



Published in final edited form as:

Drug Resist Updat. 2008 ; 11(1-2): 32–50.

Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations

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Abstract

The PI3K/Akt/mTOR pathway is a prototypic survival pathway that is constitutively activated in many types of cancer. Mechanisms for pathway activation include loss of tumor suppressor PTEN function, amplification or mutation of PI3K, amplification or mutation of Akt, activation of growth factor receptors, and exposure to carcinogens. Once activated, signaling through Akt can be propagated to a diverse array of substrates, including mTOR, a key regulator of protein translation. This pathway is an attractive therapeutic target in cancer because it serves as a convergence point for many growth stimuli, and through its downstream substrates, controls cellular processes that contribute to the initiation and maintenance of cancer. Moreover, activation of the Akt/mTOR pathway confers resistance to many types of cancer therapy, and is a poor prognostic factor for many types of cancers. This review will provide an update on the clinical progress of various agents that target the pathway, such as the Akt inhibitors perifosine and PX-866 and mTOR inhibitors (rapamycin, CCI-779, RAD-001) and discuss strategies to combine these pathway inhibitors with conventional chemotherapy, radiotherapy, as well as newer targeted agents. We will also discuss how the complex regulation of the PI3K/Akt/mTOR pathway poses practical issues concerning the design of clinical trials, potential toxicities and criteria for patient selection.

Keywords

PI3 kinase; Akt; combination; chemotherapy; cancer; wortmannin; PX-866; perifosine; mTOR inhibitors; rapamycin; RAD-001; PI-103; triciribine (API-2)

1. Introduction

1.1. Activation of the PI3K/Akt/mTOR pathway

Signaling through the PI3K/Akt/mTOR pathway can be initiated by several mechanisms, all of which increase activation of the pathway in cancer cells. Once activated, the PI3K/Akt/mTOR pathway can be propagated to various substrates, including mTOR, a master regulator of protein translation. Initial activation of the pathway occurs at the cell membrane, where the signal for pathway activation is propagated through class IA PI3K (Figure 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 (GPCRS), and oncogenes such as

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Ras. PI3K catalyzes phosphorylation of the D3 position on phosphoinositides to generate the biologically active moieties phosphatidylinositol-3,4,5-triphosphate (PI(3,4,5)P₃) and phosphatidylinositol-3,4-bisphosphate (PI(3,4)P₂). Upon generation, PI(3,4,5)P₃ binds to the pleckstrin homology (PH) domains of PDK-1 (3'phosphoinositide-dependent kinase 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 ten) antagonizes PI3K by dephosphorylating PI(3,4,5)P₃ and (PI(3,4)P₂), thereby preventing activation of Akt and PDK-1.

Akt exists as three structurally similar isoforms, Akt1, Akt2 and Akt3, which are expressed in most tissues (Zinda et al., 2001). Activation of Akt1 occurs through two crucial phosphorylation events, the first of which occurs at T308 in the catalytic domain by PDK-1 (Andjelkovic et al., 1997; Walker et al., 1998). Full activation requires a subsequent phosphorylation at S473 in the hydrophobic motif, which can be mediated by several kinases such as PDK-1 (Balendran et al., 1999), integrin-linked kinase (ILK) (Delcommenne et al., 1998; Lynch et al., 1999), Akt itself (Toker and Newton, 2000), DNA-dependent protein kinase (Feng et al., 2004; Hill et al., 2002), or mTOR (when bound to Rictor in so called TORC2 complexes (Santos et al., 2001)). Phosphorylation of homologous residues in Akt2 and Akt3 occurs by the same mechanism. Phosphorylation of Akt at S473 is also controlled by a recently described phosphatase, PHLPP (PH domain leucine-rich repeat protein phosphatase), that has two isoforms that preferentially decrease activation of specific Akt isoforms (Brognard et al., 2007). In addition, amplification of Akt1 has been described in human gastric adenocarcinoma (Staal, 1987), and amplification of Akt2 has been described in ovarian, breast, and pancreatic carcinoma (Bellacosa et al., 1995; Cheng et al., 1996). Although mutation of Akt itself is rare, Carpten *et al.* recently described somatic mutations occurring in the PH domain of Akt1 in a small percentage of human breast, ovarian, and colorectal cancers (Carpten et al., 2007).

1.2. Downstream substrates of activated Akt

Akt recognizes and phosphorylates the consensus sequence RXXRX(S/T) when surrounded by hydrophobic residues. Because this sequence is present in many proteins, numerous Akt substrates have been identified and validated (Obenauer et al., 2003). 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 pro-apoptotic genes, e.g., *Fas-L*, *IGFBP1* and *Bim* (Datta et al., 1997; Nicholson and Anderson, 2002). Additionally, Akt can directly regulate apoptosis by phosphorylating and inactivating pro-apoptotic proteins such as BAD, which controls release of cytochrome c from mitochondria, and ASK1 (apoptosis signal-regulating kinase-1), a mitogen-activated protein kinase kinase involved in stress- and cytokine-induced cell death (Datta et al., 1997; del Peso et al., 1997; Zha et al., 1996). In contrast, Akt can phosphorylate IKK, which indirectly increases the activity of nuclear factor kappa B (NF-κB) and stimulates the transcription of pro-survival genes (Ozes et al., 1999; Romashkova and Makarov, 1999; Verdu et al., 1999). Cell cycle progression can also be effected by Akt through its inhibitory phosphorylation of the cyclin-dependent kinase inhibitors, p21^{WAF1/CIP1} and p27^{KIP1} (Liang et al., 2002; Shin et al., 2002; Zhou et al., 2001), and inhibition of GSK3β by Akt stimulates cell cycle progression by stabilizing cyclin D1 expression (Diehl et al., 1998). Recently, a novel pro-survival Akt substrate, PRAS40 (proline-rich Akt substrate of 40kDa), has been described (Vander Haar et al., 2007), whereby phosphorylation of PRAS40 by Akt attenuates its ability to inhibit mTORC1 kinase activity. It has been suggested that PRAS40 may be a specific substrate of Akt3 (Madhunapantula et al., 2007). Thus, Akt inhibition might have pleiotropic effects on cancer cells that could contribute to an anti-tumor response.

The best-studied downstream substrate of Akt is the serine/threonine kinase mTOR (mammalian target of rapamycin). 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, in which mTOR is bound to Raptor, and the TORC2 complex, in which mTOR is bound to Rictor. In the TORC1 complex, mTOR signals to its downstream effectors S6 kinase/ribosomal protein S6 and 4EBP-1/eIF-4E to control protein translation. Although mTOR is generally considered a downstream substrate of Akt, mTOR can also phosphorylate Akt when bound to Rictor in TORC2 complexes, perhaps providing a level of positive feedback on the pathway (Sarbasov et al., 2005). Finally, the downstream mTOR effector S6 kinase-1 (S6K1) can also regulate the pathway by catalyzing an inhibitory phosphorylation on insulin receptor substrate (IRS) proteins. This prevents IRS proteins from activating PI3K, thereby inhibiting activation of Akt (Harrington et al., 2004; Shah et al., 2004).

1.3. Rationale for targeting the PI3K/Akt/mTOR pathway

In addition to preclinical studies, many clinical observations support targeting the PI3K/Akt/mTOR pathway in human cancer. 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 (Alkan and Izban, 2002; Choe et al., 2003; Dai et al., 2005; Ermoian et al., 2002; Gupta et al., 2002; Horiguchi et al., 2003; Hsu et al., 2001; Kanamori et al., 2001; Kreisberg et al., 2004; Kurose et al., 2001; Malik et al., 2002; Min et al., 2004; Nakayama et al., 2001; Nam et al., 2003; Perez-Tenorio and Stal, 2002; Roy et al., 2002; Schlieman et al., 2003; Sun et al., 2001; Terakawa et al., 2003; Yuan et al., 2000). 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 (Dai et al., 2005), pancreas (Schlieman et al., 2003; Yamamoto et al., 2004), liver (Nakanishi et al., 2005), prostate (Kreisberg et al., 2004), breast (Perez-Tenorio and Stal, 2002), endometrium (Terakawa et al., 2003), stomach (Nam et al., 2003), brain (Ermoian et al., 2002), and blood (Min et al., 2004). Tsurutani *et al.* recently extended these studies by using antibodies against two sites of Akt phosphorylation, S473 and T308, to show that Akt activation is selective for NSCLC tumors versus normal tissue and is a better predictor of poor prognosis in NSCLC tumors than S473 alone (Tsurutani et al., 2006). In addition, amplification of Akt isoforms has been observed in some cancers, albeit at a lower frequency (Bellacosa et al., 1995; Cheng et al., 1996; Ruggeri et al., 1998; Staal, 1987).

Another frequent genetic event that occurs in human cancer is loss of tumor suppressor PTEN function. PTEN normally suppresses activation of the PI3K/Akt/mTOR pathway by functioning as a lipid phosphatase. Loss of PTEN function in cancer can occur through mutation, deletion, or epigenetic silencing. Multiple studies have demonstrated a high frequency of PTEN mutations or deletions in a variety of human cancers, including brain, bladder, breast, prostate, and endometrial cancers (Ali et al., 1999; Aveyard et al., 1999; Dahia, 2000; Dreher et al., 2004; Li et al., 1997; Rasheed et al., 1997), making PTEN the second most frequently mutated tumor suppressor gene (Stokoe, 2001). In tumor types where PTEN mutations are rare, such as lung cancer, epigenetic silencing may occur (Forgacs et al., 1998; Kohno et al., 1998; Yokomizo et al., 1998). Several studies have also demonstrated the prognostic significance of PTEN loss in multiple human cancers, where mutation, deletion, or epigenetic silencing of PTEN correlates with poor prognosis and reduced survival (Bertram et

al., 2006; Perez-Tenorio et al., 2007; Saal et al., 2007; Smith et al., 2001; Yoshimoto et al., 2007). Collectively, these studies have established that the loss of PTEN is a common mechanism for activation of the PI3K/Akt/mTOR pathway and poor prognostic factor in human cancer.

Finally, activation of PI3K has been described in human cancers. It can result from amplification, overexpression or from mutations in the p110 catalytic or p85 regulatory subunits. Amplification of the 3q26 chromosomal region, which contains the gene PIK3CA that encodes the p110 α catalytic subunit of PI3K, occurs in 40% of ovarian (Shayesteh et al., 1999) and 50% of cervical carcinomas (Ma et al., 2000). Somatic mutations of this gene have also been detected in several cancer types and result in increased kinase activity of the mutant PI3K relative to wild-type PI3K. Mutations in the regulatory p85 subunit have also been detected (Jimenez et al., 1998; Philp et al., 2001). Because any of these alterations in individual components would result in activation of the pathway, these studies suggest that pathway activation is one of the most frequent molecular alterations in cancer.

1.4. PI3K/Akt/mTOR pathway and chemotherapeutic resistance

The rationale for targeting the PI3K/Akt/mTOR pathway in combination therapy comes from data describing constitutive or residual pathway activation in cells that have developed resistance to conventional chemotherapy and radiation (West et al., 2002), as well as to other targeted therapies such as EGFR antagonism. In these cases, combining chemotherapy or radiation with a pathway inhibitor can overcome acquired resistance to EGFR tyrosine kinase inhibitors (TKI). Some standard chemotherapeutic agents appear to directly inhibit Akt *in vitro*, and the cytotoxicity may be a direct consequence of inhibition of Akt signaling (Asselin et al., 2001; Hayakawa et al., 2002). Because Akt is integrally involved in cellular survival, many groups have investigated the effects of combining chemotherapy with pathway inhibitors. Preclinical studies that have investigated this concept will be discussed below.

2. Preclinical combination data

2.1. Combining pathway inhibitors with conventional chemotherapy and radiation

2.1.1. PI3 kinase inhibitors: LY294002 and wortmannin—Targeting PI3 kinase, the most proximal pathway component, has advantages over targeting more distal components such as Akt and mTOR. Inhibitors of PI3K diminish signaling to Rac as well as Akt, providing a broader inhibition of downstream signaling than distal inhibition. The pharmacologic agents LY294002 and wortmannin both target the p110 catalytic subunit of PI3K. Although these commercially available inhibitors effectively inhibit PI3K, poor solubility and high toxicity have limited their clinical application. However, these compounds provide powerful preclinical tools to study the cellular consequences of pathway inhibition. Both of these inhibitors of PI3K sensitize cancer cells to various types of conventional chemotherapy. LY294002 increases cytotoxicity induced by antimicrotubule agents such as taxanes and vinca alkaloids in glioma, ovarian cancer, esophageal cancer, and lung cancer cells *in vitro* and *in vivo* (Hu et al., 2002; Mabuchi et al., 2002; Nguyen et al., 2004; Shingu et al., 2003). Wortmannin has also been shown to enhance apoptosis of several cell lines when used in combination with paclitaxel (Hu et al., 2002), cisplatin (Asselin et al., 2001), gemcitabine (Ng et al., 2000), or 5-fluorouracil (Wang et al., 2002), where potentiation of apoptosis caused by wortmannin was associated with inhibition of Akt activation. In another study, wortmannin enhanced cytotoxicity of etoposide in eight tumorigenic cell lines, predominantly through inhibition of PI3K-dependent phosphorylation of protein kinase C zeta (PKC ζ) (Reis et al., 2005; Skladanowski et al., 2007). Wortmannin can also increase the efficacy of chemotherapeutic agents *in vivo*. For example, gemcitabine-induced apoptosis of orthotopic pancreatic cancer in xenografts was potentiated by treatment with wortmannin and was associated with decreased Akt

phosphorylation (Ng et al., 2001). In addition, the treatment of human ovarian cancer xenografts with wortmannin plus paclitaxel increased apoptosis and decreased tumor burden compared to either agent alone (Hu et al., 2002). Wortmannin combined with cisplatin increased the efficacy of cisplatin in an ovarian cancer model where cancer cells were injected into the peritoneum of nude mice (Ohta et al., 2006). In this study, wortmannin increased cisplatin-induced apoptosis and inhibition of intra-abdominal dissemination of cancer cells. Additionally, several studies have identified PI3K inhibitors as radiosensitizers and augmentation of radiation-induced cytotoxicity has been observed with nanomolar doses of wortmannin (Edwards et al., 2002; Gupta et al., 2003; Sarkaria et al., 1998). Although wortmannin and LY294002 are not clinically useful, newer inhibitors of PI3K such as PX-866 are being developed, but none of these have been combined with traditional chemotherapies.

2.1.2. Akt inhibitors

Perifosine: Due to feedback activation of Akt that results from mTOR inhibition, inhibiting Akt directly may have advantages over targeting more distal components of the pathway. To date, the most developed inhibitor of Akt is perifosine, a lipid-based inhibitor. *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 (Crul et al., 2002; Kondapaka et al., 2003). Additional *in vitro* data demonstrates synergistic effects of perifosine and traditional chemotherapeutic agents such as etoposide in leukemia cells (Nyakern et al., 2006), doxorubicin in multiple myeloma cells (Hideshima et al., 2006), and temozolomide in glioma cells (Momota et al., 2005). In the latter study, the combination of perifosine and temozolomide was more effective than temozolomide alone in inhibiting growth of glioma xenografts. Perifosine has also been found to sensitize cancer cells to apoptosis and cell cycle arrest induced by radiation *in vitro* and *in vivo* (Caron et al., 2005; Ruiter et al., 1999; Vink et al., 2006a).

PIAs: A relatively new group of lipid-based Akt inhibitors are the phosphatidylinositol ether lipid analogues (PIAs). PIAs were designed to interact with the PH domain of Akt and are structurally similar to the products of PI3 kinase. Although less clinically developed, PIAs are well-characterized *in vitro*. In addition to Akt inhibition, Gills *et al* recently identified several molecular targets that contribute to the cytotoxicity of PIAs, including activation of the stress kinase p38 α , and PIA-induced cytotoxicity correlates with inhibition of Akt phosphorylation in the NCI60 cell line panel. (Gills et al., 2007; Gills et al., 2006). PIAs mitigate drug resistance to several traditional chemotherapies and ionizing radiation *in vitro* (Martelli et al., 2003; Tabellini et al., 2004; Van Meter et al., 2006). PIAs also enhance the apoptosis induced by other agents such as tumor necrosis factor-related ligand (TRAIL), all-*trans*-retinoic acid (ATRA) and motexafin gadolinium (Martelli et al., 2003; Puduvalli et al., 2005; Ramos et al., 2006).

Triciribine (API-2): API-2, also known as triciribine phosphate, was identified as an Akt inhibitor after screening the National Cancer Institute (NCI)'s structural diversity set. Triciribine inhibits Akt2 phosphorylation at both sites (T309 and S474) and inhibits EGF-induced phosphorylation of all three isoforms of Akt *in vitro*. *In vivo*, treatment with low doses of triciribine stimulated apoptosis in xenografts with constitutively activated Akt or PTEN mutations, but not in tumors with low Akt activity (Yang et al., 2004). Triciribine has not been preclinically combined with standard chemotherapies or radiation.

2.1.3. mTOR inhibitors: rapamycin and its analogues—The PI3K/Akt/mTOR pathway inhibitors that are most clinically developed, target a more distal pathway component, mTOR. Rapamycin, the prototypic mTOR inhibitor, was discovered in 1975 as a potent anti-fungicide, and is produced naturally by *Streptomyces hygroscopicus* (Sehgal et al., 1975; Vezina

et al., 1975). The ability of rapamycin to inhibit the proliferation of cancer cell lines was shown over 20 years ago (Douros and Suffness, 1981; Eng et al., 1984; Houchens et al., 1983). More recently, rapamycin analogues such as CCI-779 and RAD-001 have been explicitly designed for development as anticancer drugs. These inhibitors of mTOR bind to the FK506-binding protein, FKBP-12, which then binds and inhibits mTOR. Inhibition of mTOR decreases phosphorylation of two downstream targets, 4E-BP1 and S6K, resulting in inhibition of protein synthesis.

Rapamycin and its analogues have been studied in combination with standard chemotherapies. For example, treatment of orthotopic neuroblastoma-bearing mice with rapamycin and vinblastine resulted in inhibition of tumor growth and angiogenesis, with an increase in survival compared to either drug alone (Marimpietri et al., 2007; Marimpietri et al., 2005). Similar results were observed in hepatocellular carcinoma (Ribatti et al., 2007). *In vitro*, synergy has been observed with rapamycin and paclitaxel, carboplatin, or vinorelbine. In lymphoma models, RAD-001 demonstrates *in vitro* synergy with rituximab, doxorubicin, and vincristine (Haritunians et al., 2007; Wanner et al., 2006), predominantly through induction of cell cycle arrest. Combinations of RAD-001 and anti-estrogen agents tamoxifen and letrozole also demonstrated enhanced levels of apoptosis than with either drug alone (Boulay et al., 2005; Treeck et al., 2006). Interestingly, RAD-001 sensitizes tumor cells to cisplatin-induced apoptosis in a p53-dependent manner via inhibition of mTOR function, resulting in reduced p21 translation (Beuvink et al., 2005). CCI-779, another rapamycin analogue, has been successfully combined with cisplatin, gemcitabine, and camptothecin *in vitro* and *in vivo* (Geoerger et al., 2001; Ito et al., 2006; Thallinger et al., 2007a; Thallinger et al., 2007b; Wu et al., 2005).

Rapamycin and RAD-001 are also potent radiosensitizers through mTOR-dependent enhancement of radiation-induced autophagy (Albert et al., 2006; Kim et al., 2006; Paglin et al., 2005; Moretti et al., 2007). In a recent study, RAD-001 sensitized PTEN wild-type and PTEN null cancer cells to ionizing radiation, but induced more cytotoxicity in PTEN null cells (Cao et al., 2006). RAD-001 also enhances radiation-induced damage of tumor vasculature *in vivo* through induction of apoptosis of vasculature endothelial cells (Shinohara et al., 2005). Taken together, these data indicate that combining mTOR inhibition with chemotherapy or radiation could be a potentially effective approach in cancer treatment.

2.2. Combining pathway inhibitors with other targeted therapies

Because signaling of multiple receptor tyrosine kinases (RTKs) is propagated through Akt, simultaneous inhibition of RTKs such as IGF-IR or erbB family members with pathway components such as Akt or mTOR may circumvent feedback activation seen with either approach alone. Such an approach can be considered proximal and distal signaling inhibition (Figure 2, left panel). Based on the observed feedback activation of Akt by mTOR inhibitors, it is possible that they might be most effective when combined with proximal pathway inhibitors. For example, synergistic effects between rapamycin and LY294002, an upstream inhibitor of PI3K, are commonly observed *in vitro* (Breslin et al., 2005; Sun et al., 2005; Takeuchi et al., 2005). Most recently, Fan *et al* showed that a dual inhibitor of PI3K α and mTOR, PI-103, was able to inhibit Akt activity as well as proliferation in glioma cells, regardless of PTEN or EGFR status (Fan et al., 2006). PI-103 was effective in inhibiting the growth of glioma xenografts in the absence of toxicity, most likely through a cytostatic mechanism.

Another possible approach is to combine inhibition of the PI3K/Akt/mTOR pathway with inhibition of a parallel pro-survival signaling pathway such as the MEK/ERK pathway (Tortora et al., 2007) (Figure 2, middle panel). This approach abrogates compensatory activation of other pro-survival pathways when the PI3K/Akt/mTOR pathway is inhibited. For example,

combining an inhibitor of PI3K with an inhibitor of MEK causes a synergistic increase in apoptosis in both PTEN mutant and wild-type cells (She et al., 2005). Both approaches will be discussed below.

2.2.1. Pathway inhibitors and inhibitors of receptor tyrosine kinases (RTKs)

i) Combinations with EGFR antagonists: Perhaps the most extensive data on proximal and distal signaling inhibition exists for combining PI3K/Akt/mTOR pathway inhibitors with EGFR antagonists. The epidermal growth factor receptor (EGFR/erbB1) is overexpressed or amplified in a variety of tumor types and is a major target in cancer therapy. Patients who respond to EGFR TKIs eventually develop resistance and progressive disease. Elucidated mechanisms of resistance in NSCLC include a somatic T790M mutation in the kinase domain of EGFR (Engelman et al., 2006), epithelial to mesenchymal transition (EMT) (Irie et al., 2005; Thomson et al., 2005; Yauch et al., 2005), amplification of the Met oncogene (Engelman et al., 2005; Engelman et al., 2007) and downregulation of BIM activity (Cragg et al., 2007; Gong et al., 2007). All of these mechanisms of resistance are associated with maintenance and continued activation of the PI3K/Akt/mTOR pathway.

Cancer cell lines with mutant PTEN, which have high levels of Akt are resistant to EGFR antagonists such as gefitinib. PTEN reconstitution can restore sensitivity to EGFR inhibition. She *et al.* showed that this approach inhibited growth of breast cancer xenografts, which was not seen with either EGFR inhibition or PTEN induction alone (She et al., 2005). Similar results have been observed in NSCLC, prostate, and leukemia cell lines, thereby linking PTEN status and Akt activity with sensitivity to EGFR inhibition (Festuccia et al., 2005; Janmaat et al., 2003; Janmaat et al., 2006; Kokubo et al., 2005; Li et al., 2006). In PTEN-null gefitinib-resistant cells, reintroduction of PTEN function or treatment with LY294002 restores gefitinib sensitivity (She et al., 2003).

Many different PI3K inhibitors can restore sensitivity to EGFR inhibitors (Bianco et al., 2003; She et al., 2003). Sordella *et al.* 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 LY294002 (Sordella et al., 2004). In another study with PX-866, a PI3K inhibitor selective for p110 α , PX-866 was able to abolish gefitinib resistance in NSCLC xenografts. Toxicities associated with PX-866 administration were decreased glucose tolerance and hyperglycemia, both of which were reversed upon discontinuation of drug treatment (Ihle et al., 2005).

Synergistic effects of rapamycin and EGFR TKIs have been observed in several *in vitro* systems, including glioblastoma multiforme (Hjelmeland et al., 2007; Rao et al., 2005; Wang et al., 2006), prostate cancer (Buck et al., 2006; Masiello et al., 2007), pancreatic cancer (Buck et al., 2006), squamous cell carcinoma (Jimeno et al., 2007), renal cell carcinoma (Costa et al., 2007; Gemmill et al., 2005), leukemia (Mohi et al., 2004), cervical carcinoma (Birle and Hedley, 2006), and non-small cell lung cancer (Buck et al., 2006). Several of these studies extended the efficacy of these combinations to xenograft experiments (Birle and Hedley, 2006; Buck et al., 2006; Jimeno et al., 2007). Buck *et al.* noted re-sensitization and synergistic growth inhibition with the combination of rapamycin and erlotinib in cells lines that were previously resistant to erlotinib (Buck et al., 2006). Li *et al.* noted significant regression of lung tumors in transgenic mice that possessed the secondary resistance mutation T790M when treated with the combination of rapamycin and the irreversible EGFR TKI, HKI-272 (Li et al., 2007). In human glioma cell lines with mutant PTEN, addition of the dual PI3K/mTOR inhibitor PI-103 to erlotinib was necessary to induce growth arrest, suggesting that activation of the PI3K/Akt/mTOR pathway by EGFR-independent mechanisms confers resistance to EGFR inhibitors, which can nonetheless be overcome by the addition of pathway inhibitors. Collectively, these data suggest that the use of EGFR antagonists with pathway 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 TKIs.

ii) Combinations with erbB2 antagonists: Another potentially useful combination is proximal inhibition of erbB2, also known as her-2/neu, with distal inhibition of Akt or mTOR. Inhibition of Akt phosphorylation is a requirement for the anti-proliferative effects of the her-2/neu antagonist, trastuzumab, and trastuzumab-resistant cells exhibit sustained activation of the PI3K/Akt/mTOR pathway (Chan et al., 2005a; Nagata et al., 2004; Yakes et al., 2002). A preclinical study was recently reported combining triciribine with trastuzumab in an effort to circumvent trastuzumab resistance due to loss of PTEN (Lu et al., 2007). In breast cancer cell lines and xenografts, triciribine restored sensitivity to trastuzumab, concomitant with induction of apoptosis and inhibition of tumor growth. In the same study, RAD-001 was also able to re-sensitize trastuzumab resistant cells to apoptosis *in vitro* and *in vivo*. Similar results have been observed with rapamycin (Liu et al., 2005; Wang et al., 2007), and classical PI3K inhibitors have also been successfully combined with trastuzumab *in vitro* (Chan et al., 2005a; Nagata et al., 2004).

iii) Combinations with IGF-IR antagonists: Monoclonal antibodies directed against the insulin-like growth factor 1 receptor (IGF-IR), a transmembrane RTK, have been used extensively in preclinical studies. When bound by IGF-I or IGF-II, IGF-IR is autophosphorylated and activates PI3K. Additionally, feedback activation of Akt induced by mTOR inhibition is partially mediated via upregulation of insulin receptor substrate 1 (IRS1), and subsequent signaling through IGF-IR, suggesting that dual inhibition of IGF-IR and mTOR may be more effective than mTOR inhibition alone. For example, combining rapamycin with a small molecule inhibitor of IGF-IR abrogated feedback activation of Akt and enhanced cytotoxicity of rapamycin in glioma cells (Steinbach et al., 2004). Similarly, combination of a neutralizing antibody directed against IGF-IR with RAD-001 reversed Akt phosphorylation induced by RAD-001, and resulted in additive anti-proliferative effects in leukemic cells (Tamburini et al., 2007). These data demonstrate that proximal inhibition of IGF-IR combined with inhibition of distal pathway components, such as Akt and mTOR, may abrogate feedback activation that results from mTOR inhibition alone.

2.2.2. Pathway inhibitors and other targeted agents—In addition to combining PI3K/Akt/mTOR inhibitors with agents that inhibit either the same or parallel pro-survival signaling pathways, PI3K/Akt/mTOR inhibitors have also been combined with targeted agents that defy easy categorization such as imatinib and those that do not directly affecting signaling pathways, e.g., histone deacetylase (HDAC) inhibitors and proteasome inhibitors (Landis-Piwowar et al., 2006) (Figure 2, right panel). Although the mechanisms behind the efficacy of these combinations are not completely understood, they represent potentially useful combinations for patients whose tumors do not respond to more conventional therapy regimens.

i) PI3 kinase and Akt inhibitors: PI3K inhibitors have been successfully combined with imatinib in leukemic cells (Klejman et al., 2002), as well as sulindac, a non-steroidal anti-inflammatory drug that inhibits COX-2 (Yip-Schneider et al., 2003). LY294002 and wortmannin sensitize cancer cells to histone deacetylase (HDAC) inhibitor-induced apoptosis *in vitro* and *in vivo* (Denlinger et al., 2005; Rahmani et al., 2003) (Wang et al., 2002). Rahmani et al (Rahmani et al., 2005) found that treatment with LY294002 inhibited ERK phosphorylation and p21 induction, both of which normally protect leukemic cells from HDAC inhibitor-induced apoptosis. They concluded that the latter mechanisms, rather than inhibition of Akt signaling led to increased cell death. In contrast, sensitization of A549 NSCLC xenografts by LY294002 to HDAC inhibitor-induced apoptosis resulted from Akt-dependent regulation of nuclear factor kappa B transcription (Denlinger et al., 2005). Combined treatment

with an HDAC inhibitor and LY294002 inhibited tumor growth concurrently with inhibition of Akt *in vivo*. In addition to PI3K inhibitors, the Akt inhibitor perifosine has been combined with a handful of other targeted therapies *in vitro*. Perifosine treatment of PTEN-deficient breast and prostate cancer cells enhanced growth inhibition induced by cetuximab (Li et al., 2006), as well as apoptosis induced by HDAC inhibitors in leukemic cells (Rahmani et al., 2005).

ii) mTOR inhibitors: mTOR inhibitors have also been successfully combined pre-clinically with other targeted therapies. In chronic myelogenous leukemia cells with moderate resistance to imatinib, treatment with imatinib and rapamycin or its analogue, RAD-001, resulted in synergistic inhibition of leukemic cell growth. Rapamycin has also been effectively combined in breast cancer models with targeted agents such as herceptin (Wang et al., 2007), cotylenin A (Kasukabe et al., 2005), and luteolin (Chiang et al., 2007). In multiple myeloma (MM), rapamycin sensitizes MM cells to apoptosis induced by hsp90 inhibitors (Francis et al., 2006), dexamethasone, and thalidomide analogs (Raje et al., 2004; Stromberg et al., 2004). In addition, rapamycin acts cooperatively with small molecule inhibitors of c-met and VEGF, where in the latter study, combination therapy inhibited primary and metastatic growth of orthotopic pancreatic cancer tumors, as well as liver metastasis (Ma et al., 2005; Stephan et al., 2004). mTOR inhibition can be combined with other types of therapeutic approaches. For example, rapamycin and RAD-001 enhance the efficacy of oncolytic viruses that target tumor cells in medulloblastoma and colon cancer xenografts (Lun et al., 2007; Homicsko et al., 2005). Recently, it was shown that infection with a herpes simplex viral vector activates Akt in cancer cells, and that concurrent treatment with viral particles and LY294002 enhanced the efficacy of the virus (Liu et al., 2007). This may be a common feature of pathway inhibition, whereby oncolytic viral infection activates the PI3K/Akt/mTOR pathway, and this activation is amenable to pharmacologic inhibition.

3. Clinical trials with PI3K/Akt/mTOR pathway inhibitors as single agents and in combination with other therapies

Although combinations of pathway inhibitors with various types of chemotherapy have been investigated extensively in preclinical studies, only a few clinical trials with Akt inhibitors and mTOR inhibitors have been reported up to now, while no clinical trials using PI3K inhibitors have been published. These data will be discussed below (Table 1).

3.1. Akt inhibitors

3.1.1. Perifosine—A number of phase I and II clinical trials investigating perifosine monotherapy in a variety of tumor types have been completed. In initial phase I trials employing high daily doses of perifosine, gastrointestinal toxicity led to frequent treatment discontinuations (Crul et al., 2002). More recent phase I trials utilized a loading dose followed by smaller daily maintenance doses of perifosine, allowing for rapid achievement of steady state plasma concentrations, and reduced gastrointestinal toxicity during the maintenance phase (Van Ummersen et al., 2004). The loading dose followed by maintenance perifosine has been used in Phase II clinical trials in breast cancer, pancreatic cancer, prostate cancer, head and neck cancer, melanoma, and sarcoma (Argiris et al., 2006; Bailey et al., 2006; Ernst et al., 2005; Knowling et al., 2006; Leighl et al., 2007; Marsh Rde et al., 2007; Posadas et al., 2005). In these trials, the activity of single agent perifosine in solid tumors has been disappointing, with few objective responses noted. Gastrointestinal and constitutional toxicities were problematic, particularly in a trial in advanced pancreatic cancer (Marsh Rde et al., 2007). A phase II trial of perifosine in twenty three patients with soft tissue sarcomas yielded a partial response of nine months duration in one patient with chondrosarcoma, as well as stabilization of disease in five patients at eight weeks. Despite not meeting their objectives

for progression free survival ($\geq 40\%$ at 6 months) in this trial, the investigators plan to conduct further studies of perifosine in patients with selected soft tissue sarcoma histologies, perhaps enriching their patient population with patients whose tumors bear high expression of p-Akt, and attempting to achieve high steady state plasma levels of perifosine ($>6\mu\text{g/ml}$) that appears to correlate with clinical benefit (Bailey et al., 2006).

Given the limited efficacy of perifosine monotherapy in a variety of solid malignancies in phase II trials, an alternative strategy would be to combine perifosine with chemotherapy, radiation therapy and targeted agents in an attempt to enhance cytotoxicity and overcome chemotherapeutic resistance through inhibition of the Akt pathway. A phase I trial combining perifosine and radiotherapy in advanced solid tumors demonstrated that perifosine could be safely utilized as a radiation sensitizer, and phase II trials with this strategy are in development (Vink et al., 2006b). In addition, preliminary reports from a number of phase I trials investigating the combination of perifosine with traditional cytotoxic chemotherapeutic agents such as taxanes and gemcitabine indicate that these combinations can be safely administered (Cervera et al., 2006; Ebrahimi et al., 2006; Goggins et al., 2006; Weiss et al., 2006).

Multiple myeloma (MM) is a rational target for perifosine combination therapy based upon in-vitro data in which perifosine induces cytotoxicity in MM cell lines and patient MM cells resistant to conventional therapy (Hideshima et al., 2006). Perifosine also shows antitumor activity in a human plasmacytoma mouse model (Catley et al., 2007). Preliminary results from a phase II study of perifosine alone or in combination with dexamethasone for patients with relapsed or refractory multiple myeloma revealed that single agent perifosine induced stabilization of disease in 6 of 25 evaluable patients. The addition of dexamethasone to perifosine in patients who progressed on perifosine monotherapy conferred a minor response in 3 of 9 evaluable patients and stabilization of disease in 2 of 9 patients (Richardson et al., 2006). Based upon the promising activity of perifosine as a single agent and in combination with dexamethasone, further studies of perifosine in multiple myeloma utilizing different dosing schedules as well as in combination with the proteasome inhibitor bortezomib are planned.

3.1.2. Triciribine (API-2)—In the 1980's and 1990's, a number of phase I and II clinical trials were performed utilizing triciribine as a cytotoxic agent in various advanced malignancies at different dosing schedules (Feun et al., 1993; Feun et al., 1984; Hoffman et al., 1996; Mittelman et al., 1983; O'Connell et al., 1987; Schilcher et al., 1986). Minimal efficacy was observed with few objective responses, and triciribine at high doses caused a number of serious toxicities, including hepatotoxicity, hyperglycemia and hypertriglyceridemia. Whether the hyperglycemia and hypertriglyceridemia were related to inhibition of Akt 2 is unknown. In these trials, pharmacokinetic analysis revealed erratic drug levels of triciribine, particularly with a 5-day continuous infusion dosing schedule. With the recent discovery of triciribine as a *bona fide* Akt inhibitor, phase I clinical trials are currently underway utilizing lower doses of triciribine-phosphate by weekly IV infusions in patients with metastatic solid tumors whose tumors bear high expression of phospho-AKT as well as in patients with myeloid malignancies. In addition, trials combining triciribine-phosphate with tyrosine kinase inhibitors such as erlotinib and lapatinib to overcome primary and secondary resistance mechanisms to ErbB family inhibitors are currently in development.

3.2. mTOR inhibitors: rapamycin, RAD-001, and CCI-779

3.2.1. Clinical trials of mTOR inhibitors as single agents—The mTOR inhibitors, CCI-779 and RAD-001 have been tested as single agents in Phase II trials in a variety of tumor types, and objective responses and stabilization of disease have been noted in breast cancer (Chan et al., 2005b), glioblastoma (Chang et al., 2005; Galanis et al., 2005), neuroendocrine

carcinoma (Duran et al., 2006), renal cell carcinoma (Atkins et al., 2004), mantle cell lymphoma (Witzig et al., 2005), and myeloid malignancies (Yee et al., 2006). A recent phase III trial of CCI-779 in poor risk metastatic renal cell carcinoma randomized patients to CCI-779, interferon-alpha (IFN- α) or a combination of the two. Single agent CCI-779 showed statistically significant improvement in progression free survival (5.5 vs 3.1 months) and overall survival (10.9 vs 7.3 months) as compared to IFN- α , while the combination of CCI-779 and IFN- α showed no statistically significant differences in progression free survival (4.7 months) or overall survival (8.4 months) (Hudes et al., 2007). This trial resulted in regulatory approval of single agent CCI-779 as front-line therapy in advanced renal cell carcinoma.

3.2.2. Clinical trials combining mTOR inhibitors with conventional chemotherapy and radiation—

Although responses have been observed with single agent rapamycin analogues in a variety of tumor types, in most cases activity is modest and of short duration. This may not be unexpected, as pre-clinical data have shown not only that rapamycin and its analogues are predominantly cytostatic *in vitro*, but also that feedback activation of Akt after mTOR inhibition may limit the efficacy of mTOR inhibitors as single agents. Therefore, a number of clinical trials are utilizing mTOR inhibitors in combination with chemotherapy and radiation to overcome resistance mechanisms and enhance response. A phase I trial added rapamycin to concomitant radiation and cisplatin for patients with unresectable stage III non-small cell lung cancer (Sarkaria et al., 2007). Unfortunately, this trial was terminated prematurely due to lack of further funding. Despite not reaching the maximal tolerated dose of rapamycin with combination chemo-radiation, the feasibility of utilizing mTOR inhibitors as radiosensitizing agents was established.

Other trials that combined mTOR inhibitors with conventional cytotoxic chemotherapy have revealed some unexpected toxicities. For example, a phase I trial combining CCI-779 with 5-FU and leucovorin in patients with advanced solid tumors was discontinued due to two treatment-related deaths associated with bowel perforation. Based on the overlapping mucocutaneous toxicities of CCI-779 with 5-FU, the combination of these agents at this schedule was not recommended for further development (Punt et al., 2003). Preliminary results of a phase I trial in advanced cancers with weekly gemcitabine at 600mg/m² (a dose lower than usually administered) and weekly RAD-001 revealed that the combination was not tolerated in a majority of patients due to myelosuppression (Pacey et al., 2004). Pharmacokinetic analysis of these trials did not suggest an interaction between the mTOR inhibitor and the cytotoxic agent. Clearly, based on the unexpected toxicities observed in these trials, investigators should be attentive to potential overlapping toxicities between mTOR inhibitors and conventional chemotherapy.

3.2.3. Clinical trials combining mTOR inhibitors with EGFR antagonists—

Because preclinical studies showed that PI3K/Akt/mTOR inhibitors can augment the efficacy and overcome resistance to EGFR TKIs, phase I and II clinical trials are underway testing the combination of EGFR TKI and mTOR inhibitors. A phase I trial in patients with malignant glioma combining gefitinib with rapamycin revealed that daily administration of these agents is feasible, and that rapamycin does not significantly affect gefitinib drug levels. Out of 34 pretreated patients with refractory disease, 2 attained a partial radiographic response and 13 achieved stable disease (Reardon et al., 2006). Based on these results, a number of phase II trials utilizing various combinations of EGFR TKIs and mTOR inhibitors in malignant glioma are underway. A phase I trial combining gefitinib and RAD-001 in patients with advanced NSCLC patients who had not previously been treated with an EGFR TKI yielded partial responses in 2 out of 8 evaluable patients (Milton et al., 2007). The investigators have initiated a phase II clinical trial to further assess the efficacy of this combination.

mTOR inhibitors are also being studied for their ability to overcome secondary resistance to EGFR TKI therapy in NSCLC. In NSCLC patients who progressed after initially responding to EGFR TKI therapy and were continued on the EGFR TKI with subsequent addition of RAD-001, no objective responses were seen three weeks after the addition of RAD-001 (Riely et al., 2007). In spite of these negative preliminary findings, the addition of mTOR inhibitors to EGFR inhibitors as a means of overcoming mechanisms of secondary resistance is not associated with undue toxicity and could be further investigated in clinical trials.

3.2.4. Clinical trials combining mTOR inhibitors with other targeted therapies—

A number of clinical trials are investigating the combination of mTOR inhibitors with multi-targeted tyrosine kinase inhibitors other than EGFR TKIs, such as imatinib, sunitinib and sorafenib in a variety of malignancies. Preliminary data from a phase I/II clinical trial combining RAD-001 with imatinib in 31 patients with GI stromal tumors refractory to imatinib resulted in stabilization of disease for greater than four months in eight patients. Two patients subsequently achieved partial responses, suggesting that mTOR inhibition may re-sensitize tumors to imatinib (van Oosterom et al., 2005).

Given that mTOR inhibitors have direct anti-angiogenic effects through regulation of HIF-1 α , dual angiogenic inhibition may be a rational approach. Encouraging efficacy data have been reported from a phase I trial, which combined RAD-001 with the anti-VEGF monoclonal antibody bevacizumab in various solid tumors. In a preliminary analysis, the investigators reported partial responses in 2 out of 16 evaluable patients, with an additional 8 out of 16 patients attaining minor responses or stability of disease. The combination appeared well tolerated with minimal overlapping toxicities and no dose limiting toxicities (Zafar et al., 2006).

Based on strong preclinical *in vivo* data, a number of phase II and III randomized, controlled clinical trials are underway to determine the efficacy and safety of aromatase inhibitors and mTOR inhibitors in hormone receptor positive breast cancer. Despite promising preliminary phase II data from a randomized trial of CCI-779 in combination with letrozole in postmenopausal women with hormone receptor metastatic breast cancer (Carpenter et al., 2005), a phase III trial investigating this combination in the same patient population was terminated after an interim analysis determined that the combination yielded no benefit over letrozole alone (Leary and Dowsett, 2006). Despite this negative trial, the combination of mTOR inhibitors with other molecularly targeted agents remains a promising approach to enhance cytotoxicity, overcome resistance and limit toxicity.

3.3. Prediction of response to PI3K/Akt inhibitors and pathway modulation

3.3.1. Phospho-specific antibodies in IHC and immunoblotting—The clinical use of PI3K/Akt/mTOR pathway inhibitors will be optimized by identifying biomarkers to predict response to therapy and to assess target inhibition *in vivo*. Traditional methods of assessing pathway activation include immunohistochemistry (IHC) and immunoblotting using phospho-specific antibodies that recognize pathway components when phosphorylated at specific residues. Phosphorylation at these specific sites is indicative of activation. The advantage of IHC is the ability to localize pathway proteins intracellularly, including the plasma membrane, cytoplasm and nucleus. A potential disadvantage is that IHC is not easy to quantify objectively. Several clinical trials have measured pathway components by IHC before and after drug treatment. For example, in a study of CCI-779 in neuroendocrine carcinomas, paired tumor biopsies were obtained at baseline and two weeks following treatment. The only pre-treatment marker evaluated (including PTEN, p53, phospho-S6, phospho-mTOR, phospho-Akt) that correlated with improved tumor response was an elevated baseline level of phospho-mTOR. After 2 weeks of therapy, CCI-779 effectively decreased levels of phospho-S6, validating that

the drug inhibited its intended target. Increased levels of p-AKT expression and decreased levels of p-mTOR expression after 2 weeks of treatment were associated with a statistically significant delayed time to progression (Duran et al., 2006). In another phase II study with CCI-779 in recurrent glioblastoma multiforme, increased levels of phospho-p70S6 kinase in baseline tumor specimens were shown to correlate with radiographic response (Galanis et al., 2005). From these small trials, measurement of pathway components such as phospho-Akt, phospho-mTOR and its downstream substrates may serve as predictive biomarkers for patients most likely to respond to PI3K/Akt/mTOR inhibitors, either as monotherapy or in combination with other agents. Future trials should make every effort to incorporate analysis of pathway activation and target modulation in pre- and post-treatment tumor tissue.

Depending on the tumor site, this may, however, require several invasive procedures, which are either not feasible or not safe. Therefore, immunoblotting can be used to assess biomarkers in readily accessible surrogate tissues, such as peripheral blood mononuclear cells (PBMCs). Several trials with mTOR inhibitors have incorporated analysis of downstream substrates of mTOR in PBMCs as a correlate for clinical response. Yee *et al.* analyzed PBMCs in patients treated with RAD-001 for relapsed or refractory hematologic malignancies. In 6 out of 9 samples studied, RAD-001 decreased phosphorylation of mTOR substrates, including in 3 patients who demonstrated evidence of a clinical response. Interestingly, treatment with RAD-001 led to inhibition of p-AKT in up to two thirds of specimens analyzed, including in all samples where inhibition of mTOR was noted, suggesting that feedback activation of Akt may not be clinically relevant in hematologic malignancies (Yee et al., 2006). Although less invasive than serial tumor biopsies, it is not known whether PBMCs are a valid surrogate tissue in which to measure target inhibition in non-hematologic malignancies.

3.3.2. Imaging modalities

i) Molecular imaging: Ideally, the least invasive and most sensitive way to measure inhibition of pathway components would be to quantify kinase activity of tumor tissue *in situ*. This has been achieved pre-clinically, where a reporter system was developed to quantitatively measure Akt kinase activity via bioluminescence of an Akt reporter molecule, whereby an increase in luminescence was indicative of inhibition of Akt (Zhang et al., 2007). They showed a dose- and time-dependent inhibition of Akt in several human xenografts following administration of perifosine and API-2 (triciribine) to nude mice. Use of this technology in humans would require stable integration of the reporter construct into tumor cells, which is not currently feasible. However, monitoring Akt activity within live tumor cells provides a dynamic preclinical tool with which to assess target modulation *in vivo*.

ii) FDG-PET: The use of [18F] Fluorodeoxyglucose (FDG)-positron emission tomography (PET) as a non-invasive means to assess early response to signal transduction inhibitors has been established for gastrointestinal stromal tumor patients treated with imatinib (Gayed et al., 2004). Studying FDG-PET as a pharmacodynamic marker of biochemical modulation by PI3K/Akt/mTOR inhibitors is based on evidence that activation of the pathway controls hexokinase activity and glycolysis (Majewski et al., 2004) and that FDG accumulation within tumors depends on hexokinase activity. Xenografts of (VHL null) renal cell carcinoma tumors showed a two-fold increase in FDG-PET uptake relative to parental tumor cells. This FDG-PET uptake was reduced to baseline levels 24 hours after administration of CCI-779 (Thomas et al., 2006). These data raise the possibility that FDG-PET can be used to detect modulation of the mTOR pathway in patients treated with rapamycin analogues. In a prospective assessment of the addition of RAD-001 to gefitinib or erlotinib in NSCLC, Reily *et al.* showed that 3 weeks following the addition of the mTOR inhibitor, 5 of 10 patients had a decrease in Standard Uptake Value (SUV) of >15%, with a median reduction in SUV of 18% (Riely et al., 2007).

These results need to be confirmed in additional clinical trials, where FDG-PET could be further investigated as a surrogate marker for inhibition of the PI3K/Akt/mTOR pathway.

4. Some clinical considerations for the combination of pathway inhibitors with other chemotherapies

There is substantial preclinical evidence that PI3K/Akt/mTOR pathway inhibitors can be effectively combined with chemotherapy, radiotherapy and targeted agents to enhance efficacy and overcome mechanisms of resistance. The early clinical trials suggest that pathway inhibitors may be beneficial when added to other anti-cancer therapies, particularly other targeted therapies such as EGFR TKIs, imatinib, and bevacizumab, although there remains a paucity of phase II and phase III data to corroborate these findings. Two major issues that will determine whether pathway inhibitors can be successfully used in combination with other anticancer agents are toxicity considerations and patient selection, which will be discussed below in more detail.

4.1. Toxicity concerns

The toxicity of pathway inhibitors will vary with the particular drug as well as with the *class* of inhibitor. For example, within the class of Akt inhibitors, lipid based compounds such as perifosine or the PIAs may induce more gastrointestinal toxicity than a nucleoside analogue such as triciribine. Certain toxicities, however, may be class specific to all pathway inhibitors, such as de-regulation of glucose and lipid metabolism, which have been clinically observed with Akt inhibitors such as triciribine (dose-limiting hyperglycemia and hypertriglyceridemia in phase I and II trials) as well as with mTOR inhibitors such as rapamycin and its analogues. Whether a therapeutic index can be achieved with pathway inhibitors is presently unknown, as normal cells also rely on the activation of the PI3K/AKT/mTOR pathway. However, cancer cells may have greater reliance on pathway activation for survival than normal cells because they are selectively exposed to stressors such as hypoxia or aneuploidy, which increase activation of PI3k/Akt/mTOR. Thus, pathway inhibition may result in selective cytotoxicity of cancer cells. It has yet to be determined whether the development of severe hyperglycemia and/or hyper-cholesterolemia would correlate with patient response, in a manner analogous to rash in patients responding to EGFR inhibitors. In support of this possibility in a study of CCI-779 in glioblastoma, development of grade 2 or greater hyperlipidemia was associated with a higher rate of radiographic response (Galanis et al., 2005).

A significant concern with mTOR inhibitors is immune suppression, given that rapamycin is FDA approved for the prevention of allograft rejection and blocks IL-2 induced T cell proliferation. However, there is little evidence in the literature to suggest that the mTOR inhibitors cause significant immune compromise when used as single agents. Hidalgo *et al.*, in a phase I trial of CCI-779 in advanced malignancy, found no changes in lymphocyte cell surface phenotypic markers and lymphocyte subsets. Furthermore, there was no significant change in lymphocyte proliferation assays nor was there clinical evidence of immune compromise (Hidalgo et al., 2006). In a different study, Yee *et al* noted a high frequency of infectious episodes in patients with hematologic malignancies treated with RAD-001, but no opportunistic infections were observed. The investigators noted that this increased frequency could be due to underlying immune-compromised states associated with hematologic malignancies (Yee et al., 2006).

In trials combining mTOR inhibitors with conventional chemotherapy, unexpected toxicities in two trials lead to early discontinuation of the studies (Punt et al., 2003; Pacey et al., 2004). However, overlapping toxicities were not observed in preliminary data from trials combining perifosine with conventional chemotherapy (Cervera et al., 2006; Ebrahimi et al., 2006;

Goggins et al., 2006; Weiss et al., 2006). Nevertheless, combining pathway inhibitors with conventional cytotoxic chemotherapy could result in more toxicity than when combining inhibitors with molecularly targeted agents. If overlapping toxicities with combination agents are a concern, phase I trials should be designed utilizing doses lower than established single agent doses, even if it resulted in slower achievement of biologically effective pathway inhibition *in vivo*.

4.2. Patient selection

In designing clinical trials for pathway inhibitors in combination with other agents, particularly phase II trials, investigators should stratify patients by relative strength of pathway activation, or alternatively exclude patients whose tumors do not demonstrate pathway activation. If the PI3K/Akt/mTOR pathway is not activated in tumor cells, then pathway inhibitors would not be expected to have efficacy, assuming that these agents' clinical activities will not be due to off-target effects. Of the pathway inhibitors discussed in this review, rapamycin is exquisitely specific for mTOR, and has no described off-target effects. One could argue that patients whose tumors did not exhibit mTOR activation would not be expected to benefit from an mTOR inhibitor. Certainly, any changes in design of early phase clinical trials that results in exclusion of patients based on molecular criteria must be accompanied by the development of validated assays that can reliably measure activation of pathway components.

In addition to utilizing activation state specific antibodies in IHC or immunoblotting, other methods for measuring pathway activation are in development. Recently, Saal *et al* developed a gene expression signature for PTEN loss which correlated with adverse outcomes in breast, prostate, and bladder cancer (Saal et al., 2007). Future trials could prospectively assess cancer cell gene expression signatures of key components of the pathway. Comparisons of pathway component gene expression at baseline and after therapy may be a means by which to determine if an inhibitor is altering gene expression of pathway components and to evaluate if a given gene signature is predictive of response to a pathway inhibitor. An alternative strategy to validate target modulation by pathway inhibitors early in their clinical development would be to test these agents in a patient population with uniform activation of the PI3K/Akt/mTOR pathway and accessible tissues. Such populations might include patients with PTEN hamartomatous tumor syndromes (PHTS) such as Cowden Syndrome. These are rare syndromes in which patients possess germline mutations of PTEN, leading to constitutive activation of the PI3K/Akt/mTOR pathway in benign and malignant tumors. Patients with this syndrome are at increased risk for developing certain malignancies, including thyroid, breast and endometrial cancer. Agents that effectively modulate the pathway in tissues such as PBMCs, gastrointestinal hamartomas, and skin trichilemmomas might have promise as anticancer therapeutics. Those agents that demonstrated modulation of the pathway in patients with PHTS could subsequently be tested in the general population of cancer patients whose tumors bear pathway activation.

In conclusion, the proper selection of patients for clinical trials and reliable demonstration of target inhibition *in vivo* will be critical to the development of PI3K/Akt pathway inhibitors as anticancer therapeutics.

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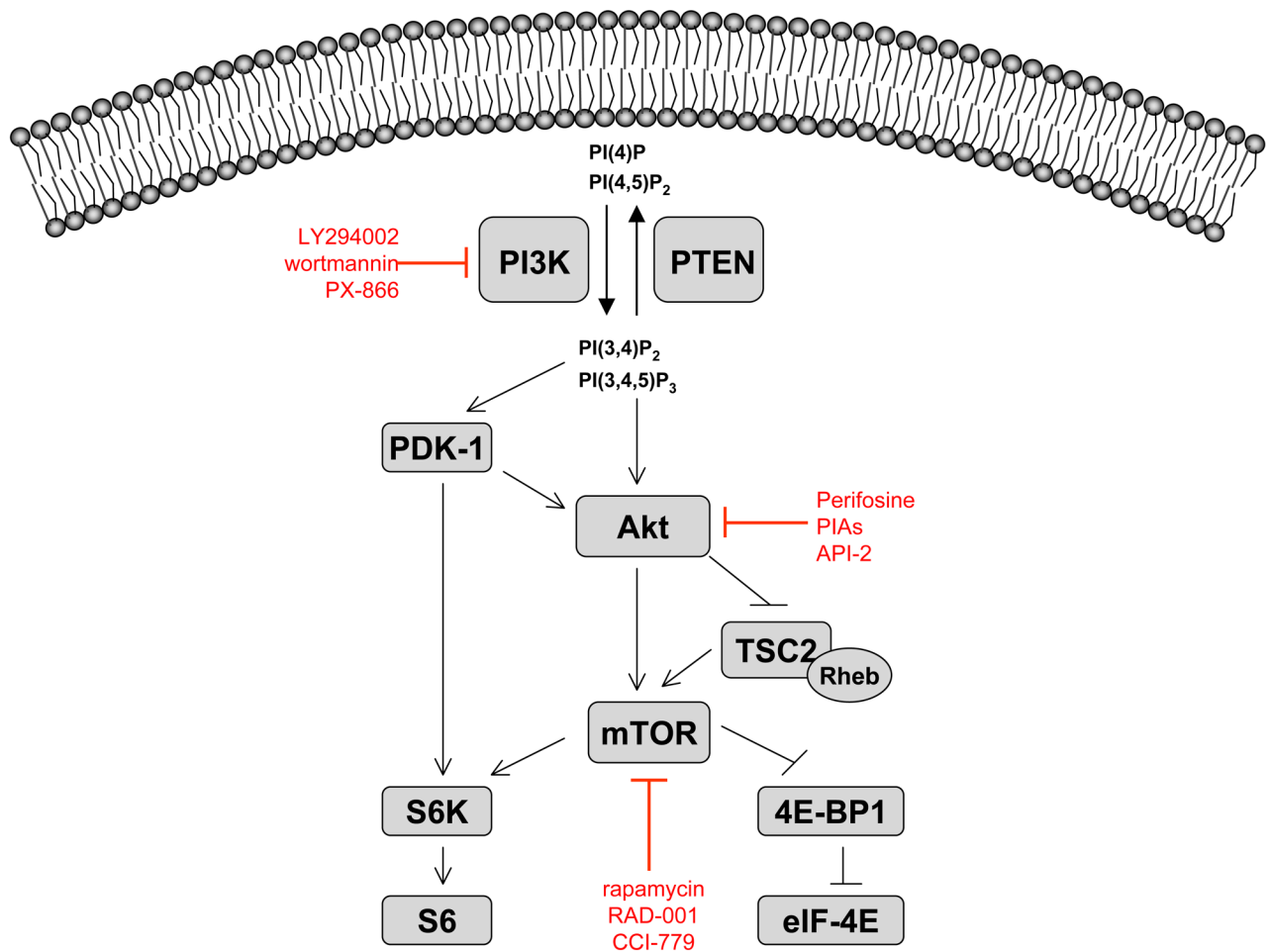


Figure 1. Pharmacological inhibition of the PI3K/Akt/mTOR pathway

Receptor tyrosine kinases (RTK) such as IGF-IR and EGFR, integrins, and G-protein coupled receptors (GPCR) can all stimulate PI3K. PI3K phosphorylates PI(4)P and PI(4,5)P₂ at the 3'-position to generate PI(3,4)P₂ and PI(3,4,5)P₃, respectively. PTEN opposes the function of PI3K by removing 3'-phosphate groups. LY294002 is a reversible small molecule inhibitor of PI3K, while wortmannin and its analogue, PX-866, are irreversible inhibitors. 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. Perifosine and phosphatidylinositol ether lipid analogues (PIAs) are lipid-based Akt inhibitors that interact with the PH domain of Akt and prevent its translocation to the membrane, while API-2, also known as tricirbine, is a small molecule that inhibits the kinase activity of Akt. 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 (TORC1) activates 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 formation of the translation initiation complex. Inhibitors of mTOR include rapamycin and its analogues, RAD-001 and CCI-779, all of which bind to the FK506-binding protein, FKBP-12, which then binds and inhibits mTOR.

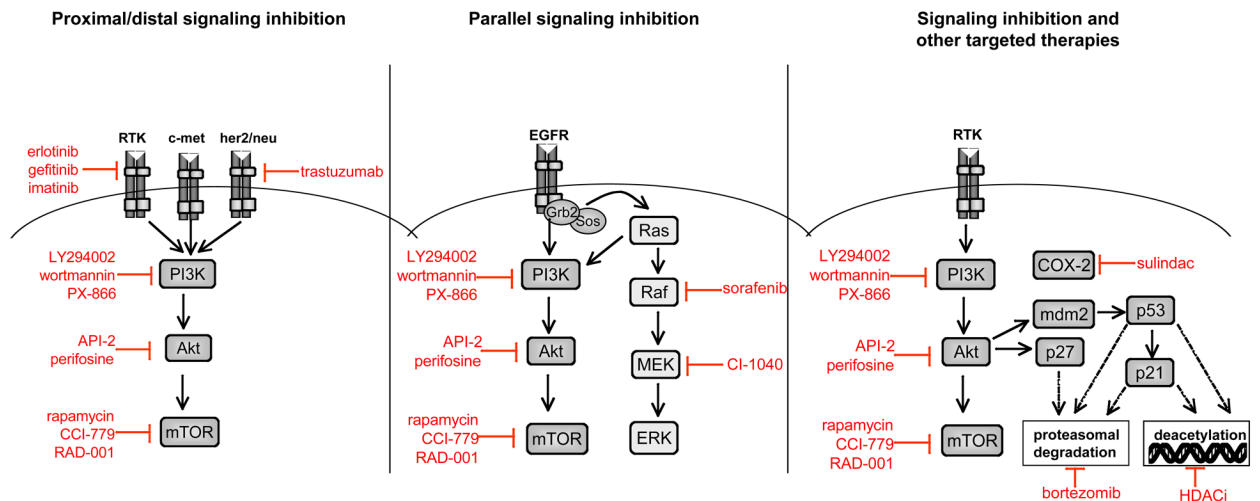


Figure 2. Combinatorial approaches with inhibitors of the PI3K/Akt/mTOR pathway
 Several approaches can be employed when combining pathway inhibitors with other targeted therapies. Inhibition of proximal pathway components, such as receptor tyrosine kinases (RTKs) and oncogenes, combined with distal inhibition of Akt or mTOR may be an effective approach to circumvent feedback activation that could occur with distal inhibition alone (left panel). Alternatively, dual inhibition of parallel signaling pathways prevents compensatory activation of redundant pro-survival pathways (middle panel). Finally, pathway inhibition can be combined with several other types of targeted therapies, including inhibition of histone deacetylase complexes (HDAC), the proteasome and cyclooxygenase-2 (COX-2) (right panel).

Table 1
Clinical trials combining PI3K/Akt/mTOR pathway inhibitors with other anti-cancer agents

Pathway Inhibitor	Combination Agent	Trial phase/ tumor type	Toxicity (CTC grade 3-5) ≥10% of patients	Response Rates	Comments	Reference
Perifosine	Radiation	Phase I Inoperable solid tumors	Nausea/ vomiting (10%), dysphagia (10%)	6/21 (29%) CR 5/21 (24%) PR	Perifosine as radiosensitizer is safe and tolerable.	Vink <i>et al.</i> , 2006
	Radiation/cisplatin	Phase I Stage III NSCLC	Dysphagia (14%) MTD not defined	Not reported	Rapamycin as radiosensitizer is safe and tolerable.	Sarkaria <i>et al.</i> , 2007
Rapamycin	Gefitinib	Phase I Recurrent malignant glioma	Mucositis (68%), diarrhea (42%), fatigue (37%), anemia (32%), leukopenia (32%), infection (26%), hypercholesterolemia (21%), hypertriglyceridemia (21%), thrombocytopenia (21%), AST/ALT (16%), nausea/vomiting (16%)	2/34 (6%) PR 13/34 (38%) SD	Increased grade 3-4 toxicity due to high doses in attempt to cross blood-brain barrier Combination of EGFR TKI and mTOR inhibitor in GBM currently in phase II	Reardon <i>et al.</i> , 2006
	5-FU/leucovorin	Phase I Advanced solid tumors	Asthenia (19%), mucositis (19%), hyperglycemia (15%), diarrhea (15%), anemia (15%), nausea/vomiting (11%)	3/26 (12%) PR 11/26 (42%) SD	Discontinued due to two treatment-related deaths. Combination of agents at this dosing schedule not recommended	Punt <i>et al.</i> , 2003
CCI-779	IFN-alfa	Phase III Advanced RCC	Asthenia (28%), anemia (38%), dyspnea (10%), infection (11%), neutropenia (15%)	8.1% CR/PR 28.1% SD	No difference OS or PFS for combination vs. IFN-a alone Single agent CCI-779 superior OS and PFS to IFN-a and combination Larger sample size (~210 patients per group)	Hudes <i>et al.</i> , 2007
	Gefitinib	Phase I Advanced NSCLC	Hypotension (11%), acidosis (11%), azotemia (11%), lymphopenia (11%), stomatitis (11%)	2/8 (25%) PR	2/2 responders seen in former smokers Currently in phase II trials	Riely <i>et al.</i> , 2007
	Bevacizumab	Phase I Solid tumors	Pain (71%), mucositis (64%), anorexia (57%), bleeding (50%), rash (50%), hyperlipidemia (43%), fatigue (43%)	2/16 (13%) PR 8/16 (50%) SD	Presented only in abstract form Currently in phase II trials	Zafar <i>et al.</i> , 2006
	Imatinib	Phase I/II GI stromal tumors	Fatigue, diarrhea, vomiting, nausea, anemia, edema, headache, and rash	2/31 (6%) PR 8/31 (26%) SD	Presented only in abstract form	Van Oosterom <i>et al.</i> , 2005

CR = complete response, PR = partial response, SD = stable disease, MTD = maximally tolerated dose, OS = overall survival, PFS = progression-free survival, TKI = tyrosine kinase inhibitor