akt1 as a suppressor of metastasis, however, is breast cancer cell type-specific and it is undetermined whether this phenomenon would be recapitulated in other cell lines or *in vivo*. Moreover, initial phases in the clinical development of Akt inhibitors would likely include patients with already established metastatic disease and it is unclear whether induction of new metastases in the setting of inhibiting established disease would adversely affect outcome. Ultimately, it has not been established which isoform(s) of Akt is most significant in cancer.

The question remains whether or not a therapeutic index can be achieved with an Akt inhibitor. On the basis of the in-vitro data showing the preferential activity of PI3K/Akt pathway inhibitors in cells that exhibit increased activation of the pathway, it is conceivable that an Akt inhibitor may cause apoptosis of cancer cells without killing normal cells. Perhaps tumor cells are selectively reliant on signaling pathways that promote survival, such as the PI3K/Akt/mTOR pathway, because they often grow in acidic or hypoxic environments, harsh conditions that would typically promote apoptosis. Inhibition of Akt in these cells that depend upon the pathway might cause apoptosis. As normal cells are not exposed to these

Table 3 Possible outcomes in clinical trials with Akt inhibitors

	Clinical response	No clinical response
Akt inhibition	Targeted drug is working as intended and Akt is important to cancer growth. Drug continues development	Targeted drug is working as intended but Akt may not important to cancer growth
No Akt inhibition	Drug is clinically effective but attribution to Akt is incorrect, if pathway inhibition is truly negative. Continue mechanistic studies	Drug is not effective clinically or mechanistically, but targeting Akt may still be valid. Test other inhibitors of same target

Tumors and surrogate tissue should be analyzed for Akt activity before, during and after administration of an Akt inhibitor. Conclusions can be made by concurrent analysis of clinical response and Akt inhibition.

conditions, they may not rely as heavily on pathway activation and might be less vulnerable to death induced by Akt inhibition. This hypothesis will be tested in the clinical trials using Akt inhibitors. If a therapeutic index is elusive, Akt inhibitors might find use as radiation or chemotherapeutic sensitizers at lower doses, where toxicities might be less likely to develop.

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