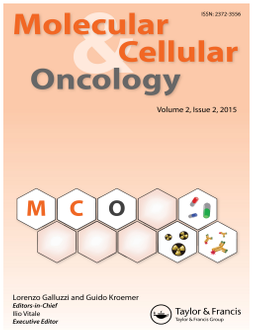

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Rationale-based therapeutic combinations with PI3K inhibitors in cancer treatment

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Keywords: AKT, *PIK3CA*, PTEN, resistance, targeted therapy

Abbreviations:

The PI3K/AKT/mTOR signaling is important for cell proliferation, survival, and metabolism. Hyperactivation of this pathway is one of the most common signaling abnormalities observed in cancer and a substantial effort has recently been made to develop molecules targeting this signaling cascade. However, it is becoming evident that PI3K inhibitors used as single agents do not elicit dramatic or durable responses. Given the numerous mechanisms mediating intrinsic and acquired resistance to these agents, hypothesis-based combinatorial strategies are probably needed to fully exploit their antitumor activity. In the first part of this review, we briefly dissect the PI3K/AKT/mTOR axis and list the most advanced compounds targeting different nodes of this cascade. The second part focuses on what we believe to be the most promising rationale-based therapeutic combinations with PI3K/AKT/mTOR inhibitors in solid tumors, with special emphasis on breast cancer.

Introduction

Recent genomic sequencing efforts have allowed investigators to decipher the molecular portraits of different types of cancer. This has led to the identification of several actionable gene alterations and paved the way toward personalized medicine.

Hyperactivation of the phosphoinositide-3-kinase (PI3K) signaling cascade, either by overexpression of upstream receptor tyrosine kinases or deregulation of several elements of the pathway, is one of the most frequent pathway alterations in cancer.

The PI3K pathway is highly conserved among species and has been proposed to play a key role in the regulation of multiple cellular events, including growth, proliferation, cell cycle progression, and survival. Direct pharmacological inhibition of the PI3K signaling is, therefore, an attractive clinical strategy and a number of PI3K pathway inhibitors are currently under clinical development. However, unlike other genomic alterations, there is no consensus regarding *PIK3CA* mutations as cancer drivers. As a consequence, inhibition of the PI3K pathway alone does not usually translate to dramatic antitumor activity. This could potentially be explained as follows:

1. The therapeutic window is narrow because normal cells also require PI3K signaling for survival. As a consequence, severe adverse effects (e.g., hyperglycemia) often manifest before full inhibition of the target in tumor cells.
2. Inhibition of the PI3K pathway leads to activation of compensatory pathways that can limit the sensitivity to these agents.[1-4]

In this article, we review the main inhibitors of the PI3K/AKT/mTOR axis, focusing on those furthest along in the clinical pipeline, and propose hypothesis-based combinations that could potentially improve their antitumor activity.

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PI3K: Structure and Biochemistry

PI3K enzymes are classified into 3 classes (Class I to III) according to their structural and biochemical properties. Because of their role in human cancer, in this review we will discuss only the Class I PI3Ks.

Class I PI3Ks are characterized by the presence of a catalytic subunit (p110) that forms a heterodimeric complex with the regulatory subunit (p85). The catalytic subunit is encoded by 1 of 4 genes, *PIK3CA* (p110α), *PIK3CB* (p110β), *PIK3CD* (p110δ), and *PIK3CG* (p110γ). All of these isoforms use phosphatidylinositol-(4,5)-biphosphate as a substrate. Whereas p110α and p110β are expressed in virtually all cell types, p110δ and p110γ are specifically enriched in leukocytes (reviewed in[5] and[6]).

The catalytic subunit p110 contains a C-terminal kinase domain that is responsible for the lipid enzymatic activity, a helical domain with a yet unknown function, a C2 domain that has been suggested to bind the cellular membrane, a Ras-binding domain (RBD), and an N-terminal adaptor-binding domain (ABD) that is responsible for the interaction with the regulatory subunit[7] (**Fig. 1A**).

Class I enzymes are further divided into 2 groups, A and B, based on the regulatory subunit that they interact with. The Class IA isoforms, p110α, β, and δ, are associated with p85α or p85β

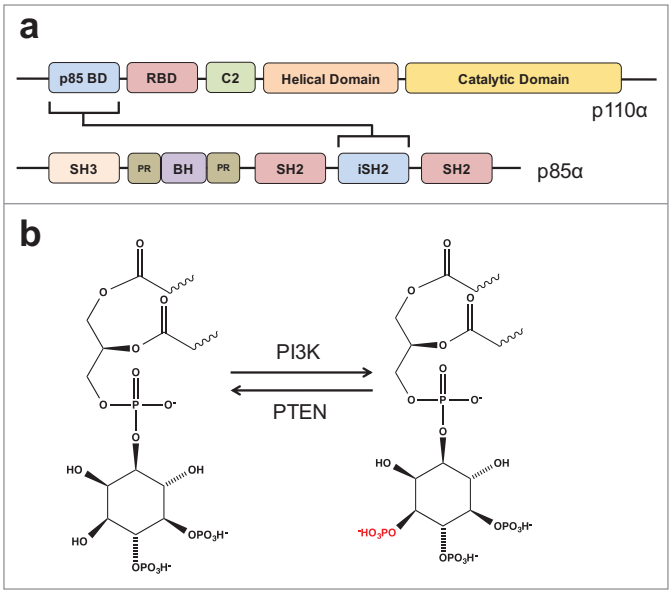

Figure 1. Structure and biochemistry of PI3K. (**A**) The domains of PI3K catalytic (p110α) and regulatory (p85α) subunits are represented. The connecting arrow indicates the domains involved in the interaction between these 2 subunits. BD (Binding Domain), RBD (Ras-BD), SH3 (SRC Homology 3), PR (Proline-Rich), BH (BcR Homology), SH2 (SRC Homology 2), iSH2 (inter-SH2). (**B**) Phosphorylation of the phosphatidylinositol 4,5-bisphosphate inositol ring at position 3-OH (red). Arrows indicate the direction catalyzed by PI3K or the phosphatase PTEN.

subunits, whereas the Class IB isoform p110γ interacts with p101 or p87.[8]

Alternative splicing of *PIK3R1*, the gene encoding p85, gives rise to 4 different isoforms with p85α being the most common. p85α contains 2 Src-homology 2 (SH2) domains that bind to phosphorylated tyrosine residues of receptor tyrosine kinases (RTKs) and an intermediate SH2 (iSH2) domain that interacts with and inhibits the p110 subunit[7] (**Fig. 1A**). Upon RTK stimulation, SH2 domains recognize the phosphorylated sites and release the inhibitory effect of the iSH2 domain on the catalytic subunit. This allows the p110 subunit to exert its enzymatic activity using the abundant phosphatidylinositol-(4,5)-biphosphate (PIP2) present in the inner plasma membrane as a substrate. Biochemically, PI3K phosphorylates the hydroxyl (-OH) group at position 3 of the inositol ring in inositol phospholipids.[9] This reaction generates the second messenger phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3) that triggers downstream signaling of the pathway. This reaction is reversed by both phosphatase and tensin homolog (PTEN, 3-phosphatase)[10] and SH2 domain-containing inositol 5'-phosphatase (SHIP, 5-phosphatase),[11] enzymes that dephosphorylate the inositol ring and convert PIP_3 to PIP_2 or phosphatidylinositol-(3,4)-biphosphate, respectively. Both PIP_3 and phosphatidylinositol-(3,4)-biphosphate have been shown to regulate the function of multiple downstream effectors by recruiting them into the plasma membrane[12,13] (**Fig. 1B**).

Downstream of PI3K

AKT, a Ser/Thr kinase that belongs to the AGC family of the human kinome, is one of the most studied kinases and is considered a key output of the PI3K pathway because of its large number of substrates. There are 3 isoforms of AKT (1–3), encoded by the respective genes *AKT1*, *AKT2*, and *AKT3*.[14] Although it remained elusive for several years, there is now some consensus about the mechanism of activation of this kinase. Upon generation of the second messenger PIP_3 in the membrane by PI3K, both phosphoinositide-dependent kinase-1 (PDK1, a constitutively active kinase) and AKT are recruited to the membrane through their pleckstrin homology domains (PHs), which recognize PIP_3 with high affinity. The proximity of these kinases allows PDK1 to phosphorylate AKT at residue T308 of the activation loop (T-loop).[15,16] Subsequently, AKT is phosphorylated at residue S473 of the hydrophobic motif by the rapamycin-insensitive mTOR complex 2 (mTORC2).[17] This phosphorylation is considered necessary to fully activate the kinase activity of AKT (**Fig. 2**); however, several reports suggest that phosphorylation at the T-loop may be sufficient to engage AKT activity on selected substrates.[18,19]

Activated AKT in turn phosphorylates several substrates involved in apoptosis and cell cycle regulation. For example, AKT is able to phosphorylate and inhibit Bad, a member of the Bcl-2 family,[20] and caspase 9,[21] 2 main regulators of the mitochondrial apoptotic pathway. It also inhibits $p21^{CIP,122}$ and $p27^{KIP,23}$ proteins that are directly involved in the inhibition of cell cycle progression. Moreover, AKT can also prevent the nuclear localization of the forkhead transcription factors FOXO1, 3, 4, and 6,[24] which are involved in the transcriptional regulation of several genes including the proapoptotic genes *CD95L*, *BCL2L11* (BIM), *BBC3* (PUMA), and genes encoding the cell cycle inhibitors *CDKN2A* ($p21^{CIP}$) and *CDKN2B* ($p27^{KIP}$). In addition to these effectors, AKT can phosphorylate PRAS40 and TSC2, 2 negative regulators of mTORC1 activity (**Fig. 2**)[25,26] thus linking the PI3K/AKT pathway with the mTORC1 pathway.

The importance of PDK1 and AKT in mediating PI3K downstream signaling has been exploited as a suitable node for pharmacological inhibition. Although PDK1 inhibitors are being used in a preclinical setting (for an excellent review see reference[27]), many AKT inhibitors are currently under clinical development.

AKT inhibitors are highly specific and potent, and consequently on-target adverse effects such as severe hyperglycemia can limit their use. One of the first inhibitors reported to inhibit AKT is the phospholipid analog perifosine, which inhibits the PH domain of AKT.[28] Despite promising clinical activity in early studies, perifosine has failed to increase overall survival in metastatic CRC when administered in combination with capecitabine.[29] Other inhibitors of AKT are being investigated, including the allosteric inhibitor MK2206[30] and the catalytic inhibitors GDC-0068,[31] AZD5363,[32] and GSK690693.[33] These orally bioavailable agents are undergoing different phases of clinical development (Phase I-III) in multiple solid and hematological malignancies. Of note, most of these trials are being tested in

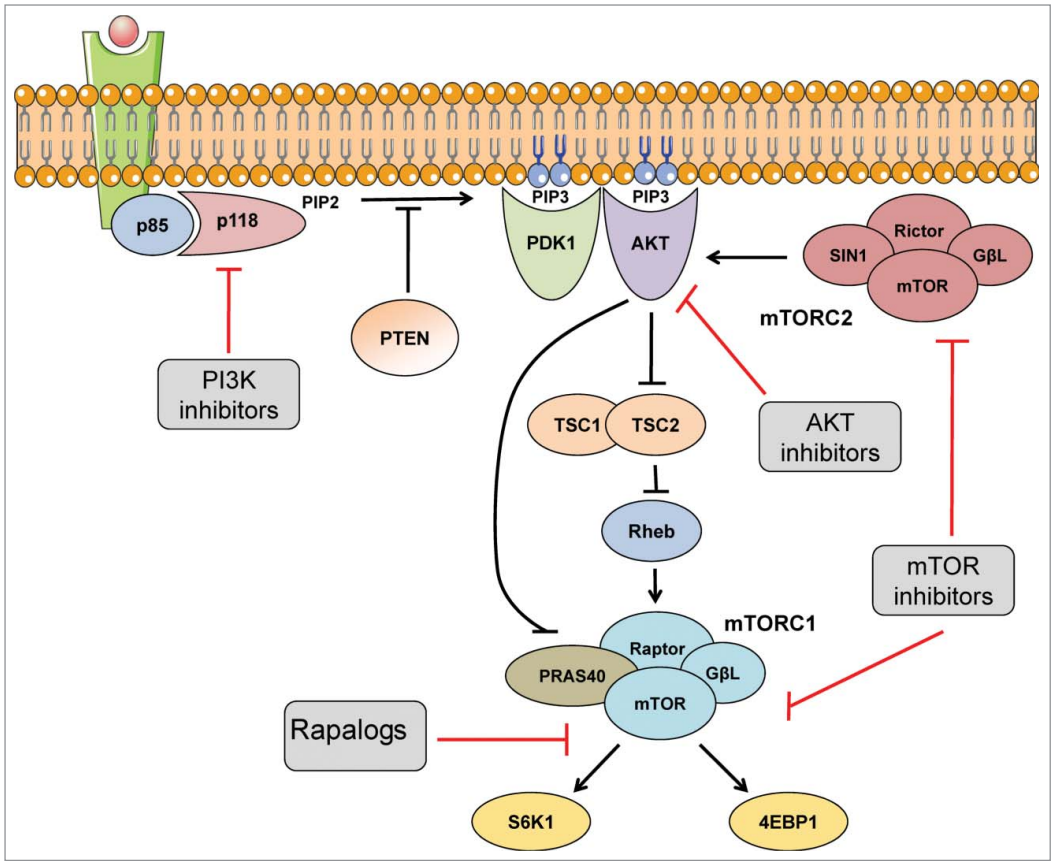

Figure 2. The PI3K/AKT pathway. Main transduction signals of the PI3K pathway. Blue phospholipids indicate PIP_3 second messenger. Arrows indicate activation while bars represent inhibition. Red bars show pharmacologic targets of the pathway. Images were taken from Servier Medical Art.

combination with other agents such as CDK, RTK, ER, and MEK inhibitors.

mTOR

mTOR, a 289-kDa serine-threonine protein kinase, is at the core of the PI3K/AKT pathway and acts as a master integrator of multiple upstream signals.[34] mTOR senses and responds to environmental cues such as nutrient availability, stress, and mitogens to regulate protein synthesis through a highly orchestrated and complex mechanism.

mTOR was originally identified in the early 1990s[35] and was later shown to form 2 discrete complexes with distinct roles in the control of cell growth.[36]

mTOR complex 1 (mTORC1), which contains mTOR, Deptor, Raptor, mLST8, and PRAS40, has been considered a master regulator of cell growth and metabolism that signals to 4E-binding protein (4EBP1) and 40S ribosomal protein S6 kinase (S6K), both of which are important in the physiological control of translation.[37] mTORC1 promotes protein synthesis by phosphorylating 4EBP1, which in turn prevents 4EBP1 from binding to the eukaryotic initiation factor 4E (eIF4E), enabling eIF4E to initiate cap-dependent translation. On the other hand, activation of S6K1 by mTORC1 leads to an increase in mRNA biogenesis and cap-dependent translation.[37] mTORC1 has also been shown to activate RNA PolI transcription and thus rRNA synthesis through a process involving the protein phosphatase 2A (PP2A) and the transcription initiation factor IA (TIFIA).[38]

The second complex (mTORC2), which is rapamycin-insensitive,[36,39] consists of at least 6 different proteins. The complex core is formed by mTOR, Deptor, and mLST8 but, instead of Raptor, it contains 3 other proteins: rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase interacting protein (mSIN1), and protein observed with Rictor-1 (Protor-1).[40] It is known that mTORC2 activity responds to growth factors, but how mTORC2 is regulated upstream and the exact molecular function of most of its interacting proteins remain elusive. Functionally, mTORC2 regulates organization of the actin cytoskeleton through the phosphorylation of protein kinase Cα and also activates AKT through phosphorylation at S473.[17,41]

Although other kinases such as DNA-PK and ATM have been suggested to phosphorylate AKT at S473,[42,43] studies in Rictor, Sin1, and LST8 knockout mice have shown that an intact mTORC2 complex is required for maximal phosphorylation and activation of AKT in mouse embryonic fibroblasts.[19,44]

Table 1. Combinations with pan-PI3K inhibitors under clinical development

Drug	Target	Company	Phase	Combinations
CH5132799	PI3K	Chugai	I	
CLR457	PI3K	Novartis	I	
GDC-0941	PI3K	Genentech	I/II	Cisplatin, paclitaxel, fulvestrant, erlotinib, bevacizumab, GDC0973.
NVP-BKM120	PI3K	Novartis	II	Radiotherapy, Chemotherapy (paclitaxel, irinotecan, carboplatin, gemcitabine, etc), RTKi (Gefitinib, Erlotinib, Cetuximab, lapatinib, rituximab), endocrine therapy (fulvestrant, abiraterone) imatinib, bevacizumab, olaparib, CDKi (LEE011), MEKi (MEK162, GSK1120212), everolimus, LDE225, INC280, BRAFi (encorafenib, vemurafinib).
PX-866	PI3K	Oncothyreon	I/II	Docetaxel, cetuximab, vemurafenib.
SF1126	PI3K	Semafore	I	
XL147	PI3K	Exelixis	I/II	Paclitaxel, carboplatin, letrozole, trastuzumab, erlotinib, XL647.
ZSTK474	PI3K	Zenyaku Kogyo	I/II	

Genomic Alterations in the Pathway: *PIK3CA* and PTEN

PIK3CA, the gene encoding p110α, is the most commonly mutated gene among the components of the PI3K pathway.[45] Although there are 3 main mutation hotspots within the helical (E545 and E542) and kinase (H1047) domains,[46] other mutations can be found across the whole gene. The E542K and E545K mutations located in the helical domain induce an important electrostatic switch in these residues by reversing the ionic charge. This modifies the interaction of the N-terminal SH2 domain of the regulatory subunit p85 with the helical and kinase domain of p110α, comparable to binding at phospho-Y residues.[47,48] In the case of the H1047R mutation within the kinase domain, it has been established that this alteration increases the kinase activity by inducing a new orientation of the C-terminal loop toward the plasma membrane, in which the active site has better access to its substrate PIP_2.[49,50]

Other mutations include the R39 (R39C and R39H) and the R88Q mutations that occur in the ABD domain. These mutations are reported to disrupt the interaction between the ABD and kinase domains of p110α, thus augmenting its activity. In addition, 2 mutations within the C2 domain, N345L and E453Q, have been proposed to alter the interaction between this domain and the iSH2 domain of the regulatory subunit, again increasing the kinase activity of p110α.[47]

Another well-studied alteration that can lead to hyperactivation of the pathway is loss of function of the tumor suppressor PTEN.[51] Low PTEN phosphatase activity results in increased levels of PIP_3 and consequent activation of downstream PI3K effectors such as AKT and mTORC1 (**Fig. 2**). PTEN is a 55-kDa enzyme containing, among other structural motifs, a phosphatase domain that controls the catalytic activity of the enzyme and a C2 domain that is responsible for lipid binding.[10] Somatic mutations of PTEN are found throughout the entire gene, although there is a slightly higher frequency at the R130 residue. Somatic PTEN mutations are relatively frequent in endometrial carcinoma and glioblastoma, and PTEN copy number loss is common in prostate, breast, and ovary cancer and glioblastoma.[52,53]

PI3K inhibitors

There are currently approximately 200 clinical trials testing the activity, safety, and efficacy of different PI3K inhibitors (www.clinicaltrial.gov). The first inhibitors, isolated almost 2 decades ago, were not specific for PI3K and commonly inhibited other kinases, especially phosphatidylinositol 3-kinase-related kinases (PIKK) such as mTOR and DNA-PK, which contain structurally similar active sites.[54]

Because PI3K uses ATP as a phosphate donor to phosphorylate the substrate PIP_2, most inhibitors work as competitive ATP mimetics.[55] The first known inhibitor of PI3K, wortmannin, is a furanosteroid derivative isolated from the fungus *Penicillium wortmanii*, which acts as a potent and irreversible pan-PI3K inhibitor ($IC_{50} \approx 4.2$ nM).[56] Unfortunately, this inhibitor also targets other members of the PIKK family and MAPK. Similarly, viridin, a natural steroid derived from *Trichoderma viridae*, has been shown to inhibit PI3K in a potent and irreversible manner ($IC_{50} \approx 5$ nM).[57] Using the structure of the furanosteroid wortmannin as a pharmacophore, multiple drugs have been developed including PX-866,[58] which is currently in Phase I/II trials. Another natural product that was shown to inhibit PI3K is the flavonoid quercetin,[55] which was used as a model in the synthesis of LY294002. Although LY294002 has been used for many years as a tool in the field of PI3K research, it actually has relatively low potency toward PI3K ($IC_{50} \approx 1.4$ μM) and co-inhibits mTOR and DNA-PK.[59] LY294002 has also been used as a model to create the β-specific inhibitors TGX-115, TGX-126, TGX-221, and TGX-286, which were initially designed as antithrombotic agents because of the involvement of PI3Kβ in hemostasis.[60] Another molecule, phenylquinazoline, inhibits PI3Kα with an IC_{50} of 1.3 μM, and is also a common pharmacophore used as a backbone to create novel PI3K inhibitors such as PI-103, a potent ($IC_{50} \approx 3.6$ nM) but not very selective inhibitor of PI3Kα and β.[61,62] The most clinically promising drug derived from a morpholino quinazoline is probably the thienopyrimidine GDC-0941, which is considered a pan PI3K inhibitor although it preferentially targets PI3Kα ($IC_{50} \approx 3$ nM).[63] This molecule has shown good pharmacokinetic properties in animals and humans and is currently in Phase II trials for metastatic estrogen receptor (ER)-positive breast cancer and non-small cell lung cancer.

Another agent derived from the heterocyclic compound quinoxalin (benzopyrazine) is XL147,[64] which is being tested in combination with other therapies including chemotherapy and targeted agents such as erlotinib and trastuzumab.

NVP-BKM120 is a pan-PI3K inhibitor that is currently in clinical development in multiple combinations. It was originally derived from the 2-morpholino-6-aminopyridyl-pyrimidine scaffold, based on the structure of PI3Kγ.[65] This dimorpholino pyrimidine derivative shows approximate equipotency toward all of the Class IA isoforms of PI3K and is currently in multiple Phase II and III trials for breast, prostate, endometrial, glioblastoma, and lung cancer, among others.

Although these early inhibitors have shown some promise in the treatment of cancer, since the elucidation of the PI3K crystal structure in 2007[7] the design of PI3K inhibitors has improved, yielding compounds of higher potency and selectivity. As a result, isoform-specific PI3K inhibitors that could potentially increase efficacy while limiting off-target toxicity have been developed and are now entering the clinic.[8]

The first reported PI3Kα specific inhibitor was the 2-aminothiazole-derivative NVP-BYL719 that inhibits the α isoform with an $IC_{50} \approx 5$ nM and shows similar potency toward the mutant versions H1047R and E545K.[66] In fact, both preclinical and clinical studies have shown that *PIK3CA* mutations are associated with response to PI3Kα inhibition with agents like NVP-BYL719,[67-70] and this compound is currently being tested in the clinic for breast, head and neck, and gastrointestinal tumors. Similarly, INK1117 (also known as MLN-1117) has been reported as a specific PI3Kα inhibitor and is currently undergoing Phase I trials.[71] Recently, a new molecule, GDC-0032, has been reported to be a PI3Kβ sparing inhibitor. This agent is a highly potent and selective inhibitor of PI3Kα and PI3Kδ ($IC_{50} \approx 0.3$ nM and 0.12 nM, respectively), but shows 3-fold greater potency toward the oncogenic mutants H1047R and E545K. GDC-0032 is currently in Phase I clinical trials and has shown promising results.[69] Finally, another notable drug is the PI3Kδ inhibitor CAL-101, a phenylquinazolin derivative that selectively inhibits the delta isoform of PI3K with an $IC_{50} \approx 70$ nM, compared to the other isoforms ($IC_{50} > 1$ μM).[72] Of note, this compound was the first PI3K inhibitor to be approved by the US Food and Drug Administration (FDA) and will be available for the treatment of chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), and follicular lymphoma (FL). In **Table 2**, we summarize the combinations with isoform-specific PI3K inhibitors that are currently under clinical investigation.

mTORC1 inhibitors: rapalogs

Much of our knowledge about mTORC1 comes from studies that elucidated the mechanism of action of rapamycin, an allosteric inhibitor that directly binds to mTORC1 and inhibits downstream phosphorylation of its substrates. Specifically, rapamycin binds to the immunophilin FKB12 to generate a highly specific complex that binds to the FKBP12-rapamycin-binding (FKB) domain of mTOR.[37]

In contrast to its effects on mTORC1, rapamycin cannot interact with mTORC2.[39] For this reason, mTORC1 is known as a rapamycin-sensitive complex and mTORC2 as a rapamycin-insensitive complex. This paradigm, however, may not be entirely accurate as it has been reported that prolonged treatment of U937 lymphoma cells and PC3 prostate cancer cells with rapamycin also inhibits mTORC2 activity.[73] This decrease in mTORC2 activity appears to be sufficient to inhibit AKT signaling.[34] Thus, rapamycin is considered to be a universal inhibitor of mTORC1 and seems to be a cell-type specific inhibitor of mTORC2 in a rapamycin time- and dose-dependent manner.

The physicochemical properties of rapamycin are not optimal for pharmacological development and as a consequence several analogs termed "rapalogs" have been developed. These analogs, such as temsirolimus, everolimus, and ridaforolimus, exhibit higher solubility and better pharmacokinetic properties than rapamycin.[75] Temsirolimus has also been used in combination with the aromatase inhibitor letrozole in patients with metastatic breast cancer in a Phase III study although the combination of drugs did not show a benefit over letrozole alone.[76]

Everolimus achieved regulatory approval for use in pancreatic neuroendocrine tumors based on a Phase III trial in which it prolonged progression-free survival when compared to best supportive care.[77] Another Phase III study demonstrated superiority for everolimus over placebo in patients with metastatic renal cell carcinoma (mRCC) with progression after vascular endothelial growth factor receptor-tyrosine kinase inhibitor therapy, leading to approval for this indication.[78] Everolimus has also been approved by the FDA for use in combination with anti-estrogen therapy in hormone-receptor positive HER2-negative breast cancer.[79] At the present time, ridaforolimus, formerly known as deferolimus, has no approved indications. All 3 agents are currently under investigation across many clinical trials.

Despite a plethora of preclinical data on rapamycin and its analogs, these molecules have not shown universal antitumor activity in clinical trials. This may be in part due to an important limitation of the rapalogs: the paradoxical increase in the AKT activity resulting from feedback loops triggered by mTORC1 inhibition. S6K (one of the key substrates of mTOR) inhibits IRS1, the adaptor protein linking the IGF-1 receptor and PI3K. This effect leads to a reduction of input into the PI3K pathway coming from the stimulation of the insulin/IGF-1 receptors. The inhibition of mTORC1 releases the S6K-IRS1-PI3K feedback inhibitory loop and results in increased AKT activity.[1] Thus, additional targeting of other key members of the pathway may be required to overcome the effects of this feedback for maximal efficacy in certain cellular contexts.

mTOR kinase inhibitors

The finding that mTORC2 has a direct role in the activation of AKT, combined with the limited clinical activity of rapalogs in many tumors, has led to the development of ATP-competitive inhibitors of mTOR kinase that potently inhibit both mTORC1 and mTORC2 complexes. Interestingly, these compounds have been shown to inhibit mTORC1 more potently than the rapalogs.[80,81] For example, the mTOR kinase inhibitor AZD8055 inhibits 4EBP1 phosphorylation more effectively than rapamycin and also effectively inhibits mTORC2 and AKT S473

Table 2. Combinations with isoform-specific PI3K inhibitors under clinical development

Drug	Target	Company	Phase	Combinations
CAL-101	p110δ	Gilead Sciences	II/III/IV	Chemotherapy (bendamustine), ofatumumab, rituximab, Bcl-2 inhibitor (GDC-0199).
GDC-0032	p110α	Novartis	I/II	Chemotherapy (docetaxel, paclitaxel), letrozole, fulvestrant.
GSK2636771	p110β	GlaxoSmithKline	I	
IPI-145	p110δ/γ	Infinity	I/II/III	Bendamustine, ofatumumab, rituximab.
MLN1117	p110α	Millennium	I/Ib	mTORi (MLN0128)
NVP-BYL719	p110α	Novartis	I/II	Chemotherapy (paclitaxel, gemcitabine, capecitabine), everolimus, RTKi (cetuximab, LJM716, AMG479, BGJ398), letrozole, T-DM1, encorafenib, MEK162, Hsp90i (AUY922), CDKi (LEE011), PIMi (LGH447).

phosphorylation.[82] However, the inhibition of AKT signaling seems to be transient, as inhibition by AZD8055 causes activation of RTKs, which in turn induce PI3K signaling and reactivate AKT activity and signaling.[82] Combined inhibition of mTOR kinase and RTKs fully abolishes AKT signaling resulting in tumor regression.[83]

INK128 is another mTORC1/2 inhibitor for which *in vitro* and *in vivo* data have demonstrated successful inhibition of mTORC1 (S6K and 4EBP1) and mTORC2 (AKT at S473).[84] Interestingly, this agent has also shown marked activity in cell lines that are resistant to rapamycin and pan-PI3K inhibitors.[71] A Phase I trial testing the activity of this molecule is ongoing in patients with solid tumors. Other compounds that aim to inhibit both mTOR complexes and potentially have a more profound antitumor activity than rapalogs are AZD2014, CC-223, and OSI-027.

Combinations of PI3K Inhibitors with RTK Inhibitors

HER2-targeted therapies have produced clinical improvements in patient survival, both in the adjuvant and metastatic setting. However, despite initial responses the emergence of resistance occurs in the vast majority of patients with metastatic disease. Activation of the PI3K pathway, either by loss of PTEN or by the presence of *PIK3CA* mutations, is perhaps the most widely accepted mechanism of resistance to anti-HER2 therapy.[84-88] Activating mutations in *PIK3CA* occur in approximately 25–30% of HER2-amplified breast cancers (TCGA) and may be important for response to HER2-targeted therapies. For example, introduction of a *PIK3CA* H1047R activating mutation in HER2-driven mammary tumors in MMTV/neu transgenic mice accelerates tumor onset and progression, and generates resistance to anti-HER2 therapy.[89] This evidence paved the way to investigate the efficacy of therapeutic strategies combining anti-HER2 agents with PI3K inhibitors. In preclinical models, this strategy proved to be synergistic in cells or tumors resistant to anti-HER2 therapy.[84,86,90–92] In a clinical setting, trastuzumab in combination with everolimus showed promising antitumor activity in heavily pretreated patients who had progressed to trastuzumab-based therapy.[93-95] Further, in a recent study testing the effect of the PI3K inhibitor NVP-BKM120 in a similar population, researchers reported clinical responses in patients with tumors possessing an activated PI3K pathway.[96] Additional trials testing other PI3K inhibitors in combination with anti-HER2 agents are currently ongoing.

Interestingly, a recent study highlighted the need to design triple inhibition strategies to effectively delay and/or avoid tumor relapse, especially in patients with large tumor burdens.[97] Although toxicity remains a limiting factor when translating these combinatorial therapeutic strategies to patients, concomitant treatment of HER2-driven cancers with synergistic combinations targeting the PI3K/AKT pathway and additional driver events might render cancer cells vulnerable to efficient eradication and lead to curative regimes for patients.

Treatment with PI3K/AKT inhibitors leads to upregulation/activation of RTKs such as EGFR, HER3, and IGFR1 that, in turn, can limit the antitumor effects of these therapies by increasing PI3K signaling or triggering the activation of other compensatory pathways.[2,4,82,98] This provides the rationale to test combinations of PI3K inhibitors with agents that block the activity of RTKs. Due to space limitations we are unable to list the large (and increasing) number of preclinical studies conducted in this regard. Likewise, many clinical trials testing the efficacy of PI3K inhibitors and anti-RTK agents (including EGFR, HER2, HER3, FGFR, IGFR, and CD20) have been launched. Details are available at www.clinicaltrial.gov and are summarized in **Table 1** and **Table 2** .

Combinations of PI3K Inhibitors with Endocrine Therapy

Up to 75% of breast tumors are hormone receptor (HR)-positive, expressing estrogen receptor (ER), progesterone receptor (PR), or both. These nuclear receptors are both targets and predictors of response to anti-estrogen therapy such as selective estrogen receptor modulators (SERMs), aromatase inhibitors (AIs), or selective estrogen receptor degraders (SERDs). Despite the fact that HR-positive breast tumors generally have a favorable prognosis and effective therapies exist, up to one-third of women diagnosed with this type of cancer will relapse after 5 years of adjuvant therapy with tamoxifen[99] and up to 20% of women treated with adjuvant AIs will undergo recurrence 10 years after the initial diagnosis.[100] Several mechanisms of resistance to hormone therapy have been proposed[101]; however, a common theme emerging from many of these studies is the importance of the activation of growth factor receptors in endocrine resistance.

Given that PI3K is the most frequently altered pathway in ER^+ breast tumors, deregulation of the PI3K pathway through

activation of upstream receptor tyrosine kinases or mutations in the downstream effectors as a potential mediator of endocrine resistance has been one of the main research areas. For example, it has been described that upregulation of ERBB2 (which signals mainly through the PI3K pathway) mediates resistance to anti-estrogen therapy[102,103] thus establishing the rationale for the combination of anti-estrogen and anti-HER2 therapy.[104] However, constitutive activation of PI3K does not seem to confer this resistant phenotype.[105-107] Despite the lack of a correlation between response to anti-estrogen therapy and PI3K alterations, there is preclinical evidence pointing to the benefit of a combination of inhibitors of ER and the PI3K pathway.[108,109] In fact, we have reported that PI3K pathway inhibition upregulates ER transcriptional activity and increases cell survival dependency on ER, which translates into a synergism between PI3K inhibitors and ER degraders with increased tumor control.[110] Furthermore, the Bolero-2 trial highlighted that inhibiting both mTOR and ER signaling with the combination of everolimus and exemestane has a superior effect in terms of progression-free survival.[79] Also, early-phase clinical trials introducing novel drugs that directly target PI3K have demonstrated that combination strategies have an excellent response rate in selected patients.[111,112]

The PI3K and androgen signaling axes are also critical drivers of the pathogenesis of prostate cancer and the 2 most commonly activated pathways in this disease. Mutation or deletion of *PTEN* and amplification of *PIK3CA* are 2 of the most common genetic aberrations observed in prostate cancer genomics studies,[113,114] and virtually all untreated primary prostate cancers initially respond to androgen deprivation therapy. Further, it has been demonstrated that these 2 signaling cascades reciprocally regulate one another, such that inhibition of one pathway induces activation of the other.[115] Thus, there is great interest in targeting these pathways simultaneously in prostate cancer. PI3K pathway inhibitors and androgen-targeted therapies have shown promising activity in preclinical studies,[115,116] and several early-phase clinical trials are ongoing.

Dual PI3K/mTOR blockade

An interest in targeting multiple important components in the PI3K/AKT/mTOR pathway has encouraged the development of dual inhibitors that might suppress the growth, proliferation, and survival of cancer cells. The activity of these compounds differs from that of rapalogs or dual mTOR inhibitors, as they are able to block both mTOR complexes in addition to the PI3K isoforms in an ATP-competitive manner. Several mTOR/PI3K dual inhibitors, such as SF1126, NVP-BEZ235, XL765, GDC-0980, PF-04691502, PKI-587, GSK2126458, and PWT3359, have shown activity in preclinical models and are in early-stage clinical trials. For example, NVP-BEZ235 has been reported to inhibit tumor growth in preclinical models of prostate, breast, pancreatic, and renal cancers, multiple myeloma, and sarcomas.[117] Other studies have shown that breast cancer cell lines with HER2 amplification and/or *PIK3CA* mutations are highly sensitive to NVP-BEZ235.[90] These and other findings have allowed NVP-BEZ235 to enter Phase I/II clinical trials in patients with advanced solid tumors, including breast cancer.[117] XL765 is a PI3K/mTOR inhibitor that showed pathway inhibition and a reduction in cell proliferation in a Phase I dose-escalation study of patients with solid tumors.[118] Another dual PI3K/mTOR inhibitor, GDC-0980, has demonstrated broad preclinical activity in breast, ovarian, lung, and prostate cancer models.[119] It has also been shown to be active against tumor cells bearing mutations in PI3K, PTEN, or KRAS[120] and is currently in Phase I clinical development.

Recent work has demonstrated that mTORC1 inhibition is required for sensitivity to p110α inhibitors in *PIK3CA*-mutant breast cancer.[67] This study demonstrated that breast cancer cell lines and patient tumors that are resistant to the PI3K inhibitor NVP-BYL719 have active mTORC1 signaling. Thus, sustained mTORC1 activation may limit the effects of NVP-BYL719 on tumor growth. This hypothesis was confirmed by the demonstration that adding everolimus to NVP-BYL719 resulted in reversal of resistance both *in vitro* and *in vivo*.[67]

Additionally, work from our laboratory showed that progressive loss of PTEN leads to clinical resistance to the PI3Kα inhibitor NVP-BYL719. Specifically, loss of PTEN in NVP-BYL719-sensitive cell lines and in patients that progress to this agent leads to resistance to PI3Kα inhibition. Since PTEN-deficient preclinical models mostly rely on the p110β subunit of the PI3K holoenzyme, concomitant inhibition of p110α and p110β isoforms of PI3K resensitizes the cells to NVP-BYL719.[121]

Combination of PI3K and MEK inhibitors

The RAS/RAF/MEK/ERK pathway is frequently dysregulated in human cancer and alterations in this pathway have been found to lead to tumorigenesis and resistance to several therapies. The PI3K/AKT/mTOR and RAS/RAF/MEK/ERK pathways are known to interact at multiple levels. Examples of this crosstalk include direct activation of PI3K by RAS via its interaction with the catalytic subunit of PI3K[122] and phosphorylation of TSC2 by ERK that suppresses TSC2 function and promotes activation of mTORC1.[123] Moreover, the 90-kDa ribosomal S6 kinase (RSK), which lies downstream of the RAS-ERK pathway, was found to phosphorylate TSC2 at Ser1798 and inactivate its tumor suppressor function leading to mTORC1 signaling and increased translation.[124] RSK was also reported to phosphorylate Raptor, providing another link between RSK and the mTOR pathway.[125]

The interaction between the PI3K/AKT/mTOR and RAS/RAF/MEK/ERK pathways may explain the modest single-agent activity of agents targeting one of these pathways. Thus, to achieve optimal anticancer effects, dual blockade of these pathways may be necessary. Indeed, studies of dual pharmacological inhibition of these pathways have shown that combination treatment increases antiproliferative activity. For example, the dual pan-PI3K and mTOR inhibitor NVP-BEZ235 in combination with a MEK inhibitor show successful synergy in shrinking KRAS-mutant lung cancers or EGFR-mutant lung cancers.[126,127] Furthermore, targeting of PI3K/mTOR in combination with MEK inhibitors was shown to be necessary to effectively inhibit growth of NRAS-mutant melanoma cells *in vitro* and *in vivo*.[128] Several Phase I and II clinical trials examining PI3K inhibitors plus MEK inhibitors in patients with advanced solid tumors are

currently undergoing or complete[129] (**Table 1**). Examples include studies of a MEK inhibitor (MEK162) plus a PI3K inhibitor (BKM120 or BYL719) in adult patients with advanced solid tumors and studies of a MEK inhibitor (GSK1120212) in combination with a AKT inhibitor (GSK2110183) in patients with solid tumors and multiple myeloma (www.clinicaltrials.gov) (**Table 1**).

Combination of PI3K inhibitors with DNA damaging agents

Aberrations in DNA damage repair (DDR) and cell cycle checkpoint proteins are present in nearly all cancers, subverting standard cell cycle arrest mechanisms and allowing genomic aberrations to persist unrepaired. Furthermore, DNA damaging therapies such as chemotherapy and radiotherapy are cornerstones of cancer therapy and are administered to the vast majority of cancer patients at some point during their disease course. The DNA damage signaling and PI3K signaling networks intersect at multiple nodes,[130] and several preclinical[131 -134] and clinical[135] studies have supported the notion that DNA damage activates PI3K/AKT pathway signaling. Further, activation of PI3K signaling has been implicated in the promotion of chemo- and radioresistance.[136 -138] Therefore, it has been hypothesized that combining therapies targeting the PI3K-AKT signaling axis with standard DNA damaging therapies in PI3K-driven tumors may induce synergistic cancer cell killing.

Perhaps the most widely studied PI3K signaling protein involved in the DDR is AKT. Numerous studies have demonstrated that AKT is phosphorylated on S473 within minutes of DNA damage.[42,139,140] Furthermore, AKT S473, but not AKT T308, accumulates at foci of DNA damage.[43,139] However, the exact mechanism of AKT activation following DNA damage and its specific role in the DNA repair cascade is controversial and may vary depending on the cell's genetic and epigenetic profile. Several studies have demonstrated that the phosphorylation of AKT on S473 is dependent on DNA-PK.[43,140,141] On the other hand, other studies have suggested that induction of AKT S473 following radiation is independent of both DNA-PK and PI3K signaling, but instead is dependent on the activation of ATM.[139] Another possible mechanism of AKT activation is provided by multiple studies demonstrating that radiation can induce activation of all 4 ErbB receptors, and therefore downstream PI3K signaling, in a ligand-independent manner.[142,143]

Given the evidence that AKT is activated by DNA damage, several studies have investigated the specific role of AKT in the DDR. In one study, AKT1 was found to form a complex with DNA-PKcs following DNA damage, resulting in enhanced activation and autophosphorylation of DNA-PKcs at S2056, thus allowing DNA-PKcs to dissociate from sites of DNA damage and other critical downstream DNA damage repair proteins to access the DNA damage sites.[144] Additionally, AKT phosphorylates multiple downstream targets, and several of these have been shown to participate in the DDR. For example, one study demonstrated that following DNA damage AKT2 mediates phosphorylation and inhibition of GSK-3β, an enzyme important for phosphorylating and activating the p53 inhibitor MDM2.[140] Further, AKT2 knockdown (but not PI3K knockdown) markedly reduced p53 accumulation in response to radiation.[140] AKT-mediated inhibition of GSK-3β has been also shown to upregulate expression of MRE11, a component of that MRN complex that is one of the earliest proteins recruited to sites of DNA double-strand breaks.[145]

However, the relationship between AKT and DNA damage signaling is complex, and many other targets that are inhibited by AKT actually enhance DNA damage checkpoints and repair. For example, FOXO3A has been shown to directly associate with ATM, p53, and Chk2, enhancing the activation of these proteins,[146,147] and to regulate both the G1[148] and G2/M[149] DNA damage checkpoints. Thus, FOXO3A inactivation through AKT phosphorylation might be expected to impair the DDR. Further, AKT has also been shown to inhibit Chk1[150] and Wee1[151] through inhibitory phosphorylations on S280 and S642, respectively, thus promoting progression through the cell cycle. It is unclear, therefore, whether hyperactivation of AKT signaling can abrogate the ability of cells to arrest at the G2/M checkpoint and repair DNA damage prior to mitosis.

Despite these complexities, most preclinical evidence suggests that the net effect of AKT loss is increased sensitivity to DNA damaging agents. For example, $AKT^{-/-}$ mice exhibit decreased survival following total body irradiation compared with their AKT-proficient littermates.[152] Similarly, $AKT^{-/-}$ thymocytes are more susceptible to irradiation-induced apoptosis than wild-type thymocytes.[152] Several studies have also shown that AKT1 knockdown sensitizes cancer cells to radiotherapy.[134,139,145,153]

Given the extensive evidence that PI3K signaling is induced following DNA damage, for many years there has been interest in using PI3K inhibitors to sensitize to DNA damaging therapies. In fact, multiple studies have demonstrated that pharmacologic inhibitors of the PI3K pathway are capable of sensitizing cancer cells to DNA damaging therapies *in vitro* and *in vivo*.[83,134,144,154–156] However, the vast majority of these studies relied heavily on PI3K pathway inhibitors like LY294002, wortmannin, PI103, and especially NVP-BEZ235, which have significant activity against DNA-PK and lower activity against ATM and ATR.[157,158] In fact, in a study from Kevan Shokat's group, inhibitors of PI3K were found to frequently inhibit DNA-PK, and p110α was found to cluster with DNA-PK in target space by structural activity relationship analysis despite sharing limiting sequence homology.[158] Additionally, 5 of the 6 chemotypes that inhibited p110α with an IC_{50} lower than 5 μM also potently inhibited DNA-PK in this study. Given that DNA-PK plays a central role in the DDR, particularly in non-homologous end joining, caution should be used when drawing conclusions from studies that rely predominantly on these inhibitors to demonstrate enhanced efficacy from PI3K pathway inhibition and DNA damaging agents compared to either alone. Studies using more selective p110α inhibitors with limited activity against DNA-PK, such as NVP-BYL719 and GDC-0032, will help define the relationship between PI3K inhibition and sensitivity to DNA damaging agents.

Two recent studies have demonstrated a direct role for PI3K in homologous recombination via regulation of BRCA1 expression,[159]

thereby inhibiting recruitment of Rad51 to foci of DNA damage[160] in patients with triple-negative breast cancer. These findings have led to a Phase I clinical trial combining the pan-PI3K inhibitor NVP-BKM120 and the PARP inhibitor olaparib in patients with triple-negative breast cancer or high-grade serous ovarian cancer.[161] However, in addition to the role of p110α in the DNA damage response, activation of p110β by DNA damage seems to play a critical role in recognition of DNA double-strand breaks and recruitment of NBS1 to sites of DNA damage.[162] Loss of p110β leads to impaired recruitment of downstream DNA damage repair mediators such as ATM, RAD17, γ-H2AX, and 53BP.[162]

In contrast to AKT, several reports have shown that mTORC1 signaling is actually downregulated as a result of DNA damage.[163-165] Oxidative stress was shown to downregulate mTORC1 signaling by activating ATM, leading to a phosphorylation chain resulting in sequential activation of LKB1, AMPK, and finally TSC2.[165] Given that DNA damage is also a major regulator of ATM signaling, the mechanism of DNA damage-induced downregulation of mTORC1 signaling may be similar. This suggests that both AKT and ATM signaling may converge on TSC2, and that mTORC1 output may depend on the relative contribution of both signals.

In summary, the relationship between DNA damage repair and PI3K signaling is complex and bidirectional, with each network influencing the other in multiple ways. However, the bulk of evidence supports the notion that DNA damage enhances AKT activation, thereby leading to activation of a signaling network that generally promotes survival following DNA damaging therapies like radiation and chemotherapy. Although the use of selective PI3K inhibitors to enhance the efficacy of DNA damaging agents represents a promising approach for some cancers, it is imperative to define molecular predictors for which patients are most likely to benefit from this approach in order to maximize the chance that this approach will be successful in the clinic.

Concluding Remarks

Although the precise biology and the mechanisms of oncogenicity of the PI3K/AKT pathway are still under investigation, this signaling cascade is an attractive target for cancer therapy because of the high frequency of aberrations seen across a wide spectrum of cancers. Nevertheless, PI3K inhibitors have limited efficacy when used as a single agent, in part due to the activation of compensatory pathways. Thus, combinatorial strategies seem to be the best approach in order to obtain the maximal therapeutic advantage from these agents.

In this review, we have discussed what we believe are some of the most promising strategies for combinatorial therapy with PI3K inhibitors. However, it should be mentioned that this field is continuously evolving with the development and testing of new combinations that we have not specifically discussed. Such approaches include cosuppression of cyclin-dependent kinases,[166,167] Smoothened,[168] BCL-2,[169] histone deacetylase,[170] Janus kinase,[171] heat shock protein 90,[172] and many others.

Ultimately, given the intra- and inter-tumoral molecular heterogeneity underlying cancer, a single specific combinatorial strategy is unlikely to be uniformly effective for all patients. Thus, understanding which tumors are most prone to respond to specific combinations of PI3K inhibitors with other targeted agents will be critical to their successful implementation in the clinic.

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No potential conflicts of interest were disclosed.

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