

Handicapping the Race to Develop Inhibitors of the Phosphoinositide 3-Kinase/Akt/Mammalian Target of Rapamycin Pathway

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Abstract The phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway controls many cellular processes that are important for the formation and progression of cancer, including apoptosis, transcription, translation, metabolism, angiogenesis, and cell cycle progression. Genetic alterations and biochemical activation of the pathway are frequent events in pre-neoplastic lesions and advanced cancers and often portend a poor prognosis. Thus, inhibition of the PI3K/Akt/mTOR pathway is an attractive concept for cancer prevention and/or therapy. Inhibitors of individual components, such as PI3K, PDK-1, Akt, and mTOR, are being developed at a rapid pace and have promise for improving the care of cancer patients. Here, we review the published data on inhibitors of the pathway and discuss relevant issues, such as the complex regulation of the pathway, the design of clinical trials, and the likelihood of finding a therapeutic index when targeting such a critical signaling pathway.

Phosphoinositide 3-Kinase/Akt/Mammalian Target of Rapamycin Pathway

Signaling through the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway can be initiated by several mechanisms that lead to the generation of 3'-phosphoinositides by PI3K (Fig. 1). The tumor suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN) opposes the action of PI3K by dephosphorylating 3'-phosphoinositides. Once generated, 3'-phosphoinositides bind to the pleckstrin homology domains of 3'-phosphoinositide-dependent kinase-1 (PDK-1) and Akt and cause translocation of each kinase to the plasma membrane, where both are activated. Akt is partially activated through PDK-1-mediated phosphorylation in the catalytic domain, but full activation requires a second phosphorylation event in the hydrophobic motif, which can be mediated by several kinases. Activated Akt propagates its signal to numerous substrates that control key cellular processes, such as transcription, translation, cell cycle progression, and apoptosis. Although no single critical Akt substrate for tumor development and maintenance has been identified, perhaps the best studied downstream substrate is the serine/threonine kinase, mTOR. Akt activates mTOR through at least two mechanisms, direct phosphorylation of mTOR itself, and via phosphorylation and inactivation of TSC2. Inactivation of TSC2 allows for maintenance of Rheb in its GTP-bound state, increasing activation of mTOR.

The PI3K/Akt/mTOR pathway has traditionally been viewed as linear. However, considerable crosstalk and feedback regula-

tion exists within the pathway. For example, PDK-1 can regulate translation independently of Akt by directly phosphorylating and activating a downstream effector of mTOR, S6 kinase-1 (S6K1). Negative feedback regulation of Akt can occur through S6K1, which can catalyze an inhibitory phosphorylation on insulin receptor substrate proteins, abrogating activation of PI3K. In addition, mTOR can phosphorylate Akt at its hydrophobic motif when bound to Rictor, potentially providing a level of positive feedback on the pathway (1). Continued study of this pathway will likely reveal further layers of complexity that will need to be considered during the development of pathway inhibitors and in the analysis of clinical trials with pathway inhibitors.

Rationale for Targeting the PI3K/Akt/mTOR Pathway

The hypothesis that inhibition of the PI3K/Akt/mTOR pathway might provide benefit for the prevention and treatment of cancer comes from many observations. First, activation of PI3K/Akt/mTOR pathway kinases is common in many cancers, and can occur through multiple mechanisms, including mutation or decreased expression of the tumor suppressor PTEN, mutation or amplification of PI3K, amplification of Akt, and activation of receptors or oncogenes upstream of the PI3K/Akt/mTOR pathway (Table 1; ref. 2). Second, pathway activation is an early event in carcinogenesis. Activation can occur rapidly in normal cells in response to carcinogen exposure and can cause increased proliferation and survival, suggesting that pathway activation may be permissive for carcinogenesis by increasing the threshold for apoptosis of damaged cells. Increased activity can also be detected in pre-neoplastic lesions (3–8). Third, activation of pathway components is a poor prognostic factor in many cancers (9–18). Fourth, pathway activation contributes to therapeutic resistance because its inhibition by biochemical or genetic means increases the efficacy of chemotherapy and/or radiation *in vitro* and *in vivo* (19–22). Several standard chemotherapeutic and

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

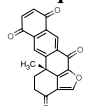
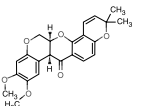
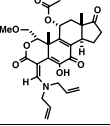
Agent/ Structure	Mechanism of action	<i>In vivo</i> Efficacy*	<i>In vivo</i> Target mod [†]	Clinical Status	Refs.
LY294002 	Reversible; inhibits p110 ATP binding site	Yes	Yes	n/a	(22, 28, 30)
Wortmannin 	Irreversible; binds p110 subunit	Yes	No	n/a	(31, 125)
IC486068	Inhibits p110 δ ; other isoforms at high concentration	Yes	No	n/a	(36)
Halenaquinone 	p110 δ inhibitor (inhibits other isoforms at higher concentrations)	No	No	n/a	(37, 38)
Deguelin 	Inhibits PI3K	Yes	No	n/a	(39-41)
PX-866 	Inhibits p110 ATP binding site	Yes	Yes [‡]	n/a	(35)

Fig. 1. Development of inhibitors of PI3K for cancer therapy. *, ability to reduce tumor burden *in vivo*. †, showed target modulation in tumor or surrogate tissue *in vivo*. ‡, target inhibition seen above doses required to inhibit tumor growth. Refs., references; n/a, not applicable (clinical trials have not been reported with this compound).

chemopreventive agents inhibit the PI3K/Akt/mTOR pathway as a consequence of administration *in vitro*, and in some cases, inhibition of Akt is directly responsible for the cytotoxicity of these agents (23). The efficacy of some new, targeted drugs, such as erlotinib and trastuzumab, which inhibit upstream components can also be correlated with inhibition of the PI3K/Akt/mTOR pathway. These observations with erlotinib and trastuzumab highlight the importance of inhibition of the pathway, but because inhibition by these drugs is mediated by altering upstream components, it is an indirect mechanism and will not be covered in this review. For reviews on targeted drugs

that indirectly inhibit the PI3K/Akt/mTOR pathway, the reader is referred elsewhere (24, 25). Taken together, the rationale to develop inhibitors is strong and has potential to decrease tumorigenesis, cancer growth, and therapeutic resistance. Approaches that directly target the pathway are discussed below.

Reconstituting PTEN

PTEN is a dual phosphatase that functions as a tumor suppressor through its lipid phosphatase activity. Loss of PTEN increases activation of the pathway, and PTEN mutation or

Table 1. Genetic alterations resulting in activation of the PI3K/Akt/mTOR pathway

Target	Mechanism	Type of cancer	References
PTEN	Mutation or deletion	Brain, bladder, breast, prostate, and endometrial cancers	(103–108)
	Epigenetic silencing	NSCLC, endometrial and gastric cancer, prostate, ovarian, and melanocytic tumors	(109–112)
PI3K	Amplification of PIK3CA (encodes p110 α)	Ovarian and cervical cancers	(113–115)
	Mutation in PIK3CA	Colorectal, gastric, breast, ovarian cancers, glioblastoma and hepatocellular carcinoma	(115–118)
Akt	Mutation in p85 α	Colon and ovarian cancers	(119)
	Akt1 amplification	Gastric adenocarcinoma	(120)
	Akt2 amplification	Ovarian, breast, and pancreatic cancers	(121–123)
	Akt3 amplification	Hormone-independent breast and prostate cancer	(124)

Abbreviations: NSCLC, non-small cell lung cancer; PIK3CA, phosphoinositide-3-kinase, catalytic, α polypeptide.

silencing is characteristic of many cancers, indicating that loss of PTEN function is a common event in cancer (26). Although there is merit to considering whether reconstitution of PTEN might have therapeutic value, the safety concerns over gene therapy will limit clinical application of this approach for the foreseeable future. Nonetheless, gene therapy with wild-type PTEN has been done *in vivo* in a mouse model of lung cancer in which aerosolized PTEN contained in an adenovirus was delivered through the nasal passages (27). PTEN overexpression in lung epithelium was associated with the induction of apoptosis and decreased phosphorylation of Akt and mTOR. Because wild-type PTEN was overexpressed in a wild-type background, further studies are needed to determine if PTEN reconstitution will be useful in tumors driven by the loss of PTEN function.

PI3K Inhibitors

The lipid kinase PI3K is a proto-oncogene that generates 3'-phosphoinositides at the cell membrane. The best-characterized inhibitors of PI3K are LY294002 and wortmannin, which are commercially available compounds that target the p110 catalytic subunit of PI3K (Fig. 1). LY294002 effectively inhibits the growth of many types of tumor cells *in vitro* and *in vivo*, which is associated with inhibition of PI3K and downstream components of the pathway (28–30). Wortmannin is also effective *in vitro* and *in vivo*, but some studies showed that its *in vivo* antitumor activity did not correlate with differences in PI3K activity or *in vitro* cytotoxicity (31, 32). Combining LY294002 or wortmannin with chemotherapy can be effective and less toxic, providing a mechanism for circumventing possible narrow therapeutic indices for these compounds and suggesting that the combination of a PI3K inhibitor with conventional chemotherapies might provide a therapeutic option for cancers that have become resistant to standard therapies (19, 22). Despite the antitumor efficacy of LY294002 and wortmannin, poor solubility and high toxicity have limited the clinical application of these older compounds. To address these issues, derivatives of LY294002 and wortmannin are being developed (33, 34). The wortmannin derivative, PX-866, is more potent and less toxic than wortmannin, and inhibited the growth of human lung, ovarian, and colon cancer xenografts (35). Inhibition of Akt phosphorylation was only observed at doses that exceeded those that caused growth delay, suggesting the cytotoxicity of PX-866 may be independent of PI3K inhibition. Further optimization of wortmannin or LY294002 may yield inhibitors that are tolerable and effective.

Other inhibitors of PI3Ks are also being developed. For example, IC486068 is an isoform-specific inhibitor of the p110 δ catalytic subunit of PI3K (36). *In vitro*, IC486068 inhibits radiation-induced Akt phosphorylation as well as endothelial cell migration and tube formation. IC486068 also sensitized endothelial cells and tumors to radiation-induced growth delay *in vivo*. Another PI3K inhibitor, halenaquinone, has been isolated from the marine sponge *Xestospongia exigua* in a screen of natural products. Halenaquinone induced apoptosis of cancer cells *in vitro* and inhibited PI3K kinase activity and may also block progression into mitosis through inhibition of Cdc25B (37, 38). The naturally occurring plant rotenoid, deguelin, is an effective chemopreventive agent in murine tumorigenesis studies and has been described *in vitro* as a PI3K

inhibitor (39–41). However, inhibition of Akt activation by deguelin occurs only after many hours, suggesting that the effects on the pathway might be indirect.

Although current inhibitors of PI3K are not attractive for clinical use, inhibition of PI3K, a proximal component in the pathway, has a hypothetical advantage in that feedback activation of the pathway that is observed with inhibition of distal components, such as mTOR (see below), would not be observed. Given the lack of specificity of LY294002 and wortmannin, it is unclear whether any of these newer compounds will have the requisite specificity and activity to emerge as promising drug candidates for further development. The development of PI3K inhibitors that work through mechanisms other than inhibition of ATP binding, such as through inhibition of the association of the regulatory subunit p85 with growth factor receptors or with the catalytic subunit p110, might identify candidate inhibitors that possess better specificity, efficacy, and toxicity profiles.

PDK-1 Inhibitors

As the kinase responsible for phosphorylating Akt at T308, which contributes to activation of Akt, PDK-1 plays a critical role in activation of the pathway. Because PDK-1 also activates other kinases that regulate cell proliferation and survival, such as PKC, S6K, SGK, and PKA, compounds that target PDK-1 may indirectly inhibit these other kinases. This may be advantageous from a therapeutic standpoint but obviously complicates their development as compounds that specifically inhibit the PI3K/Akt/mTOR pathway. Derivatives of two well-characterized drugs, staurosporine and celecoxib, are being developed as PDK-1 inhibitors (Fig. 2). The 7-hydroxy-derivative of staurosporine, UCN-01, mediates Akt inhibition through direct inhibition of PDK-1 (42), which correlates with apoptosis (43). UCN-01 is a potent inhibitor of many kinases, including PKC α , PKC β , PKC γ , Chk1, and PDK-1. UCN-01 has synergistic effects on apoptosis when combined with cytotoxic chemotherapy *in vitro* and *in vivo* (44–53), but this synergism may be due to combined effects on Chk1 not PDK-1 (54). In a phase I trial with UCN-01, a partial response in a patient with melanoma and long-term stabilization of disease in a patient with anaplastic large cell lymphoma was observed (55). Hyperglycemia was a dose-limiting toxicity, which might be related to the regulation of glucose uptake by the PI3K/Akt/mTOR pathway. Phase II trials with UCN-01 are ongoing. Despite positive results in clinical trials, the ability of UCN-01 to inhibit many kinases makes it difficult to consider UCN-01 as a targeted pathway inhibitor because clinical outcomes have not been associated with inhibition of PI3K/Akt/mTOR pathway. Determining a critical target will greatly assist its classification and development.

The celecoxib derivatives OSU-03012 and OSU-03013 were designed to minimize cyclooxygenase-2 inhibition and maximize PDK-1 antagonism (56). These analogues are more potent PDK-1 inhibitors than celecoxib and achieve total growth inhibition at a mean concentration of $\sim 3 \mu\text{mol/L}$ in 60 different human tumor cell lines. However, the celecoxib derivatives also have other biological activities, because they can delay G₂-M cell cycle progression and induce apoptosis independently of PDK-1 inhibition (57), suggesting that like UCN-01, off-target effects contribute to their anticancer activity.




Agent/ Structure	Mechanism of action	<i>In vivo</i> Efficacy*	<i>In vivo</i> Target mod†	Clinical Status	Refs.
UCN-01 	Competitive inhibitor of ATP binding domain	Yes	No	Phase II	(42, 126, 127)
OSU03012 	Unknown	No	No	n/a	(56, 57)
BX-320 	Binds to PDK1 catalytic domain	Yes	No	n/a	(58)

Fig. 2. Development of inhibitors of PDK-1 for cancer therapy. *, ability to reduce tumor burden *in vivo*. †, showed target modulation in tumor or surrogate tissue *in vivo*. Refs., references; n/a, not applicable (clinical trials have not been reported with this compound).

Other PDK-1 inhibitors, such as BX-795, BX-912, and BX-320, have been developed by identification in high-throughput screens and subsequent chemical modification (58). These compounds can bind to the catalytic domain of PDK-1 and inhibit kinase activity with IC_{50} s in the low nanomolar range. Although BX-320 inhibited the growth of melanoma xenografts and inhibited PDK-1-mediated signal transduction *in vitro*, inhibition of PDK-1 and/or its downstream targets was not validated *in vivo*. Correlation of target modulation with cellular outcome will help validate these compounds as targeted antitumor agents.

Optimism for the clinical development of PDK-1 inhibitors as cancer therapeutics is fueled by many factors, including the initial success of UCN-01, the potency of most inhibitors in this class, and the relative lack of toxicity of these drugs compared with available inhibitors of PI3K. However, all of the compounds in this class have targets outside the PI3K/Akt/mTOR pathway that may not be related to inhibition of PDK-1 but nonetheless might contribute to associated antitumor effects. Determining the critical target(s) of these drugs will help guide their development as targeted therapeutics.

Akt Inhibitors

The serine/threonine kinase Akt, also known as protein kinase B, contributes to tumor formation and tumor maintenance through the phosphorylation of many substrates that contribute to cell survival, cell cycle progression, and increased protein translation (Fig. 3). Lipid-based inhibitors of Akt were the first to be developed, but other approaches, such as high-throughput screening of small molecule libraries and rational design of peptide-based Akt inhibitors, have also been employed (Fig. 4).

Lipid-based inhibitors of Akt include perifosine, phosphatidylinositol ether lipid analogues, and D-3-deoxy-phosphatidylinositol-1-[(R)-2-methoxy-3-octadecyloxypropyl hydrogen phosphate] (PX-316). The best-characterized 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 (59, 60). Two phase I studies have been reported. In the initial phase I trial, the dose-limiting toxicity of oral administration of perifosine was gastrointestinal toxicity (60). More recently, another phase I trial with peri-

fosine was conducted in patients with incurable solid malignancies (61). Using a larger loading dose/maintenance dose schedule, toxicities were minimized. Although nausea, diarrhea, dehydration, and fatigue were seen during the loading phase, these toxicities were ameliorated with the use of prophylactic medications (61). In this study, perifosine had clinical activity in a patient with sarcoma and a patient with renal cell carcinoma (61). However, in neither study was modulation of Akt assessed. Phase II trials assessing perifosine in refractory cancers of the breast, pancreas, prostate, head and neck, and lung are ongoing.

Another group of lipid-based Akt inhibitors are phosphatidylinositol ether lipid analogues, which were designed to interact with the pleckstrin homology domain of Akt. Phosphatidylinositol ether lipid analogues inhibit Akt activation and translocation, selectively induce apoptosis in cancer

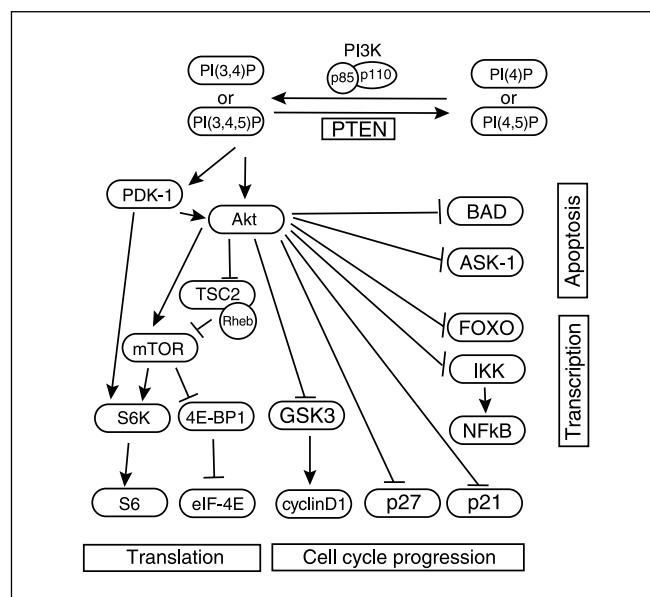
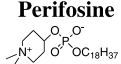
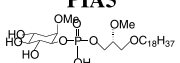
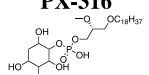
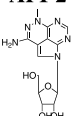
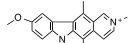
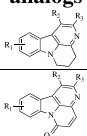
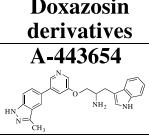
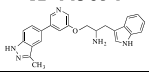
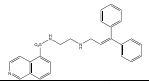


Fig. 3. The PI3K/Akt/mTOR pathway. G-protein-coupled receptors, growth factor receptors, integrins, activated Ras, or PTEN loss stimulates the PI3K/Akt/mTOR pathway. The products of PI3K, including PIP_3 , stimulate phosphorylation and activation of Akt. Once active, Akt can control key cellular processes by phosphorylating substrates involved in apoptosis, transcription, cell cycle progression, and translation. Arrowheads indicate activating phosphorylation events; perpendicular lines indicate inhibitory phosphorylation events.

Fig. 4. Development of inhibitors of Akt for cancer therapy. *, ability to reduce tumor burden *in vivo*; †, showed target modulation in tumor or surrogate tissue *in vivo*. Refs., references; n/a, not applicable (clinical trials have not been reported with this compound).

Agent/ Structure	Mechanism of action	<i>In vivo</i> Efficacy*	<i>In vivo</i> Target mod†	Clinical Status	Refs.
Perifosine 	Inhibition of translocation	Yes	No	Phase I,II	(59-61)
PIA5 	Inhibition of translocation	No	No	n/a	(62, 128)
PX-316 	Binds PH domain; prevents membrane translocation	Yes	Yes	n/a	(65)
API-2 	Unknown	Yes	Yes	n/a	(66, 67)
API-59CJ-OMe 	Unknown	No	No	n/a	(70)
Cathine alkaloid analogs 	Bind Akt at site other than PH-domain; inhibition is PH-domain dependent; some isoform specificity	Yes	No	n/a	(72-74)
Doxazosin derivatives 	Unknown	No	No	n/a	(76)
A-443654 	Binds catalytic site of Akt	Yes	Yes	n/a	(71)
Akt-in (peptide) AVTDHPDRLWAWEKFH	Binds Akt; inhibits translocation	No	No	n/a	(77)
Pseudo-substrate Ac-VELDPEFEPARERTYAFG H	Binds Akt substrate binding site	No	No	n/a	(78)
NL-71-101 	Binds catalytic site of Akt	No	No	n/a	(75)
Anti-Akt ScFv	Competitive inhibitor of substrate binding to catalytic site	Yes	Yes	n/a	(79)

cell lines that have high levels of constitutively active Akt, and can increase the efficacy of many therapeutic agents or radiation (62, 63). Currently, phosphatidylinositol ether lipid analogues are being evaluated for pharmacokinetic properties, *in vivo* efficacy, and determination of secondary targets. PX-316, another lipid-based inhibitor of Akt, also binds to the pleckstrin homology domain of Akt, preventing membrane localization (64). In preclinical studies, daily i.p. administration of PX-316 inhibited Akt and slowed the growth of MCF-7 xenografts but was only moderately effective at inhibiting the growth of HT-29 xenografts (65). The pharmacokinetic variables and specificity of PX-316 have not been assessed. Key issues for the development of lipid-based Akt inhibitors are oral bioavailability and hemolysis, a side effect that may determine which of these lipid-based inhibitors emerges as the front runner.

In addition to the lipid-based inhibitors, several Akt antagonists have been identified using high-throughput screen-

ing of chemical libraries. These inhibitors include Akt/protein kinase B signaling inhibitor-2 (API-2), 9-methoxy-2-methyl-ellipticinium acetate (API-59CJ-OMe), the indazole-pyridine A-443654, and isoform-specific canthine alkaloid analogues. Following its identification in a screen of the National Cancer Institute diversity set, API-2 was shown to inhibit Akt kinase activity and stimulate apoptosis of xenografts of human cancer cells exhibiting high Akt activity (66). API-2 is a tricyclic nucleoside that previously showed antitumor activity in phase I and phase II trials conducted over 20 years ago, but multiple toxicities, including hepatotoxicity, hyperglycemia, thrombocytopenia, and hypertriglyceridemia, precluded further development (67–69). 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.

Another Akt inhibitor that was identified in an analysis of the National Cancer Institute's anticancer 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 (70). Because inhibition of Akt and the induction of apoptosis were only measured after 48 or 72 hours of treatment, it is unclear whether Akt inhibition was a direct effect of compound administration or occurred as a secondary event after cellular damage or apoptosis.

The indazole-pyridine A-443654 was synthesized from a lead compound following a chemical library screen and was shown to inhibit the growth of PC-3, MiaPaCa-2, and 3T3-Akt1 xenografts (71). At doses that inhibited tumor growth, inhibition of signaling downstream of Akt was observed in tumors, but increased phosphorylation of Akt itself at S473 was observed. These paradoxical results raise questions about this compound's mechanism of action and the use of measuring S473 alone to assess Akt activity.

Isoform-specific Akt inhibitors have also been identified in a high-throughput kinase activity screen. Canthine alkaloid analogues that specifically inhibit Akt1, Akt2, or both Akt1 and Akt2 *in vitro* with IC₅₀s in the micromolar range were synthesized from lead compounds (72, 73). Treatment with the Akt1 or Akt2 inhibitor sensitized cancer cells to chemotherapy-induced cell death, but maximum sensitization was seen with the Akt1/Akt2 dual inhibitor (74). These studies are notable because this is the only published data on isoform-specific Akt inhibitors. Further studies to test the efficacy of these compounds and the consequences of inhibiting both Akt1 and Akt2 *in vivo* are needed, but the concept of isoform-specific Akt inhibitors is interesting because such an approach might mitigate toxicity (see Discussion).

Other putative Akt inhibitors have been generated by chemically modifying existing compounds, such as the PKA inhibitor, H89, and the α_1 -adrenoreceptor antagonist doxazosin. NL-71-101 is an analogue 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 (75). Structural modification of doxazosin has yielded compounds that both inhibit Akt and induce apoptosis in PC-3 cells *in vitro* (76). However, because Akt inhibition by these compounds is only observed after several hours, it is unclear if this is a direct or indirect effect. NL-71-101 and doxazosin analogues have only been tested in a few cell lines and have not been tested in animal models; therefore, their potential as therapeutics is unclear.

Peptide-based inhibitors of Akt are also being developed. AKT-in, a 15-amino-acid peptide that was designed to mimic the interaction between Akt and a binding protein, TCL1, directly binds and inhibits Akt. Although it inhibits cellular proliferation in preclinical studies, it has poor oral bioavailability and poor cellular penetration (77). Nonetheless, its description is provocative because it suggests that the interaction of Akt with its inhibitory binding proteins could be exploited to inhibit Akt. 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. In another approach, Luo et al. used the consensus

sequence of Akt to develop pseudopeptide substrates of Akt that have been shown to inhibit Akt and the growth of cancer cells *in vitro* (78). 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* (79). Although the concept of peptide-based inhibitors is exciting, their development is currently hampered by their low bioavailability and stability *in vivo*.


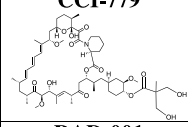
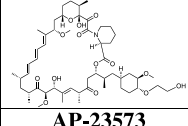
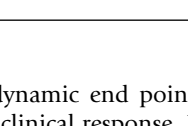
Similar to adenoviruses that overexpress PTEN, adenoviruses expressing a mutant inhibitory form of Akt have been developed (80). These adenoviruses selectively caused apoptosis in cancer cell lines expressing high levels of endogenous Akt activation. Direct intratumoral injection of this construct inhibited 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.

The ability of Akt to propagate activation of the pathway widely to many downstream substrates, thereby controlling cellular processes that contribute to tumor development and maintenance, makes it an attractive target for cancer prevention and therapy. Inhibition of Akt directly has advantages over targeting more downstream components. For example, Akt inhibition would be expected to inhibit most, if not all, Akt substrates. Because all substrates of Akt have not been completely identified and the "critical substrates" can vary with cell type, inhibition of individual downstream components may miss key substrates that are responsible for Akt-mediated survival or proliferation. Thus, Akt inhibition may offer greater efficacy, albeit at the expense of potential greater toxicity. Of the myriad inhibitors, lipid-based compounds, such as perifosine, are the best developed and have shown efficacy and tolerability in early clinical trials. However, optimal dosing must be addressed, and as with other Akt inhibitors, clinical effects need to be correlated with inhibition of the pathway.

mTOR Inhibitors

mTOR is a serine/threonine kinase downstream of Akt in the PI3K/Akt/mTOR pathway that controls protein synthesis, angiogenesis, and cell cycle progression. Inhibitors of mTOR are the most developed class of inhibitors of the PI3K/Akt/mTOR pathway. Although rapamycin is Food and Drug Administration approved as an immunosuppressant, the rapamycin analogues CCI-779, RAD-001, and AP-23573 have been explicitly designed for development as cancer drugs (Fig. 5). Preclinical studies with these compounds have shown potent activity as single agents or in combination with cytotoxic chemotherapy or radiation. In phase I and II trials, rapamycin analogues have shown activity against many types of cancer. Specifically, partial responses and stable disease have been observed in non-small cell lung cancer, breast, cervical, and uterine carcinomas, as well as sarcoma, mesothelioma, mantle cell lymphoma, and glioblastomas (81–91). Notably, many of these trials have incorporated analysis of phosphorylation of two downstream substrates of mTOR (4-EBP1 and S6K-1) in

Fig. 5. Development of inhibitors of mTOR for cancer therapy. *, ability to reduce tumor burden *in vivo*; †, showed target modulation in tumor or surrogate tissue *in vivo*. Refs., references.

Agent/ Structure	Mechanism of action	<i>In vivo</i> Efficacy*	<i>In vivo</i> Target mod†	Clinical Status	Refs.
Rapamycin 	Binds FKBP-12; complex binds to mTOR	Yes	Yes	Phase I	(129, 130)
CCI-779 		Yes	Yes	Phase III	(92,131-134)
RAD-001 		Yes	Yes	Phase I-II	(135, 136)
AP-23573 		n/a	n/a	Phase II	

peripheral lymphocytes as a pharmacodynamic end point to correlate inhibition of the pathway with clinical response. In a phase II trial of patients with renal cell carcinoma, a tumor type refractory to conventional chemotherapy, administration of CCI-779 resulted in one complete response, several patients with partial responses, and stabilization of the disease for >24 weeks in approximately half of the patients (92). The results of this study have prompted a phase III trial that is currently investigating treatment with CCI-779 versus IFN α in patients with stage IV recurrent renal cell carcinoma.

In addition to investigating the effect of rapamycin analogues as single agents, several clinical trials have also examined the efficacy of these drugs in combination with other anticancer agents, including gefitinib, imatinib mesylate, 17-AAG, letrozole, and NVP-AEW-541, an insulin-like growth factor-I receptor antagonist. Recent phase I/II trials examined the effect of RAD001 administration in combination with imatinib or gefitinib in patients with gastrointestinal stromal tumors refractory to imatinib or with non-small cell lung cancer, respectively. Partial responses were observed in both trials, and prolonged stable disease was observed in the gastrointestinal stromal tumor trial, suggesting that RAD001 may resensitize tumors to imatinib and that the combination may be beneficial for a subset of patients (93, 94). This result provides evidence that the use of signal transduction inhibitors in combination may be an effective treatment strategy in tumors that exhibit therapeutic resistance. Based on the results with the imatinib/RAD001 trial, many phase I studies combining RAD001 with other signal transduction inhibitors are planned or open for accrual.

The most recent rapamycin analogue, AP-23573, is a phosphorus-containing derivative currently in phase II clinical trials. Although preclinical data that has been presented in abstract form seem promising, the efficacy of AP-23573 as a targeted therapy cannot be fully evaluated because these studies have not been published as peer-reviewed articles.

Taken together, the results from preclinical studies and clinical trials using mTOR inhibitors are encouraging. Rapamycin and its analogues are extremely specific, and the long clinical history with rapamycin will facilitate development of CCI-779, RAD001, and AP-23573. The fact that mTOR inhib-

itors have induced clinical responses in a wide variety of tumors suggests that targeting the PI3K/Akt/mTOR pathway could be successful in many types of cancers. However, the fact that rapamycin and its analogues frequently induce cytostatic responses raises the possibility that more proximal inhibitors of PI3K, PDK-1, or Akt might result in greater inhibition of more components in the pathway and greater cytotoxicity. The use of rapamycin analogues might be further complicated by the observation that feedback activation of Akt due to mTOR inhibition could decrease the response to mTOR inhibitors as single agents (see below).

Considerations in Developing Inhibitors of the PI3K/Akt/mTOR Pathway

Clinical trial design. The success of tyrosine kinase inhibitors, such as erlotinib and imatinib, and the strong rationale to target the PI3K/Akt/mTOR pathway has fed optimism that inhibitors of serine/threonine kinases, such as PI3K, PDK-1, Akt, or mTOR, might have clinical use for cancer patients. Because these drugs are predicted to modulate a target and do not cause direct DNA damage like most standard chemotherapies, the design of clinical trials with PI3K/Akt/mTOR pathway inhibitors should reflect their biological activities. Based on numerous preclinical studies, pathway inhibitors are likely to be most effective in patients whose tumors bear activation of the pathway. Thus, phase I protocols with inhibitors of the PI3K/Akt/mTOR pathway should determine the biologically effective dose and the maximum tolerated dose and should determine the relationship between these doses. Although it can be argued that determining a tolerated biologically effective dose is sufficient for a targeted therapeutic, dose escalation to the maximum tolerated dose may help define the therapeutic index and may help identify dose-dependent "off-target" effects. 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 exclusion of patients who might obtain clinical benefit because of secondary, unknown, yet cytotoxic effects of the pathway modulator, but a priori, effects on tumors

that bear activation of the pathway should be shown. If efficacy against the intended pathway component is shown in initial trials, then consideration should be given to other trials that do not exclude patients who lack pathway activation.

How could the biologically effective dose of an inhibitor of the PI3K/Akt/mTOR pathway be measured? Noninvasive, molecular imaging of kinase inhibition would be ideal but is not currently possible. Existing assays that could be used to determine pathway inhibition include immunohistochemistry with phosphospecific or native antibodies that recognize levels of active or total protein, respectively, as well as immunoblotting, kinase assays, and/or flow cytometry using these same antibodies. Only one of these assays need be positive to show that the pathway inhibitor is hitting its desired target.

Although immunohistochemistry is not inherently quantitative, establishing an arbitrary cutoff for intensity and/or distribution of staining could provide a basis for quantification. As long as consistent methods are used throughout trials within a given institution for a single institution trial, or within each member of a cooperative group for a multi-institutional trial, meaningful comparisons of pathway activation could be made despite the lack of standard assays. The current absence of standard assays for evaluating inhibition of the PI3K/Akt/mTOR pathway in clinical pathology laboratories should not hinder efforts to assess changes in pathway components using immunohistochemistry or novel techniques. This may require close collaborations between scientific experts in the PI3K/Akt/mTOR pathway and clinical investigators, but this type of collaboration can only enhance the quality of clinical investigation of pathway inhibitors.

Once a pathway inhibitor is administered in a clinical protocol, tumor and/or surrogate tissues should be obtained to monitor changes in pathway components. Changes in activation of pathway components in surrogate tissues, such as skin, lymphocytes, or buccal mucosa, may not reflect changes within tumor tissues, but evaluation of the PI3K/Akt/mTOR pathway in surrogate tissues could provide some information on biological effectiveness, even if accession of tumor tissue is not feasible. One could advocate that early-stage clinical trials with pathway inhibitors might be best done in tumor types where repetitive analysis of tumor tissue is most feasible (e.g., hematopoietic tumors). Similar to the issue of how to measure activation of pathway components for enrollment in protocols, how is inhibition of the pathway measured as a response to a targeted inhibitor? Clearly, the same assay(s) should be used at each point in a trial to monitor pathway inhibition over time. The biggest problem arises with quantification. Is a 10% change in phosphorylation of Akt or mTOR a positive response? That is a difficult question, but the most important aspect here is to make every effort to evaluate the pathway, because this type of analysis has not been done consistently in early clinical trials with other targeted therapies.

The same principles outlined for therapeutic trials would apply to prevention studies that would use molecular end points as outcomes, not primary or secondary prevention. Inhibitors of the pathway should be tested first in patients whose precursor lesions bear activation of the pathway. A successful trial would show a correlation between inhibition of the pathway and regression of the precursor lesions. Implementation of these recommendations would establish that inhibition of the PI3K/Akt/mTOR pathway correlates with

clinical use of new agents targeting pathway components. Moreover, this approach could reveal which pathway targets are most commonly associated with clinical responses, which drugs are most effective, and the patient populations most likely to respond.

Biological complexity. Recent studies have identified two feedback mechanisms between components of the PI3K/Akt/mTOR pathway that may have implications for the efficacy of drugs that target the pathway, especially mTOR inhibitors. First, it was recently shown that short-term rapamycin administration can drive formation of an mTOR/Rictor complex that can phosphorylate and activate Akt (1). If rapamycin-induced Akt activity led to increased survival, this could counteract the effects of mTOR inhibition. This is unwanted if the desired effect is to decrease survival of malignant cells in cancer patients. Second, studies have also shown that prolonged exposure to rapamycin can lead to down-regulation of Akt phosphorylation. This is based on the observation PDK-1 can phosphorylate and activate the mTOR substrate S6K-1 independently of mTOR. Once active, S6K-1 can decrease Akt activity through phosphorylation and down-regulation of insulin receptor substrate proteins (95). Whether these mechanisms will be observed in cancer patients treated with inhibitors of the PI3K/Akt/mTOR pathway is unclear, but the existence of these feedback mechanisms emphasizes the need to evaluate upstream and downstream components when using an inhibitor such as rapamycin. Ultimately, this knowledge could help explain therapeutic responses to inhibitors of the pathway.

Therapeutic index. Because the PI3K/Akt/mTOR pathway plays a central role in many normal physiologic functions and is activated in many normal tissues, pathway inhibition may cause serious side effects and might lead to a limited therapeutic index. Murine models clearly show that loss of PI3K, Akt, or mTOR causes discernable phenotypes, which might provide insight into potential toxicities in humans. Specifically, Akt2 knock-out mice (Akt2^{-/-}) develop a diabetic phenotype (96), Akt1 knock-out mice (Akt1^{-/-}) are small in size and the males are infertile (97), and Akt3 knock-out mice (Akt3^{-/-}) develop smaller brains (98, 99). Because these side effects are related to mouse development, they may not be indicative of toxicities in humans receiving pathway inhibitors. Nevertheless, complications, such as hyperglycemia and insulin resistance, might be commonly observed with many inhibitors of the pathway and could be managed medically. Interestingly, neither Akt1^{-/-} nor Akt3^{-/-} mice develop a diabetic phenotype, which suggests that a diabetic phenotype may be less likely with isoform-specific Akt1 or Akt3 inhibitors. Loss of fertility caused by pathway inhibitors is less likely to be of importance after childbearing years, which is when most cancer occurs. Because preclinical data has shown that activation of the PI3K/Akt/mTOR pathway can protect against neurologic diseases, such as glaucoma, Alzheimer's disease, Parkinson's disease, and schizophrenia, it is possible that inhibition of the pathway might exacerbate or even cause these conditions (100–102). Although important to consider, these toxicities may not be manifest with short-term therapy, or they may reverse following withdrawal of pathway inhibition. Obviously, any observed toxicities would have to be weighed against the benefits of receiving a potentially effective cancer therapy. A key issue that has not been adequately addressed in *in vitro* studies,

much less *in vivo* studies, is how long does the pathway need to be inhibited to cause cell cycle arrest or apoptosis in cancer cells? Shorter exposures in patients might be associated with less toxicity. It is unclear whether continual exposure can be tolerated, and whether comparable responses can be achieved with long-term, low-dose exposure versus short-term, high-dose exposure. Studies that couple pharmacokinetic analysis with pharmacodynamic monitoring of biological end points could assist in answering these critical questions.

Is there reason to expect that a therapeutic index with inhibitors of the PI3K/Akt/mTOR pathway can be achieved? *In vitro* data show that pathway inhibitors are preferentially cytotoxic in tumor cells that exhibit increased activation of the pathway, suggesting that death of cancer cells without death of normal cells may be possible. One explanation for this observation is the concept of oncogene addiction, or selective reliance of tumor cells on activation of the PI3K/Akt/mTOR pathway. Tumor cells frequently have constitutive activation of the pathway, and therefore might be selectively reliant on signaling pathways that promote survival because they often grow in harsh conditions, such as acidic or hypoxic environments, that can activate the PI3K/Akt/mTOR pathway but

would otherwise induce apoptosis. Inhibition of the pathway in cells that depend upon it for survival might cause apoptosis but would not kill normal cells that do not grow in these conditions and therefore do not have the same dependence. Selective reliance of tumor cells on activation of the PI3K/Akt/mTOR pathway will be assessed in clinical trials with these inhibitors in patients with solid tumors, and the balance of toxicity and efficacy will show whether a therapeutic index can be achieved. If a therapeutic index is elusive when inhibitors are used as single agents, then these agents might nevertheless find use as radiation or chemotherapeutic sensitizers at lower doses, where toxicities might be less likely to develop.

In conclusion, the race to develop inhibitors of the PI3K/Akt/mTOR pathway is led by the mTOR inhibitors, rapamycin and its analogues, and the Akt inhibitor perifosine. Behind these leaders are a pack of interesting but relatively untested inhibitors of components proximal to mTOR in the pathway. These "unknowns" offer potentially greater efficacy, but at what cost? Intelligent clinical trial design, coupled with rigorous scientific evaluation, will determine how many inhibitors of the PI3K/Akt/mTOR pathway are able to finish the race and become useful therapeutics.

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