Pharmacodynamic Biomarker Development for PI3K Pathway Therapeutics

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ABSTRACT: The phosphatidylinositol 3-kinase (PI3K) signaling pathway is integral to many essential cell processes, including cell growth, differentiation, proliferation, motility, and metabolism. Somatic mutations and genetic amplifications that result in activation of the pathway are frequently detected in cancer. This has led to the development of rationally designed therapeutics targeting key members of the pathway. Critical to the successful development of these drugs are pharmacodynamic biomarkers that aim to define the degree of target and pathway inhibition. In this review, we discuss the pharmacodynamic biomarkers that have been utilized in early-phase clinical trials of PI3K pathway inhibitors. We focus on the challenges related to development and interpretation of these assays, their optimal integration with pharmacokinetic and predictive biomarkers, and future strategies to ensure successful development of PI3K pathway inhibitors within a personalized medicine paradigm for cancer.

KEYWORDS: PI3K, biomarkers, therapeutics

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Introduction

Phosphatidylinositol 3-kinases (PI3Ks) are lipid kinases that act as intermediate signaling molecules, translating signals from extracellular stimuli into intracellular signals that regulate multiple signal transduction pathways.[1] These pathways regulate many essential cell processes, including cell growth, differentiation, proliferation, motility, and metabolism.[2–5] Somatic mutations that result in the activation of PI3K are frequently detected in cancer. Indeed, reports suggest that deregulation of the PI3K signaling pathway is associated with tumor development in >30% of cancers.[6–9] As a result, a large body of research has focused on developing potent, selective, and efficacious PI3K pathway inhibitors, which are currently at different stages of development.[5,10,11]

With the advent of rationally designed molecular cancer therapeutics, our understanding of drug discovery in cancer has undertaken a paradigm shift.[12] The goal of drug development strategies is to obtain maximal biological effect on the target, which will translate into therapeutic efficacy. Therefore, a significant need has emerged for molecular biomarkers that can accurately assess the underlying mechanisms of action and pharmacodynamic (PD) effects of the drug. These so-called PD biomarkers provide confirmation of the pharmacologic effects of a novel antitumor compound on its intended target, pathway, and downstream biological processes and often assess whether a compound is engaging its molecular target in the expected manner. In clinical trials, PD biomarkers may allow (i) proof of mechanism (ie, evidence that the drug hits its intended target), (ii) proof of concept (evidence that hitting the drug target alters the biology of the tumor), (iii) selection of optimal biological dosing, and (iv) understanding of response/resistance mechanisms. In addition, PD biomarkers, in association with pharmacokinetic (PK) parameters of drug exposure, can be linked to therapeutic effects in what is known as a *pharmacological audit trail*.[12]

Other biomarkers that are also applied in clinical drug development include (i) predictive biomarkers, which can guide the selection of patients likely to respond to a particular therapy, (ii) prognostic biomarkers, which estimate the likely disease course and, hence, the most appropriate management strategy, and (iii) surrogate response biomarkers, which monitor a patient's response to treatment.

Here, we review the biomarkers that have been applied in early-phase clinical trials of PI3K pathway inhibitors. We

focus primarily on PD endpoints that demonstrate target modulation, including invasive molecular assays, circulating biomarkers, and functional imaging technology.

The PI3K/AKT/mTOR Signaling Pathway

The PI3K family consists of three distinct classes (I–III), each with different structures and substrate specificities. Class I PI3Ks are further divided into subclasses IA and IB, and it is the IA subclass that is most frequently activated in cancer.[13,14] Class IA molecules are heterodimers consisting of a p110 catalytic subunit complexed with a p85 regulatory subunit.[15] There are three isoforms of the p110 catalytic subunit (α, β, and δ), encoded by *PIK3CA, PIK3CB,* and *PIK3CD* genes, respectively. In addition, the p85 regulatory subunit consists of five isoforms, encoded by three *PIK3R* genes (Fig. 1).[16,17]

In the absence of an extracellular activating signal, p85 interacts with p110, which results in inhibition of p110 kinase activity. Following receptor tyrosine kinase (RTK) or G-protein-coupled receptor activation, the p85–p110 heterodimer is recruited to the plasma membrane, together with interaction between RTK phosphotyrosine residues and SH2 domains on p85, resulting in release of the basal p85 inhibition of the p110 catalytic subunit and activation of class IA PI3K. Activated PI3K phosphorylates phosphatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3), which then acts as a second messenger leading to the recruitment of AKT and its subsequent phosphorylation by PDK1 and mammalian target of rapamycin complex 2 (mTORC2).[18] AKT stimulates glycolysis by activating glycolytic enzymes and regulating glucose transporters.[19] This mechanism drives tumor cells to avidly consume glucose as a source of ATP.[20] In addition, activated AKT promotes cell growth and survival by a number of mechanisms, including (1) inhibition of proapoptotic proteins of the B-cell leukemia/lymphoma-2 (BCL-2) family; (2) transcription of antiapoptotic genes, *BCL2-Like 11* (*BIM)* and *Fas Ligand* (*FASLG)*, via transcription

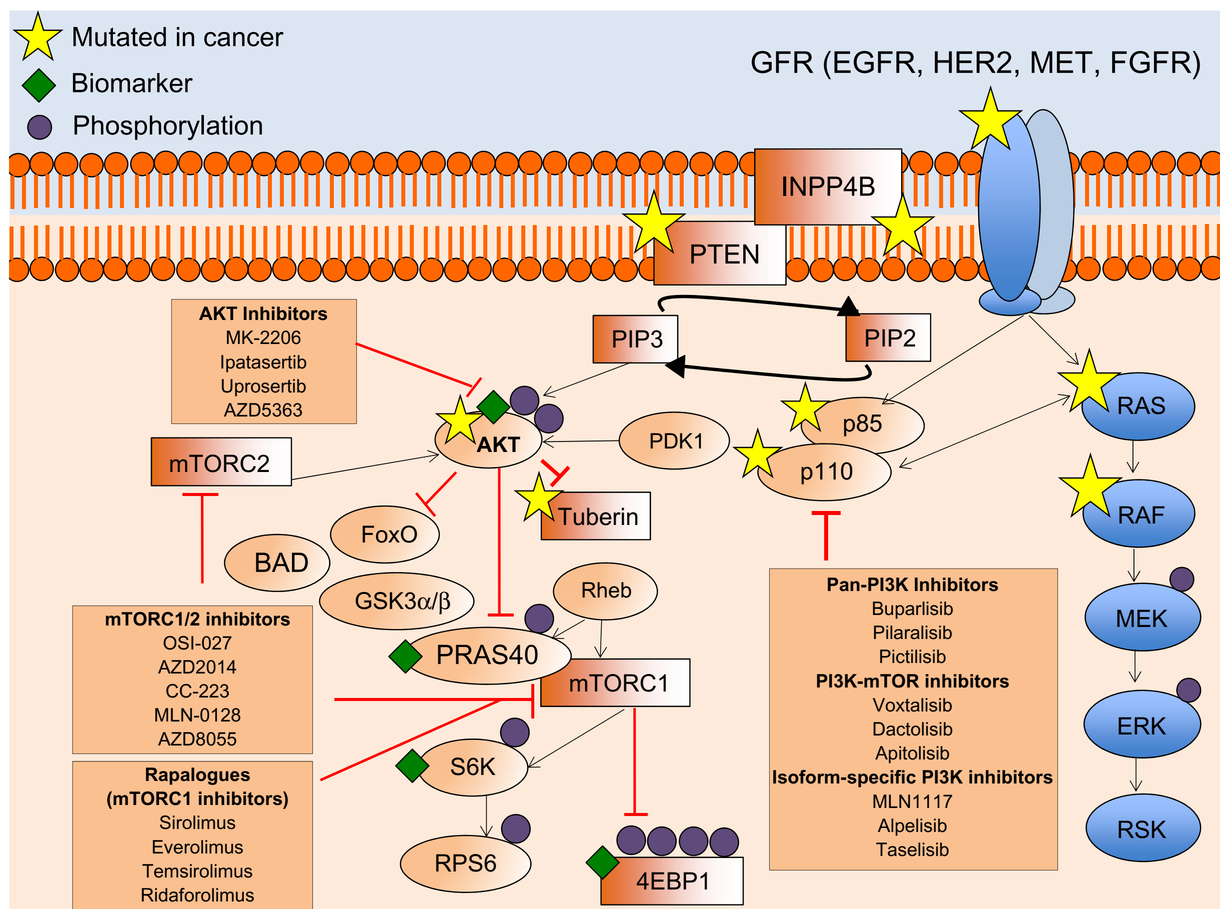

Figure 1. The PI3K/AKT/mTOR signaling pathway and examples of drugs targeting each of its components.

Note: RSK, 90 kDa ribosomal protein S6 kinase. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Clinical Oncology, Rodon et al.[28], copyright 2013.

Abbreviations: 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; BAD, BCL-2 antagonist of cell death; CDKN1, cyclin-dependent kinase inhibitor 1; FASLG, Fas antigen ligand; FoxO, forkhead box O; GFR, growth factor receptor; GSK3, glycogen synthase kinase-3; HIF1, hypoxia-inducible factor 1; INPP4B, type II inositol 3,4-bisphosphate 4-phosphatase; mTORC, mTOR complex; PDK1, 3-phosphoinositide-dependent protein kinase 1; PIP2, phosphatidylinositol (4,5)-biphosphate; PIP3, phosphatidylinositol (3,4,5)-triphosphate; PRAS40, proline-rich AKT1 substrate 1; PTEN, phosphatase and tensin homolog; RPS6, 40S ribosomal protein S6.

factors forkhead box O (FoxO) and nuclear factor-kappaB; (3) enhanced degradation of proapoptotic p53 via increased cytoplasmic availability of mdm2[18,21,22] regulation of the cyclin-dependent kinase inhibitors such as CDKN1A and CDKN1B (also known as p21 and p27) through activation of cyclin D1 and cyclin E1 and transcription factors such as Jun proto-oncogene (JUN) and V-Myc avian myelocytomatosis viral oncogene homolog (MYC).

The mammalian target of rapamycin complex 1 (mTORC1)/S6 kinase (S6K) axis and downstream effectors, such as eukaryote translation initiation factor 4E-binding protein 1 (4E-BP1) and 40S ribosomal protein S6 (RPS6), regulate some of these downstream functions of AKT.[23] mTORC1 is a complex consisting of raptor, mLST8, and proline-rich Akt substrate 40 (PRAS40). mTORC1 is activated by AKT via the inhibition of tuberous sclerosis 1/2 (TSC1/2). AKT phosphorylates TSC2, thus inhibiting TSC1/2; it also phosphorylates PRAS40, thus stimulating mTORC1.

Finally, the tumor suppressor molecules phosphatase and tensin homolog protein (PTEN) and inositol polyphosphate, 4-phosphatase type II, a protein encoded by *INPP4B*, are the most important downregulators of the PI3K pathway.[24,25] PTEN dephosphorylates PIP3 to PIP2, whereas INPP4B dephosphorylates PIP2 to PIP.[21] Since both these products inhibit PI3K-dependent AKT activation,[26] the loss of *PTEN* or *INPP4B* results in activation of AKT.

PI3K/AKT/mTOR Pathway Aberrations in Cancer

The PI3K signaling pathway is frequently deregulated in human cancer.[17,27] The comprehensive review by Rodon et al.[28] provides a full list of mutations and the frequency of each alteration in different tumors. Aberrant activation of PI3K signaling occurs by a number of mechanisms, the most important of which are (i) loss of function of *PTEN* through mutation, microRNA expression, or epigenetic silencing, (ii) mutation or amplification of *PIK3CA*, (iii) amplification or mutation of *AKT* isoforms, or (iv) pathway activation by RTKs and Ras.[1,2]

There are several mechanisms through which decreased PTEN expression can occur, including loss of heterozygosity on chromosome 10q and *PTEN* mutation.[29] Unlike other tumor suppressor genes, such as p53, biallelic inactivation is not required for the suppression of PTEN activity; rather, haploinsufficiency may suffice in promoting tumorigenesis. Alternative mechanisms of somatic loss of *PTEN* activity include homozygous deletion and epigenetic silencing via promoter methylation. Functional PTEN loss has been found in a number of cancers, including endometrial cancer, melanoma, prostate cancer, and glioblastoma, and appears to be important in cancer progression.[29–34]

Mutations or amplifications of *PIK3CA*, encoding the catalytic p110α subunit of class IA PI3K, are common in several human cancers including cervical cancer and squamous lung cancer, providing cells with a growth advantage and promoting tumor progression.[1,16,34,35] Although mutations affecting the p85a regulatory subunit are less frequent, they have been found in up to 10% of human glioblastomas and <5% of colon and ovarian carcinomas.[36,37]

A single amino acid substitution, E17K, in the lipid-binding PH domain of AKT1 has been observed in seven human cancers, including breast, colorectal, lung, and ovarian cancers.[38] In addition, AKT2 overexpression has been identified in colorectal cancers. It is proposed that AKT2 promotes cellular survival and growth and that loss of AKT1 promotes cellular invasion and metastasis, possibly by shifting the balance of signaling through AKT2.[39,40]

In addition to somatic mutations of *PTEN, PIK3CA, PIK3R1*, and *AKT,* some cancers have amplifications of *AKT1*, *AKT2*, and *PIK3CA*; however, it is not clear if these amplifications have a significant impact on clinical outcome.

Inhibitors of the PI3K/AKT/mTOR Signaling Pathway

Numerous compounds have been developed to inhibit different nodes in the PI3K/AKT/mTOR signaling pathway. These include PI3K inhibitors (subdivided into pan-PI3K inhibitors, isoform-selective PI3K inhibitors, and dual PI3K/mTOR inhibitors), mTOR inhibitors (divided into allosteric inhibitors [rapalogues] and ATP-competitive inhibitors), and AKT inhibitors (including allosteric inhibitors and ATP-competitive AKT inhibitors). A description of each of these classes of inhibitor and a discussion of some of the PD biomarkers used in THEIR evaluation IN phase I trials are explained later. Table 1 provides a comprehensive summary of PD biomarker evaluations reported in phase I studies of all PI3K/AKT/mTOR signaling pathway inhibitors in current development.

Inhibitors of the PI3K/AKT/mTOR signaling pathway have similar toxicity profiles, which include rash, hyperglycemia, gastrointestinal disturbances (eg, nausea, vomiting, diarrhea, anorexia, and dysgeusia), alopecia, mucositis, fatigue, thromboembolism, cytopenias, and liver enzyme elevations.

PI3K inhibitors. *Pan-class I PI3K inhibitors.* The earliest agents to enter clinical development were the pan-class I inhibitors of PI3K, which directly inhibit p110 kinase activity by acting as ATP mimetics, binding competitively and reversibly to the p110 ATP-binding pocket. Of the pan-class I PI3K inhibitors in clinical development, BKM120 (buparlisib) is the furthest developed and is being extensively evaluated in hormone-positive breast cancer, often in combination with endocrine therapy.[41,42] Buparlisib has potent, pan-class I PI3K inhibitory PROPERTIES against p110-α, -β, -δ, and -γ enzymes at IC_{50} of 52, 166, 116, and 262 nM, respectively.[43] Other pan-class I PI3K inhibitors in clinical development include XL147 (IC_{50} of 39, 36, 23, and 383 nM against p110-α, -β, -δ, and -γ, respectively), GDC-0941 (pictilisib; IC_{50} of 3 nM against p110-α and -δ enzymes), BAY80–6946 (copanlisib; IC_{50} of 0.469 nM against p110-α and 3.72 nM against p110-β), PX-866 (sonolisib; IC_{50} of 0.1–88 nM), and CH5132799 (IC_{50} of 14 nM against p110-α).[44–49]

Table 1. PD biomarker evaluations reported in phase I studies of all PI3K/AKT/mTOR signaling pathway inhibitors in current development.

AGENT (COMPANY) *NAME*	PD BIOMARKERS EVALUATED IN SURROGATE TISSUE	PD BIOMARKERS EVALUATED IN TUMOUR TISSUE	IMAGING PD BIOMARKERS	SERUM PD BIOMARKERS	REFERENCE
Pan PI3K Inhibitors					
BKM120 (Novartis) *Buparlisib*	Decreased levels of pRPS6 in skin in >40% patients	Decreased levels of pRPS6, pAKT, p4EBP1 and Ki-67 (in selected cases)	FDG-PET (9/19 patients had a metabolic PR)	Increased levels of serum C-peptide and fasting blood glucose (dose-dependent)	Bendell et al, 2012[41]
	Decreased levels of pS6 in skin	Decreased levels of pS6, pAKT, p4EBP1 and Ki-67 (in selected cases)	FDG-PET (21/52 patients had a metabolic PR)	Increased levels of serum C-peptide (dose-dependent) Increased levels of serum M30 and M65 from baseline to cycle 3 (high intra-patient variability) Decreased levels of serum BFGF, PLGF, SVEGFR1, SVEGFR2 and VEGF from baseline to cycle 1 D28 (high intra-patient variability)	Rodon et al, 2014[42]
XL147 (Exelixis) *Pilaralisib*	Decreased levels of PI3K proximal biomarkers ($pAKT^{S473}$, $pPRAS40^{T246}$ and $pAKT^{T308}$) and downstream biomarkers ($p4EBP1^{T70}$ and $pS6^{S240/S244}$) in skin and hair sheath cells (in selected cases)	Decreased levels of $pAKT^{T308}$, $p4EBP1^{T70}$, pERK and ki67 (in selected cases)	Not reported	Increased fasting and food-induced plasma insulin levels	Shapiro et al, 2014[48]
GDC-0941 (Genentech/ Piramed Pharma) *Pictilisib*	Decreased levels (dose- and concentration-dependent) of $pAKT^{S473}$ in PRP	Decreased levels of $pS6^{235/236}$ and $pAKT^{S473}$ (in selected cases)	FDG-PET (7/32 patients had a metabolic PR)	Increased plasma insulin and glucose levels	Sarker et al, 2015[44]
BAY80–6946 (Bayer) *Copanlisib*	Not reported	Not reported	FDG-PET (selected cases)	Increased post-infusion fasting insulin levels	Patnaik et al, 2011[46]
PX-866 (ProlX Pharmaceuticals) *Sonolisib*	Decreased levels of RPS6 ribosomal protein and mTOR phosphorylation in PBMCs	Not reported	Not reported	No change in glucose or insulin levels	Jimeno et al, 2010[47]
CH5132799 (Chugai Pharma Europe)	Decreased levels of pAKT in PRP	Decreased levels of pAKT (selected cases)	FDG-PET (17/23 patients had a decrease in FDG avidity)	Not reported	Blagden et al, 2014[45]
GDC-0032 (Genentech) *Taselisib*	Not reported	Inhibition of the PI3K pathway as assessed by reverse phase protein array	FDG-PET (7/13 patients had a metabolic PR)	Not reported	Juric et al, 2013[49]
Isoform-specific PI3K Inhibitors					
MLN1117; p110α (Intellikine/Millenium)	Decreased levels of p4EBP1 and pS6 in skin	Not reported	Not reported	Not reported	Juric et al, 2015[59]
CAL-101; p110δ (Gilead Sciences) *Idelalisib*	Not reported	Not reported	Not reported	Decreased plasma concentrations of chemokines CCL22 and CCL17	Kahl et al, 2010[60]
	Not reported	Decreased levels of $pAKT^{T308}$ in CLL cells	Not reported	Normalized plasma concentrations of CCL3, CCL4, and CXCL13.	Coutre et al, 2011[61]

AZD8186; p110β (Astra-Zeneca)	Not yet reported	Not yet reported	Not yet reported	Not yet reported	Siu et al, 2015[53]
GSK2636771; p110β (GlaxoSmithKline)	Decreased levels pAKT in surrogate tissue	Not reported	Not reported	Not reported	Arkenau et al, 2014[54]
SAR260301; p110β (Sanofi)	Maximal inhibition of pAKT/tAKT (total AKT) in platelets correlated with exposure at steady state	Not reported	Not reported	Not reported	Bedard et al, 2015[55]
IPI-145; p110γ + δ (Infinity) *Duvelisib*	Not reported	Not reported	Not reported	Reductions in serum cytokines and chemokines known to support the malignant B-cell microenvironment	Flinn et al, 2014[56]
AMG319; p110δ (Amgen)	Not reported	Not reported	Not reported	Not reported	Lanasa et al, 2013[57]
PI3K-mTOR Inhibitors					
XL765 (Sanofi) *Voxtalisib*	Time-dependent inhibition of $p4EBP1^{T70}$ $pAKT^{S473}$, $pPRAS40^{T246}$, and $pS6S^{240/244}$ in hair sheath cells, and inhibition of $pAKT^{T308}$, $pAKT^{S473}$, $pPRAS40^{T246}$, $p4EBP1^{T70}$, and $pS6^{S240/244}$ and Ki67 in skin biopsies	Decreased levels of $pAKT^{T308}$, p4EBP1, and pERK $pAKT^{S473}$ and ki67 in selected cases	Not reported	Increased plasma insulin levels	Papadopoulos et al, 2014[63]
BEZ235 (Novartis) *Dactolisib*	Decreased levels of pRPS6 in skin and sVEGFR2 (dose-dependent)	Decreased levels of pRPS6 (in selected cases)	FDG-PET (8/37 patients had a metabolic PR with QD dosing and 4/9 with BID dosing)	Dose-dependent elevations of plasma C-peptide	Burris et al, 2010[66]
GDC-0980 (Genentech) *Apitolisib*	Decreased levels of pAKT in PRP	Not reported	FDG-PET (5/6 patients had a metabolic PR)	Not reported	Wagner et al, 2011[65]
BGT226 (Novartis)	Reductions in $pS6S^{240/244}$ and $pAKT^{S473}$ levels in skin	Reductions in $pS6S^{240/244}$ and $pAKT^{S473}$ levels (in selected cases)	Not reported	Increased levels of serum M30 and M65 (markers of apoptosis/cell death)	Markman et al, 2012[126]
PKI-587 (Pfizer) *Gedatolisib*	Not reported	Reductions in $pAKT^{S47}$	Not reported	Dose-dependent increase in blood glucose levels, C-peptide and insulin levels Increases in cholesterol and triglycerides levels	Shapiro et al, 2014[48]
PF04691502 (Pfizer)	Decreased levels of $pAKT^{S473}$, $pPRAS40^{T246}$, and $pSTAT3^{Y705}$ in hair follicles, followed by rebound in signaling	Reductions in $pAKT^{S473}$, $pAKT^{T308}$, $pFKHR^{T24}$/ $FKHRL1^{T32}$ and $pSTAT3^{Y705}$	Not reported	Increased fasting glucose, insulin and C-peptide levels	Britten et al, 2014[64]
Allosteric mTOR Inhibitors (Rapalogues)					
RAD001 (Novartis) *Everolimus*	Decreased levels of $pAKT^{S473}$, $p4EBP1^{T70}$, $peIF\text{-}4G^{S1108}$, $pS6^{S235/236}$, $pS6^{S240/244}$, and proliferation marker Ki-67 in skin.	Decreased levels of $pAKT^{S473}$, $p4EBP1^{T70}$, $peIF\text{-}4G^{S1108}$, $pS6^{S235/236}$, $pS6^{S240/244}$, and proliferation marker Ki-67 in tumour.	Not reported	Not reported	Tabernero et al, 2008[80]

(*Continued*)

Table 1. (*Continued*)

AGENT (COMPANY) *NAME*	PD BIOMARKERS EVALUATED IN SURROGATE TISSUE	PD BIOMARKERS EVALUATED IN TUMOUR TISSUE	IMAGING PD BIOMARKERS	SERUM PD BIOMARKERS	REFERENCE
MK-8669 (Merck and ARIAD Pharmaceuticals) *Ridaforolimus*	Decreased levels of p4E-BP1 Ser65/Thr70 in PBMCs	Not reported	Not reported	Not reported	Mita et al, 2008[76]
ATP-competitive mTOR Inhibitors					
OSI-027 (OSI Pharmaceuticals)	Decreased levels of p4EBP1$^{T37/T46}$ in PBMCs	Not reported	Not reported	Not reported	Tan et al, 2010[84]
AZD2014 (Astra Zeneca)	Decreased levels of pAKT^{S473} in PRP, p4EBP1$^{T37/T46}$ in PBMCs	Decreased levels of pRPS6$^{S235/236}$, pAKT^{S473}, and Ki-67 (selected cases)	FDG-PET (3/11 patients demonstrated partial metabolic response)	Not reported	Basu et al, 2015[82]
CC-223 (Celgene)	Inhibition of pRPS6 (B cells), p4EBP1 (T cells) and pAKT (monocytes)	Not reported	Not reported	Not reported	Shih et al, 2012[85]
MLN-0128/INK-128 (Intellikine)	Decreased levels of p4EBP1 in PMBCs, and decreased levels of p4EBP1, pRPS6 and pPRAS40 in skin	Not reported	Not reported	Not reported	Infante et al, 2012[86]
AZD8055 (Astra Zeneca)	Decreased levels of p4EBP1 and pAKT in PMBCs	No conclusive evidence of biomarker modulation for pAKT, p4E-BP1 and pS6.	FDG-PET (8/26 patients demonstrated partial metabolic response)	Not reported	Naing et al, 2012[83]
AKT Inhibitors					
MK-2206 (Merck)	Sustained decreased levels of pPRAS40^{t246}/total PRAS40 ratio in hair follicles	Decreased levels of pAKT^{S473}, in 9 patients	Not reported	Not reported	Yap et al, 2011[71]
GDC-0068 (Array BioPharma) / *patasertib*	Decreased levels of pGSK3β in PRP (dose-dependent) of >70% compared with baseline	Decreased levels of pGSK3β and increased levels pAKT. Decreased levels of pPRAS40 and cyclin D1 (dose-dependent)	Not reported	Not reported	Yan et al, 2011[72]
GSK-795 (GlaxoSmithKline) *Uprosertib*	Not reported	Decreased levels of pPRAS40, Increased levels of pAKT (in selected patients)	FDG-PET (7/8 patients had a metabolic PR)	Increased post-prandial blood glucose levels, or a delay in the return to baseline of post-prandial glucose levels	Burris et al, 2011[73]
AZD5363 (Astra Zeneca)	Not reported	Increased levels of pAKT and reduced levels of pGSK3β and pPRAS40	Not reported	Not reported	Banerji et al, 2015[74]

Abbreviations: PRP, platelet-rich plasma; PBMC, peripheral blood mononuclear cell; p, phosphorylated.

In the phase I dose-escalation and expansion study of BKM120 in patients with advanced solid tumors, there was clear demonstration of inhibition of phosphorylation of AKT, 4E-BP1, and S6 in posttreatment tumor biopsies, confirming that BKM120 was capable of inhibiting the PI3K pathway in tumor tissue. Furthermore, BKM120 induced partial metabolic responses by fluorodeoxyglucose positron emission tomography (^{18}F-FDG PET) in 21 of 54 evaluable patients after 28 days of treatment, demonstrating the capability of ^{18}F-FDG PET as a PD biomarker; however, no association with progression-free survival, best response as per Response Evaluation Criteria in Solid Tumors (RECIST), or best percent change in CT scan was identified.[42] Dose-dependent inhibition of phosphorylation of S6 in the skin with single-agent BKM120 has been reported.[41,42,50] Indeed, the greatest reduction in phosphorylated S6 was associated with the best clinical response, highlighting phosphorylated S6 levels in the skin as a potentially useful surrogate biomarker of response to PI3K inhibition.[50]

In the phase I study of pictilisib, in addition to the decreases in AKT phosphorylation in platelet-rich plasma (PRP) and tumor, and the reduction in S6 phosphorylation observed in tumor (described earlier) following pictilisib treatment, other PD biomarkers also demonstrated evidence of target modulation.[44] Following treatment, there was a statistically significant increase in plasma insulin and glucose levels at one hour from baseline, and ^{18}F-FDG PET imaging showed a reduction from baseline of ^{18}F-FDG-tracer uptake at dose levels ≥45 mg.[44]

Isoform-selective PI3K inhibitors. Novel PI3K inhibitors that are isoform specific have generated much enthusiasm owing to the hypothesized advantage that they may have fewer toxicities when compared with the pan-class I inhibitors, allowing these agents to be tolerated at doses that may result in more complete inhibition of kinase activity. In addition, data suggest that the pro-tumor effects of different genetic alterations of the PI3K pathway may signal preferentially through specific isoforms of p110. For example, HER2-amplified breast carcinoma may depend primarily on p110α,[51] and the effects of *PTEN* loss largely depend on p110β in models of prostatic intraepithelial neoplasia.[52] Therefore, there is interest in the activity of PI3Kα inhibitors (such as BYL719 [alpelisib] and MLN1117) in cancers with *PIK3CA* mutations and PI3Kβ inhibitors (such as AZD8186, GSK2636771, and SAR260301[53–57]) in tumors with *PTEN* loss. Furthermore, p110δ is thought to be a dominant isoform in the lymphocytic lineage; indeed, PI3Kδ inhibitors (CAL-101 [Idelalisib] and AMG319) have shown promise in patients with chronic lymphocytic leukemia.[57] A randomized double-blind placebo-controlled phase III study of CAL-101 (idelalisib) in combination with rituximab in patients with relapsed CLL demonstrated that the combination of rituximab and idelalisib led to greater disease-free survival, treatment response rate, and survival compared to rituximab plus placebo.[58]

In the first-in-human dose-escalation study of MLN117, doses of 200–900 mg suppressed phosphorylation of 4E-BP1 and S6 in the skin by up to ~100% and 70%–90%, respectively, at ~3 hours post single dose.[59] However, evaluation of target inhibition in the tumor itself has not yet been reported for this drug.

'For PI3Kδ inhibitors in hematological malignancies, reductions in plasma chemokine concentrations have been reported as important PD biomarkers.[60,61] For example, plasma concentrations of CLL-derived chemokines such as CCL3, CCL4, CCL22, and CCL17, which were elevated at baseline, demonstrated significant decreases within one cycle of CAL-101 (idelalisib) treatment.[62]

Dual PI3K/mTOR inhibitors. The development of dual PI3K/mTOR inhibitors was based on the known structural similarities between the ATP-binding domain of p110 and the catalytic domain of mTOR. Unlike rapalogue mTOR inhibitors, these agents are active site inhibitors of mTOR and have the advantage of inhibiting the kinase activity of mTOR regardless of whether it is in complex with TORC1 or TORC2. Since mTOR responds to a variety of signals besides PI3K/AKT, these dual inhibitors are thought to have a broader activity in cancers in which PI3K/AKT is not the primary driver of mTOR activity. Finally, unlike the rapalogues, these agents might be able to inhibit TORC1 activity while preventing feedback activation of PI3K. Several dual PI3K/mTOR inhibitors, such as XL765 (voxtalisib; IC_{50} of 157, 39, 113, 9, and 43 nM for mTOR, p110-α, -β, -γ, and -δ, respectively), GDC-0980 (apitolisib; IC_{50} of 17, 5, 27, 7, and 4 nM for mTOR, p110-α, -β, -γ, and -δ, respectively), BEZ235 (dactolisib; IC_{50} of 6, 4, 5, 7, and 75 nM for mTOR, p110-α, -β, -γ, and -δ, respectively), and PF04691502 (IC_{50} of 16, 1.8, 2.1, 1.6, and 1.9 nM for mTOR, p110-α, -β, -γ, and -δ, respectively), are now in clinical development.[63–66] However, although some objective tumor responses have been observed, the activity of these agents thus far has been modest, and no clinical trial with either a pan-class I PI3K inhibitor or dual PI3K/mTOR inhibitor has reported robust clinical activity, even in tumors with known genetic alterations.[67] Possible reasons for this include (1) uncertainty as to the extent of downstream phosphoprotein biomarker modulation required to result in efficacy in patients; (2) insufficient target inhibition; (3) inappropriate biomarker selection; or (4) inappropriate patient selection. Furthermore, it is conceivable that continuous inhibition of PI3K/mTOR may result in an adaptation favoring cell growth.[64] For certain conditions harboring driver mutations, such as BCR-ABL in chronic myeloid leukemia, the continued inhibition of kinase activity provided by higher trough concentrations of targeted therapy results in more favorable clinical outcomes.[68,69] However, in other malignancies, models of drug resistance are challenging the requirement for continuous inhibition.[70] For dual PI3K/mTOR inhibitors, further studies are required to determine whether selective pressures are in operation.

The phase I study of PF04691502 exemplified the use of diverse PD biomarkers in a single study.[64] The analyses were performed on paired tumor biopsies, paired hair follicle samples, and blood. Posttreatment tumor biopsies and hair follicle samples demonstrated reductions in phosphorylation of AKT^{S473}, AKT^{T308}, $FKHR^{T24}/FKHRL1^{T32}$, and $STAT3^{Y705}$or AKT^{S473}, $PRAS40^{T246}$, and $STAT3^{Y705}$ on day 21 of treatment cycle 1. In addition, increases in blood glucose, c-peptide, and insulin levels, which generally occurred by day 8 of the first treatment cycle, were also consistent with PI3K/mTOR pathway inhibition.[64]

AKT inhibitors. AKT inhibitors in clinical development are of two main classes: allosteric inhibitors such as MK2206 and ATP-competitive AKT inhibitors such as GDC-0068, GSK795, and AZD5363.[71–74] Thus far, these agents are all pan-isoform inhibitors of AKT. In early-phase clinical trials, the clinical activity of these agents has been modest thus far, and questions regarding appropriate patient selection remain. For example, it was suggested that certain *PIK3CA* mutations result in relatively low activation of AKT in comparison to *PTEN* loss.[75] Thus, these agents may be more appropriately directed to those cancers with AKT alterations and *PTEN* loss.

The first-in-man phase I trial of MK2206 incorporated detailed PK-PD studies in both normal and tumor tissues to confirm adequate drug exposure with concomitant target and pathway blockade.[71] Average steady-state trough MK2206 concentrations at the recommended phase II dose (RP2D) were greater than the concentrations required for 70% inhibition of AKT^{S473} phosphorylation in whole blood, a level identified in preclinical models as associated with antitumor activity. PD analyses demonstrated AKT signaling blockade in both tumor and surrogate tissues. Studies assessing phosphorylation of AKT^{S473} in tumor samples provided evidence of target blockade at the RP2D. In addition, studies in hair follicles also indicated that the phosphorylated Thr246 signal on PRAS40 was effectively blocked at this dose level.[71]

mTOR inhibitors. *Allosteric inhibitors (rapalogues).* Allosteric inhibitors describe rapamycin (sirolimus) and its analogs, temsirolimus, everolimus, and ridaforolimus (formerly known as deforolimus).[76] They inhibit the mTORC1 kinase by binding to an abundant intracellular protein, FKBP12, forming a complex that inhibits mTOR signaling. Rapalogues initially demonstrated efficacy as a single agent for the treatment of renal cell carcinoma and progressive advanced pancreatic neuroendocrine tumors.[77,78] Other indications with significant clinical activities include mantle cell lymphoma, sarcoma, and ER-positive breast cancer in combination with hormone therapy.[79]

A phase I study of everolimus used the PD effects on mTOR-dependent pathways (4E-BP1 pathway: phosphorylated 4E-BP1 and eIF4G; S6K1 pathway: phosphorylated S6) in paired pre- and on-therapy tumor and skin biopsies, in addition to the safety profile of the drug, in order to determine the optimal dose and schedule of everolimus.[80] Tabernero et al demonstrated that daily dosing resulted in near complete inhibition of phosphorylation of S6 at both dose levels tested, while inhibition of phosphorylation of eIF4G and 4E-BP1 was more profound at the 10 mg dose level when compared with the 5 mg dose level. In the weekly schedule, complete inhibition of S6 phosphorylation was again seen at all the studied dose levels. However, complete and prolonged inhibition of phosphorylation of eIF4G was only observed at doses ≥50 mg. Based on these data, the authors recommended everolimus treatment at either 10 mg/day or 50 mg/week.

ATP-competitive inhibitors. mTOR catalytic site inhibitors directly target the kinase domain of mTOR and, therefore, impede the activity of both mTORC1 and mTORC2 kinases. The theory behind dual mTORC1/2 inhibition is that it may prevent compensatory feedback activation of AKT upon mTORC1 inhibition as occurs with rapalogues.[81] mTORC1/2 inhibitors in clinical development include OSI-027, AZD2014, CC-223, MLN-0128/INK128, and AZD8055.[82–86]

In the phase I study of AZD2014, the PD profile demonstrated target engagement in both surrogate and tumor tissues.[82] Proof-of-mechanism biomarkers of mTORC1 and mTORC2 inhibition, such as A 40%–45% reduction in levels of phosphorylated 4E-BP1 in peripheral blood mononuclear cells (PBMCs) and A 37%–62% reduction in phosphorylated AKT in PRP, respectively, were seen at 2 and 8 hours but recovered at 24 hours following a single dose of AZD2014. Therefore, this, together with the PK profile demonstrating an elimination half-life of approximately three hours supported a twice-a-day schedule. Importantly, at the maximum tolerated dose (MTD), the authors also demonstrated reduced levels of phosphorylated S6 (~20%–100%) in all evaluable posttreatment biopsies and reduction of phosphorylated AKT levels (20%–50%) in 3/4 assessable posttreatment biopsies.[82] In addition to these proof-of-mechanism PD biomarkers, proof-of-concept biomarkers such as reduction of proliferation (Ki67) and reduction in metabolism (^{18}F-FDG PET) also supported evidence of target inhibition in tumor.[82] At the MTD, five of nine patients showed reduction in Ki67 expression of 50%–100%. In addition, 8 of 11 patients showed a reduction of change in maximum standardized uptake value (SUV_{max}), with three patients attaining a partial response (30% reduction in SUV_{max}).[82]

In the phase I trial of OSI-027, an oral dual mTORC1/2 inhibitor, there was inhibition of phosphorylation of $4E\text{-}BP1^{T37/T46}$ by >60% in PBMCs, but no evidence of objective responses.[84] In contrast, the phase I study of the dual mTORC1/mTORC2 inhibitor, AZD8055, used phosphorylation of AKT in PBMCs as a biomarker of mTORC1 and mTORC2 inhibition. Unfortunately, the assay in PBMCs was rendered challenging due to very low levels of AKT phosphorylation at baseline; therefore, no conclusions could be drawn on mTORC2 inhibition in PBMCs in this study.[83]

PI3K/AKT/mTOR Pathway Biomarkers

Clinical trials of PI3K/AKT/mTOR pathway inhibitors have utilized several biomarker strategies, including PD biomarkers of signaling output, eg, inhibition of downstream phosphorylated proteins, indirect PD biomarkers of metabolic effect, eg, glucose metabolism markers, and functional imaging monitoring biomarkers, eg, ^{18}F-FDG PET (Table 2). In addition, many trials are now using predictive biomarkers, eg, *PIK3CA* or *AKT* mutations, and *PTEN* loss, for the purpose of identifying subpopulations of patients who are most likely to respond to PI3K/AKT/mTOR pathway inhibitors.

Biomarkers of PI3K pathway signaling. During early-phase clinical trials of novel PI3K/AKT/mTOR pathway inhibitors, the degree and duration of PI3K pathway inhibition

Table 2. PI3K/AKT/mTOR pathway biomarkers and their potential disadvantages.

BIOMARKER	USE	POTENTIAL DISADVANTAGES
Biomarkers of PI3K pathway signaling		
Phosphorylation of AKT at the residues Thr308 and Ser473;	Demonstrates target modulation	Imperfect at predicting efficacy in patients since only modest response rates are observed despite achievement of predicted tumour target inhibition of phosphorylation
Phosphorylation of the AKT substrate PRAS40 at Thr246	Demonstrates target modulation	
Phosphorylation of 4EBP1 at Ser65 and Thr70	Demonstrates target modulation	
Phosphorylation of RPS6 at Ser240 and Ser244	Demonstrates target modulation	
Biomarkers of metabolic effect		
Fasting plasma glucose levels	Indirect determination of pathway modulation	Insulin and C-peptide may be superior to fasting glucose levels, because increased insulin/C-peptide release can effectively compensate for decreased glucose transport and metabolism due to PI3K inhibition at lower doses
Plasma insulin levels	Indirect determination of pathway modulation	
Plasma C-peptide levels	Indirect determination of pathway modulation	
Functional imaging biomarkers		
^{18}F-FDG PET	(i) Indirect determination of pathway modulation (ii) Surrogate marker of response	Uncertain role as a predictive biomarker due to lack of association between ^{18}F-FDG-PET changes and tumour response evaluated by standard cross-sectional imaging (eg, CT).
^{18}F-FLT PET	(i) Indirect determination of anti-proliferative effects (ii) Surrogate marker of response	Not yet been utilized in a trial of a PI3K pathway inhibitor
Magnetic resonance spectroscopy	Surrogate marker of response	Not yet been utilized in a trial of a PI3K pathway inhibitor
Diffusion-Weighted- and Dynamic Contrast Enhanced-MRI	Surrogate marker of response	Not yet been utilized in a trial of a PI3K pathway inhibitor
Circulating biomarkers		
Circulating tumour cells	(i) Surrogate marker of response (ii) Molecular characterisation for 'real time' demonstration of target modulation	Cells in the blood will be exposed to plasma drug concentrations, which may or may not be the same as drug levels achieved in solid tumours.
Cell-free DNA/Circulating tumour DNA	(i) Surrogate marker of early response (ii) Predict sensitivity to PI3K/AKT/mTOR pathway inhibitors	Low plasma DNA levels may mean mutations are missed, and clonal evolution of detected mutations
Circulating markers of cell death and angiogenesis	Surrogate marker of cell death and angiogenesis	Intrapatient variability is high
Predictive biomarkers		
Mutations in *PIK3CA*	Predict sensitivity to PI3K/AKT/mTOR pathway inhibitors	(i) The complexities of the pathway and its feedback loops mean that clear prediction of response to genotype is difficult. (ii) Use of incorrectly standardized or unvalidated assays may mean that driver mutations are missed (iii) Coexistence of mutations related to resistance (iv) Presence of intratumour heterogeneity
Loss of PTEN expression	Predict sensitivity to PI3K/AKT/mTOR pathway inhibitors	

has been established by measuring PD biomarkers primarily through evaluating the degree of inhibition of phosphorylation in downstream proteins. Since quantification of a biomarker in close proximity to PI3K (eg, PIP3) has not been feasible in the clinical setting, and measurement of AKT in tissues requires stringent sampling and handling conditions to avoid variability (which may not be possible in the context of a multicenter clinical trial), other targets have been preferable in biomarker development for PI3K/AKT/mTOR pathway inhibitors.[28,41] These have included assessment of (i) phosphorylation of AKT at the residues Thr308 and Ser473, (ii) phosphorylation of the AKT substrate PRAS40 at Thr246, (iii) phosphorylation of 4E-BP1 at Ser65 and Thr70, and (iv) phosphorylation of RPS6 at Ser240 and Ser244.[28] However, it is important to note that phosphorylation of 4E-BP1 and RPS6 can also be regulated by enhanced RAS/RAF/ERK/mTORC1 activity, thereby potentially masking PD effects.[23] Nevertheless, biomarker analyses from phase I studies have consistently demonstrated a dose- and time-dependent decrease in phosphorylation of markers such as AKT, 4E-BP1, and RPS6 when PI3K/AKT/mTOR inhibitors are used at the MTD, thereby demonstrating target modulation.

Preclinical tumor target phosphorylation data (percentage inhibition of phosphorylation and duration of inhibition of phosphorylation) are often employed to predict the duration and magnitude to which the pathway should be inhibited in patients, in order to achieve meaningful efficacy. However, in a number of phase I studies of PI3K/AKT/mTOR pathway inhibitors, although predicted tumor target inhibition of phosphorylation was achieved at the RP2D, only modest response rates were observed. This highlights the imperfection of phosphoprotein biomarker modulation at predicting efficacy in patients and demonstrates the need to utilize separate biomarkers to define PD effect and predict therapeutic efficacy.[44]

Biomarkers of metabolic effect. Given the role of the PI3K pathway in physiological glucose metabolism, many studies of PI3K/AKT/mTOR inhibitors have included biomarkers of metabolic effect, which are indirect PD biomarkers.[87,88] Fasting glucose, insulin, and C-peptide levels in plasma, and glucose uptake using ^{18}F-FDG PET (see later), have all been evaluated in the determination of target engagement and pathway modulation.[41] Inhibition of PI3K abrogates the actions of insulin, mainly mediated by the PI3K p110α isoform, resulting in hyperglycemia and a compensatory release of insulin and C-peptide. Hyperglycemia has, therefore, commonly been used as a PD biomarker of PI3K pathway inhibition; however, Bendell et al suggest that reduced plasma C-peptide levels may provide a better, indirect biomarker, since in their phase I study of BKM120 in patients with advanced solid tumors, increases in fasting C-peptide were detected at doses lower than those associated with hyperglycemia, suggesting that increased insulin/C-peptide release can effectively compensate for decreased glucose transport and metabolism due to PI3K inhibition at lower doses.[41]

In a more recent phase I study of BKM120 in patients with advanced solid tumors, the analysis of glucose metabolism biomarkers (fasting plasma glucose, insulin, and C-peptide) supported the observation that BKM120 inhibits the PI3K pathway and perturbs glucose metabolism; however, a clear relationship between these biomarkers and the dose of BKM120 administered or the degree of pathway inhibition was not established.[42] Further work is ongoing to establish the utility of biomarkers such as C-peptide as indicators of PI3K inhibitor activity.

Functional imaging biomarkers. Given the issues related to acquisition of tumor tissue for evaluation of PD biomarkers (including the need for repeated invasive biopsies, sampling errors, and bias due to tumor heterogeneity) and the limitations of surrogate tissue (see later), functional imaging has emerged as a novel method for evaluating the PD effects of PI3K pathway drugs.[89,90] Functional imaging biomarkers have the potential to quantify biological characteristics of tumors and measure on- and off-target effects and allow serial, noninvasive assessments of whole tumor, which is particularly important in the context of inter- and intrapatient tumor heterogeneity. In addition, utilization of functional imaging could be used to guide assessment of both optimal biological dose and drug schedule.

Functional surrogate response imaging biomarkers for PI3K pathway inhibitor drugs have included ^{18}F-FDG PET, 39-deoxy-39-[^{18}F]-fluorothymidine (^{18}F-FLT) PET, MR spectroscopy, and diffusion-weighted- (DW) or dynamic contrast-enhanced- (DCE) MRI.

^{18}F-FDG PET. Since AKT activation disrupts transcription of the glucose transporter GLUT1 and its translocation to the plasma membrane, and also promotes glucose utilization independent of the effects on cell proliferation, ^{18}F-FDG PET has been proposed as a PD biomarker for assessing efficacy of on-target inhibition of the PI3K/AKT pathway.[91] In addition, early reductions in uptake on ^{18}F-FDG PET (SUVmax from baseline) have been demonstrated to be a predictor of change in tumor burden, and therefore ^{18}F-FDG PET can also be utilized as a *predictive* biomarker.[92] ^{18}F-FDG PET has been incorporated into biomarker evaluations in several preclinical and clinical studies of PI3K and/or MEK inhibitor therapy. For example, it has been shown to be a surrogate marker of sensitivity to PI3K inhibition by the dual PI3K/mTOR inhibitor NVP-BEZ235 and the pan-class I PI3K inhibitor, NVP-BKM120, in human head and neck squamous cell carcinoma and mouse mammary 3D tumor spheroids *in vitro*.[93] In addition, ^{18}F-FDG PET has been shown to be a surrogate marker of response following treatment with the pan-isoform PI3K inhibitor LY294002 or the dual PI3K/mTOR inhibitors PF04691502 and NVP-BEZ235 in colorectal, lung, and ovarian tumor xenografts and/or mouse models *in vivo*.[94–96]

Furthermore, a decrease in uptake on ^{18}F-FDG PET has been observed in several phase I clinical trials following PI3K/AKT/mTOR inhibitor treatment.[41,42,46] However, whether

the cause of this decrease is related to a direct effect of PI3K inhibition on glucose uptake (therefore, acting as a PD biomarker), or an antitumor effect (thus, acting as a predictive biomarker) is not yet known, and in some cases, both can have a role, as seen with mTOR inhibitors.[91,97]

In a recent phase I study, BKM120 administration instigated partial metabolic responses detected by ^{18}F-FDG PET (a >25% decrease in ^{18}F-FDG uptake) in 39% of evaluable patients after 28 days of treatment; however, no association with progression-free survival or best response was identified. The authors suggested that the effect of PI3K inhibitors on glucose metabolism, the small number of patients and responses observed, and the highly heterogeneous range of patients and tumor types treated might have explained why ^{18}F-FDG PET was unable to predict response to therapy in this study, despite demonstrating capability as a PD biomarker.[42]

In the phase I study of pictilisib (GDC-0941), partial metabolic responses detected by ^{18}F-FDG PET were observed in 7 of 32 evaluable patients.[44] ^{18}F-FDG PET imaging showed a reduction from baseline of tracer uptake at dose levels ≥45 mg with an overall median change in SUV_{max} of –13%, thereby confirming some degree of pathway modulation.[44] However, an association between ^{18}F-FDG PET changes and RECIST response was not detected in this trial, highlighting the uncertainties of the role of ^{18}F-FDG as a predictive biomarker.

^{18}F-FLT PET. The fluorine-modified thymidine analog, ^{18}F-FLT, also represents a promising proof-of-concept antiproliferative PD and surrogate response biomarker for PI3K pathway inhibitor therapy. ^{18}F-FLT PET is used for detecting antiproliferative effects, since it is a thymidine analog, whose accumulation in cells is determined by the expression and activity of the enzyme thymidine kinase 1 and specific nucleoside transporters, both of which are under the control of S-phase cell cycle regulators.[98] Furthermore, the uptake of ^{18}F-FLT PET has been shown to correlate with standard proliferation markers, such as Ki67, TK1, and BrdU uptake.[99–102] Using ^{18}F-FLT PET, changes in proliferation compared to baseline have been demonstrated in a variety of human tumor xenografts as early as 18, 24, and 120 hours after using either single-agent class I selective PI3K inhibitor GDC-0941 (pictilisib) or MEK inhibitor PD0325901.[89,90,103,104] Furthermore, in human xenograft tumor-bearing mice, the combination of the class I selective PI3K inhibitor pictilisib with the MEK inhibitor PD0325901 resulted in superior efficacy when compared with controls, and this correlated with a subsequent decrease in tumor ^{18}F-FLT uptake measured by PET just two days after treatment.[98] ^{18}F-FLT PET has not yet been utilized in a trial of a PI3K pathway inhibitor; however, it has been incorporated into a clinical trial of the MEK inhibitor AZD6244 (selumetinib) as a single agent.[105] In this pilot study, ^{18}F-FLT PET scans were performed in four patients at baseline and after two weeks of treatment with selumetinib. FLT uptake in tumor was compared to CT scans at baseline and eight weeks to evaluate the utility of ^{18}F-FLT PET as an early predictor of response. In two patients, changes in FLT uptake as early as after two weeks of treatment were consistent with CT results after eight weeks.[105]

Magnetic resonance spectroscopy. Cancer cells are known to reprogram their metabolism to facilitate tumor growth and survival by alteration of signaling pathways, which lead to alterations in glucose, glutamine, and lipid metabolism.[20,106] As discussed earlier, the PI3K/AKT/mTOR signaling pathway is a master regulator of enzymes involved in glucose, glutamine, and lipid metabolism.[107,108] Therefore, inhibition of the PI3K signaling pathway impacts on the levels and/or activities of these enzymes.[109] Magnetic resonance spectroscopy (MRS) is a technique that has recently been used in the clinical setting to study cancer metabolism. It offers the opportunity to investigate metabolic components of cells and tissues in physiological environments, noninvasively and without the use of radioactive reagents. In addition, since it produces spatial mapping of metabolites, MRS can overcome the challenges of tumor heterogeneity. These data are represented by a spectrum, in which the peaks correspond to different metabolites wherein peak areas can be measured and metabolite concentrations quantified. MRS is now being increasingly used for monitoring tumor cell metabolism and alterations in response to therapy in cultured cells, animal models, and patients.

Metabolic effects of PI3K inhibition in cancer have been studied *in vitro* and *in vivo*.[110] Using nuclear magnetic resonance (NMR), altered choline metabolism has been demonstrated in response to inhibition of the PI3K signaling pathway with LY294002, wortmannin, and the selective dual pan-class I PI3K/mTOR inhibitor PI-103 in adult human cancer cell models.[111,112] Distinct metabolic changes have been demonstrated using *in vitro* ^{1}H- and phosphorus (^{31}P)-NMR following PI3K pathway inhibition by PI-103 and pan-class I PI3K inhibitor GDC-0941 in pediatric glioblastoma cell lines. These included a decrease in the levels of lactate, phosphocholine (PC), and total choline.[109] Moestue et al demonstrated, using ex vivo high-resolution magic angle spinning MRS, that response to PI3K inhibition in a breast cancer basal-like xenograft was associated with reduced lactate concentration and increased concentration of PC, glycerophosphocholine (GPC), and glucose.[113] The magnitude of the metabolic response was reflected by the inhibition of cancer cell proliferation and the reduction in phosphorylation of AKT ser473 level.[113] Lactate, PC, and GPC can potentially be imaged noninvasively *in vivo* using MRS and may, therefore, be valuable biomarkers for early monitoring of response to PI3K inhibition. However, to date, the authors are not aware of any utilization of MRS in early-phase clinical trials for PI3K pathway inhibitors.

Diffusion-weighted- and dynamic contrast-enhanced-MRI. DW-MRI can be used to measure the apparent diffusion coefficient (ADC) of water molecules and has been proposed as a marker for tissue cellularity.[114] Treatment-induced cell death can be reflected by increased ADC values even before

significant tumor volume changes occur,[114,115] and DW-MRI is, therefore, suggested as a method for measuring early treatment response.[116] DCE-MRI investigates the vascularization of a tissue by measuring signal enhancement curves after intravenous administration of a contrast agent and can be used to measure the changes in tumor blood flow, vascular permeability, and extracellular extravascular and vascular volumes.[117,118] Both DW-MRI and DCE-MRI have been proposed as tools for measuring response to PI3K pathway inhibitors and have been evaluated in preclinical *in vivo* models.[119–121]

In a recent study, ADC was found to be a useful biomarker for response to the dual PI3K-mTOR inhibitor BEZ235 in an ovarian cancer xenograft model.[119] In the same study, DCE-MRI, which provides information on tumor perfusion and vascular permeability also proved to be a useful biomarker for response. The parameter v_e is a measure of the extravascular extracellular space and, similar to ADC, may be related to the cellular density of the tumor tissue. In another recent study, Sampath et al.[122] showed that response to the PI3K/mTOR inhibitor GDC-0980 was associated with increased v_e.[122] Despite these promising data, this functional imaging modality has not yet been used in clinical trials in this setting.

Tumor versus surrogate biomarkers. Assessments of PD biomarkers of signaling output have classically required the collection of serial tumor biopsies; however, for solid tumors, this can be challenging. Therefore, researchers have explored using normal tissues, such as the skin, PBMCs, PRP, and plucked hair follicles, as POTENTIAL SURROGATES for tumor tissues. These minimally invasive sampling methods may reduce the risks associated with repeated tumor biopsies and enable serial determinations of drug effects, thus minimizing the impact of inter- and intrapatient variability on such results. However, such surrogate methods are hampered by (i) requirement of the therapeutic target of interest to be highly expressed in normal tissues; (ii) differences in drug penetration and concentrations between normal and tumor tissues due to likely differences in tissue architecture; (iii) possible differences in gene expression between tissues; (iv) normal tissues lacking somatic mutations in the oncogenic target; (v) mutant enzymes in tumor tissue leading to significant differences in drug sensitivity when compared with wild-type enzymes in normal tissues; and (vi) possible differences in signal transduction pathway regulation in tumors to normal cells.[123–125]

For example, in the phase I study of the pan-PI3K inhibitor pictilisib (GDC-0941), a 90% decrease in AKT phosphorylation was detected in PRP at up to three hours postdose at the RP2D.[44] In patient tumors, although at the highest level of drug exposure a >75% decrease in S6 phosphorylation and 100% reduction in AKT phosphorylation were detected, a direct correlation between pictilisib exposure and decrease in S6 and AKT phosphorylation in tumors was less clear.[44] In contrast to the consistent dose–response relationship in PRP, this lack of consistent target modulation in tumor at lower drug exposures was thought to be possibly related to different assay conditions, together with disparities in drug concentrations between normal and tumor tissues due to likely differences in tissue architecture and hemodynamics.

In a phase I study of the dual PI3K-mTOR inhibitor BGT226, the observed PD effects were also inconsistent between tumor tissue and skin.[126] At BGT226 doses >80 mg, PI3K pathway inhibition was evident in skin biopsies as determined by a reduction in phosphorylation of S6 (Ser240/244) by 37%–64%. However, reductions in phosphorylation of S6/AKT in tumors were not seen in all samples, and no biopsies were performed at doses >80 mg, making further interpretation difficult.[126] This example illustrates that as collection of solid tumor biopsies is challenging, it is imperative to ensure that tumor tissue samples are always obtained at the MTD.

These differences also highlight both the importance of evaluating multiple PD biomarkers to comprehensively evaluate the overall pharmacological effects of the drug, and the danger of overinterpretation results from a single PD marker evaluation.

Circulating biomarkers. Alternative methods for the evaluation of PD biomarkers of PI3K/AKT/mTOR pathway inhibition include circulating tumor cells (CTCs), cell-free DNA/circulating tumor DNA (ctDNA), and circulating markers of cell death and angiogenesis.

Circulating tumor cells. A promising development in translational cancer medicine has been the emergence of CTCs as a minimally invasive multifunctional biomarker. CTCs in peripheral blood originate from solid tumors and are involved in the process of hematogenous metastatic spread to distant sites to establish metastases. The potential use of CTCs as biomarkers is not only confined to their enumeration (and therefore as a surrogate of response) but also includes their routine molecular characterization. The assessment of CTC-based PD biomarkers has the potential for rapidly demonstrating proof of mechanism during the clinical development of molecularly targeted anticancer therapeutics in *real time*.

However, one potential challenge in using CTCs for PD studies is that, similar to other surrogate tissue biomarkers, cells in the blood will be exposed to plasma drug concentrations, which may or may not be the same as the drug levels achieved in solid tumors. Therefore, when interpreting PD data from CTCs, the PK data are critical and must be used in conjunction.

Western blot analyses of S6K1, phosphorylated-S6K1, and mTOR in CTCs were used as PD biomarkers in a recent phase II trial of the mTOR inhibitor sirolimus given in combination with trastuzumab for HER2-positive metastatic breast cancer.[127] Unfortunately, no statistically significant correlation between response and posttreatment change in levels of the mTOR pathway biomarkers was detected in CTCs.[127] CTC analyses have otherwise not yet been used in clinical trials in this setting.

Cell-free DNA/ctDNA. Cell-free fragments of DNA are shed into the bloodstream by cells undergoing apoptosis or necrosis, and in patients, a small proportion (<1%) of the fragmented DNA in the circulation is derived directly from the tumor.[128] Recent advances in sequencing technologies have enhanced the sensitivity and accuracy of DNA analysis, allowing for the genotyping of somatic genomic alterations in circulating cell-free DNA. Cell-free ctDNA contains genetic defects identical to those of the tumors themselves, enabling the detection of cancer-associated genetic alterations, including point mutations, chromosomal rearrangements, amplifications, and aneuploidy. Indeed, ctDNA can be identified and distinguished from normal cell-free DNA by testing for genetic mutations that are tumor specific. Furthermore, ctDNA levels have correlated with changes in tumor burden in a number of different malignancies.[128,129] Thus, ctDNA has the potential to serve as a noninvasive tool, as a biomarker of early response, and as a predictive biomarker.

Potential drawbacks with this method include low plasma DNA levels, which may mean that mutations are missed, and also clonal evolution of detected mutations. As yet, in the context of PD biomarkers of PI3K/AKT/mTOR pathway inhibition, both CTC and ctDNA approaches have been more exploratory. However, these biomarker strategies are likely to be increasingly utilized in the future.

Circulating markers of cell death and angiogenesis. Circulating full-length and caspase-cleaved cytokeratin 18 (CK18) are considered biomarkers of anticancer therapy-induced cell death, measured using a combination of M30 and M65 enzyme-linked immunosorbent assays (ELISAs).[130] M30 measures caspase-cleaved CK18 produced during apoptosis, and M65 measures the levels of both caspase-cleaved and intact CK18, the latter of which is released from cells undergoing necrosis. Circulating M30 and M65 and markers of angiogenesis (BFGF, PLGF, SVEGFR1, SVEGFR2, and VEGF) have been evaluated in early-phase clinical trials of PI3K pathway inhibitors.[42,126] In general, intrapatient variability in these markers has been found to be high, and therefore, statistical relationships between pre- and post-treatment levels have not been confirmed.[42]

Predictive biomarkers. Preclinical data of PI3K pathway inhibitors have supported the hypothesis that tumors with *PIK3CA* mutations or PTEN loss are more sensitive to PI3K/AKT inhibitors.[131–133] However, the complexities of the pathway and its feedback loops mean that clear prediction of response to genotype is difficult. For example, for the pan-class I PI3K inhibitor BKM120, studies demonstrated that inhibition of PI3K had significant effects on cells carrying mutations of *PIK3CA*, whereas cells with *PTEN* or *KRAS* aberrations were not as sensitive.[134] Although triple-negative breast cancer subtypes with *PIK3CA* mutations were found to be sensitive to the dual PI3K/mTOR inhibitor, BEZ235, again the loss of PTEN did not predict response.[135]

Nevertheless, these preclinical studies have encouraged the enrichment of clinical trials with patients whose tumors harbor mutations in *PIK3CA* and *PTEN* or have loss of PTEN expression.[131,136–138] Some pooled analyses of these clinical studies have suggested a correlation between molecular alterations in the PI3K pathway and antitumor effect,[41,47,71] whereas others have suggested no such correlation.[139] The inconclusive predictive value of *PIK3CA*, *PTEN*, *KRAS,* or *BRAF* mutations for delineating the clinical value of PI3K/AKT/mTOR pathway inhibitors may be due to several reasons. First, tumors without PI3K alterations might have responded because early detection methods were based on a limited number of assays and were unable to detect other alterations that could be driving sensitivity, such as alterations in *AKT1/2*, *PIK3R1*, *LKB1*, or *NF1*, or were using improper assays or thresholds. Second, tumors described as having PI3K alterations may not have responded because of the coexistence of mutations related to resistance, such as mutations in *KRAS*,[51,140] intratumor heterogeneity,[75] or the use of incorrectly standardized or unvalidated assays. Finally, an important recent study designed to decipher whether actionable driver mutations are found in all, or a subset of tumor cells found that 15% of mutations in genes of the PI3K/AKT/mTOR signaling axis across all tumor types were subclonal, rather than truncal.[141] This frequent presence of subclonal driver mutations in the PI3K/AKT/mTOR signaling axis may explain the inconclusive predictive value of these mutations and suggests the need to stratify PI3K-directed therapy response according to the proportion of tumor cells in which the driver mutation is identified.[141]

In a study of >1600 patients with diverse advanced cancers enrolling onto phase I trials, *PIK3CA* mutations and/or *PTEN* aberrations were detected in ~20% of patients.[142] *PTEN* aberrations were mostly determined by loss of staining on immunohistochemistry (95% of patients with PTEN aberration), as only 5% of patients were tested for PTEN mutations. They also demonstrated that, in colorectal and gynecological cancers, *PIK3CA* mutations often coexisted with mutations in the MAPK pathway such as *KRAS* and *BRAF* mutations, which can abrogate response to PI3K/AKT/mTOR pathway inhibitors.[95,142–145]

Levels of phosphorylated S6K and AKT may be predictive biomarkers for inhibitors of the mTOR pathway. Indeed a high level of phosphorylated S6K has been associated with poor prognosis, and the levels of phosphorylated S6K and AKT have been shown to predict a favorable response to rapamycin or rapamycin analogs in breast cancer cells lines and other tumors.[146–148] Furthermore, high levels of phosphorylated AKT, GSK3β, and TSC2 have also been demonstrated to correlate with increased sensitivity to RAD001 (everolimus).[149]

Another potential predictive biomarker is *INPP4B*, a tumor suppressor that regulates PI3K/AKT. Its deletion may be seen with *PTEN* loss and it correlates with poor prognosis.

Tumors with *INPP4B* loss may also be candidates for targeting with PI3K inhibitors.[150]

It must be remembered that in addition to their direct therapeutic action on cancer cells, PI3K inhibitors can have effects on tumor angiogenesis, immune cells, and other tumor microenvironmental interactions; hence, it is conceivable that there may not be a single biomarker of sensitivity but rather a predictive molecular signature. Critical information will come from molecular profiling of clinical tumor material, including global cancer genome sequencing and gene expression analysis, followed by the correlation of such data with therapeutic response and outcome to various PI3K inhibitors.

Conclusions

The PI3K pathway is one of the most commonly deregulated in cancer and is currently a major focus for anticancer drug development. As cancer medicine moves toward an increasingly *personalized* paradigm, PI3K pathway inhibitors are likely to form a critical part of the therapeutic strategy for many cancers. For these drugs to be optimally used, it is of critical importance that early-phase trials include comprehensive PD evaluation as part of a broader strategy incorporating PK, predictive, and pharmacogenetic biomarkers. It is clear from the studies conducted to date that a multimodality PD approach is optimal, evaluating both inhibition of phosphorylation of downstream proteins and also the metabolic and immunological effects of the drug. Increasingly, technological advances in functional imaging and circulating biomarkers (eg, ctDNA) will allow for more detailed PD evaluation.

As the studies described in this review have largely demonstrated, use of single-agent PI3K pathway inhibitors is usually associated with modest therapeutic efficacy. These drugs are more likely to be optimally used in combination with other molecularly targeted THERAPIES, cytotoxics, or hormonal therapies. Preclinical modeling and molecular profiling strategies will help determine the most effective combinations, with increasing use of patient-derived tissue to allow a more personalized therapeutic strategy. PD biomarkers will be critical in helping to determine optimal dosing by allowing assessment of differing dosing regimens, including pulsatile schedules, which could potentially offset the toxicity concerns around many PI3K combination regimens.

In conclusion, comprehensive PD evaluation together with robust predictive biomarkers is likely to be critical to the successful development of PI3K pathway inhibitors and for their integration into personalized treatment strategies.

Author Contributions

Wrote the first draft of the manuscript: DJ, DS. Contributed to the writing of the manuscript: DJ, DS. Jointly developed the structure and arguments for the paper: DJ, DS. Made critical revisions and approved final version: DJ, DS. Both authors reviewed and approved of the final manuscript.

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