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Authors
Fruman, David A
Rommel, Christian

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PI3K and Cancer: Lessons, Challenges and Opportunities

David A. Fruman [1]* & Christian Rommel [2]*

[1] Department of Molecular Biology & Biochemistry, and Institute for Immunology, University of California, Irvine, Irvine, CA 92697 USA. [2] Amgen, Inc., Thousand Oaks, CA 91320 USA

* Address correspondence:

David A. Fruman, University of California, Irvine, Department of Molecular Biology & Biochemistry, 3242 McGaugh Hall, Irvine, CA 92697-3900 Tel: 949-824-1947 Fax: 949-824-8551 dfruman@uci.edu

OR

Christian Rommel, Amgen, Inc., One Amgen Center Drive, CA 91320-1799, Tel: 805-447-2022, crommel@amgen.com

Summary/Abstract

The central role of phosphoinositide 3-kinase (PI3K) activation in tumor cell biology has prompted a sizeable effort to target PI3K and/or downstream kinases such as AKT and mTOR in cancer. However, emerging clinical data show limited single agent activity of PI3K/AKT/mTOR inhibitors at tolerated doses. One exception is the response to PI3Kδ inhibitors in chronic lymphocytic leukemia, where a combination of cell-intrinsic and -extrinsic activities drive efficacy. Here we review key challenges and opportunities for clinical development of PI3K/AKT/mTOR inhibitors. Through a greater focus on patient selection, increased understanding of immune modulation, and strategic application of rational combinations, it should be possible to realize the potential of this promising class of targeted anti-cancer agents.

Introduction

The signaling network defined by PI3K, AKT and the mechanistic target of rapamycin (mTOR) controls most hallmarks of cancer: cell cycle, survival, metabolism, motility, and genomic instability [1]. The pathway also contributes to cancer-promoting aspects of the tumor environment such as angiogenesis and inflammatory cell recruitment (**Fig. 1**) [2-4]. The lipid second messenger produced by PI3K enzymes, phosphatidylinositol-3,4,5-trisphosphate (PIP_3), is elevated constitutively in most cancer cells and recruits cytoplasmic proteins to membrane-localized "onco" signalosomes [5,6]. The oncogenic signaling proteins recruited in this way include members of the AGC kinase family (e.g. AKT, Fig. 1), TEC family tyrosine kinases, and various modulators of small GTPase activity [7]. Cancer genetic studies suggest that the pathway is the most frequently altered in human tumors: the *PIK3CA* gene encoding the PI3K catalytic isoform

p110α is the second most frequently mutated oncogene, and *PTEN* encoding the major PIP_3 phosphatase is among the most mutated tumor suppressor genes [8,9]. In accord, a recent genomic study of head and neck cancer found the PI3K pathway to be the most frequently mutated [10]. Indeed, even in cancer cells expressing normal PI3K and PTEN genes, other lesions are present that activate the PI3K signaling network (i.e. activated tyrosine kinases, RAS, AKT; loss of LKB1 (STK11), INPP4B, TSC) [11]. This strong genetic evidence and the druggability of various components in the network provided the original rationale and excitement for targeting PI3K/AKT/mTOR signaling in oncology. The signaling network was seen as an opportunity to combat tumor complexity and genomic heterogeneity through a central, common oncogenic driver fundamental to all cancer cells. However, counterbalancing this opportunity is the challenge of targeting enzymes that are also active and critical in normal cells and tissues.

Groundbreaking structural studies of PI3K enzymes [12-18], together with extensive medicinal chemistry efforts [19-21] have led to the discovery of compounds targeting one or more nodes in the network. Several of them harbor favorable drug properties and suppress tumor growth in preclinical models of cancer [20-24]. The challenge is to translate these findings into a meaningful activity at acceptable tolerability in cancer patients. The early results from trials in advanced solid tumors are rather sobering, showing limited single agent activity of PI3K and mTOR inhibitors [25, 222], especially when compared to agents targeting driver oncogenes such as *BCR-ABL*, *ALK* or *BRAF*. Pharmacology plays an important part in clinical efficacy, in that doses high enough and over a long enough exposure period to achieve cancer eradication might not be tolerated due to mechanism based on-target toxicities. Yet the pathway itself might not be as essential to cancer cells as originally proposed, at least at an advanced stage of tumorigenesis. Indeed, blockade of the pathway generally fails to induce cancer cell death and selects for

compensatory pathways that maintain survival and restore tumor growth [26-28]. Furthermore, refinement of genetically engineered mouse models suggests that *PIK3CA* mutants expressed at endogenous levels do not strongly drive tumor development like some other oncogenes [29,30]. In essence, "oncogene addiction" to PI3K/AKT/mTOR signaling is not absolute. Therefore, unleashing the full potential of PI3K/AKT/mTOR inhibitors in oncology will require earlier treatment, dose/schedule optimization, and rational combinations with other therapeutic approaches.

It will also be important to identify biomarkers that can guide patient selection, and to determine which tumor types/genetic profiles benefit from blocking single nodes/isoforms versus multiple targets. Encouragingly, the p110δ-selective inhibitor GS-1101 (formerly CAL-101 and currently in phase 3 development) produces dramatic responses in some B cell malignancies [31,32]. This proves the principle that a potent and selective PI3K inhibitor can improve survival of selected cancer patient populations. Yet, GS-1101 has an unusual mechanism of action: the drug is not directly cytotoxic to malignant B lymphoma cells and its efficacy arises in part from modulating the tumor immune environment [31,32,223]. This illustrates the importance of understanding PI3K pathway biology in immune cells and in physiological models of tumor immunity (or immunology). The success of antibody therapies targeting immune checkpoints (CTLA-4, PD-1) [33,34] emphasizes the potential of targeting immune-inhibitory pathways in cancer and the importance of evaluating immune effects of small molecule kinase inhibitors.

The goal of this review is to reset both expectations and directions. Our understanding of the complexity of the PI3K/AKT/mTOR signaling network and its role in cancer has significantly increased, establishing the pathway as a challenging yet viable target in oncology. Much can be learned from clinical failures and the limited successes to date, to chart a course for next

generation strategies. In our opinion, enthusiasm and commitment towards targeting such an important pathway in cancer should not be reduced.

Overview of the PI3K/AKT/mTOR signaling network

Key features of the PI3K/AKT/mTOR signaling network that illustrate both the promise and the challenges for targeting the pathway in cancer have been previously discussed [11,21,35,36]. Below, we will provide a brief overview of the functions and signaling mechanisms of members of the family of PI3K enzymes, highlighting their roles in cancer and issues faced in therapeutically targeting them.

There are eight mammalian PI3K enzymes, grouped into three classes [36]. The most important in cancer are the four class I enzymes, termed PI3Kα, PI3Kβ, PI3Kγ and PI3Kδ. These are heterodimers of a catalytic subunit of 110 kDa (p110α, p110β, p110γ or p110δ) and a regulatory subunit. The catalytic isoforms share considerable sequence homology and produce the same lipid product (PIP_3), and each can receive activation inputs from both tyrosine kinases and from GTPase signaling [36,37]. However, the details of these inputs differ (see *Box 1*). The distinct activation mechanisms of the class I PI3K isoforms suggest that each has unique biological functions, a model supported by abundant evidence from targeted gene inactivation in mice [36,38-41]. It follows that targeting single isoforms might have therapeutic effects. On the other hand, functional redundancy in maintaining cell survival has been documented in various cell types including cancer cells [42]. Furthermore, mouse genetic models have caveats and do not always accurately predict the response to acute target inhibition by pharmacological agents.

Of the four class I catalytic isoforms, only *PIK3CA* (encoding p110α) is frequently mutated in human cancer [8,11]. Although many *PIK3CA* mutations exist, there are two hotspots that cause

elevated PI3K enzyme activity through distinct mechanisms [15]. Transforming mutations in the ubiquitously expressed gene *PIK3CB* (encoding p110β) are rare [43], perhaps due to this isoform's distinct mode of interaction with regulatory subunits [18]. Mutations in class I regulatory subunit genes (*PIK3R1* or *PIK3R2*) are also found in cancer cells and cause increased PI3K activity [44,45]. In cell transformation assays, p110α plays a dominant role in the oncogenic potential of *PIK3R1* mutants [46]. This observation provides further support for a unique role of the p110α isoform in tumorigenesis. In addition, p110α has a cell-extrinsic role in tumor angiogenesis (**Fig. 1**) and possibly stromal fibroblasts [3,4], another potential advantage for targeting this isoform. In cancer cells with wildtype PI3K genes, there are usually oncogenic lesions in upstream tyrosine kinases (TKs) and/or RAS that cause constitutive signaling through PI3K [11]. Loss of lipid phosphatases *PTEN* and/or *INPP4B* is an alternative path to elevated PI3K lipid products, but inactivation of these tumor suppressors is not mutually exclusive with mutations in PI3K or RAS [44,47]. Indeed, a mouse model demonstrated that loss of *PTEN* cooperates with *PIK3CA* mutations to cause ovarian tumors [29]. *PIK3CA* mutations or *PTEN* loss can also co-exist with oncogenic TKs [48,49].

The mTOR serine/threonine kinase functions at two distinct nodes in the PI3K signaling network (**Fig. 2A**) [50,51]. mTOR complex-2 (TORC2) phosphorylates key residues to activate AKT and other kinases. TORC2 appears to have basal activity that is stimulated by growth factors and through association with ribosomes [52]. mTOR complex-1 (TORC1) is a central regulator of cellular metabolism and biosynthesis, and is subject to complex regulation by growth factors, nutrients and cellular stresses [51]. When conditions are favorable for cell growth, TORC1 phosphorylates several substrates to promote anabolic processes (ribosome biogenesis, translation, and synthesis of lipids and nucleotides) and suppress catabolic processes (e.g. autophagy) [51]. One of the key control nodes for TORC1 activity is the TSC complex containing

TSC1, TSC2 and TBC1D7 proteins [53,54]. By phosphorylating TSC2, AKT suppresses the inhibitory effect of the TSC complex on TORC1. Although the *MTOR* gene is not frequently mutated in human tumors, there is evidence for "non-oncogene addiction" to mTOR function in cancer cells. For example, tissue specific deletion of mTOR in mouse prostate inhibits tumor formation driven by PTEN loss without disrupting normal prostate tissue [55,56]. Also, mTOR catalytic inhibitors can achieve anti-leukemic effects at doses that preserve the function of normal bone marrow and peripheral lymphocytes [57,58].

Feedback control is a common feature of cellular signaling systems, and the PI3K/mTOR network provides many examples (**Fig. 2A**). An important consequence of feedback is that inhibitors of AKT or mTOR tend to cause elevated expression and activity of growth factor receptors, leading to increased PI3K activity and RAS signaling, and alternative survival pathways in cancer cells [59,60]. There are several potential strategies to overcome the "rebound" signaling in response to PI3K/AKT/mTOR inhibitors, including vertical inhibition of several signaling nodes and combination approaches.

There is also crosstalk between elements of the PI3K signaling network and components of other oncogenic pathways (**Fig. 2B**). A key consequence is that PI3K and AKT are not the dominant regulators of TSC1/2 and TORC1 in some cells. ERK and RSK are two effector kinases downstream of RAS that can promote TORC1 activity by phosphorylating TSC2 on residues distinct from AKT phospho-acceptor sites [61-63]. GSK3 and AMPK can also phosphorylate TSC2 [64]. Another example of crosstalk is that ERK and TORC1 provide distinct and complementary inputs to eIF4E, a central regulator of cap-dependent mRNA translation [65,66]. The PI3K/AKT/mTOR and RAS/RAF/MEK/ERK networks also converge to stabilize protein expression of the MYC oncoprotein [67]. Therefore, oncogenic compensation by RAS can severely

limit the anti-cancer efficacy of PI3K/AKT/mTOR inhibitors. Conversely, active PI3K signaling is likely a central mechanism of resistance to various targeted therapies.

Clinical trial results and associated challenges

There are six general classes of agents in clinical trials that target the PI3K/AKT/mTOR network: pan-class I PI3K inhibitors, isoform-selective PI3K inhibitors, rapamycin analogs (rapalogs), active-site mTOR inhibitors, pan-PI3K/mTOR inhibitors, and AKT inhibitors. Supplementary **Table 1** lists many of the compounds currently in oncology clinical trials according to clinicaltrials.gov. Rapalogs are not broadly effective as single agents, though they have obtained FDA approval for treatment of a few tumor types where modest therapeutic effects can be achieved. Clinical trial data for the other five classes remain largely unpublished; however, results presented at conferences allow some preliminary conclusions. The most impressive results have been achieved with the p110δ-selective inhibitor GS-1101 (Idelalisib), which causes dramatic responses in chronic lymphocytic leukemia (CLL) and certain other B cell malignancies (*Box 2*). Overall, other agents targeting PI3K/AKT/mTOR have not yielded broad responses when given to advanced cancer patients at tolerated doses. By comparison, early trials of currently approved drugs targeting oncogenes like BCR-ABL, mutant B-RAF or ALK revealed marked single agent activity even in phase I trials, albeit in prospectively selected patient populations. A recent review by Tabernero and colleagues provided a detailed discussion of emerging clinical trial data for PI3K pathway inhibitors, including safety profiles and pharmacodynamic markers [25]. Below we highlight four central challenges/issues in the field of PI3K/AKT/mTOR drug development arising from clinical studies so far.

Pan-PI3K versus isoform-selective inhibition

Several pan-class I PI3K inhibitors in clinical trials target all four class I PI3K isoforms with similar potencies (Supp **Table 1**). The main argument for pan-PI3K inhibitors is that most cancer cells express multiple PI3K isoforms with redundant functions in oncogenic signaling [42]. Another factor driving early development of pan-PI3K compounds was that these efforts proceeded before PI3K isoform structures were available to aid the design of isoform-selective compounds. However, pan-PI3K inhibitors are blunt tools that are not specifically aligned with the disease biology and context. The main concern with pan-PI3K inhibitors is that doses needed to fully block all class I PI3Ks for extended periods might not be tolerated. For this reason, it is possible that trials to date have missed an "all or nothing" threshold for tumor responses due to dose-limiting toxicities. A related concern is that the first-in-class compounds that have entered oncology trials are not sufficiently selective for PI3K. Compared to isoform-selective inhibitors, compounds targeting all class I PI3Ks seem more commonly to have off-target effects on members of the PI3K-related kinase (PIKK) family (mTOR, DNA-PK, ATM, ATR) and other cell components. For example, BKM120 at concentrations needed to fully inhibit PI3K (5X the IC50) has off-target effects on tubulin and causes general cellular toxicity [68]. Filling the competitive landscape with inadequate compounds might have discouraged later entry of "best-in-class" agents with the needed selectivity to deliver on the potential of the target biology.

Isoform-selective PI3K inhibitors (Supp **Table 1**) have the potential to block the relevant target more completely, while limiting toxicities associated with broader inhibition profiles. Indeed, GS-1101 is well tolerated in most patients at doses that maintain drug exposure levels sufficient to suppress p110δ activity to a level that translates into anti-tumor activity [32]. Yet the therapeutic activity of p110δ inhibitors was unexpected. These compounds deviate from the traditional paradigm for targeting a kinase that is required for the cancer cell but not its normal

counterpart. In this case, the target (*PIK3CD*) is not mutated in cancer but is required for survival of normal B cells (**Fig. 1**). GS-1101 efficacy derives from an unusual confluence of factors: a very selective drug with a target whose expression is restricted, and a dual role for the target in the cancer cell and the tumor immune environment. This emerging paradigm for leukemia/lymphoma treatment also applies to Ibrutinib, an inhibitor of the tyrosine kinase BTK that acts downstream of p110δ in B cells [69,224].

Other than p110δ inhibitors, the most advanced isoform-specific compounds are selective for p110α (Supp **Table 1**). The prevalence of *PIK3CA* mutations in human cancer provides a potentially rapid and cost-effective development path analogous to BRAF or ALK inhibitors. Yet questions remain about the best patient selection strategy for p110α-selective inhibitors. One approach is to design "basket" trials grouping patients with *PIK3CA* mutant tumors across several histologies, and let the data guide expanded trials. This idea builds on experience attained from the use of BRAF inhibitors, which provided efficacy in BRAF mutant melanoma but not colorectal cancer [70,71]. Similarly, GS-1101 efficacy in CLL emerged from empirical testing in a broad range of B cell malignancies [31,32]. Another approach is to include tumor types that are *PIK3CA* wildtype but in which p110α plays a critical signaling role (e.g. HER2, KRAS, PIK3R1) [46,72,73]. With either approach, drugs targeting p110α should be tested in patients at an earlier stage of disease with less tumor complexity and reduced toxicity load of prior treatments.

There is also an opportunity, so far untapped, to develop irreversible p110α inhibitors, as this is the only isoform with a reactive cysteine residue near the ATP binding site [74]. The clinical success of the covalent BTK inhibitor Ibrutinib provides encouragement for this pharmacological approach [69,224]. Another way to improve the therapeutic index might be to develop inhibitors that are selective for the common *PIK3CA* "hotspot" mutant enzymes such as H1047R, E542K and

E545K. However, such compounds would lose cell-extrinsic activity (e.g. angiogenesis), they would not act on wild-type p110α downstream of receptors and RAS, and they might select for other mutants. Agents targeting hotspot *PIK3CA* mutants might find an alternative use in the treatment of inherited overgrowth syndromes caused by somatic *PIK3CA* mutations [75-77]. Another clinical use of isoform-selective agents outside of oncology might be p110δ inhibitors in patients with newly identified immunodeficiency syndromes caused by activating mutations in *PIK3CD* [78,79].

Some studies suggest that p110β activity is essential in cancer cells lacking PTEN (**Fig. 1**), particularly in prostate and breast cancer [80-82], suggesting that p110β inhibitors would be more effective than p110α inhibitors in patients with PTEN-deficient tumors. However, another study reported that p110α and p110β have overlapping functions in various PTEN-deficient tumor models [83]. p110α also has the aforementioned role in tumor angiogenesis [3,4]. Ultimately, the success of targeting p110β alone in PTEN mutant advanced tumors might depend on whether the tumor also harbors mutations in upstream receptors or RAS that activate p110α.

Arguments can be made for compounds targeting two of the four class I isoforms, and now it seems technically feasible due to advances at the level of structural biology and medicinal chemistry. A dual p110α/p110β inhibitor might work in tumors lacking PTEN, or in *PIK3CA* mutant tumors that have grown resistant to single p110α inhibition. Yet, targeting both p110α and p110β is likely to recapitulate most of the toxicity profile seen with pan-PI3K inhibitors. A compound targeting p110α and p110δ might overcome resistance to GS-1101 in B cell malignancies, since in some cases resistance correlates with elevated p110α expression and activity [84]. A dual p110α/δ inhibitor, BAY 80-6946, was recently described [225]. Combined

targeting of p110γ and p110δ has potential in T cell leukemias, in which p110γ and p110δ have redundant functions [85]. The compound IPI-145 is selective for p110γ and p110δ (though 10-fold more potent towards p110δ), has activity in autoimmunity models [226,227], and is in clinical trials for both B and T cell malignancies.

Most solid tumor cells express p110α and/or p110β. but usually not p110γ or p110δ. Nevertheless, inhibiting p110γ or p110δ may suppress tumor viability by modulating leukocyte subsets in the tumor environment (**Fig. 1**). In mouse models, blocking p110γ activity reduces recruitment of inflammatory cells to tumor sites and suppresses tumor growth [86]. Whether p110γ inhibition can shrink established tumors is uncertain, but this approach could be employed to prevent regrowth or metastasis. Inhibiting p110δ suppresses the function of regulatory T cells, enabling increased cytotoxic T cell responses to tumors (Khaled Ali and Bart Vanhaesebroeck, personal communication). Thus, targeting p110α with p110δ in solid tumors seems a particularly promising approach that would have cell-intrinsic anti-cancer effects while promoting a favorable immune environment and avoiding some toxicities of pan-PI3K inhibition. A downside of pan-PI3K inhibitors is that they suppress function of mouse and human lymphocytes to a much greater degree than p110α inhibition alone, or p110α/δ combinations [87]. It is likely that compounds with seemingly subtle differences in potency against different isoforms will provide significantly different efficacy and tolerability based on cancer cell-extrinsic effects.

Single node versus pan PI3K/mTOR inhibition

mTOR is structurally related to PI3Ks, and many ATP-competitive compounds inhibit mTOR and PI3K with similar potencies. In fact, the broadly used experimental PI3K inhibitors wortmannin and LY294002 also inhibit mTOR directly [88-90]. Several pan-PI3K/mTOR inhibitors

with improved pharmacological properties are now in clinical trials (Supp **Table 1**). The rationale for this compound class is to overcome crosstalk and feedback through "vertical" inhibition of the pathway at three key nodes: PI3K, TORC1 and TORC2 (**Fig. 2A**). This approach circumvents a limitation of selective PI3K inhibitors, that other inputs maintain considerable TORC1 activity even when PI3K and AKT are shut off [91]. Pan-PI3K/mTOR inhibitors should also prevent the rebound activation of PI3K that occurs in cells treated with rapalogs or active-site mTOR inhibitors.

It seems unlikely that pan-PI3K/mTOR inhibitors will provide a better efficacy window compared to targeting single nodes as there is obviously potential for greater toxicity at effective doses. Another consideration is that pan-PI3K/mTOR inhibitors do not always provide better efficacy than selective active-site mTOR inhibitors in preclinical tumor models [58]. Combined targeting of mTOR and one PI3K isoform (for example, p110α in *PIK3CA* mutant tumors) might improve tolerability relative to pan-PI3K/mTOR inhibitors and increase efficacy compared to single PI3K inhibition. The greater efficacy of a combination of the RAF and MEK inhibitors (Dabrafenib/Trametinib) in metastatic melanoma compared to monotherapy provides proof of concept in a different oncogenic pathway [92]. In addition to suppressing intra-pathway feedback, such approaches may achieve synergistic suppression of key downstream effectors using synchronized doses that partially inhibit the two upstream targets. A phase 1b trial (NCT01899053) has been initiated (by Millennium Pharmaceuticals) to test a combination of inhibitors selective for p110α and mTOR (MLN1117 and MLN0128). Providing further support for such combinations, resistance of *PIK3CA* mutant breast cancers to BYL719 correlated with persistent TORC1 signaling [93]. In the Dabrafenib/Trametinib trial, pERK was a reliable

downstream PD marker and we would anticipate that pS6 and p4EBP1 offer similar potential as PD markers for TORC1 activity.

Rapalogs versus TOR-KIs

Rapalogs (everolimus, temsirolimus, deforolimus) are structural analogs of rapamycin with improved pharmacological properties [94]. Their acceptable safety profile [95] has allowed the completion of a large number of trials testing rapalogs as single agents or in combination. Although most single agent trials have not demonstrated therapeutic benefit, rapalog monotherapy does significantly extend survival for some cancer patients. Currently one or more rapalogs are FDA-approved for use in renal cell carcinoma, mantle cell lymphoma and neuroendocrine tumors.

Incomplete mTOR inhibition contributes to the limited efficacy of single agent rapalogs in cancer (see *Box 3*). A second strategy to target mTOR is through ATP-competitive inhibitors that completely block mTOR kinase activity in both complexes, TORC1 and TORC2 [20,96,97] (*Box 3*). Termed active-site mTOR inhibitors, these compounds cause greater suppression of biosynthetic pathways compared to rapamycin, and generally cause a more marked cytostatic effect in cell lines [20,96-98]. Active-site mTOR inhibitors have shown cytotoxic effects in some but not all preclinical cancer models [58,99,100]. A key question for clinical development is whether active-site mTOR inhibitors should be tested first in malignancies in which rapalogs have some clinical benefit, since mTOR is a validated target in those diseases. An alternative is to test more broadly to see whether complete TORC1/TORC2 inhibition is effective in diseases where partial TORC1 inhibition is not. It is important to note that the most encouraging preclinical results with active-site mTOR inhibitors have been achieved in combination with TKIs in human tumor xenograft

models in mice [58,101]. These findings argue for early evaluation of active-site mTOR inhibitors in combination with other targeted agents. A note of caution is that advanced tumors often have an increased ratio of eIF4E to 4EBPs, a known mechanism of resistance to active-site mTOR inhibitors [102,103]. Therefore, high eIF4E expression might be a useful negative prognostic biomarker for patient selection.

Recent findings have renewed interest in clinical application of rapalogs. A large combination trial (BOLERO-2) of everolimus with anti-estrogen therapy (aromatase inhibitors) showed a significant survival benefit for patients with hormone receptor-positive breast cancer [104]. Rapalogs are useful in the treatment of subependymal giant cell tumors and angiomyolipomas in patients with tuberous sclerosis caused by inherited mutations in TSC1 or TSC2 [105,106]. Another important study used deep genomic sequencing of a rare responder tumor to identify TSC1 loss as a biomarker of everolimus sensitivity in bladder cancer [107]. Novel tumor suppressors (NPRL2, DEPDC5) have been identified in the GATOR1 complex that regulates amino acid sensing by TORC1, and human cancer cell lines lacking these components are very sensitive to rapamycin [108,109]. Additional clinical studies are needed to determine whether loss of TSC or GATOR1 components predict sensitivity to rapalogs in a broad range of tumors. Finally, two recent studies identified TORC1 signaling in resistance to PI3K or BRAF inhibitors [228,229].

The strong immunosuppressive properties of rapamycin have led to extensive investigation of mTOR function in the immune system. Genetic and pharmacological studies in mice have shown remarkable complexity of mTOR function in different immune cell types and at different stages of cell activation [110-112]. Although mTOR blockade reduces proliferation and effector differentiation of CD4 T cells, mTOR inhibition enhances generation of CD8 T cell memory [113-115]. In addition, mTOR inhibition can augment inflammatory cytokine production by innate

immune cells [116]. Remarkably, mTOR inhibition can either promote or suppress the function of regulatory T cells depending on the timing and experimental conditions [115,117,118]. Therefore, modulating the schedule of mTOR inhibitor therapy has the potential to promote anti-tumor immune responses while providing some tumor intrinsic activity.

Tolerability and alternative targets

A recurring theme above is the challenge of achieving a therapeutic window for compounds targeting PI3K and/or mTOR. PI3K signaling is linked to many physiological processes, and mTOR is a non-redundant sensor of nutrients and growth factors in dividing cells [51,119,120]. For these reasons, many investigators have evaluated other targets in the PI3K/mTOR signaling network. The best studied of these is AKT. This kinase is commonly overexpressed or mutated in tumors and was first discovered as the oncogene of a transforming virus [121]. Since AKT is one of many PI3K effectors linked to cell-specific physiological functions, it is conceivable that direct AKT inhibition would attack cancer cells with greater selectivity than PI3K inhibition. However, the data so far suggest that this might not be the case. AKT inhibitors cause severe rash similar to some TKIs, and cause hyperglycemia in both mice and humans [122,123]. Genetic studies in mice have shown that the AKT2 isoform is required for insulin signaling [124], and most clinical AKT inhibitors block both the AKT1 and AKT2 isoforms [125]. On the other hand, some AKT inhibitor candidates have off-target effects and a recent study suggested that a pharmacologically optimized AKT inhibitor causes only transient and reversible hyperglycemia [126]. Hyperglycemia can be managed with approved drugs like metformin, and can also be a useful biomarker of target modulation. Interestingly, metformin and related compounds have

been suggested to provide direct anti-tumor effects by activating AMP kinase leading to reduced TORC1 signaling [127].

Several other cellular components associated with the PI3K/mTOR network might be useful targets for anti-cancer therapeutics (**Table 1**). Based on the central role of cap-dependent translation in cancer cells, drugs targeting eIF4E have been developed and show promise in preclinical studies [128]. For example, the compound 4EGI-I interferes with the eIF4E-eIF4G interaction and has anticancer activity in cell lines [129]. Selective inhibitors of S6 kinases have been identified [130-132], and could attack cancer by restricting protein synthesis and other anabolic growth processes mediated by S6Ks. Supporting this concept, genetic targeting of S6K1 delayed leukemogenesis in a PTEN loss model [133]. MNK and PIM kinases also promote protein synthesis and are under evaluation as targets in oncology [134,135]. MNK kinases phosphorylate eIF4E to increase cap-dependent translation and promote survival [136], and PIM kinases increase translation through phosphorylating several substrates including eIF4B [135]. Inhibiting RAS function in RAS-driven cancers would be expected to diminish signaling through PI3K as well as ERK and other RAS effectors. Though targeting RAS has long been an unrealized dream in molecular medicine, novel strategies have recently been [137,138] described.

Emerging rational combination strategies

The typical drug development path for a targeted anti-cancer drug involves establishing single agent efficacy before testing the drug in combination. However the initial results from PI3K/AKT/mTOR inhibitor trials suggest that deep and sustained responses to single agents are infrequent. Given limited resources, some promising drugs might be halted in development because they do not significantly prolong survival of a selected patient population. An alternative

approach would be to initiate combination trials as soon as pharmacodynamic (PD) activity can be established at a tolerated dose. Development of robust and informative PD markers remains a challenge, as discussed in reference [25]. Moreover, it is essential to choose rational combinations that are most likely to provide synergy (a "1 + 1 = 3" effect), to overcome the expected increases in toxicity and justify the costs and complexity of combination trials. The BOLERO-2 clinical trial combining everolimus with endocrine therapy provided proof of principle that the PI3K/AKT/mTOR pathway can be targeted in rational combinations to achieve real therapeutic benefit to a large patient population [104]. What other combinations can be envisioned (**Table 2**)?

There is ample mechanistic rationale to test combinations of PI3K/AKT/mTOR inhibitors with TKIs. It is useful to consider this issue from two perspectives (**Fig. 3**). First, cancers harboring active or overexpressed receptor tyrosine kinases (RTKs) such as EGFR or HER2 can display resistance to TKIs through PI3K signaling [139,140]. This knowledge provides justification for adding PI3K/AKT/mTOR inhibitors to initial TKI treatments to prevent the emergence of resistance, even in tumors with a high initial response rate to TKIs. In line with this view, a review from Rexer and Arteaga discussed strategies to incorporate PI3K inhibitors into treatment regimens for $HER2^+$ breast cancer [141]. Such approaches should be considered for other TK-driven cancers, as combining GDC-0941 with imatinib produced more durable remissions than imatinib alone in a xenograft model of gastrointestinal stromal tumor (GIST) driven by BCR-ABL [142]. A second consideration is that single agent PI3K/AKT/mTOR inhibitors increase RTK expression through FOXO-mediated feedback [27,60]. From this point of view, TKIs act as the second agent to augment the efficacy of a PI3K/AKT inhibitor. Supporting this concept, targeting EGFR-family receptors with lapatinib increased the efficacy of a p110α-selective inhibitor in *PIK3CA* mutant breast cancer cells [143]. A challenge is that the feedback tends to

increase expression of multiple RTKs, such that selective TKIs would have minimal efficacy. In hematopoietic malignancies driven by non-receptor TKs such as BCR-ABL or JAK2, PI3K/AKT/mTOR inhibitors strongly synergize with TKIs [58,144]. A possible explanation is that blood cancer survival is maintained in part by cytokines and stromal cell contacts that signal through the PI3K pathway. There is also evidence for interdependence of PI3K signaling and the JAK/STAT pathway, with agents targeting STAT3 or upstream kinases holding promise to enhance efficacy of PI3K inhibitors [145].

Targeting the RAS/RAF/MEK/ERK cascade is an attractive strategy for combination therapies with PI3K/AKT/mTOR inhibitors (**Fig. 2B**). Both networks can promote cell proliferation and survival, and there is extensive crosstalk between the pathways. Thus, TORC1 inhibition tends to increase ERK phosphorylation [146,147] whereas MEK inhibition reduces PTEN membrane localization and increases AKT activity [148]. Synergy of a MEK inhibitor with the dual PI3K/mTOR inhibitor NVP-BEZ235 was shown first in a *KRAS*-driven lung cancer model [26]. Similar findings have been observed in many subsequent reports including a study of *NRAS*-mutant melanoma cells [149]. One mechanism for synergistic cell killing appears to be through complementary effects on pro-apoptotic proteins: MEK/ERK inhibition stabilizes BIM, whereas PI3K/AKT inhibition upregulates PUMA via FOXO transcription factors [150]. Both pathways also converge on the pro-apoptotic protein BAD [151]. Such combinations might also achieve synergy at the level of metastasis suppression. MEK inhibitors can suppress the epithelial-mesenchymal transition (EMT) that is a critical step in the evolution of metastatic tumor cells [152,153]. Active-site mTOR inhibitors decrease translation of mRNAs encoding proteins involved in prostate cancer invasion and EMT [154]. A major concern is whether a therapeutic window can be achieved with combinations of PI3K/AKT/mTOR and MEK inhibitors [155]. To overcome likely toxicities, it

might be necessary to experiment with dose and schedule, such as high dose intermittent treatments or alternating sequences. Validation of downstream or parallel effectors (such as eIF4E, S6K, MNK, PIM and RSK kinases) might lead to more tolerable combinations with anti-cancer efficacy. One setting in which toxicity should be minimized is in colorectal cancers harboring B-RAF^{V600E}. Selective inhibitors of mutant B-RAF (vemurafinib) are well tolerated but are ineffective due to compensatory signaling. Combining B-RAF inhibitors with a PI3K/mTOR inhibitor caused apoptosis and tumor regression in a model of colorectal cancer driven by mutant B-RAF [156]. A very recent study showed persistent TORC1 activity in vemurafinib-resistant melanomas [157], providing rationale for also combining vemurafinib with mTOR inhibitors in this setting.

The *MYC* oncogene is frequently amplified in cancer, and can confer resistance to PI3K/AKT/mTOR inhibitors independent of the RAS pathway [27,28]. Recent breakthroughs indicate that it might be possible to suppress the MYC transcriptional program indirectly by targeting BET proteins (bromo and extra terminal (BET) proteins) such as BRD2 and BRD4, which are transcriptional regulators required for efficient expression of MYC [158-161]. Inhibition of the BET-histone interaction by small molecules blocking the bromo-domain binding site (so called BET inhibitors) can downregulate expression of MYC and its target genes in tumor cells [158-161]. Combining BET inhibitors with PI3K/AKT/mTOR inhibitors is a sensible strategy, particularly in hematopoietic malignancies where MYC cooperation with PI3K has been established [162]. *MYC* is the defining oncogene of Burkitt lymphoma, and there is evidence for a cooperative role for PI3K activation in human specimens and in a mouse model [163,164]. Inhibiting mTOR or eIF4E strongly impaired MYC lymphomagenesis in mice [165]. In T-cell acute lymphoblastic leukemia (T-ALL), two common lesions are loss of PTEN and activating NOTCH

mutations that elevate MYC activity [166,167]. Hence, either NOTCH inhibitors or BET inhibitors could be combined with PI3K/AKT/mTOR inhibitors in T-ALL trials.

Blocking autophagy might provide another avenue to augment cancer cell killing by PI3K/AKT/mTOR inhibitors [168]. Autophagy is a process by which cells recycle organelles and macromolecules to survive under conditions of starvation or other stresses. Inhibition of mTOR causes an autophagy response comparable to nutrient starvation. In glioma, leukemia and other cancer cell types, chemical inhibitors of autophagy potentiate apoptosis by active-site mTOR inhibitors or dual PI3K/mTOR inhibitors [169,170]. A combination trial of temsirolimus with an autophagy inhibitor in renal cell carcinoma is underway [168]. However, current autophagy inhibitors are nonspecific agents that generally act by inhibiting lysosomal degradation. Discovery of compounds inhibiting specific components of the autophagy machinery will be helpful for testing the potential of combination approaches with PI3K/AKT/mTOR inhibitors. A related issue is how PI3K/AKT/mTOR inhibitors will affect the response to emerging therapies targeting cancer cell metabolism. Considering that the PI3K/AKT/mTOR pathway drives many of the metabolic hallmarks of cancer cells, it is possible that pathway inhibition will reduce sensitivity to metabolic interventions.

Emerging evidence connects the PI3K/AKT/mTOR network to maintenance of genome integrity. PI3K is involved in sensing double strand breaks [171,172] and in maintaining expression of BRCA1 and BRCA2 that participate in homologous recombination [173]. Exploiting these findings, two groups showed that PI3K inhibitors increase DNA damage and sensitize triple-negative breast cancer (TNBC) cells to inhibitors of polyADP-ribose polymerase (PARP) [173,174]. A phase I trial of the PARP inhibitor olaparib with the panPI3K inhibitor BKM120 has been initiated, enrolling patients with TNBC or high-grade serous ovarian cancer. Paradoxically,

PTEN also has a role in protecting cells from genotoxic stress mediated by a nuclear pool of the phosphatase [175]. Elevated PI3K survival signaling in PTEN-deficient cells protects them from accumulated DNA damage. This property renders PTEN-deficient tumors sensitive to the combination of PI3K inhibitors and DNA damaging agents in preclinical studies [175]. Loss of PTEN might also sensitize to PARP inhibitors, similar to BRCA1-deficient tumors. It is worth noting that several DNA repair enzymes are members of the PIKK family: ATM, ATR and DNA-PK [176]. Some inhibitors developed against class I PI3Ks have off-target effects on PIKK family members, and this property might enhance synergy with DNA-damaging agents [177]. Inhibiting mTOR can also promote DNA damage through suppression of the FANCD2 and other mechanisms [178-180]. Thus, an important area for continuing study is to investigate how inhibitors acting at different levels of the PI3K/AKT/mTOR network affect the cellular response to radiation and chemotherapeutic drugs that are currently the standard of care in many cancers. Also worth considering is that off-target effects on DNA-PK and ATM are probably a liability rather than an advantage, if not combined with DNA damaging agents or radiation, by increasing genomic instability that tends to accelerate drug resistance.

The BOLERO-2 trial illustrated the potential of PI3K/AKT/mTOR inhibitors to prevent or overcome targeted therapy in hormone-dependent cancers. There is evidence for positive feedback between hormone receptors and the PI3K network [181]. The ligand-bound estrogen receptor (ER) interacts directly with PI3K, augmenting PI3K/AKT activity. In turn, AKT and S6K1 can phosphorylate the hormone receptor to increase its activity. Hormone-dependent cancers frequently exhibit high basal PI3K activity through loss of PTEN, *PIK3CA* mutation or other mechanisms [182,183]. Thus, inhibitors acting at multiple levels of the PI3K/AKT/mTOR network might supplement the anti-cancer effects of hormonal therapy. In advanced prostate

cancer with loss of PTEN, it would be interesting to test a p110β inhibitor in combination with an androgen receptor antagonist. There is also rationale for a dual p110β/δ inhibitor in this setting, based on a report that B cell infiltrates sustain prostate cancer survival after hormone withdrawal [184]. This strategy would act via tumor intrinsic effects (p110β) together with extrinsic effects on the immune infiltration (p110δ).

A conceptually simple approach to sensitize cancer cells to PI3K pathway-targeted agents is to combine with agents that increase mitochondrial priming for death. BCL2 family members (BCL2, BCL-X_L, MCL-1, A1) maintain mitochondrial integrity by blocking the pro-apoptotic function of BAX and BAK [185]. A large family of pro-apoptotic proteins homologous to BCL2 can sequester the pro-survival proteins or directly activate BAX and BAK [186]. Priming refers to suppressing the activity of pro-survival factors at the mitochondria, such as BCL2 and MCL-1, relative to pro-apoptotic proteins like BIM and PUMA [187]. ABT-263, a small molecule inhibitor of BCL2 and BCL-X_L, has entered clinical trials for cancer and shown some promise in CLL [185]. By increasing mitochondrial priming, BCL2 antagonists should lower the threshold for apoptosis in response to PI3K/AKT/mTOR inhibition (**Figure 4**). A growing body of work supports the synergistic anti-tumor effects of PI3K/AKT/mTOR inhibitors combined with BCL2 antagonists [150,188-190].

Future directions

Building on the discussions above, we envision four key strategies that will maximize the potential of PI3K/AKT/mTOR inhibitors in oncology.

Biomarker identification through next generation sequencing

A limited response rate with a single agent strategy at an early stage of development should not necessarily mean that a clinical trial has failed, especially when targeting a genetically validated target or disease biology. The advent of next generation sequencing allows significant knowledge to be gained from the rare responders in a trial. The "n = 1 response" matters. An exciting report from Solit and colleagues used exome sequencing to identify TSC1 inactivation in a rare bladder cancer that responded to everolimus [107]. Targeted sequencing of additional tumors from everolimus trials showed a significant delay in recurrence for samples with TSC1 mutations [96]. As sequencing costs decline and technologies improve, it should be feasible to apply this approach in trials of PI3K/AKT/mTOR inhibitors as single agents or in combinations. This idea should not replace patient selection based on the drug target, cancer genetics and disease biology. It should, however, be applied in parallel to select additional genetic markers for subsequent trials.

Initial emphasis on hematologic malignancies

Leukemia, lymphoma and myeloma are a diverse set of cancers that are individually less common than solid tumors like lung, breast and colon cancer. Few blood cancers carry activating mutations in RAS or PI3K. Nevertheless, there are several reasons to devote resources to PI3K/mTOR inhibitor trials in blood cancer. For one thing, leukemia and lymphoma models often show non-oncogene addiction to mTOR [58,165]. In addition, blood cancers generally express p110δ and/or p110γ that should in principle confer responsiveness to agents targeting these isoforms, as illustrated by GS-1101. Unlike many solid tumors, hematopoietic cancer cells are in constant contact with the immune system and might be especially sensitive to immune enhancing effects of PI3K/mTOR inhibitors. It is also easier to access tumor cells for PD monitoring in

patients with blood cancers, and plasma analysis can provide useful information about immune modulation [191]. Lastly, treating rare blood cancers effectively can be rewarding, as proven by BCR-ABL inhibitors that have saved the lives of an ever-expanding population of CML patients who must continue on therapy.

Harness immune effects

Though cancer is a genetic disease of aberrant cells, it is also a chronic immune disease (**Fig. 1**) [33,192]. The immune system restrains tumorigenesis but eventually the tumor enforces a state of immune tolerance and exhaustion. Recently there has been exciting progress treating human tumors with immunotherapies to overcome tolerance and exhaustion [33,34,193]. There is also an increasing appreciation for how small molecules targeting the cancer cell affect the immune context of the tumor [194,195]. The efficacy of GS-1101 emphasizes how a drug targeting both the tumor and the immune system can act as an all-in-one combination therapy. How is it possible to harness anti-tumor immunity through a pathway defined by a target of the immunosuppressive drug rapamycin?

Extensive studies of the PI3K/AKT/mTOR network in immune cells have shown that PI3K activation is not a simple on/off switch [110,111,196,197]. Inhibiting the pathway can either suppress or enhance immune responses, through effects on diverse subsets of innate and adaptive immune cells. In theory it should be possible to implement treatment regimens that increase immune rejection of tumors in concert with direct anti-tumor effects. A significant factor limiting progress in this area is that preclinical drug development programs mainly use xenograft tumor models. These models are convenient and are useful to assess drug pharmacology, providing valuable information about PD in the context of PK and general tolerability. Yet these models

overlook any modulation of adaptive immunity, since growing human tumor cells in mice requires host strains lacking functional lymphocytes. Interactions of xenograft cells with innate immune components might also fail to recapitulate events in the development of human tumors. For these reasons it is essential to test candidate inhibitors in genetically engineered mouse (GEM) models, and to extensively monitor infiltration and activity of diverse immune subsets including macrophages, T cells and natural killer cells. Such systems would be useful to test ideas such as "dialing in" activity against p110δ and/or p110γ to create a more favorable immune environment. One can even imagine that inhibiting p110δ and/or p110γ alone in solid tumors would provide significant therapeutic benefit and tolerability without any direct effect on the PI3K isoforms expressed within the cancer cell (based on the work of Ali and Vanhaesebroeck, personal communication).

It will also be crucial to determine which agents targeting PI3K/AKT/mTOR enhance or suppress the efficacy of emerging immunotherapies and cancer vaccines. In mouse models, both PI3K and mTOR inhibitors can enhance the efficacy of immune-directed therapies [198-200]. It is relevant to consider that isoform-selective agents minimize immune suppressive effects on lymphocytes compared to pan-class I inhibitors [201]. Hence, pan-PI3K inhibitors are more likely to enforce or accelerate the immune exhaustion state. Eventually, the best combination therapies might turn out to be isoform-selective PI3K or mTOR inhibitors with immunotherapies or cancer vaccines. Matching patients to the right combinations will require knowledge of the genomic driver and the immune fingerprint of the tumor.

Combination trials

Above, we proposed several rational combinations to increase cancer cell killing by PI3K/AKT/mTOR inhibitors. However, the process of drug development always faces tension between what should be done and what can be done. Developing combination therapies costs more resources and time and might ultimately result in challenges for reimbursement. On the other hand, experience with BRAF inhibitors shows that combinations will be justified even for therapies that provide an impressive initial response in selected patients. In the short term, the plan should be to prioritize approaches based on feasibility, pragmatism and the likelihood of a meaningful therapeutic advance. It makes sense to start by combining PI3K/AKT/mTOR inhibitors with approved targeted agents that are standard of care for specific malignancies. Using a companion drug whose safety profile and optimal dosing is well understood will reduce the complexity of the combination trial. It will also allow the incorporation of PI3K/AKT/mTOR inhibitors into treatment regimens at earlier stages of disease, rather than only in patients with relapsed or refractory tumors. The limited success of single agents to date might be explained in part by the polygenic and polyclonal nature of advanced tumors, some of which is caused by prior therapies. Preclinical data support testing of several combinations with approved TKIs: BCR-ABL inhibitors in Ph^+ leukemias and GIST [142], EGFR inhibitors in lung and colon cancer [139,140], and agents targeting HER2/ErbB3 in breast cancer [202]. Adding a PI3K inhibitor to a BRAF inhibitor might enhance efficacy in melanoma and produce responses in colorectal cancers carrying mutant B-RAF [156,203,204].

Sometimes key biological insights from preclinical data can justify combination trials of two experimental agents. Agents that show synthetic lethality with PI3K pathway inhibitors in cancer cell lines and patient-derived xenografts, but not in normal cells, should be given priority for

clinical testing. An example discussed above is the combination of PI3K and PARP inhibitors for TNBC (Table 2), in which trials were quickly initiated after remarkable preclinical results [173,174].

In cases where GEM models exist for driving oncogenes and tumor types, it should be possible to design synchronous "co-clinical" trials that help in identifying genetic and pharmacodynamic markers of responsiveness [205]. These are especially powerful when paired with studies using patient-derived primary tumor tissue analysis. Regulatory approval of immunotherapies in certain cancers also sets the stage for testing the immune enhancing potential of PI3K or mTOR inhibitors.

Conclusions

The rationale for targeting the PI3K/AKT/mTOR network in cancer remains anchored on a solid foundation of cancer genetics and cell biological studies. Despite many challenges, measurable advances have been achieved in the clinic. Rapalogs are useful in some advanced cancers and as adjuvants to hormone therapy in breast cancer. Inhibitors of PI3Kδ and BTK are on track for FDA approval in certain B cell malignancies. Other agents are advancing through development. Nevertheless, early hopes have been tempered by the realization that targeting PI3K/AKT/mTOR alone will not be a cure-all for diverse cancers.

How can we reset strategies to maximize the potential of PI3K/AKT/mTOR inhibitors? Previous experience with successful oncology drug development shows the importance of: (1) targeting genetic drivers in selected patient populations; (2) understanding the biology of crosstalk and feedback to employ effective combinations; (3) stimulating an immune environment that favors tumor eradication. Thoughtful application of these principles will light the path towards effective cancer control by PI3K/AKT/mTOR inhibitors.

Figure legends

Figure 1: **Targets in the signaling network and their role in tumor biology.** This diagram shows a highly simplified scheme of the signaling pathway leading from PI3K to AKT to mTOR. The four isoforms of class I PI3K are shown in dark gray boxes. Blue italics illustrate cancer cell-intrinsic functions of isoforms: p110α is a frequent genetic driver (*PIK3CA* mutations); basal activity of p110β is implicated in tumors with PTEN loss; p110δ has a fundamental role in survival of normal B cells and malignancies of this lineage. PI3K and mTOR drive tumor metastasis by promoting cell motility and epithelial-mesenchymal transition (EMT). Purple arrows represent cell extrinsic functions of various components in the network. p110α drives angiogenesis. p110γ, p110δ and p110β have important functions in inflammatory cells. p110δ and mTOR control key aspects of adaptive immunity including lymphocyte activation, differentiation and tolerance. Drugs in clinical development that target nodes in this network are listed in Supplementary Table 1.

Figure 2: **Complexity, crosstalk and feedback in the PI3K/AKT/mTOR signaling network.** (**A**) The TORC1-TORC2 network and key feedback mechanisms. The mTOR serine-threonine kinase forms two multi-protein complexes whose defining subunits are raptor (TORC1) and rictor (TORC2). TORC2 activity is stimulated by association with ribosomes and by growth factors through a poorly defined mechanism, which may involve PI3K. TORC2 promotes stability and activity of AKT and other kinases including serum- and glucocorticoid-induced kinases (SGKs) and protein kinase C (PKC). TORC1 is a signal integrator whose activity is tuned by diverse inputs. Growth factors, energy sensors and cellular stress converge at the level of the TSC complex (TSC1/TSC2/TBC1D7), a negative regulator of TORC1 with GAP activity

towards the Rheb GTPase. Amino acids regulate TORC1 through the Ragulator and GATOR complexes. TORC1 promotes anabolic programs through many substrates, of which three classes are shown: S6 kinases, eukaryotic initiation factor-4E (eIF4E)-binding proteins (4E-BPs), and autophagy regulators (ULK1, etc.). TORC1 activity exerts feedback control on growth factor signaling. One canonical feedback pathway is initiated by S6 kinase-1 (S6K1), a TORC1 substrate, which phosphorylates adaptor proteins of the insulin receptor substrate (IRS) family to attenuate growth factor receptor signaling to PI3K and RAS. In parallel, TORC1 suppresses growth factor receptor signaling by phosphorylating the GRB10 adaptor protein. AKT activity triggers a feedback mechanism that suppresses growth factor receptor expression and signaling. Through phosphorylation and inactivation of Forkhead Box Subgroup O (FOXO) transcription factors, active AKT reduces the transcription of FOXO target genes including several growth factor receptors. (**B**) Redundancy and feedback between the RAS-RAF-MEK-ERK and PI3K/AKT/mTOR signaling networks. ERK and downstream kinase RSK can compensate for AKT in the activation of TORC1 via inhibitory TSC phosphorylation; GSK3 and AMPK phosphorylation of TSC2 increase its ability to suppress TORC1 activity. MNK kinases phosphorylate eIF4E to provide a distinct signal to increase cap-dependent translation. ERK and mTOR independently promote accumulation of MYC oncoproteins. Mutual feedback inhibition is a feature of the two pathways: MEK activity suppresses PI3K signaling by promoting PTEN membrane localization, while TORC1 activity suppresses RAS activation through mechanisms shown in Figure 3.

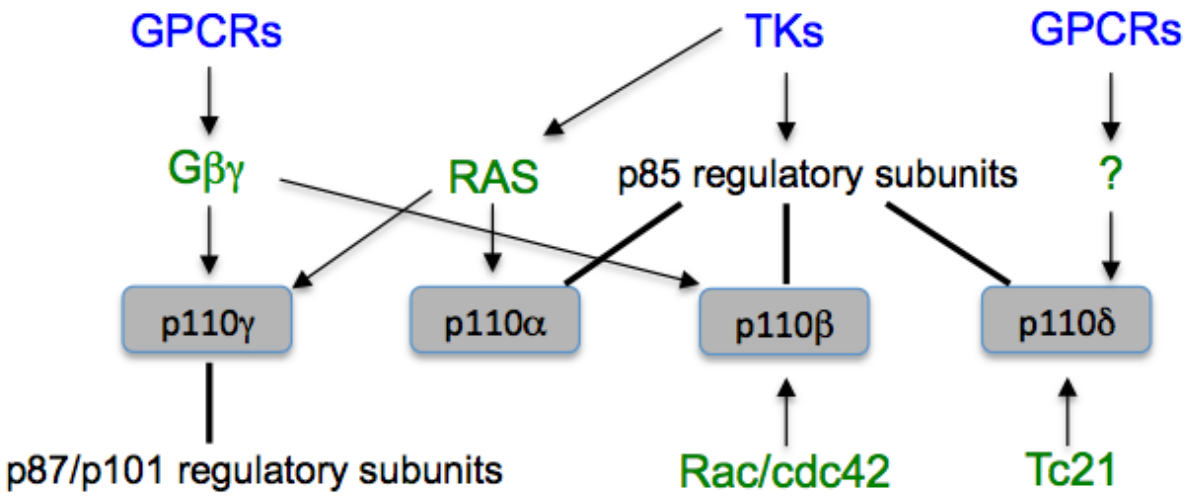
Figure 3: **Two arguments for combining TKIs with PI3K/AKT/mTOR inhibitors.** Left: In cancers driven by activated tyrosine kinases, TKI resistance can develop through alternative

pathways that maintain PI3K signaling such as compensatory growth factor (GF) receptors, PTEN loss, *PIK3CA* mutation or RAS activation. Combined targeting of PI3K can prevent or overcome drug resistance. Right: In cancers driven by lesions in PI3K or PTEN, inhibiting PI3K or AKT or TORC1/TORC2 can cause elevated GF receptor signaling through FOXO-dependent gene expression. Adding a TKI can ameliorate this compensatory signaling mechanism.

Figure 4: **Rationale for BCL2 antagonist combination.** The balance of pro-survival and pro-apoptotic BCL2 family members at the mitochondria is a primary factor controlling cell survival versus apoptosis. PI3K/AKT/mTOR signaling suppresses expression and activity of multiple pro-apoptotic proteins (i.e. BAD, BIM, PUMA, and death receptors) and can increase expression of pro-survival factors (MCL-1). However, PI3K/AKT/mTOR inhibition does not necessarily tip the balance towards apoptosis. Combining with small molecule antagonists of pro-survival proteins (BCL2, BCL-X_L) increases mitochondrial "priming" for death, lowering the threshold for apoptosis induction by PI3K/AKT/mTOR inhibitors.

Boxes

Box 1: *Inputs from GTPases and tyrosine kinases to PI3K.* Each of the class I PI3K catalytic isoforms has a segment known as the RAS-binding domain (RBD). Knock-in mouse studies established p110α as a *bona fide* downstream effector of oncogenic RAS [73], and demonstrated that the RBD of p110γ is required for PI3K signaling in neutrophils [206]. Recent work indicates that the RBD of p110β does not bind to RAS or its close relatives; instead, the RBD of p110β interacts with GTP-bound Rac/cdc42, establishing p110β as a novel effector of these GTPases [37]. The RBD of p110δ interacts with the Tc21 small G protein [207,208]. Tyrosine kinases activate p110α, p110β and p110δ via interaction of their regulatory subunits with tyrosine phosphorylated (pTyr) peptide motifs, and can activate p110γ in some cell types via RAS [86]. GPCRs directly stimulate p110β and p110γ via βγ subunits of heterotrimeric G proteins [206,209], and can activate p110δ in B cells by an unknown mechanism [210,211].



Box 2: *Selective inhibitors of PI3Kδ or the PI3K effector BTK.* The PI3Kδ isoform is mainly expressed in immune cells and is absent from most solid tumors. Gene targeting in mice has established essential functions for PI3Kδ in mature B cells, and in other immune cell types [41,212]. A key downstream effector of PI3Kδ in B cells is BTK, a member of the TEC family of nonreceptor tyrosine kinases. PI3Kδ and BTK are activated by signals from the B cell receptor (BCR), chemokines and cytokines to drive survival, proliferation and adhesion to supportive stromal cells. However, activating mutations in PI3Kδ and BTK are not present in B cell tumors, and inhibitors of these enzymes were initially developed for application in immune diseases. Unexpectedly, phase I clinical trials of a PI3Kδ inhibitor (CAL-101, renamed GS-1101) and a BTK inhibitor (PCI-32765) showed dramatic and durable responses in a subset of human patients with indolent B cell malignancies [31,32,69,224]. Even greater efficacy was achieved in combination studies with Rituximab and/or Bendamustine. Both the PI3Kδ inhibitor and BTK inhibitors have shown acceptable safety profiles. These compounds, now called Idelalisib and Ibrutinib, have progressed to phase II/III trials and are likely to be the first FDA-approved agents targeting the PI3K pathway. The FDA has granted Breakthrough Therapy Designation for Ibrutinib in three diseases: mantle cell lymphoma, Waldenström's macroglobulinemia, and chronic lymphocytic leukemia with deletion at chromosome 17p (note added in proof: Ibrutinib was approved Nov. 13, 2013, for relapsed mantle cell lymphoma).

Box 3: Two distinct classes of mTOR inhibitors. Rapalogs act through an allosteric mechanism and cause only partial inhibition of TORC1, with more marked effects on certain TORC1 substrates (i.e. S6K1) than others (i.e. 4E-BPs). This profile causes weak inhibition of cap-dependent translation and releases negative feedback, leading to "rebound" activation of upstream signaling. Rapalogs do not directly inhibit TORC2, allowing continual survival signaling by AKT and other TORC2 substrates. In accord, extensive cell line surveys consistently show that rapamycin and analogs are cytostatic and not cytotoxic. Active-site mTOR inhibitors fully block phosphorylation of all known TORC1 and TORC2 substrates.

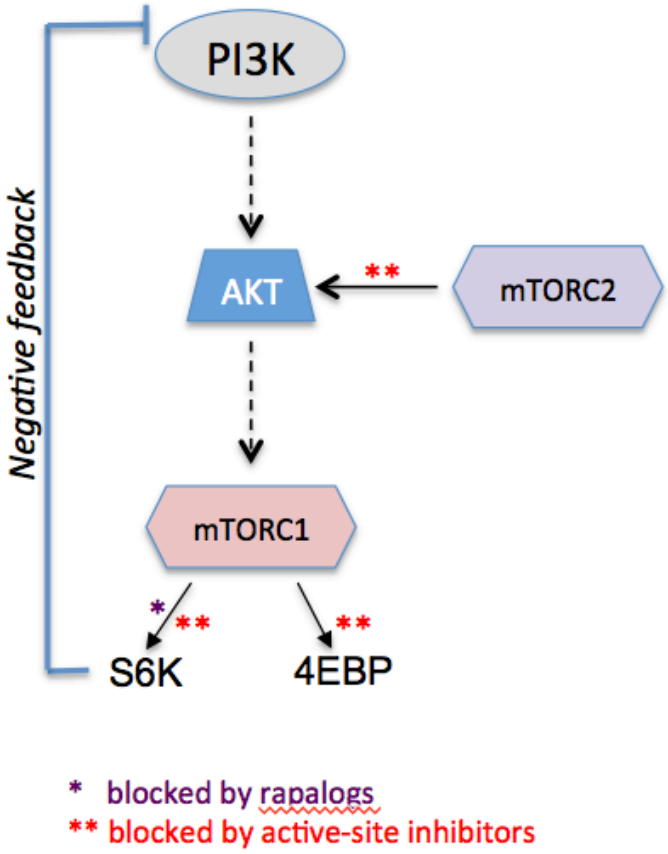


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Table 1: Emerging Targets within the PI3K signaling network

Target	Upstream activators	Effectors/Substrates	Tool Compounds (Refs.)
eIF4E	mTORC1, MNK	Cap-dependent translation	4EGI-I [129] 4ei-1[213]
S6K	mTORC1, PDK1	S6, PDCD4, eIF4B, eEF2K, SKAR	PF-4608671 [132] DG2 [131] LYS6K2 [130]
MNK	ERK	eIF4E	CGP57380 [214] AST 487 [215] Cercosporamide [216]
PIM	Growth factor-mediated increase in transcription	eIF4B, 4EBP1, BAD, p27	SMI-4a [217] ETP-45299 [218] SGI-1776 [219] Pimi-14J [220] K00135 [221]

Table 2: Selected PI3K Pathway Combination Strategies

Target for combo	*Tumor stratification*	*References*
TKs	Active or overexpressed TK	[58,139-144]
MEK	Active RTK, RAS mutant	[26,149,150,155]
$BRAF^{V600E}$	Melanoma, colon	[156,157]
MYC	MYC amplification, NOTCH mutant	[27,28,163-165,167]
Autophagy	Glioma, leukemia, others	[168-170]
PARP	TNBC	[173,174]
Aromatase inhibitors	ER-positive	[104,181]
BCL2 antagonists	Leukemia, lymphoma, others	[188-190]