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Novel approaches to inhibitor design for the p110 β PI 3-kinase

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Abstract

Phosphoinositide 3-kinases (PI 3-kinases) are essential regulators of cellular proliferation, survival, metabolism and motility that are frequently dysregulated in human disease. The design of inhibitors to target the PI 3-kinase/mTOR pathway is a major area of investigation by both academic laboratories and the pharmaceutical industry. This review focuses on the Class IA PI 3-kinase p110 β , which plays a unique role in thrombogenesis and in the growth of tumors with deletion or loss-of-function mutation of the Phosphatase and Tensin Homolog (PTEN) lipid phosphatase. Several p110 β -selective inhibitors that target the ATP binding site in the kinase domain have been identified. However, recent discoveries on the regulatory mechanisms that control p110 β activity suggest alternative strategies by which to disrupt signaling by this PI 3-kinase isoform. This review summarizes the current status of p110 β -specific inhibitors, and discusses how these new insights into p110 regulation might be used to devise novel pharmacological inhibitors.

Class I PI3-Kinases and PTEN

PI3-kinases are classified based on sequence homology among catalytic subunits and on lipid substrate specificity [1, 2]. The class I PI3-kinases consist of one of four catalytic subunits (p110 α , p110 β , p110 δ and p110 γ) associated with one of seven regulatory subunits (p85 α , p55 α , p50 α , p85 β , p55 γ , p101 and p87). These enzymes are activated downstream of receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs) and use PI-4,5-P₂ as a substrate to generate PI-3,4,5-P₃ *in vivo* [3]. Among the PI3-kinases, p110 β is unique in signaling downstream of both RTKs and GPCRs [4–6] (Figure 1). p110 β is also unusual in that it binds to the GTP-bound form of the endosomal small GTPases Rab5 [7, 8]. This interaction has been linked to kinase-independent roles of p110 β in endocytosis and autophagy [9, 10] (Figure 1).

Inappropriate activation of the PI3-kinase pathway has been strongly associated with human cancer, with studies showing common mutations and deletions in p110 α catalytic subunit, the p85 α and p85 β regulatory subunits, and in the PI 3-kinase antagonist PTEN [11–13]. p110 β , p110 δ and p110 γ are rarely mutated, and overexpression of these isoforms in their wild type state is sufficient to cause transformation [14]. In contrast, p110 α only causes transformation when mutated. Interestingly, p110 β is specifically required for proliferation in prostate cancer cell lines that are defective for PTEN function [15], whereas other tumors characterized by a PTEN loss of function, such as thyroid tumors and pheochromocytoma, require p110 α [16]. Recent studies suggest that pharmacological inhibition of p110 β might

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be effective in treating some PTEN-deficient tumors [17]. Inhibitors of p110 β may also be useful in the treatment of thrombotic disease and inflammation [18–20].

Current Class I PI3K inhibitors: the ATP binding site

Most PI 3-kinase inhibitors target the ATP binding site of the kinase domain and act as competitive inhibitors [21, 22]. The first PI 3-kinase-specific inhibitors, wortmannin and LY294002, were not clinically useful, although modifications such as PEGylation and linkage to biological molecules, such as an RDGS integrin binding element, are in clinical trials [23]. There has been enormous progress in the development of pan-PI 3-kinase inhibitors, PI 3-kinase plus mTOR inhibitors, as well as isoform-specific inhibitors for p110 α , p110 δ and to a lesser extent p110 γ [24, 25]. The first isoform selective inhibitor of p110 β to be characterized was TGX221 [26]. Since then, KIN-193 has been shown to inhibit proliferation in a wide array of PTEN-deficient tumors in mice, and AZD6482 has shown anti-platelet activity in humans and is in clinical trials [17, 27].

There has been extensive debate on whether pan-PI3K inhibitors would be advantageous over isoform-specific inhibitors. The discovery of negative feedback loops in the regulation of PI 3-kinase signaling, particularly the inhibition of upstream PI 3-kinase activators by mTORC1 signaling, has led to interest in inhibitors that target both PI 3-kinase and mTOR [28]. A number of these inhibitors have now entered clinical trials [22]. Studies on inhibitors of oncogenic mutants of the B-Raf kinase has raised the possibility of mutation-specific inhibitors [29], and a recent report suggests that mutation-selective inhibitors for p110 α may also be feasible [30].

With regard to inhibition of p110 β , it is important to note that some functions of the enzyme are independent of kinase activity. For example, whereas p110 β knockout mice show embryonic lethality, mice expressing kinase-dead p110 β are viable, although infertile [9, 10]. Interestingly, kinase-independent functions of p110 γ in the heart have also been described [31]; it may not be coincidence that the two p110 isoforms known to interact with G β γ both show kinase-independent scaffolding functions. If the kinase independent functions of p110 β and p110 γ signaling involve targeting by G β γ subunits downstream from GPCRs, then inhibitors designed to disrupt G β γ binding could display a different clinical spectrum from inhibitors that target the ATP binding site of the kinase; this is discussed in more detail below.

The p85-p110 β interface

Class IA catalytic subunits form stable obligate heterodimers with p85 regulatory subunits [32]. p85 binding is required for the thermal stability of the associated p110, inhibition of its basal activity [33, 34], and recruitment to the membranes by binding to tyrosine-phosphorylated receptors and receptors substrates [3]. The best understood regulatory interactions within the p110/p85 dimer involve inhibitory contacts between the p85 SH2 domains and p110 (Figure 2). The nSH2 domain makes a charge-based inhibitory contact with the helical domain of p110 subunits, with the contact site in p85 coincident with the SH2 binding site [35, 36]. Disruption of this interface by the binding of tyrosine phosphorylated peptides or proteins, or by mutation, relieves the inhibition and increases the catalytic activity of p110. In p110 β /p85, the p85 cSH2 domain makes an inhibitory contact with the C-terminus of the p110 β kinase domain, with the contact site involving Tyr677 in p85 α and Leu1043 in p110 β [37]. Like the nSH2 domain, disruption of this contact by tyrosine-phosphorylated protein binding relieves the inhibition and activates the p110 β /p85 dimer. Interestingly, the cSH2 phosphopeptide binding site is not precisely coincident with the binding site for p110 β . Thus, although a minimal nSH2-binding tetra-peptide will activate p110/p85 via disruption of the nSH2 domain contact, disruption of the cSH2

domain contact with p110 β requires peptides of at least 6 amino acids [37]. This means that a short molecule or peptidomimetic compound that occupies the cSH2 domain without disrupting the cSH2 kinase domain contact would block subsequent activation. This would not be true of the nSH2 domain, where the phospho-binding site and the helical domain contact site are identical [36].

Based on a comparison of the relevant crystal structures, Williams and colleagues have proposed that the cSH2 domain-kinase domain contact is present in p110 β /p85 and p110 δ /p85, but not in p110 α /p85 [37, 38]. This is because the loop corresponding to K α 7/K α 8 is significantly longer in p110 α , presumably preventing the inhibitory contact. In this model, a short peptidomimetic ligand for the cSH2 domain of p85 would specifically reduce activation of p110 β (and p110 δ , in cell types that express this isoform). It is worth noting, however, that this model is not consistent with older data showing that mutation of the nSH2 domain phosphotyrosine binding site in a p110 α /p85 dimer only partly reduced activation by phosphotyrosine peptides [39]; significant phosphopeptide activation via the cSH2 domain was still observed, whereas mutation of both nSH2 and cSH2 domains abolished activation. These data would suggest that cSH2 occupancy does in fact contribute to the activation of p110 α /p85. Whether short versus long phosphopeptides cause differential activation via occupancy of the cSH2 domain in the p110 α /p85 dimer is not yet known.

Interactions with small GTPases

Direct binding of Ras-GTP to the p110 α /p85, p110 δ /p85 and p110 γ /p87 isoforms leads to activation of these PI 3-kinases [40–48], whereas this has not been documented for p110 β /p85. In contrast, p110 β does bind strongly to Rab5-GTP [7, 8]. This interaction has been suggested to explain the role of p110 β in endocytic trafficking [9, 10], and Zerial and colleagues have shown that p110 β -mediated production of PI[3,4,5]P₃, followed by dephosphorylation of the lipid product, can provide an alternative source of PI[3]P in endosomes [49]. Endosomal PI[3]P mediates the recruitment of FYVE domain-containing effectors such as EEA1, the sorting nexins, and Rabenosyn 5 [50]. Whereas endosomal PI[3]P is thought to act during sorting and recycling steps in the endocytic system, the reported endocytic phenotype in p110 β ^{-/-} MEFs appears instead to involve the internalization step [9, 10]. Thus, the relationship between Rab5-mediated p110 β recruitment to endosomes and the endocytic phenotype of p110 β ^{-/-} MEFs is not yet clear.

If the endocytic role of p110 β does involve its binding to Rab5, the potential therapeutic benefit of interfering with this interaction is uncertain. Although some aspects of signaling by the EGFR require its delivery to endosomes, signaling by other RTKs is amplified by inhibition of internalization [51, 52]. A growing literature suggests that disruptions in clathrin-mediated and clathrin-independent receptor internalization may contribute to increased proliferative signaling, whereas they may inhibit migration and invasion by interfering with integrin recycling [53]. Finally, recent work has also suggested that p110 β is required for the activation of the macroautophagy pathway in starved cells [54]. If binding to Rab5 is required for this activity of p110 β , then inhibitors of Rab5-p110 β binding would presumably inhibit macroautophagy. Macroautophagy is usually viewed as a physiologically important process whose disruption or attenuation leads to adverse health effects [55]. Although there have been discussion of whether acute inhibition of autophagy might be effective as adjunct chemotherapy in the treatment of cancer [56], it is too early to tell what the net physiological effect of such an intervention would be.

The p110 β -G β γ interface

Studies over the past 15 years have clearly shown that p110 β can be activated both by phosphotyrosine binding to SH2 domains and by G β γ subunits downstream of activated

GPCRs [4–6]. The relative contribution of these two mechanisms to the net activation level of p110 β *in vivo* is not yet clear. *In vitro* data shows synergistic activation of p110 β /p85 by both phosphotyrosine-containing peptides and G $\beta\gamma$ subunits [5, 57], whereas *in vivo* studies suggest that p110 β /p85 primarily acts downstream of GPCRs [4, 9, 10]. G $\beta\gamma$ subunits can interact directly with p110 β , as activation of p110 β is observed in the absence of p85 *in vitro* [57]. This is unlike the G $\beta\gamma$ -mediated activation of p110 γ , which requires contacts with both p101 and p110 γ [57–59].

The relative contribution of RTKs versus GPCRs to p110 β -dependent signaling has been difficult to analyze until recently, as no methods were available to distinguish the two inputs in cells. However, a recent study has identified the G $\beta\gamma$ binding site in p110 β using a combination of sequence alignment and deuterium exchange/mass spectrometry (HDX-MS) [60]. The binding site is a flexible surface-exposed loop in the C2 domain-helical domain linker, residues 514–537 (Figure 3A). Mutation of conserved residues in the loop, or replacement of the loop with the corresponding region of p110 δ , abolished G $\beta\gamma$ activation but had no effect on basal activity or activation by phosphotyrosine peptides. With regard to signaling by p110 β , point mutants that interfere with G $\beta\gamma$ binding blocked p110 β -mediated enhancement of Akt activation, chemotaxis, and transformation. Surprisingly, a peptide derived from the G $\beta\gamma$ -binding loop in p110 β was an effective inhibitor of G $\beta\gamma$ -mediated activation of p110 β /p85 *in vitro*, and a cell-permeant version of the peptide blocked p110 β -stimulated chemotaxis, invasion, and transformation in cells, and blocked the proliferation of PTEN-null prostate and endometrial cancer cells. The peptide appeared to be specific for p110 β /p85, as it had no effect on G $\beta\gamma$ activation of p101/p110 γ , adenylyl cyclase, or PLC β .

The efficacy of a peptide derived from the G $\beta\gamma$ -binding site in p110 β in these cell-based experiments suggests that site-specific targeting of G $\beta\gamma$ might provide a novel approach to PI 3-kinase inhibition. HDX-MS mapping of the binding site for p110 β within G $\beta\gamma$ (Figure 3B) revealed two peptides that showed protection: residues 31–45 in the linker between the Nterminal alpha helix and the first blade of the β -propeller, and residues 85–99 at the second blade and extending onto the top of the β -propeller [60]. The second p110 β peptide is close to a region known to interact with canonical G $\beta\gamma$ effectors, and which partially overlaps with the SIGK binding site [61], whereas the first region has not been previously implicated in G $\beta\gamma$ signal transduction. Although the binding site of the p110 β -derived peptide is not yet known, its apparent specificity for G $\beta\gamma$ -p110 β interactions suggests that small molecule inhibitors could uniquely disrupt G $\beta\gamma$ -p110 β interactions. Indeed, small molecule inhibitors specific for G $\beta\gamma$ -mediated activation of PLC β 2, PLC β 3 and p110 γ have been described [61, 62]. In some cases, inhibition of p110 β /p85 targeting by binding to membrane bound G $\beta\gamma$ might be more efficacious than inhibitors targeting kinase activity, as some activities of p110 β are kinase independent [9, 10].

In contrast to the defined role of p110 γ in HHV8 vGPCR-driven transformation [63], p110 β has not been shown to mediate any GPCR-dependent transformation in tissues. However, p110 β has been shown to be required for driving tumorigenesis in situations of loss of PTEN function, particularly in the prostate [10, 15]. The finding that inhibition of p110 β binding to G $\beta\gamma$ blocks the growth of PTEN null cells suggests that GPCR signaling may play an unappreciated role in these tumors. Consistent with this idea, a study using PTEN null PC3 cells in a xenograft model showed that blocking G $\beta\gamma$ signaling by expressing a peptide sequence derived from the C-terminus of G protein-coupled Receptor Kinase 2 (GRK2) resulted in decreased PC3 cell growth in response to serum *in vitro* and reduced tumor growth *in vivo* [64]. It will be interesting to determine whether the GRK peptide interferes with activation of p110 β /p85. If GPCR stimulation of p110 β is in fact required for the growth of these tumors *in vivo*, the identification of the specific GPCRs that drive tumor growth will provide additional drug targets for the management of these tumors.

The *in vivo* significance of the GPCR-input to p110 β in driving tumors has not yet been tested in xenografts or genetic mouse models. However, a recent study showed that a p110 β -specific kinase inhibitor has dramatic effects in blocking growth of PTEN null tumors [17]. Peptidomimetic or small molecule inhibitors disrupting the G $\beta\gamma$ -p110 β interface could have similar efficacy but fewer side effects due to the sparing of RTK-mediated p110 β activation. Alternatively, the surprising result that p110 β -derived peptides were better inhibitors of PC3 cell growth than the kinase inhibitor TGX221 [60] suggests that some of the growth-promoting GPCR inputs to PTEN-null cells may require scaffolding functions of p110 β . In this case, agents aimed at disrupting G $\beta\gamma$ -p110 β interactions might actually have greater efficacy than ATP-competitive kinase inhibitors.

Concluding remarks

The search for inhibitors of p110 β has been motivated by the finding that p110 β plays a crucial role in thrombosis and in the growth of tumors displaying a loss of PTEN function. Recent studies have shown the efficacy of p110 β -specific kinase inhibitors in blocking growth of PTEN^{-/-} prostate cancers in mice [17] as well as in anti-thrombotic therapy [27]. A more complete understanding of the regulation and functions of p110 β , both kinase-dependent and kinase-independent, will be important in order to better target this isoform. The identification of the binding sites in p110 β for G $\beta\gamma$ and, eventually, for Rab5, will greatly expand the options for targeting this complexly regulated PI 3-kinase. In addition, studies implicating GPCR signaling in the growth of tumors with diminished PTEN expression or activity will hopefully lead to the identification of the relevant receptors, whose pharmacological inhibition might provide a novel approach to the management of these tumors.

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Signaling by p85/p110 β

Figure 1. Signaling by p110 β /p85 dimers

The p110 β /p85 dimer is activated both by binding to tyrosine phosphorylated receptors and their substrates, via the SH2 domains of p85, as well as by direct p110 β binding to G β γ subunits, in response to activation of G-protein coupled receptors. p110 β /p85 dimers are also targeted to Rab5-positive early endosomes. p110 β /p85 dimers signal in part by the production of PI[3,4,5]P3, which activates the Akt/mTOR pathway, TEC-family tyrosine kinases, Rho-family GTPases, and other downstream effectors. The targeting of p110 β /p85 dimers to early endosomes may also contribute to PI[3]P production in this organelle, via the dephosphorylation of PIP3. However, kinase independent signaling of p110 β /p85 dimers contributes to proliferation of PTEN-null tumor cells, as well as regulation of endocytic trafficking and autophagy.

Figure 2. Specific targeting of the cSH2-kinase domain interface

Cartoon of the p85(nSH2-iSH2-cSH2)/p110 β dimer. The phosphopeptide binding sites in the nSH2 and cSH2 domains, and the inhibitory contact sites in the helical and kinase domains of p110 β , are stippled. Whereas the nSH2 phosphopeptide binding site is exactly coincident with the helical domain contact site, the cSH2 phosphopeptide binding site is adjacent to the kinase domain inhibitory site. Short phosphopeptides could block the cSH2 domain binding site without activating p110 β .

Figure 3. Regulation of p110β/p85 dimers by Gβγ

A. Cartoon (left) and crystal structure (right) [37] showing the Gβγ-binding site in the p110β/p85(iSH2-cSH2), which is defined as a surface loop in the C2 domain-helical domain linker. The ends of the loop only are seen in the crystal structure. Mutagenesis of the loop abolished Gβγ activation of p110β/p85 dimers. B. The crystal structure of Gβγ (green and cyan) bound to the SIGK peptide (magenta), which blocks interactions with canonical Gβγ effectors [61, 65]. Regions that interact with p110β are shown in red. While p110β interacts with some residues near the canonical effector-binding site, it also binds to regions not known to interact with canonical Gβγ effectors.