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Therapeutic Potential of Phosphoinositide 3-Kinase Inhibitors

Review

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At least one Holy Grail for many academic researchers and pharmaceutical research divisions alike has been to identify therapeutically useful selective PI3K inhibitors. There are several different but closely related PI3Ks which are thought to have distinct biological roles. Until now, however, researchers have been frustrated by poor selectivity of the available pharmacological inhibitors, which are unable to distinguish the different isoforms of PI3K adequately. Fortunately, recently published work gives cause for optimism; there are now several patent specifications published that describe new PI3K inhibitors, including some that are more selective for the delta isoform of PI3K. Given the involvement of PI3Ks in a plethora of biological settings, such isoform-selective inhibitors may have immense potential use for the treatment of patients with inflammatory and autoimmune disorders as well as cancer and cardiovascular diseases.

Introduction

Phosphoinositide 3-kinases (PI3Ks) were first identified as an activity associated with various oncoproteins and growth factor receptors [1, 2], and evidence soon accumulated that PI3Ks can provide a critical signal for cell proliferation, cell survival, membrane trafficking, glucose transport, neurite outgrowth, membrane ruffling, and superoxide production as well as actin reorganization and chemotaxis [3, 4]. The diverse nature of PI3K functional effects is reflected in its activation by multiple receptors, the existence of three classes and several isoforms of PI3K, and multiple effector proteins that can interact with PI3K lipid products by distinct structural motifs [3, 4].

The term PI3K is applied to a family of lipid kinases that can be classified into three subfamilies according to structure and substrate specificity (Table 1) [4]. The class I group of PI3Ks consists of two subgroups. The prototypical class IA PI3K consists of an 85 kDa regulatory subunit (responsible for protein-protein interactions via SH2 domain interaction with phosphotyrosine residues of other proteins) and a catalytic 110 kDa subunit. There are three catalytic isoforms (p110α, p110β, and p110δ) and five regulatory isoforms (p85α, p85β and p55γ encoded by specific genes and p55α and p50α that are produced by alternate splicing of the p85α gene). A distinct lipid kinase termed PI3Kγ (or p110γ) is activated by G protein-coupled receptors, and this is the only characterized member of the class 1B G protein-coupled PI3K family. The class IB PI3Ks are stimulated by G protein $\beta\gamma$ subunits and do not interact with the SH2-containing adaptors that bind class IA PI3Ks. Instead, the only identified member of this family, p110γ, associates with a unique p101 adaptor molecule [5]. Nevertheless, there is some evidence that G protein-coupled receptors (GPCR), such as receptors for chemokines and lysophosphatidic acid, are also able to activate the p85/p110 heterodimeric PI3Ks [6, 7]. In this respect, the p85/p110 heterodimer has been demonstrated to be synergistically activated by the $\beta\gamma$ subunits of G proteins and by phosphotyrosyl peptides [8].

The class I PI3Ks are the most extensively studied of these lipid kinases and can potentially generate three lipid products, namely phosphatidylinositol 3-monophosphate [PI(3)P], phosphatidylinositol 3,4-bisphosphate [PI(3,4)P_2], and phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P_3] [4]. In vivo, the major products of class I PI3Ks appear to be PI(3,4)P_2 and PI(3,4,5)P_3, as these are transiently induced following cell stimulation. They can selectively bind certain pleckstrin homology domains, modular segments of about 100 amino acids found in many signaling proteins. The ability of PH domains within different proteins to discriminate PI(3,4)P_2 and PI(3,4,5)P_3 from each other and from other PI lipids could be central to the specificity of PI3K signaling. It is these PH-domain-containing proteins that are able to propagate and drive further downstream signaling events, and the best characterized PI3K effector molecule is protein kinase B (PKB/Akt). The PI3K family is completed by the class II C2-domain-containing PI3Ks and the class III PtdIns-specific 3-kinases [4]. The class II PI3Ks (comprising α, β, and γ isoforms) are characterized by the presence of a C2 domain at the carboxyl terminus and utilize predominantly PI and PI(4)*P* as substrates in vitro [9]. In contrast to members of the other PI3K classes, PI3K-C2α is refractory to inhibition by the PI3K inhibitor wortmannin [9]. Recent evidence has indicated that clathrin functions as an adaptor for class II PI3K-C2α, binding to its N-terminal region and stimulating its activity [10]. The class III PI3Ks utilize only PI as a substrate (e.g., mammalian PI 3-kinase and yeast Vps34p) [4]. A clearer picture is now emerging of the importance of individual catalytic isoforms in distinct biological effects, which will be highlighted later in this review.

The termination of PI3K signaling by degradation of PI(3,4,5)P_3 can be mediated by at least two different types of phosphatases, namely SH2-containing inositol 5-phosphatase (SHIP) [11] and the 3′ phosphatase termed phosphatase and tensin homology deleted on chromosome ten protein (PTEN) [12, 13]. Dephosphorylation of PI(3,4,5)P_3 by SHIP impairs some downstream

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Table 1. PI3K Family Members

Class	Catalytic Subunit	Adaptor/Binding Partner	Distribution
IA	p110α	p85α, p50α, p55α	Broad
	p110β	p85β	Broad
	p110δ	p55γ	Leukocytes[a]
IB	p110γ	p101	Leukocytes[a]
II	PI3K-C2α/β/γ[c]	Clathrin	Broad[b]
III	PtdIns 3-kinase	p150	Broad

[a] p110δ and p110γ have been considered leukocyte specific, but recent work suggests that they are more broadly expressed.
[b] Type II C2α is the only PI3K that is substantially resistant to the inhibitor wortmannin.
[c] PI3K-C2γ is liver specific.

effects of PI3K, although the metabolic product of SHIP phosphatase activity is $PI(3,4)P_2$, which can also mediate PI3K-dependent responses.

First Generation PI3K Inhibitors

The availability of PI3K inhibitors has contributed greatly to our current understanding of the biological role of PI3Ks and their effector proteins. The major pharmacological tools that have been used in this respect are assessed below.

Wortmannin

Despite limitations in selectivity, the fungal metabolite wortmannin isolated from *Penicillium wortmanni* (structure 1, Figure 1), has proven to be an invaluable tool with which to assess the functional relevance of PI3K activation in many settings. The concentration of wortmannin required to inhibit PI3Ks ranges from 1–100 nM (reviewed in [14]). Irreversible inhibition of lipid and serine kinase activity of class 1 and class III PI 3-kinases occurs by covalent modification of the catalytic sites [15]; class II PI3Ks are also inhibited at higher concentrations (e.g., >100 nM). The highly electrophilic C-20 position of the furan ring of wortmannin is thought to be responsible for alkylation of the protein, so that nucleophilic attack of Lys802 of p110α results in formation of an enamine (at C-20) achieving covalent modification of PI3K (Figure 1) [15]. Nucleophilic residues equivalent to Lys802 are present in all PI3Ks and protein kinases, such as myosin light chain kinase, protein kinase A, and DNA-dependent protein kinase catalytic subunit as well as TOR-related proteins, and probably account for the poor selectivity of wortmannin.

Bioflavanoid-Related Compounds

The bioflavanoid quercetin (structure 2, Figure 1) was initially shown to effectively inhibit PI3K with an IC_{50} of 3.8 μM but has poor selectivity, inhibiting related enzymes such as PI 4-kinase and several tyrosine and serine/threonine kinases [16]. Using quercetin as a model compound, several chromenones were synthesized and evaluated for their ability to inhibit PI3Ks. The placement of a phenyl moiety at the 8 position of the chromenone ring resulted in a potent compound, LY294002 [17] (structure 3, Figure 1), that completely inhibits PI3K at 100 μM [17]. Both of these compounds are competitive inhibitors at the ATP binding site of PI3K, but unlike quercetin, LY294002 has no detectable effect on other ATP-requiring enzymes such as protein kinases and PI 4-kinase [17]. Despite a reported IC_{50} about 500-fold higher than wortmannin, LY294002 has the advantage of being more stable in solution and has been widely used in cell biology, being almost as instrumental as wortmannin in characterizing the biological importance of PI3K signaling pathways. Although PI3K-C2α is refractory to both inhibitors, neither wortmannin nor LY294002 exhibit any degree of selectivity for individual PI3K isoforms.

Lessons from Structural Analysis

Most protein kinase inhibitors in development for pharmaceutical application work by competing with ATP binding. Despite the overall similarity of the ATP binding sites among protein kinases, it has been possible to exploit local variation in the mode of ATP binding in order to develop protein kinase-specific inhibitors that

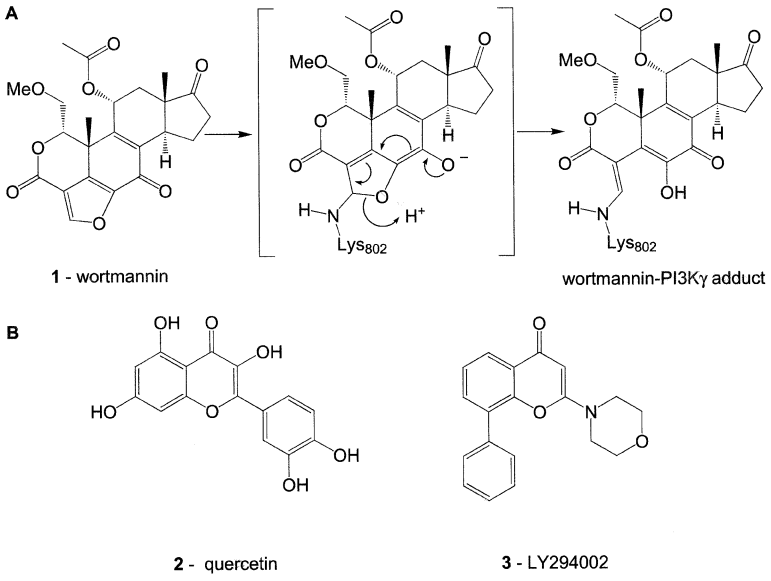

Figure 1. First Generation PI3K Inhibitors

(A) Structure of wortmannin highlighting the region responsible for nucleophilic attack of Lys802 of PI3K. (B) Structures of the bioflavanoid quercetin and its derivative LY294002.

have led to promising clinical applications [18, 19]. This approach has proven highly successful in the development of EGF-R inhibitors (e.g., IRESSA, developed by AstraZeneca) for the treatment of solid tumors and cAbl inhibitors (e.g., Glivec, developed by Novartis) that are among the vanguard of attack in treating patients with chronic myeloid leukemia.

Interesting insights into the possible development of isoform-selective PI3K inhibitors have been provided by determination of the crystal structure of PI3Kγ [20]. This structure reveals a multidomain organization with the catalytic domain of PI3K having a similar fold to that of the protein kinases. The catalytic domain has two lobes: a smaller N-terminal lobe consisting of a five-stranded β sheet flanked by three α helices and a larger primarily helical C-terminal lobe. The ATP cosubstrate binds between these two lobes in a manner similar to ATP binding in protein kinases, with many of the enzyme-ATP contacts involving residues in the linker region between the two lobes. X-ray crystallography of PI3Kγ bound to various PI3K inhibitors has revealed how these compounds fit into the ATP binding pocket. Wortmannin, LY294002, and quercetin all bind to the ATP binding site of PI3Kγ such that the aromatic portion of the compound occupies the space that is normally occupied by the adenine base, with a hydrogen-bond acceptor in a position approximating to the N1 atom of PI3Kγ-bound ATP. Wortmannin binds irreversibly to the enzyme and causes a small distortion in the packing of the neighboring side chains in the catalytic domain. The close shape complementarity between the PI3K active site and wortmannin together with the irreversible modification are important determinants of the low nanomolar IC_{50} for this compound. However, neither LY294002 nor quercetin causes conformational changes, although remarkably, they bind PI3K in different orientations that are related to each other by 180° rotations.

Isoform Selective Inhibitors: The Next Generation

Insights into the structural determinants of PI3K inhibitors as outlined above should provide a valuable impetus for the design and development of isoform-selective inhibitors. However, binding properties of individual compounds and the degree to which structural plasticity of the enzyme might be exploited by any therapeutic lead compound are hard to predict. It is worth repeating the caution noted by Cohen and coworkers that the selectivity of protein kinase inhibitors cannot be taken for granted by only testing against structurally related enzymes, as inhibitors previously reported as selective have often been shown to inhibit protein kinases not closely related in primary structure to the original target. For example, wortmannin is known to potently inhibit myosin light chain kinase, while LY294002 has been shown to potently inhibit casein kinase-2 [21].

Recently, a number of patent specifications have been published which describe inhibitors of PI3K, including compounds that exhibit some selectivity for individual p110 catalytic isoforms (Figure 2 and Table 2). It is important to stress that the patents which describe these so-called second generation inhibitors do not necessarily provide detailed information as to the biological characterization of the compounds, and the compounds are not commercially available. Nevertheless, the compounds reported in these patents do represent promising lead compounds for the future development of PI3K isoform-selective inhibitors. In this respect, Yamanouchi has described several imidazopyridine derivatives, (e.g., compounds of general structure 4, Figure 2). These are claimed to exhibit excellent PI3K inhibitory activities, especially against p110α, although no isoform selectivity data are provided [22]. Thrombogenix disclosed a series of morpholino-substituted compounds closely related to LY294002 that for the first time were able to elicit any degree of isoform selectivity [23]. They described quinolone and pyridopyrimidine compounds (structures 5 and 6, Figure 2) that are approximately 100-fold more potent against α/β isoforms compared to γ isoforms, exhibiting submicromolar activity against p110α/β compared to micromolar activity against γ (Table 2). A recently published patent application from the ICOS Corporation describes compounds that have high selectivity for inhibition of p110δ [24]. They report several quinazolinone compounds (e.g., structures 7 and 8, Figure 2) that display selectivity for p110δ versus PI3Kγ and p110α and β, with the most potent exhibiting around 40-fold greater selectivity for p110δ versus the other isoforms (Table 2). A recent publication reports that methylxanthines such as caffeine and theophylline (structures 9 and 10, Figure 2) are selective for p110δ isoforms over p110α and p110β isoforms, although their activity is rather low [25]. To date, no PI3Kγ-selective inhibitor has been reported in the literature.

Therapeutic Potential for PI3K Inhibitors

Inflammation and Autoimmune Disease

The catalytic subunit isoforms p110γ and p110δ have sparked a great deal of interest among immunologists and pharmaceutical companies alike, because their expression is largely restricted to leukocytes, and there is increasing evidence that these isoforms in particular play key roles in innate and adaptive immunity. This view has been borne out by several studies, some of which are mentioned here. A hallmark of inflammatory responses is the migration of leukocytes to the inflammatory lesion in response to chemokines and other chemoattractants. Several studies suggest that the polarized activation of PI3K at the leading edge of a migrating cell and subsequent PI(3,4,5)P_3-dependent recruitment of PKB and possibly other PH-domain-containing proteins is a crucial and early event in the detection of a chemoattractant gradient [26–29]. The phenotype of two-knockout mice models has reinforced the view that PI3K isoforms can significantly contribute to inflammatory responses. First, studies of mice lacking PI3Kγ have shown that this isoform is essential for PI(3,4,5)P_3 production and PKB activation as well as superoxide production in neutrophils exposed to the chemoattractants that act via GPCRs (e.g., fMLP, C5a, and IL-8). Chemotaxis of cells involved in mounting an inflammatory response (e.g., neurophils, macrophages, and T lymphocytes) was also impaired in the absence of PI3Kγ, both in vitro and in vivo [30–32]. Second, SHIP$^{-/-}$ mice suffer from lethal infiltration of the lungs by macrophages and

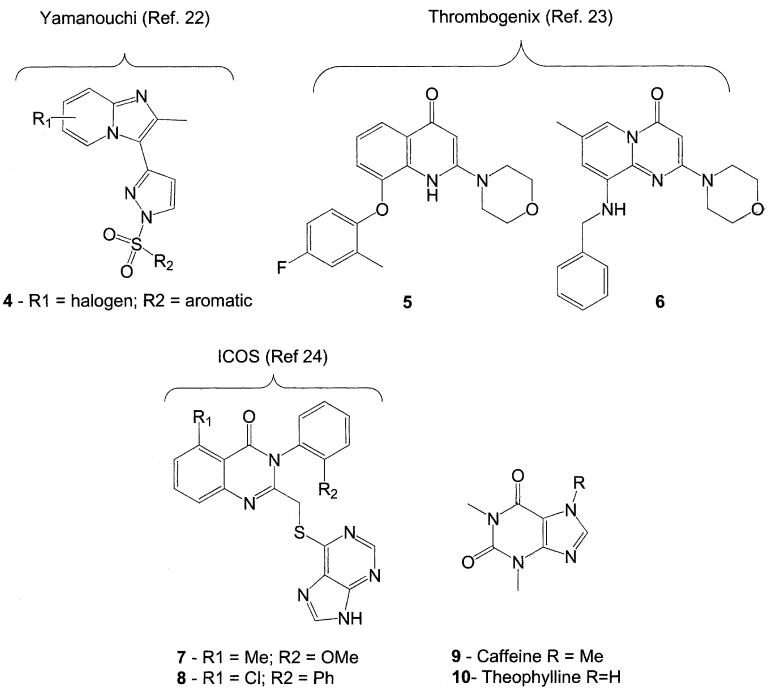

Figure 2. Second Generation PI3K Inhibitors

Structure of new PI3K inhibitors: imidazopyridines (general structure 4) reported by Yamanouchi; quinolone (structure 5) and pyridopyrimidine (stucture 6) described by Thrombogenix; quinazolinone (structures 7 and 8) reported by ICOS as well as caffeine (structure 9) and theophylline (structure 10).

neutrophils. Thus, persistently high levels of the $PI(3,4,5)P_3$ and subsequent activation of its downstream effectors might lead to excessive inflammation [33]. Given that PI3Kγ has been proposed to be the major isoform responsible for producing $PI(3,4,5)P_3$ in response to chemoattractants, it seems likely that selective inhibitors of PI3Kγ would be potentially useful in preventing inflammatory cell recruitment in a range of inflammatory diseases including asthma, rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease.

Insights into the biological role of p110δ have also been provided by an elegant study in which a new strategy was adopted to address the role of the p110δ isoform [34]. Instead of deletion of a particular p110 isoform gene which alters expression of other isoforms, knockin mice expressing a mutated and catalytically dead p110δ were generated. This approach prevented changes in the expression levels of the other PI3K catalytic and regulatory subunits. These mice exhibit impaired antigen receptor signaling in T and B lymphocytes as well as impaired in vivo immune responses, suggesting a key role for p110δ in immunity [34]. The selective attenuation of immune function in these p110δ mutant mice suggests that a specific inhibitor of p110δ could effectively suppress B and T cell mediated autoimmunity and possibly B and T cell transformation.

A p110δ isoform-specific inhibitor would be predicted to have similar effects as expression of a catalytically dead p110δ mutant. Indeed, p110δ-specific compounds also significantly inhibited IgM and IgG production from B cells and B cell proliferation in response to IgM stimulation and inhibited T cell proliferation in response to CD3 and CD28 costimulation [24]. The p110δ-specific compounds also provide an insight into the role of this isoform in a variety of other cellular functions. In neutrophils, for example, δ-specific inhibitors prevent the production of superoxide by *N*-formyl-Met-Leu-Phe (fMLP) as well as fMLP-stimulated elastase release and fMLP-induced cell migration. This is rather surprising given that the G protein $\beta\gamma$ subunit-dependent p110γ catalytic isoform has been suggested to be the sole contributor

Table 2. Relative Potency of Second Generation PI3K Inhibitors against Individual Isoforms

Inhibitor	IC_{50} μM				Reference
PI3K α/β Selective	PI3K α	PI3K β	PI3K γ	PI3K δ	
Quinolone (structure 5)	0.02		5	ND	23
Pyridopyrimidine (structure 6)	0.05		>10	ND	23
PI3K δ Selective					
Caffeine	400	400	1000	75	25
Theophylline	300	800	800	75	25
Quinazolinone (structure 8)	>2.0	>2.0	>2.0	0.05	24

IC_{50} values are derived from different studies in which there is likely to be variation in source of enzyme and experimental conditions. Refer to Figure 2 for structures and source of compounds. ND, not determined.

to lipid accumulation in response to several different ligands that activate distinct GPCRs [31, 35]. It seems, therefore, that p110δ can also participate in GPCR signaling, indicating that some redundancy of function may exist between the two isoforms.

Cardiovascular Disease

Platelet activation involves cytoskeletal rearrangements and granule secretion and converts the fibrinogen receptor (the integrin $\alpha IIb\beta 3$) from low- to high-affinity binding state. Platelets express p110α, β, and γ (but not δ) that are believed to play important roles in aggregation [36]. Indeed, aggregation in response to thrombin and phorbol ester stimulation is sensitive to wortmannin. However, many soluble platelet stimuli, including ADP, thromboxane A2, and thrombin, exert their physiological actions through GPCRs. It is interesting to note, therefore, that platelets lacking the G protein-activated PI3Kγ isoform exhibit impaired aggregation and $\alpha IIb\beta 3$ fibrinogen receptor activation as well as reduced thromboembolism when modeled in vivo [37]. Thus, pharmacological targeting of PI3Kγ as well as p110α and β may have potential use as antithrombotic therapy.

Some impressive advances in our understanding of the crucial role PI3K isoforms play in cardiomyocyte contractility have recently been achieved with the use of cardiac-specific PTEN knockout mice. Amazingly, these mice exhibited spontaneous cardiac hypertrophy as well as decreased contractility when either whole hearts or single cardiomyocytes were examined [38]. Analysis of double-mutant mice that were deficient in PTEN and also expressed dominant-negative mutants of either p110α or PI3Kγ revealed that the cardiac hypertrophy and contractility defects could be genetically uncoupled. Thus, the increase in cell size found in PTEN-deficient hearts could be reversed in mice that were deficient in PTEN but also transgenically overexpressed a heart-specific dominant-negative mutant of p110α. Overexpression of a dominant-negative p110α on a wild-type background affects heart size but has no effect on heart function, while the PTEN-deficient mice expressing the p110α mutant still displayed a marked decrease in contractility. This latter observation suggested the involvement of a distinct PI3K isoform in the regulation of heart contractility. Indeed, the deficits in cardiomyocyte and whole-heart contractility from PTEN-deficient mice were reversed when a dominant-negative form of PI3Kγ was coexpressed in PTEN-deficient mice, whereas mice deficient in PI3Kγ activity alone exhibited enhanced cardiomyocyte and whole-heart contractility [38]. Although expression of PI3Kγ has been generally considered to be leukocyte restricted, this study reported strong expression of PI3Kγ protein in total heart extracts of mice and in isolated cardiomyocytes. It transpires that PI3Kγ regulates cardiac function by negatively regulating cAMP levels upon GPCR-coupled β2-adrenergic receptor stimulation [38]. In cardiomyocytes, increased cAMP levels result in enhanced contractility via activation of protein kinase A and subsequent phosphorylation of phospholamban in the sarcoplasmic reticulum. This suggests that downregulation of cAMP levels by PI3Kγ-dependent mechanisms impairs contractility and leads to the exciting prospect that selective inhibition of PI3Kγ may induce better cardiac contractility during heart failure.

There is a growing body of evidence that PI3Ks play an important role in regulating vascular tone as well as myocardial contractility and cell size. Consequently, dysfunction of PI3K-dependent signaling pathway may be important in the pathogenesis and pathology of other cardiovascular diseases such as hypertension. The spontaneous tone generated in hypertensive rats was eliminated by the PI3K inhibitors LY294002 and wortmannin, whereas hypertensive rat aortas showed enhanced PI3K activity associated with increased expression of the PI3K subunits, p110β and p110δ [39, 40]. Thus, selective inhibitors for p110 isoforms may be useful in treatment of hypertension.

Cancer

Evidence for the role of PI3K in the progression of human cancers is largely circumstantial. However, bearing in mind that many oncogenic signaling pathways are mediated by PI3K and that PTEN, the 3′ phosphatase that breaks down $PI(3,4,5)P_3$ is a tumor suppressor, it would seem likely that there is potential benefit from developing PI3K inhibitors for the treatment of cancer. Increased PI3K activity may lead to aberrant control of the cell cycle, increased survival from apoptosis, and promotion of a metastatic phenotype. PI3K inhibitors may block each of these facets of tumorigenesis.

Amplification of the *PIK3CA* gene has been observed in several carcinomas [41, 42]. Mutation of the lipid phosphatase PTEN is one of the most common mutations in human cancer [43]. In addition, PKB amplification and increases in PKB kinase activity have been widely reported in tumor tissue [44]. The number of PKB substrates is also rapidly expanding, and many of these proteins are well-characterized modulators of cell growth and survival. The cyclin-dependent kinase $p27^{Kip1}$, which normally resides in the nucleus, is located in the cytoplasm in a variety of tumors. Recent studies have shown that p27 phosphorylation by PKB blocks entry into the nucleus and that p27 cytosolic localization correlated with poor patient survival [45–47]. Another substrate of PKB is the oncoprotein Mdm2, phosphorylation of which promotes its nuclear entry, leading to the degradation of the tumor suppressor, p53 [48].

The PI3K inhibitors wortmannin and LY294002 have been shown to block the proliferation of numerous cancer cell lines in vitro and are effective in xenograft models [49, 50]. In addition, PI3K inhibitors have been shown to enhance the effects of radiation and cytotoxic agents [51, 52]. These effects may be partially due to the inhibition of the closely related kinases ATM and DNA-PK but may also occur due to an enhancement of apoptosis in treated cells. The prospects for PI3K inhibitors in the treatment of cancer look promising. One advantage of such an approach would be the opportunity to interfere with multiple signaling pathways, allowing the blockade of both tumor proliferation and angiogenesis mediated by growth factors such as vascular endothelial growth factor (VEGF). The level of PI3K isoform selectivity that is required in such inhibitors and the optimal dosing regimen remain to be determined. Finally, chemokine receptors have been shown to be involved in breast cancer metastasis [53]. Given that these receptors are strongly coupled to PI3K isoforms [7], this would suggest that PI3K inhibitors might reduce the risk of cancer metastasis.

Conclusion

Clearly, the therapeutic potential for isoform-selective PI3K inhibitors is huge. However, there are still some questions about redundancy of function between PI3K isoforms, and it is perhaps worth emphasizing that even isoform-selective inhibitors may present problems. For example, it is worth noting that p110δ mutant mice developed a mild inflammatory bowel disease, suggesting not only that the human p110δ gene may be a human inflammatory bowel disease susceptibility gene, but also that p110δ may be required for homeostatic immune responses to normal gut flora. In addition, *chronic* activation of p110α and PKB using transgenic approaches reveals a spectrum of phenotypes ranging from no cardiomyopathic changes, moderate cardiac hypertrophy with preserved systolic function, to massive cardiac dilatation and sudden death [54–56]. There is strong evidence, however, that *acute* PKB activation protects cardiomyocytes from apoptosis in vitro and in vivo and dramatically reduces infarction and cardiac dysfunction 24 hr after transient ischaemia [57, 58]. Thus, the use of p110α- and β-selective inhibitors as therapeutic agents may well be tempered by their potential to increase susceptibility to infarct. Such issues will have to be given careful consideration, but for now, the availability of promising lead compounds should provide the momentum to develop new compounds with better spectra of selectivity for future therapeutic use.

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Note Added in Proof

Another quinazolinone-related compound, IC87114, has been reported recently by ICOS to have an IC_{50} for p110δ of 0.5 μM, whereas IC_{50} values for p110α, p110β, and PI3Kγ were $>$100, 75, and 29 μM, respectively: Sadhu, C., Masinovsky, B., Dick, K., Sowell, C.G., and Staunton, D.E. (2003). Essential role of phosphoinositide 3-kinase delta in neutrophil directional movement. J. Immunol. *170*, 2647–2654.