
Role of Phosphatidylinositol 3,4,5-Trisphosphate in Cell Signaling

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Abstract

Many lipids present in cellular membranes are phosphorylated as part of signaling cascades and participate in the recruitment, localization, and activation of downstream protein effectors. Phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P₃) is one of the most important second messengers and is capable of interacting with a variety of proteins through specific PtdIns(3,4,5)P₃ binding domains. Localization and activation of these effector proteins controls a myriad of cellular functions including cell survival, proliferation, cytoskeletal rearrangement, and gene expression. Aberrations in the production and metabolism of PtdIns(3,4,5)P₃ have been implicated in many human diseases including cancer, diabetes, inflammation, and heart disease. This chapter provides an overview of the role of PtdIns(3,4,5)P₃ in cellular regulation and the implications of PtdIns(3,4,5)P₃ dysregulation in human diseases. Additionally, recent attempts at targeting PtdIns(3,4,5)P₃ signaling via small molecule inhibitors are summarized.

Keywords

Phosphatidylinositol (3,4,5)-trisphosphate • PI3-kinase • Akt • PTEN • Tumorigenesis

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7.1 Introduction

Phosphoinositides (PIs), phosphorylated derivatives of phosphatidylinositol (PtdIns), have been investigated as agonist-dependent second messengers since their discovery in the mid 1950s by Hokin and Hokin [1]. Initial focus was made on PtdIns(4)P and PtdIns(4,5)P₂ and the enzymes responsible for their synthesis and degradation. The discovery of a family of PI3-kinases and its lipid products PtdIns(3)P, PtdIns(3,4)P₂ and most

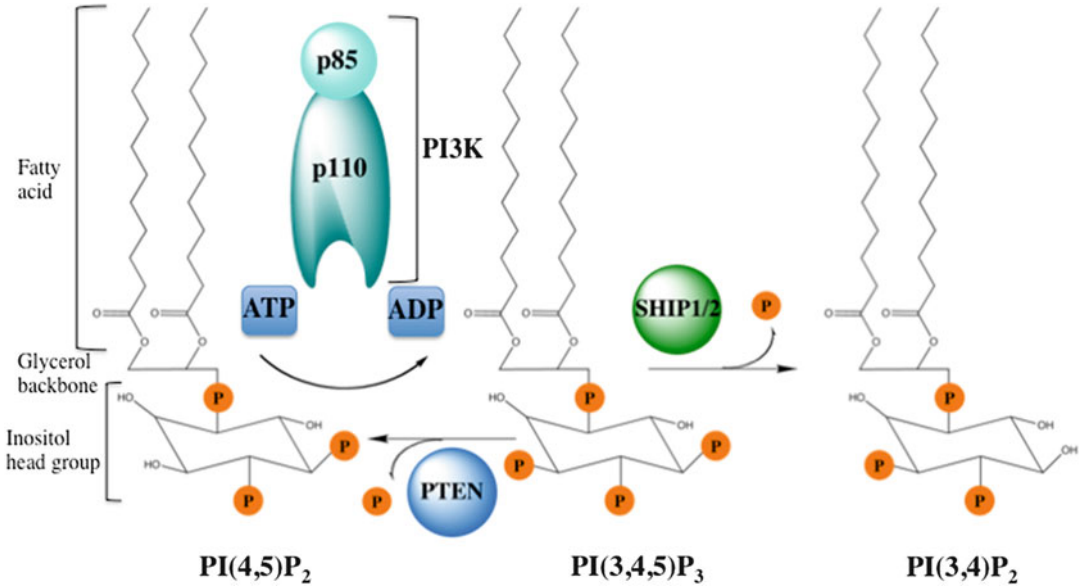


Fig. 7.1 Phosphatidylinositols are composed of long chain fatty acid groups attached to a phosphorylated inositol head group via a glycerol moiety. Fatty acids anchor the molecule in the membrane, while the inositol is exposed to the cytoplasm. Various kinases catalyze the addition or removal of phosphates to the head group at

specific locations. Above, PI3K phosphorylates PtdIns(4,5)P₂ (shown as PI(4,5)P₂) at the 3' position to form the second messenger PtdIns(3,4,5)P₃ (shown as PI(3,4,5)P₃). The phosphatases PTEN and SHIP breakdown PtdIns(3,4,5)P₃ by removing phosphates from the 3' and 5' positions respectively

importantly phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P₃) uncovered additional complexity to PI-mediated signal transduction [2–4]. Further work led to the elucidation of the mechanisms of PI3-kinase signaling and the critical importance of this class of lipid kinases in a variety of cellular functions including directional motility, metabolism, growth, and cell survival. Additionally, many diseases can be traced back to aberrations in the PI3-kinase pathways. For this reason, efforts have been made to understand the complex regulation of PIs and PtdIns(3,4,5)P₃, in particular. PI3Ks are subdivided into three classes: I, II and III, differing in their structure, products, and regulation. PtdIns(3,4,5)P₃ is the product of Class I of PI3-kinases, which will be referred to as PI3K throughout the text.

PtdIns(3,4,5)P₃ is a member of the PI class of membrane lipids. Typically, PtdIns found in cell membranes account for approximately 5–10 % of the total lipid with only approximately 1 % being phosphorylated, indicating that their primary function is regulatory in nature [5]. PtdIns can be

phosphorylated at either the 3', 4' or 5' hydroxyl sites (Fig. 7.1). These hydroxyl groups are phosphorylated alone or in combination to create seven possible PIs with individual stereospecificity and charge. PIs are localized on the endosomal, Golgi, nuclear, and plasma membranes, depending on their structure. Of the 7 PIs only PtdIns(4)P and PtdIns(4,5)P₂ are found in relatively high abundance [6]. Unique inositol head groups coupled with extremely low PI concentrations in the cell offer deliberate, specific, sensitive, and localized signaling.

Various kinases and phosphatases regulate the formation and turnover of PIs depending on cellular stimuli [7]. Of the PtdIns present in the cell only 0.25 % are phosphorylated at the 3' position indicating a highly specific regulatory function [5]. The PI3-kinase family of proteins is responsible for the formation of these PIs and four separate lipid products can be formed: PtdIns(3)P, PtdIns(3,4)P₂, PtdIns(3,5)P₂, and PtdIns(3,4,5)P₃. PtdIns(3,4,5)P₃ is predominately synthesized by the phosphorylation of PtdIns(4,5)P₂ by class

I PI3K in response to extracellular stimuli [8]. The precursor of $\text{PtdIns}(3,4,5)\text{P}_3$, $\text{PtdIns}(4,5)\text{P}_2$, has been shown to have some affinity for various effector proteins and to control the activity of several integral membrane proteins. However, it primarily serves as a pool for formation of second messengers: inositol trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$), diacylglycerol (DAG), and $\text{PtdIns}(3,4,5)\text{P}_3$ [9–11]. Though only present at less than 1 % of all plasma membrane phospholipids, $\text{PtdIns}(4,5)\text{P}_2$ is found at high localized concentrations due to its accumulation into sphingolipid/cholesterol-based rafts [12].

$\text{PtdIns}(3,4,5)\text{P}_3$ functions both as a protein activator as, for example, in the case with Akt (also known as Protein Kinase B) and as a membrane anchor, recruiting proteins to the plasma membrane where they localize and perform their actions.

7.2 $\text{PtdIns}(3,4,5)\text{P}_3$ Signaling Pathway

$\text{PtdIns}(3,4,5)\text{P}_3$ lipid second messengers serve to amplify and propagate signals from receptor tyrosine kinases (RTKs), G protein-coupled receptors (GPCRs), and the small GTPase Ras, as well as to confer signaling specificity by its temporal and spatial distribution in cellular membranes [13]. Tight regulation of $\text{PtdIns}(3,4,5)\text{P}_3$ levels in the plasma membrane is critical for the prevention of hyper- or hypo-responsiveness to many extracellular stimuli. Since the original discovery of a novel PI3K activity associated with cellular transformation by polyoma middle T antigen in 1985 [2], major insights have been gained into the mechanism of $\text{PtdIns}(3,4,5)\text{P}_3$ signaling, including initiation and termination of the signal, its effector preferences and its role in cellular regulation.

7.2.1 Signal Initiation and Termination

$\text{PtdIns}(3,4,5)\text{P}_3$ signaling is initiated by extracellular stimulation of a variety of plasma membrane receptors, including RTKs and GPCRs, leading to

the activation of Type I PI3K (Fig. 7.2). Cellular homeostasis is dependent in part on tight regulation of $\text{PtdIns}(3,4,5)\text{P}_3$ levels in the plasma membrane and is controlled by growth hormones, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin, and insulin-like growth factor I (IGF-I). PI3Ks specifically phosphorylate the D-3 inositol ring position of PIs in the plasma membrane, generating $\text{PtdIns}(3)\text{P}$, $\text{PtdIns}(3,4)\text{P}_2$, and $\text{PtdIns}(3,4,5)\text{P}_3$. Synthesis of $\text{PtdIns}(3,4,5)\text{P}_3$ is the primary cellular function of Type I PI3K, while Type II and III PI3Ks are the key regulators of the cellular $\text{PtdIns}(3)\text{P}$ pool. Dephosphorylation of $\text{PtdIns}(3,4,5)\text{P}_3$ by SHIP phosphatases is an important mechanism of $\text{PtdIns}(3,4)\text{P}_2$ generation [14].

Type I PI3Ks are heterodimers consisting of the p110 catalytic subunit and the p85 regulatory subunit. The two SH2 domains in the p85 subunit recruit the p110 subunit to the cytoplasmic domains of activated receptors at the plasma membrane through a variety of adaptor molecules. These SH2 domains are connected by a coiled coil domain, which constitutively interacts with and stabilizes the p110 subunit. There exist three isoforms of Type I_A PI3K (p110 α , β and δ) and one Type I_B PI3K isoform (p110 γ), which is controlled by an alternative regulatory subunit (p101) and was previously believed to be activated exclusively by GPCRs, but more recently was found to respond to RTKs as well [15]. Type I_A PI3Ks are mainly activated by growth factor receptors and Ras proteins. p110 α and β isoforms are expressed ubiquitously, while p110 γ and p110 δ are expressed primarily in leukocytes [16].

Under basal conditions, the levels of $\text{PtdIns}(3,4,5)\text{P}_3$ in the plasma membrane are extremely low and almost undetectable, constituting about 0.0001 % of the plasma membrane's lipid content. Phosphorylation of $\text{PtdIns}(4,5)\text{P}_2$ by PI3K results in a rapid, approximately 40-fold increase of $\text{PtdIns}(3,4,5)\text{P}_3$, to about 10 % of the basal level of $\text{PtdIns}(4,5)\text{P}_2$, which is relatively abundant in the plasma membrane with an approximately 500-fold greater concentration than basal $\text{PtdIns}(3,4,5)\text{P}_3$ levels [17]. The low level of $\text{PtdIns}(3,4,5)\text{P}_3$ reflects its potent effect on key cellular regulatory pathways, requiring a tight

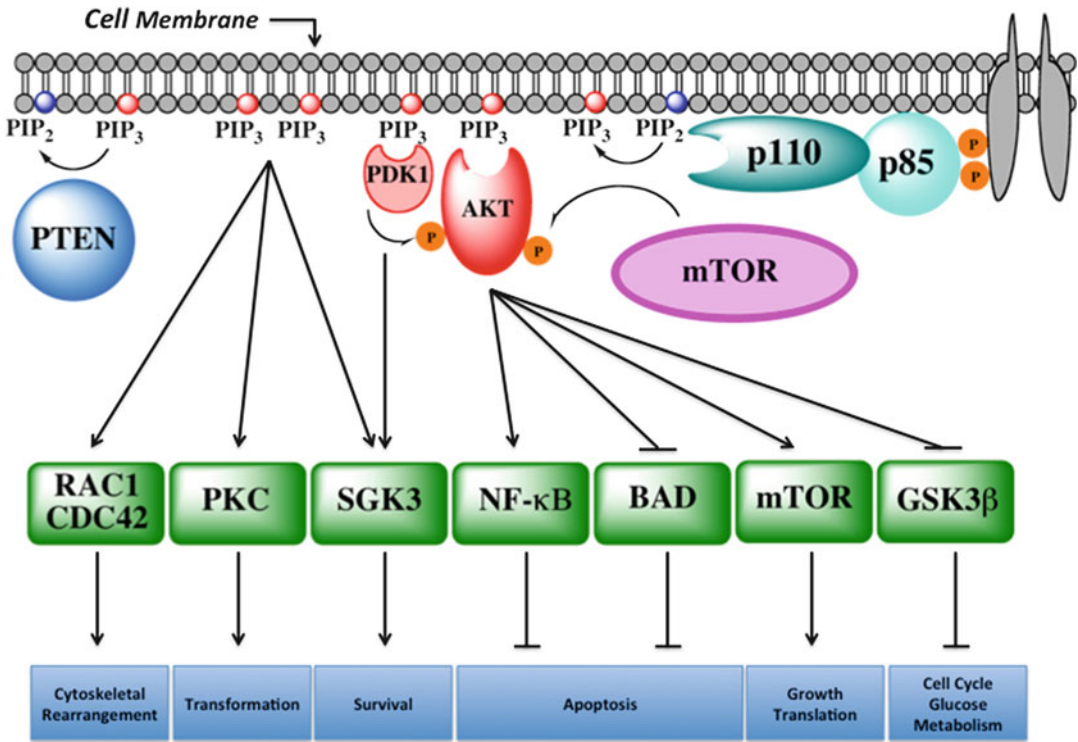


Fig. 7.2 The general model of PtdIns(3,4,5)P₃ (shown as PIP₃) activation and downstream signaling. Upon growth hormone stimulation of receptors in the plasma membrane, PI3K phosphorylates PtdIns(4,5)P₂ to PtdIns(3,4,5)P₃, leading to the recruitment of PH-domain containing

effector proteins, such as Akt/PKB and PDK1. Activation of these effector proteins leads to signaling cascades controlling a multitude of cellular pathways, including cell survival, metabolism, and cell migration. PTEN and other phosphatases quench PtdIns(3,4,5)P₃ signaling

control to prevent hyperactivation by extra- and intracellular cues.

Dephosphorylation of PtdIns(3,4,5)P₃ is required to prevent its accumulation and constitutive signaling. This is under the control of the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and SH2-containing inositol 5-phosphatases (SHIPs), which produce PtdIns(4,5)P₂ and PtdIns(3,4)P₂, respectively [17]. The structural basis of PtdIns(4,5)P₂ recognition and membrane binding of PTEN is discussed in detail in Chap. 6. The main function of PTEN is as a lipid phosphatase, dephosphorylating the D-3 position of PtdIns, and negatively regulating the PtdIns(3,4,5)P₃ pathway [18]. It is well established to serve as one of the most important tumor suppressors, which is inactivated as frequently as p53 in some forms of cancer. The phosphatase consists of a central

phosphatase domain, two C-terminal PEST sequences that serve to stabilize the protein, and an N-terminal tensin- and auxilin-homology region. The gene coding for PTEN is located on human chromosome 10q23, and is often found affected in a multitude of disorders [19]. While SHIP1 is present primarily in hematopoietic cells, SHIP2 is ubiquitously expressed and utilizes PtdIns(3,4,5)P₃ as a main substrate [20]. There is some evidence that PTEN plays a more important role in controlling basal levels of PtdIns(3,4,5)P₃, while SHIPs counteract stimulus-induced increases [21]. It is also worth noting that another phosphatase, Inositol Polyphosphate 4-phosphatase type II, has emerged more recently as an important tumor suppressor [22]. This enzyme dephosphorylates PtdIns(3,4)P₂, which can bind both Akt and PDK1. This lipid is either synthesized *de novo*

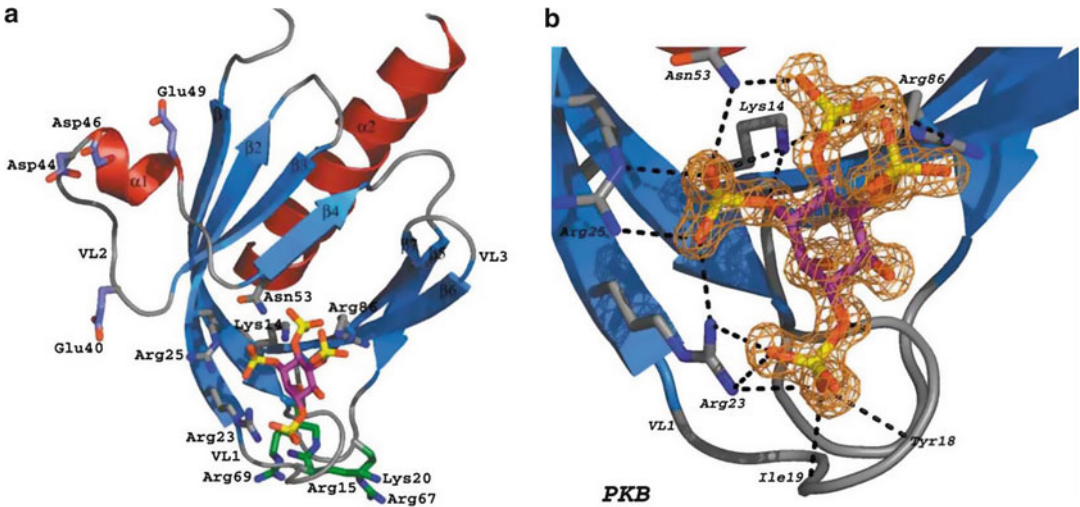


Fig. 7.3 Ribbon drawings of PtdIns(3,4,5)P₃ bound to the PH domain of Akt. (a) Seven-stranded beta-barrel structure formed from the β 2- β 3, β 4- β 5, and β 6- β 7 loops (blue), capped at one end by an amphipathic α -helix (red), and bound to the inositol (1,3,4,5)-phosphate head group

of PtdIns(3,4,5)P₃ (purple). The basic residues proposed to bind PtdIns(3,4,5)P₃ are drawn in green. (b) Close up of the PtdIns(3,4,5)P₃ head group interactions, with dotted lines indicating hydrogen bonds (Reproduced with permission from Ref. [1])

from PtdIns(3)P and PtdIns(4)P, or is a result of SHIP dephosphorylation of PtdIns(3,4,5)P₃. Therefore, concerted actions of PI3Ks and several phosphatases control signaling by multiple PI species in a highly dynamic fashion in response to growth factor signals.

7.2.2 PH Domains: Primary Sequence vs. Protein Folding and Recognition Preferences

PtdIns(3,4,5)P₃ serves to anchor its effector proteins to the membrane surface, as well as to activate, stabilize, and co-localize proteins. The best-known mechanism of action involves direct binding of the PtdIns(3,4,5)P₃ inositol head-group to the pleckstrin homology (PH) domain of proteins resulting in their recruitment to the plasma membrane [17]. The effector network of PtdIns(3,4,5)P₃ includes only about 40 of the approximately 250 known PH domain-containing proteins, and the members bind PtdIns(3,4,5)P₃ with varying degrees of specificity and affinity. The most commonly

studied of these include Akt/Protein kinase B, phosphoinositide-dependent kinase-1 (PDK1), guanine nucleotide-exchange factors (GEFs) GRP-1 and ARNO, and Bruton's tyrosine kinase (Btk), all of which are considered relatively specific for PtdIns(3,4,5)P₃.

The PH domain consists of approximately 120 amino acid residues arranged into a bowl-like binding pocket that closely fits the inositol head group of PtdIns(3,4,5)P₃. While the tertiary structure of this pocket remains highly conserved between different classes of PtdIns(3,4,5)P₃ effector proteins, the primary structure is known to vary significantly. It is believed that this variation contributes to the specificity and affinity for PtdIns(3,4,5)P₃ versus other membrane PIs [23]. The binding pocket is a seven-stranded anti-parallel beta-barrel formed by the β 2- β 3, β 4- β 5, and β 6- β 7 loops and capped at one end by an amphipathic α -helix [24] (Fig. 7.3). Basic residues creating a highly positive electrostatic environment capable of attracting negatively charged PtdIns(3,4,5)P₃ line the interior of the PH domain. The pattern of phosphate groups on the inositol head group is also very important in binding [17].

7.2.3 Mechanisms of Effector Activation – Localization and Conformational Change

PtdIns(3,4,5)P₃ in cellular membranes serves to recruit effector proteins from the cytoplasm and anchor them by binding to their PH domains. The action of binding to PtdIns(3,4,5)P₃ often induces a conformational change, allowing protein activation and propagation of downstream signaling. The induced conformational change, along with membrane localization, both contribute to activation of PtdIns(3,4,5)P₃ effectors.

Activation of Akt by PtdIns(3,4,5)P₃ has been studied extensively and is best understood. Binding of PtdIns(3,4,5)P₃ to Akt produces a conformational change within the protein that allows for its phosphorylation, and full activation, by PDK1 and mammalian target of rapamycin complex 2 (mTORC2) at residues Thr308 and Ser473, respectively. PDK1 is also bound and recruited to the membrane to allow full Akt activation following growth factor signal. Mutation of the PDK1 PH domain inhibits protein kinase B/Akt, leading to small size and insulin resistance in mice [25], by preventing its co-localization with Akt and subsequent activation downstream pathways. Membrane localization is not the only mechanism of Akt activation. Rather, PtdIns(3,4,5)P₃ binding also promotes dissociation of the auto-inhibitory binding of PH domain to the catalytic domain, thus exposing the catalytic loop residue Thr308 to PDK1 phosphorylation [26, 27].

Regulation of other PH domain-containing proteins is less well characterized. However, complex regulation involving changes in protein conformation and localization, acting in concert, is emerging as a common theme. In the case of PDK1 kinase, initial work has suggested that PtdIns(3,4,5)P₃ binding to its PH domain does not affect catalytic activity of the kinase [28]. Hence, inability of the PH domain mutant of PDK1 to promote activation of Akt in response to growth factor signaling primarily reflected defects in membrane localization. The normal cytosolic phosphorylation of S6K by PDK1 in PDK1 PH domain mutants indicates that they retained its kinase activity [29]. However, further detailed

analysis suggested a complex role of the PH domain in the regulation of PDK1 activity in the cells. The PH domain has been proposed to both promote Ser241 autophosphorylation, which is required for activity, and to autoinhibit the catalytic activity of phosphorylated kinase under basal conditions, creating a pool of primed kinase [30]. Binding of PtdIns(3,4,5)P₃ releases PH domain-dependent inhibition, allowing PDK1 to affect its substrates.

In case of another PH-domain containing factor, Grp1, elements proximal to PH domain have been shown to potently autoinhibit the Sec7 exchange domain of this Arf6 GEF. Membrane localization through PtdIns(3,4,5)P₃ binding in concert with complex formation with membrane-targeted Arf6-GTP was shown to result in full catalytic activation of Grp1 towards Arf6-GDP molecules [31].

7.3 Cellular Processes Controlled by PtdIns(3,4,5)P₃

PtdIns(3,4,5)P₃ is directly involved in a variety of processes within mammalian cells, including cell cycle progression, regulation of cell death, cytoskeleton rearrangement, chemotaxis, and metabolic control. The role of PtdIns(3,4,5)P₃ in both plasma and nuclear membranes has been identified. As new cutting edge cell imaging techniques have become widely available, increasingly intricate roles and mechanisms of action for PtdIns(3,4,5)P₃ are being elucidated. It is clear that tight regulation of PtdIns(3,4,5)P₃ is fundamental to cell function, and deregulation along any of its effector pathways leads to pathogenesis, including cancer, autoimmune disease, cardiovascular problems, and diabetes.

7.3.1 Cellular Growth, Proliferation, and Apoptosis

Regulation of cell growth is one of the key functions of PtdIns(3,4,5)P₃ pathway. The growth of cells requires coordinated regulation of proliferation,

cell death, and metabolism, all processes involving signaling from PI3K.

7.3.1.1 Progression Through the Cell Cycle

During interphase of the cell cycle, cells increase in size, synthesize lipids and proteins, and replicate DNA, before the cell can enter the division phase, also known as mitosis (or meiosis in the case of reproductive cells). PtdIns(3,4,5)P₃ is involved in the regulation of the critical factors (cyclins and cyclin-dependent kinase (CDK) inhibitors) involved in the regulation of the progression through interphase. The necessary conditions for a cell to transition from the G₁ checkpoint to the S phase of interphase are dependent in part on the activity of Forkhead-box Class O (FoxO) transcription factors in the nucleus, which are responsible for the repression of cyclins D1 and D2 as well as the transcription of CDK inhibitors, including p21^{Cip1} and p27^{Kip1} [32]. Cyclins make up of a family of proteins that oscillate throughout the cell cycle, controlling its progression via interactions with CDKs. Cyclin D is G₁/S-specific molecule and is a regulatory subunit of CDK4 and CDK6 [33]. FoxOs also promote the synthesis of cyclin G2, an atypical cyclin highly expressed in quiescent cells.

The binding of growth factors activates the PI3K/PtdIns(3,4,5)P₃/Akt pathway, followed by a translocation of active Akt to the nucleus, where it phosphorylates FoxO and promotes its exclusion from the nuclei [34]. Phosphorylation of different FoxOs by Akt occurs at the conserved T1, S1, and S2 residues and promoting the coordinated action of nuclear export machinery (Crm1, Ran GTPase) and 14-3-3 regulatory proteins resulting in the export FoxO into the cytosol and preventing the phosphorylated protein from re-entering the nucleus. Absence of FoxO proteins from the nuclei releases inhibition of cyclin D synthesis, while also decreasing levels of CDK inhibitors (p21, p27, etc.), thus, triggering a G₁-S phase transition [35].

Another mechanism involved in the promotion of proliferation by Akt is its direct phosphorylation of p21^{Cip1} at Thr145 [36]. Interestingly,

Akt signaling has been recently shown to also increase expression of cell cycle inhibitor p57^{Kip2}, limiting proliferative responses of breast epithelial cells to insulin and IGF-1, thus, highlighting a system of checks and balances established by Akt in controlling proliferative responses by growth factors [37].

7.3.1.2 Cell Growth

The majority of cell types spend most of their cell cycle growing in size and preparing to proliferate. Nutrients and growth factors in the extracellular matrix initiate the PI3K/PtdIns(3,4,5)P₃ pathway, leading to increased biosynthesis of lipids and proteins, which are required for rapid cell growth.

PtdIns(3,4,5)P₃-activated Akt phosphorylates and inhibits the action of tuberlin, which binds to hamartin, generating a protein complex known as the tuberous sclerosis complex (TSC). The TSC complex suppresses activity of Complex 1 of mTOR (mTORC1) [38]. Two of the best studied downstream effectors of mTORC1 are p70S6Ks, AGC kinases required for cell growth and progression through G₁, and 4EBP1, which are inactivated in the absence of mTORC1 activity through the binding to eukaryotic initiation factor 3 (eIF3) [39]. Activated mTORC1 binds to eIF3 and phosphorylates p70S6K and 4EBP1, relieving their inhibition. p70S6K, in turn, phosphorylates multiple effectors required for cell growth and protein biosynthesis including ribosomal S6, promoting translation of a subset of mRNAs containing an oligopyrimidine tract at the 5' end [40]. Another important target of mTORC1 is 4EBP1, which dissociates from its target eIF4E upon phosphorylation, leading to an increase in cap-dependent translation.

Though regulation of translation is an important function of mTORC1 on cell growth, it is not the only contribution. It has also been linked to the control of the availability of endogenously produced amino acids needed for biosynthesis, mitochondrial biogenesis, and *de novo* lipogenesis [41, 42].

Cellular metabolism is also altered in the transition of cells from quiescence to rapid proliferation. Activation of PI3K/PtdIns(3,4,5)P₃/Akt

pathway plays a major role in these changes as well, activating glucose uptake, promoting aerobic glycolysis, and suppressing fatty acid oxidation, thus preserving a source of lipids for rapid membrane expansion [41, 42].

7.3.1.3 Cell Survival and Apoptosis

PI3K, acting through a PtdIns(3,4,5)P₃-dependent activation of Akt kinases, provides one of the most important growth and survival signaling circuits in a wide range of cell types. Overactivation of PI3K/PtdIns(3,4,5)P₃/Akt signaling plays a central role in the enhanced proliferation and reduced cell death of tumor cells (see below). PI3K signaling also plays a key role in mediating growth factor-dependent “tonic” survival signals in many lineages of normal cells. For example, important early insights into the growth and survival signaling by PI3K and Akt were obtained in the models of interleukin-dependent survival of hematopoietic cells [43–46].

Akt has many well-characterized connections to the basic apoptotic machinery. Early findings suggested that Akt phosphorylates the pro-apoptotic BH3-only Bcl-2 family member Bad, leading to its retention in the cytosol through the binding to 14-3-3 and blocking translocation to mitochondria. Thus, interaction with other Bcl-2 family members is prevented resulting in the inhibition of the mitochondrial step in apoptosis activation [44, 47]. Notably, Akt phosphorylation of Ser-136 residues of Bad, acts in concert with other pro-survival kinases, such as A-kinase anchor proteins, p90 ribosomal S6 kinase and Protein kinase A, to control pro-apoptotic activity of Bad [47–52]. Another critical role of Akt in apoptosis is mediated by the phosphorylation of FoxO transcription factors, including ubiquitously expressed FoxO1, FoxO3, and FoxO4 [53–55]. FoxOs promote transcription and synthesis of a variety of proteins involved in apoptosis, including TNF α family members TRAIL and Fas-ligand, and pro-apoptotic BH3-only family members BIM and PUMA [32].

Glycogen synthase kinase 3 (GSK3) is another class of direct Akt target, playing important and complex roles in the regulation

of cell death as well as many other cellular functions. Energy metabolism, transcription, microtubule dynamics and Wnt and Hedgehog pathways are all influenced by GSK3 activity [56]. Akt phosphorylates GSK3 α/β on Ser-21/9 residues respectively, targeting them for ubiquitination and degradation. Akt has also been shown to directly promote nuclear factor (NF)- κ B activation in response to growth factors and TNF α activation through association with I κ B kinase (IKK) complex and subsequent phosphorylation of the Thr23 residue of IKK α [57, 58].

Akt is not the only PtdIns(3,4,5)P₃ target involved in the control of cell growth and survival. Bruton’s tyrosine kinase (Btk), expressed in B lymphocytes, is recruited into activated B-cell receptor “signalosome” in a PtdIns(3,4,5)P₃-dependent manner where it undergoes activating phosphorylation by the Src family Lyn and Syk kinases [59]. Btk, in turn, activates phospholipase C γ (PLC γ), which regulates Ca²⁺ mobilization and activation of mitogen-activated protein kinase (MAPK) and NF- κ B pathways [60]. Activation of NF- κ B in a protein kinase C β (PKC β)-dependent manner is one of the more important mechanisms of cell survival regulation by Btk [61]. Activity of Btk is required for cell survival during development and provides “tonic” pro-survival signaling in mature resting B cells [62, 63]. Btk has been also reported to promote survival macrophages, but only following stimulation with lipopolysaccharide (LPS) or interferon- γ (IFN γ) [64].

PDK1 is another PtdIns(3,4,5)P₃ binding kinase critically involved in the regulation of cell survival [65]. PDK1 plays a key role in the survival of cancer cells with perturbed PI3K signaling, which is mediated by Serum/glucocorticoid regulated kinase 3 (SGK3), rather than Akt activation [66]. In general, PDK1 regulates a large number of downstream kinases linked to the regulation of growth and survival, including Akt, several PKC isoforms, p70S6Ks and SGKs. However, only Akt and SGK3 have been linked to date to PDK1 signaling in plasma membrane, indicating the involvement of PtdIns(3,4,5)P₃.

7.3.2 Cytoskeleton Rearrangement and Chemotaxis

Human cell migration is an important process beginning with conception and continuing throughout adulthood. Cell migration is a critical process in a variety of biological responses including cell renewal and tissue repair, immune and inflammatory responses, and angiogenesis. Aberrant changes in cell migration directly contribute to a variety of pathologies. Most importantly, tumor vascularization, aberrant immune responses, osteoporosis, chronic inflammatory diseases, and loss of correct neuronal network formation are all characterized by dysregulation of cellular migration. In most cells, cell migration is highly dependent on the actin cytoskeleton [67]. PtdIns(3,4,5)P₃ along with other regulators such as cAMP and cGMP, is the key signaling molecule contributing to the regulation of polymerization and rearrangement of the actin cytoskeleton that occurs during chemotaxis, with a marked enrichment of PtdIns(3,4,5)P₃ at the leading edge of migrating cells [68].

The appropriate direction of migration is established by receptor molecules at the cell surface, which sense the locations and intensities of extracellular signals, subsequent activation of the receptors and signal propagation leads to a rearrangement of the actin cytoskeleton. This rearrangement is regulated by Rho GTPases, a subfamily of small signaling G-proteins in the Ras superfamily, including Rho, Rac, and Cdc42 [69]. Activation of PI3K by extracellular cues initiates binding of PtdIns(3,4,5)P₃ to GEFs, which, in turn, activate Rho GTPases. Formation of protrusions at the front of the migrating cell, specifically lamellipodia and filopodia, are stimulated by Rac and Cdc42 respectively, while Rho is responsible for the cells posterior retraction [67]. Activation of Cdc42 and Rac has been shown to decrease the activity of Rho at the leading edge, as well as enhance PI3K activity. Cdc42 is also implicated in microtubule growth and recruitment of vesicles to the leading edge, a process dependent on the localization of the microtubule-organizing center and the Golgi apparatus between the nucleus and the front of the cell [70].

The final downstream effectors of Rho GTPase signaling are the Wiscott-Aldrich syndrome protein (WASP) and the WASP verprolin homologous proteins (WAVE), which form links between the GTPases and the actin cytoskeleton. PtdIns(4,5)P₂-activated WASP binds via its newly exposed VCA (verprolin homology, cofilin homology and acidic) region to the actin-related protein (Arp)2/3 complex, leading to rapid actin polymerization [67]. Of the three WAVE isoforms present in human tissues, WAVE2 is ubiquitously expressed, localizes to the plasma membrane, possibly by specific binding to PtdIns(3,4,5)P₃ to its basic region (rather than through a canonical PH domain), and has been shown to be critical for lamellipodia formation at the leading edge of the cell [69]. More recent evidence indicated that PtdIns(3,4,5)P₃ acts in concert with the microtubule binding complex of EB1 and stathmin in promoting localization of WAVE2 to the leading edge of the cell [71]. Furthermore, another PtdIns(3,4,5)P₃ target IRSp53, rather than WAVE2 itself, may directly interact with PtdIns(3,4,5)P₃ and is responsible for membrane localization of WAVE2, its constitutive binding partner [71].

Regulation of actin polymerization is not the only contribution of PtdIns(3,4,5)P₃ to directional migration. The actin motor myosin has also been found to interact with PtdIns(3,4,5)P₃ and contributes to lamellipodia formation. Non-muscle myosin IIA [72], several Class I myosins in *Dictyostelium* and Class IF myosin in mammalian cells are all able to bind PtdIns(3,4,5)P₃ through their tail homology domain 1 [73].

An impressive aspect of cell migration is the ability of cells to respond to very shallow chemical gradients, at times experiencing less than 10 % variation in concentration between the leading and trailing edge of the cell. It is thought that this is possible due to positive feedback loops that amplify the signal at the leading edge of the cell, as well as sequestration of PTEN to the sides and rear of the cell, resulting in an anterior focusing the Rho GTPase signaling [70].

It is also worth noting that Akt, PDK1, and PTEN are all also implicated in the regulation of cell migration and motility. For example, Akt1

has been shown to inhibit cell migration through phosphorylation of actin-binding factor, paxillin, as well as through the regulation of nuclear factors of activated T-cells, Erk, and TSC2. Alternatively, Akt2-specific regulation of $\beta 1$ integrin can promote cell migration [74]. *Pdk1*^{-/-} cells display defects in cell migration [75]. The mechanisms of this regulation have been proposed to involve direct binding of PDK1 to ROCK1 and PKN kinases, the latter being a direct substrate of PDK1 [76, 77]. However, the role of PtdIns(3,4,5)P₃ in the regulation of this function of PDK1 has not been investigated. Curiously, while PTEN certainly contributes to the regulation of migration through the control of PtdIns(3,4,5)P₃, its lipid-independent protein phosphatase activity towards Focal adhesion kinase (FAK) and SHC-transforming protein 1 (Shc) has also been proposed to play an important role in the regulation of cell migration and metastasis [78]. These data establish very profound, but complex roles of PtdIns(3,4,5)P₃ and its effectors in the regulation of cell migration, motility, chemotaxis, and metastasis.

7.3.3 Nuclear Function

The discovery of the existence of PtdIns(3,4,5)P₃ in cellular membranes other than the plasma membrane has led to an investigation of the role of this lipid in other cellular compartments, especially in the nucleus. Components of the PI3K signaling cascade, including PI3K itself, Akt, and PDK1, have been detected in nuclear membranes. In one reported mechanism, nerve growth factor (NGF) stimulation was found to activate PI3K through the activity of nuclear GTPase, PIKE, acting as an equivalent to the cytoplasmic PI3K activator, Ras. PtdIns(3,4,5)P₃ in the nuclear membrane plays a critical role in several processes, including cell survival, cell cycle regulation, and DNA repair [79]. Similar to the plasma membrane pool, dephosphorylation of PtdIns(3,4,5)P₃ is coordinated by PTEN and SHIP phosphatases, although the nuclear pool of PtdIns(3,4,5)P₃ may be less sensitive to PTEN compared to that observed at the plasma membrane

[80]. It is interesting to note that while PTEN is found localized to the nucleus in many primary cells, its nuclear pool is dramatically reduced in a number of tumor cells, including exocrine pancreatic tumors and melanomas [81].

It has been established that following growth factor stimulation at the plasma membrane, activated Akt detaches from the plasma membrane and translocates to the nucleus. Nuclear Akt appears to retain activity independently of PtdIns(3,4,5)P₃, and was shown to promote cell survival, in part by direct interaction with the nuclear PtdIns(3,4,5)P₃ target, nucleophosmin (NPM)/B23. NGF-stimulation promotes PtdIns(3,4,5)P₃ binding to B23, recruiting the protein from the nucleoli to the nucleosomes, where it can interact with and stabilize nuclear Akt [82]. Nuclear PtdIns(3,4,5)P₃, Akt, and B23 have been shown to protect PC12 cells from apoptosis via the formation of a complex capable of inhibiting DNA fragmentation activity of caspase-activating DNase (CAD). It is also clear that the spatial and temporal dynamics of PtdIns(3,4,5)P₃ following NGF stimulation are critical to the interaction between B23 and Akt, and deregulation of these dynamics leads to impaired biological function [83].

Class I PI3K β is also involved in the DNA damage response (DDR) that eukaryotic cells utilize to repair double-stranded breaks (DSB), which are among the most disastrous lesions that affect the human genome. Experiments carried out on irradiated NIH 3 T3 cells revealed that PI3K β and PtdIns(3,4,5)P₃ localize to damaged DNA sites, and are necessary for the early detection of DSB. PI3K β functions to promote recruitment of the Mre11-Rad50-Nbs1 (MRN) complex, of which Nbs1 is considered the earliest sensor of DNA damage, and is critical for the proper association of the complex. It was also shown that PI3K β is necessary for the activation of G2/M cell cycle arrest, during which DNA damage is assessed and repaired, or else apoptosis is induced. Loss of PI3K β function results in an impairment of MRN recruitment and inefficient G2/M arrest, resulting in an accumulation of DSB in irradiated cells. While the exact role of local PtdIns(3,4,5)P₃ accumulation to DSB sites is not

yet completely understood, it is thought that besides its normal role in protein recruitment, it may serve to stabilize DNA in an open conformation in order to facilitate the DDR, either by repelling the negatively charged DNA strands, or by recruiting positively charged histones to chromatin [84].

Finally, a role of the nuclear PtdIns(3,4,5)P₃ pool in the differentiation of myeloid cells through a mechanism involving association of the p85 subunit of PI3K with tyrosine phosphorylated Vav1 GEF has also been proposed [85]. Notably, Vav1 is a PH domain-containing protein, which can be activated by PtdIns(3,4,5)P₃ *in vitro* [86, 87]. Novel nuclear PtdIns(3,4,5)P₃ targets continue to emerge, making it clear that it has important functions in the control of nuclear processes.

7.3.4 Neuronal Development and Function

The typical characteristic morphology of a neuronal cell consists of one long axonal process, through which the signal is transmitted, and several shorter tapered dendrites, through which neighboring cells activate the neuron. Once a neuronal progenitor cell attaches to the substratum, a five-stage process of neuronal differentiation is initiated [88]. The first stage involves the formation of several lamellipodia along the surface of the cell, which in stage 2 develops into immature neurites of approximately equal length. During the third stage, the neuron becomes polarized with one of the immature neurites lengthening rapidly into a long neurite possessing axonal characteristics (*i.e.*, growth cone). Following development of the axon, the neuron enters the fourth stage, in which the remaining neurites transform into dendrites and all processes continue to mature. In the fifth and final stage, the neuron forms synaptic connections with surrounding neurons and a neuronal network is established [89].

While the exact trigger for the development of a single neurite into a mature axon remains largely unknown, it has been shown that PI3K/

PtdIns(3,4,5)P₃/Akt signaling is involved in this process, as well as in the maintenance of neuronal polarity [88]. Akt regulates neuronal polarity by inhibiting GSK3β. Inhibition of GSK3β occurs equally among all the neurites during the first two stages of development. During the third stage, Akt localizes to the cell body (the soma) and to tip of the neurite newly designated as the axon. This results in the inhibition of GSK3β primarily at the axonal tip. It has been experimentally shown that the expression of constitutively active Akt throughout the neuron leads to the formation of multiple axons [90] and inhibiting GSK3β in existing dendrites causes their conversion to axons [88]. Thus, spatially-specific accumulation of PtdIns(3,4,5)P₃ and activation of Akt is critical for neuronal polarization and development of neuronal networks.

The activation of Akt at the distal tip of neurites is dependent on the local accumulation of PtdIns(3,4,5)P₃. Transport of PtdIns(3,4,5)P₃ to the neurite tip occurs via guanylate kinase-associated kinesin (GAKIN), a member of the kinesin-3 family of microtubule-based motor proteins, which binds the PtdIns(3,4,5)P₃ binding protein (PtdIns(3,4,5)P₃-BP), also called centaurin-α, via its forkhead-associated (FHA) domain. PtdIns(3,4,5)P₃-BP exhibits ADP-ribosylating factor GTPase activating protein (Arf GAP) activity, and contains two PH domains through which they specifically interact with PtdIns(3,4,5)P₃. Using *in vitro* motility assays, it has been shown that the accumulation of PtdIns(3,4,5)P₃ at the distal tip of developing neurons occurs at least in part via transport of PtdIns(3,4,5)P₃-containing vesicles bound to PtdIns(3,4,5)P₃-BP complexed to GAKIN [91].

7.4 Implications in Disease

As discussed in detail above, PtdIns(3,4,5)P₃ mediated signaling is a highly ordered and conserved pathway in a variety of tissues and cells. The relatively low abundance and precise control over the levels of PtdIns(3,4,5)P₃ ensure ordered and deliberate activation of downstream targets, which contribute to many core functions in the

cells. It is therefore not surprising that a significant number of human diseases can be linked to perturbations in PtdIns(3,4,5)P₃ signaling. In fact, approximately 15–30 % of human cancers have been shown to have disruptions in this pathway [92]. Additionally, diabetes, cardiovascular diseases and various inflammatory disorders have also been linked to PtdIns(3,4,5)P₃ signaling abnormalities.

In normal cells, proper signaling is reliant on low basal levels of PtdIns(3,4,5)P₃ with rapid and controlled production by PI3K in response to extracellular signals. Additionally, a return to basal levels requires the activity of either PTEN or SHIP phosphatases. Activating mutations in PI3K (or its downstream activators) or the loss of PTEN result in elevated cellular levels of PtdIns(3,4,5)P₃. Promiscuous and constitutively present PtdIns(3,4,5)P₃ is a trademark of tumorigenesis in cells of many tissue types [92]. Alternatively, a loss of PI3K function has been implicated in insulin resistance and the resulting diabetic phenotype [93]. A discussion of specific alterations in PtdIns(3,4,5)P₃ signaling and their implications in diseases such as cancer, inflammation, cardiac diseases, and diabetes are presented below.

7.4.1 Cancer

Cancer is a complex disease requiring genetic alterations in the cell resulting in diverse changes including enhanced proliferation, uncontrolled growth, resistance to apoptotic signals, and invasion of surrounding tissues [94]. PtdIns(3,4,5)P₃ signaling is implicated in these oncogenic traits and is demonstrably overactivated in a wide range of tumor types [95]. Many excellent in-depth reviews, detailing the role of PI3K signaling pathway in cancer, are available [93, 95, 96], therefore just the major points will be outlined.

As previously discussed, PtdIns(3,4,5)P₃ production results in the activation of various downstream proteins, most notably Akt, and is responsible for enhanced cell growth and proliferation, survival, and motility. Upon PtdIns(3,4,5)P₃ production and Akt activation, multiple

downstream processes promote cell survival and resistance to apoptosis [97]. This occurs through both activation of cellular signaling and transcriptional changes. Akt phosphorylates BAD, thus inhibiting its association with downstream apoptosis factors and decreasing apoptotic tone [98]. Additionally, Akt has been shown to activate NF-κB resulting in transcription of pro-survival and proliferation genes [58, 99]. The FoxO family of transcription factors is phosphorylated by Akt, leading to their sequestration in the cytosol and attenuation of transcription of pro-apoptotic proteins including BIM and Fas-ligand [100], thus, any increase in Akt activity has major implications on the ability of cells to undergo apoptosis. Tumorigenic effects of Akt are not limited to the regulation of cell survival. Activation of Akt pathway contributes to major remodeling of cellular metabolism promoting aerobic glycolysis, reducing β-oxidation of fatty acids and maximizing lipid biosynthesis, all critical for rapid proliferation of cancer cells [101, 102]. Akt is also a major mediator of mTORC1 regulation downstream from growth factor and oncogenic signals, providing a critical link to the robust requirement for protein biosynthesis during proliferation. Inhibition of the p21^{Cip1} and p27^{Kip1} CDK inhibitors, through direct phosphorylation by Akt, as well as transcriptional regulation of p27 and Cyclin D1 contribute to increased cellular proliferation [103]. More recent evidence indicates a critical role of Akt2 in epithelial-mesenchymal transition through the regulation of specific miRNAs [104]. These are some of the key examples of the multitude of pro-oncogenic mechanisms controlled by overactivated Akt pathway in cancer cells [95].

Multiple factors contribute to the overactivation of PtdIns(3,4,5)P₃ synthesis in cancer cells. Genetic mutations resulting in increased expression and/or activity of RTKs, PI3K, or loss of PTEN activity are some of the most common alterations of the PtdIns(3,4,5)P₃ pathway noted to drive tumorigenesis. These mutations can often occur redundantly [92]. RTKs have been repeatedly shown to be dysregulated in a wide variety of cancers [105]. The epidermal growth factor receptor (EGFR) in particular is commonly

overexpressed or mutated in gliomas and non-small cell lung cancer [106, 107]. HER2 is overexpressed in approximately 25 % of breast cancer patients and is commonly associated with high recurrence rates and increased mortality [108]. Gain-of-function mutations in the tyrosine-protein kinase c-Kit are evident in gastrointestinal-stromal tumors, acute myeloid leukemia, mast cell leukemia, and melanoma [109, 110]. These are examples of some of the frequent RTK mutations leading to an increased PI3K activation and subsequent PtdIns(3,4,5)P₃ production.

Since the discovery of cancer-specific mutations in the *PI3KCA* gene encoding the p110 α subunit of PI3K, oncogenic mechanisms of mutated PI3K has been intensely investigated [111]. Mutations in *PI3KCA* gene have been implicated in head and neck, squamous cell lung carcinoma, gastric, and cervical cancers [93]. Three hot spot, non-synonymous, missense mutations of amino acids in the helical (E542K and E545K) or kinase (H1047R) domains make up approximately 80 % of the mutations in the *PI3KCA* gene [112–114]. PtdIns(3,4,5)P₃ generation is amplified several fold as a result of increased PI3K kinase activity [115–120]. Increase in PtdIns(3,4,5)P₃ concentration is the primary driver of oncogenic signaling [121]. Additionally, constitutively active PI3K uncouples PtdIns(3,4,5)P₃ production from growth factor-induced RTK activation. Several mutations in *PI3KRI*, the gene encoding p85, have also been shown to induce oncogenic transformation in the cell [122]. These mutations typically occur in regions of the protein responsible for p110 binding, thus preventing inhibitory effects on the catalytic activity of p110 [123].

Since the discovery of its role as a potent tumor suppressor in 1997, decreased PTEN function has been correlated with a number of human cancers [124, 125]. Subsequent studies of *Pten* knockout mice confirmed PTEN tumor suppressor functions in a variety of tissues [126–128]. Additional studies of *Pten*^{+/-} mice revealed that PTEN functions as a haploinsufficient tumor suppressor gene [129, 130]. Thus, any inhibitory effect on the expression or activity of PTEN has implications in cancer with PTEN levels inversely

correlating with disease severity [131, 132]. Additionally, many molecular mechanisms have been associated with decreased expression and/or activity of PTEN, making any correlations of simple step-wise changes in the expression of *Pten* on cancer progression incomplete [133]. For example, allelic loss, epigenetic silencing, functional mutations, miRNA silencing, and post-translational modifications have all been demonstrated to decrease PTEN expression and/or function. These discoveries have led to the proposal of a “continuum model of tumor suppression” relating overall level and function of PTEN with tumor suppression [131, 134]. The obvious role of PTEN as an inhibitor of PtdIns(3,4,5)P₃ signaling is well established, though novel functions of PTEN are continuously being elucidated [133]. For example, nuclear localization of PTEN is associated with tumor suppression independent of its phosphatase activity [80, 135, 136]. Additionally, PTEN has been implicated in fibroblast-mediated shaping of the tumor microenvironment [137, 138].

While Akt plays a critical role in PtdIns(3,4,5)P₃ contribution to tumorigenesis, other mechanisms contribute as well. The contribution of PtdIns(3,4,5)P₃ to the increased cell motility through regulation of the activity of small GTPases and actin cytoskeleton remodeling should not be discounted. Recent siRNA screens revealed, unexpectedly, that PDK1 may be an even more critical driver than Akt in *PI3KCA* tumors. This has been linked to the Akt-independent regulation SGK3 as mentioned above [66].

The obvious role of PtdIns(3,4,5)P₃ signaling in cancer has led to a dramatic increase in research into the mechanisms of increased PtdIns(3,4,5)P₃ production and expression, while therapies targeting this pathway are concomitantly being developed. Several drugs are currently on the market targeting RTK activity upstream of PtdIns(3,4,5)P₃ production and there is a race to develop isoform specific inhibitors of PI3K. Additionally, increased PtdIns(3,4,5)P₃ signaling has been associated with drug resistance in tumors, therefore, druging this pathway is extremely important

clinically [139, 140]. Further discussion of these efforts will be presented below.

7.4.2 Inflammation

There are four different isoforms of Class I PI3K, depending on the identity of the catalytic subunit. Class I_A PI3K α and PI3K β isoforms are ubiquitously expressed while class I_A PI3K δ and class I_B PI3K γ are predominately expressed in the hematopoietic cells [141]. As a result of their restricted expression, it has been proposed that these isoforms are important regulators in different populations of immune cells including neutrophils, macrophages, mast cells, eosinophils, T-cells, and B-cells. Current research indicates that PI3K δ and PI3K γ -mediated production of PtdIns(3,4,5)P₃ is an important druggable target in inflammatory diseases [142]. In the immune system, PtdIns(3,4,5)P₃ signaling is initiated in response to antigen, cytokine, and chemokine receptors [19]. Increased PtdIns(3,4,5)P₃ signaling results in differing phenotypes depending on the cell type.

Neutrophils and macrophages respond to microbial invasion and are crucial players in inflammatory reactions. In response to inflammatory signals, these cells migrate from the circulation to the site on injury through a process known as chemotaxis [143]. Extracellular chemokine signaling results in the local activation of cellular receptors translating into dramatic localized increases in PtdIns(3,4,5)P₃. PtdIns(3,4,5)P₃ then recruits and activates GEFs for Rac and Arf GTPases, promoting actin cytoskeletal rearrangement and directional cell movement [144]. Several studies have indicated that this process is dependent on PI3K γ [145–147]. The precise role of PI3K δ is still unclear. An isoform specific inhibitor of PI3K δ was shown to inhibit chemotaxis in neutrophils [148]. On the other hand, subsequent studies have supported the function of PI3K γ as the primary isoform responsible for cellular migration in response to chemoattractants [149]. PTEN stability and activity is essential for proper inflammatory cell migration, further illustrating the importance

of PtdIns(3,4,5)P₃ signaling [150]. In addition to chemotaxis, the production of reactive oxygen species (ROS) at the site of inflammation also depends on PtdIns(3,4,5)P₃ signaling [145–147, 151]. PDK1 and Akt are both implicated in the phosphorylation of various NADPH oxidase proteins leading to their assembly, subsequent production, and release of ROS [152]. Increased neutrophil and macrophage activation has been associated with atherosclerosis, lupus, and rheumatoid arthritis indicating PtdIns(3,4,5)P₃ signaling as a potential druggable target in these inflammatory diseases [153–155].

Mast cells are an additional player in mediating inflammatory activities in response to infection or parasites and are important in amplifying adaptive immunity [156]. These cells are perhaps best known for their detrimental effects in allergic diseases [146]. In response to allergens, the high-affinity IgE receptor is crosslinked and immunoreceptor tyrosine-based activation motifs are phosphorylated. Class I PI3Ks are then activated upon binding to the phosphorylated immunoreceptor tyrosine-based activation motifs. PtdIns(3,4,5)P₃ production results in the activation of Btk and, subsequently, PLC γ , ultimately initiating the opening of plasma membrane Ca²⁺ channels and granule release [157]. Initial degranulation and release of cytotoxic effector molecules is dependent on PI3K δ activity while subsequent waves of degranulation are dependent on PI3K γ [158, 159]. In addition, shRNA knockdown or genetic deletion of PTEN increased PtdIns(3,4,5)P₃ signaling in mast cells leading to mastocytosis and heightened allergic responses in mice [160, 161]. Shenker et al. have demonstrated that a chimeric toxin containing a PtdIns(3,4,5)P₃ phosphatase was able to inhibit mast cell degranulation, further illustrating the importance of this pathway in mast cell pro-inflammatory responses [162]. Increased PtdIns(3,4,5)P₃ signaling in mast cells has implications in a variety of immune disorders including allergic disease, asthma, anaphylaxis, autoimmunity, and mastocytosis [163].

T-cells and B-cells play a central role in the adaptive immune responses. T-cells are responsible for cell-mediated immunity while B-cells

produce antibodies and comprise humoral immunity. Class I PI3K have been shown to be activated in response to T-cell receptors, cytokines such as IL-2, IL-4, IL-7 and IFN γ , and CD28 signaling [16]. PI3Ks are also essential for proper lymphocyte development [164, 165]. Okkenhaug and Fruman review the importance of PI3K in the development of lymphocytes, in particular, illustrating the importance of p110 δ [165]. Thymocyte development is dependent on both class I_A and class I_B PI3Ks. Loss of class I_A PI3K δ results in aberrant pre-T-cell receptor signaling, though it has no effect on overall thymocyte numbers [166]. PI3K γ knockout mice display an increase in thymocyte apoptosis and double knockout (PI3K δ/γ negative) mice display a dramatic reduction in thymocyte numbers [167, 168]. Additionally, PI3K δ regulates the differentiation and expansion of helper T-cells [169]. The role of PtdIns(3,4,5)P₃ in directional lymphocyte migration is still a matter of debate, but it has been suggested that PI3K γ mediates chemotaxis in T-cells while PI3K δ is responsible for B-cell migration [170, 171]. Notably, PI3K γ has not been implicated in B-cell development or survival and all PtdIns(3,4,5)P₃ signaling has been shown to be dependent on PI3K δ [172].

Counterintuitively, both increases and decreases in PtdIns(3,4,5)P₃ signaling have been implicated in the development of autoimmunity [173–175]. This is likely due to the alterations in the attenuation of peripheral immune functions mediated by regulatory T-cells. Several studies have linked the contribution of PtdIns(3,4,5)P₃ signaling in autoimmunity to its regulation of functions of regulatory T-cells (reviewed in [19]).

The evidence outlined above gives plenty of weight to the importance of PtdIns(3,4,5)P₃ signaling in immune function and disease. Current research in this area is focused on elucidating the role and importance of PI3K isoforms and effects of PTEN/SHIP activity on the immune system, in particular, defining the areas where drug intervention could provide the most benefit, given the complex role of PI3K signaling in immune regulation as well as in many other tissues [157, 176]. Targeting the correct PI3K isoform is critical

and several isoform-specific inhibitors are currently under clinical investigation for various inflammatory disorders [142].

7.4.3 Cardiovascular Disease

PtdIns(3,4,5)P₃ plays an important role in cardiac physiology and pathophysiology of heart disease. PI3K and PTEN are expressed throughout the heart including cardiomyocytes, fibroblasts, endothelial cells, and vascular smooth muscle cells [177]. Cardiac PtdIns(3,4,5)P₃ signaling has been shown to have an important role in cell survival, hypertrophy, contractility, metabolism, and mechanotransduction [178]. If normal PtdIns(3,4,5)P₃ signaling is enhanced through either overactivation of PI3K or a loss of PTEN, myocardial hypertrophy and decreased contractility can result, thus impairing normal cardiac function. In contrast, a decrease in PtdIns(3,4,5)P₃ signaling results in increased areas myocardial infarction and prevents myocardial preconditioning.

Class I_A and class I_B PI3Ks have both been demonstrated to have functions in cardiac PtdIns(3,4,5)P₃ signaling. Class I_A PI3K catalytic isoforms p110 α and p110 β are expressed in the heart and vasculature [179–181] while the class I_B isoform p110 γ is found in cardiomyocytes, cardiac fibroblasts, vascular smooth muscle cells, and endothelial cells [177, 182, 183]. Each isoform has a distinct role in cardiac regulation and function. Class I_A isoforms are thought to regulate physiologic growth mediated by activation of RTKs [177, 179, 184]. Class I_B γ isoform is required for proper contractility of the myocardium [179, 185]. Termination of PtdIns(3,4,5)P₃ signaling in the heart is dependent on the function of PTEN. Loss of PTEN leads to increased PtdIns(3,4,5)P₃ signaling ultimately resulting in various pathologies including hypertrophy and decreased contractility [177, 179, 186, 187]. The downstream targets of PtdIns(3,4,5)P₃ signaling described above are similarly involved in cardiac regulation. In particular, Akt/GSK3 pathway plays an important role in heart disease [188, 189].

All three Akt isoforms are expressed in the heart, though Akt1 and Akt2 are the most prevalent [179, 190, 191]. Normal Akt activation in the myocardium is required for cell proliferation, metabolism and inhibition of apoptosis [192]. A transgenic mouse model with cardiac specific knockdown of *Akt1* resulted in a reduced number of cardiomyocytes and smaller overall heart sizes, though there was no effect seen on contractility [193]. At the same time, overexpression or increased Akt activation can result in cardiac hypertrophy and abnormal contractility [193–195].

Both GSK3 isoforms, GSK3 α and GSK3 β are expressed in the heart though more emphasis has been placed on the impact of GSK3 β [189]. GSK3 β is a constitutively active enzyme, phosphorylating and inactivating glycogen synthase and, thus, inhibiting glycogen synthesis [196]. Upon PtdIns(3,4,5)P₃ production and Akt activation, GSK3 β is phosphorylated and rendered inactive, thus promoting glycogen synthesis. GSK3 β is also shown to be a regulator of important transcription factors, in particular, nuclear factors of activated T-cells, β -catenin, CREB, and the Jun family of proteins [189]. Overall, constitutively active GSK3 β has been shown to act as a hypertrophic restraint in the heart [197], while induction of hypertrophy by PtdIns(3,4,5)P₃ has been linked to the inhibition of GSK3 β [189].

As discussed above, PtdIns(3,4,5)P₃ signal alteration has variable effects on the physiology of the myocardium. PI3K α signaling in response to exercise has been shown to provide protective effects in patients with heart failure [198]. Notably, myocardial ischemic preconditioning, a process whereby a brief period of ischemia is able to protect the heart from further ischemic events, has been linked to the changes in PI3K pathway [199–201]. This occurs through the reduction in apoptosis and increased proliferation of preconditioned cells. Activation of PtdIns(3,4,5)P₃ signaling pathways, or loss of PTEN, has been shown to mimic the cardio-protective nature of ischemic preconditioning [200, 202–204]. Additional heart pathologies such as diabetic cardiomyopathy [205], adriamycin-induced cardiomyopathy [206], chronic β -adrenergic receptor

stimulation [184], or pressure overload induced hypertrophy [207] have all been shown to involve altered PI3K/Akt signaling.

Overall, similar to immune regulation, PtdIns(3,4,5)P₃ signaling pathway has multiple effects on the regulation and pathophysiology of the cardiac system. There is still much work to be done to unravel the exact role of each PI3K isoform in the pathophysiology of different heart diseases. Particular care should be taken to avoid cardiovascular toxicity following prolonged exposure to various isoform specific PI3K inhibitors [208].

7.4.4 Diabetes

A number of tissues are involved in the complex process of metabolic regulation. Liver, skeletal muscle, adipose tissue, pancreatic beta cells, and several CNS neuronal populations are all involved in glucose sensing and regulating insulin signaling. Insulin receptors (IR) are coupled to downstream effects in part by inducing PI3K production of PtdIns(3,4,5)P₃ [93]. Recent research has implicated the generation and breakdown of PtdIns(3,4,5)P₃ as a prime mediator of insulin receptor signaling and alterations in this pathway have been shown to result in metabolic syndromes and the diabetic phenotype [209]. Potential inhibition of glucose metabolism is one of the most important considerations in developing PI3K inhibitors.

Insulin receptors belong to the RTK class of receptors. Upon activation by their ligands and subsequent dimerization, IR and IGF receptors phosphorylate insulin receptor substrate (IRS) proteins [210]. Several IRS proteins displaying differences in tissue expression have been identified [211]. Tyrosine phosphorylated IRS proteins bind to the SH2 domains of the regulatory p85 subunit of Class IA PI3Ks. The p110 α catalytic subunit is thought to be the primary subunit involved in IR/PI3K metabolic signaling as inactivation of this subunit results in embryonic death. Heterozygote mice display small body size, insulin resistance, and glucose intolerance [212]. Similarly, isoform-selective p110 α inhibitors

blocked IR/PI3K signaling, while p110 β inhibitors showed little effect [213]. On the other hand, emerging evidence indicates p110 β may still play a role by changing the kinetics of PtdIns(3,4,5)P₃ production, prolonging insulin induced signaling, resulting in the prolonged Akt activation [214].

Peripheral insulin resistance is thought to be the product of disrupted PI3K signaling in the effector cells [210, 215]. Any mechanism resulting in decreased PtdIns(3,4,5)P₃ production or expression can result in diabetic phenotypes. Mice lacking either p110 α or p110 β die in early embryogenesis while mice heterozygous for p110 α and p110 β display glucose insensitivity [212, 216]. In contrast, mice lacking PTEN expression in either muscle, adipose, or liver tissue leads to an increase in insulin sensitivity and glucose tolerance [217, 218]. Additionally, studies of SHIP2 heterozygous deletion mice have been shown to result in increased insulin sensitivity indicating SHIP2 is a key regulator of glucose homeostasis [219].

Several reports suggested that decreased expression of p85 regulatory subunit of PI3K resulted in enhanced insulin sensitivity in mice [220]. Subsequent investigation uncovered an IR/PI3K inhibitory function of p85 [221]. p85 is present in excess to p110 in many cell types and free p85 has been shown to sequester activated IRS1 in the cytoplasm, preventing IRS1 from interacting with PI3K at the membrane [222]. Notably, elevated levels of p85 have been found in pregnancy-induced diabetes in women and in skeletal muscles of type-2 diabetic patients [223, 224]. Interestingly, the levels of p110 were not altered indicating the importance of the p85-p110 ratio. At the same time, a complete loss of p85 in either the muscle or liver resulted in compromised insulin signaling in those tissues due to its critical role in linking PI3K to IR [225, 226]. Additionally, chronic inflammation present in adipose tissue of obese patients leads to release of TNF α , which can activate JNK and block IRS thus decreasing PtdIns(3,4,5)P₃ signaling [227, 228]. p70S6K activation downstream of mTOR is also able to inhibit PtdIns(3,4,5)P₃ signaling by phosphorylation of IRS-1 thus providing a

negative feedback and contributing to insulin resistance [229, 230].

Following activation of IR/PI3K and production of PtdIns(3,4,5)P₃, a variety of downstream events are regulated by Akt, including GLUT4-mediated glucose uptake [231], glycogen synthase activation through GSK3 inhibition [196], and inhibition of FoxO-mediated gene transcription [232]. In particular, Akt2 has been shown to play a key role in glucose homeostasis. Disruption of *Akt2* in mice results in the diabetic phenotype [233]. In addition, full activation of Akt in response to insulin signaling is reliant on PtdIns(3,4,5)P₃-dependent phosphorylation by PDK1. Genetic mutation of the PH domain of PDK1 in mice resulted in glucose and insulin intolerance, indicating the critical role of PtdIns(3,4,5)P₃ in the connection between PDK1 and Akt [25].

PtdIns(3,4,5)P₃ presence in the membrane is able to both propagate IR/PI3K signaling and initiate events leading to the attenuation of the signal. O-linked β -N-acetylglucosamine(O-GlcNAc) transferase (OGT) has been recently shown to bind PtdIns(3,4,5)P₃ and to translocate to the membrane upon insulin induced PtdIns(3,4,5)P₃ production [234]. Once at the membrane, OGT is able to catalyze the addition of GlcNAc to the key insulin signaling intermediates downstream of PtdIns(3,4,5)P₃ such as ribosomal proteins. It also competes with other PH-containing proteins for PtdIns(3,4,5)P₃ binding at the membrane, resulting in attenuation of their signal [235]. O-GlcNAc levels were also shown to be elevated under hyperglycemic conditions. Another protein with affinity for PtdIns(3,4,5)P₃ is prohibitin, a protein shown to bind PtdIns(3,4,5)P₃ and modulate insulin signaling *in vitro* [236].

Although there is ample evidence to implicate perturbed PtdIns(3,4,5)P₃ signaling in diabetes, it remains a difficult pathway to directly target therapeutically. The complex pathophysiology of diabetes covers several tissue types, each with individual expression of PI3K isoforms and unique pathway elements. Furthermore, any attempts to increase PtdIns(3,4,5)P₃ signaling could have oncogenic implications, as this pathway is highly associated with tumorigenesis.

Conversely, any PI3K pathway inhibitors should be analyzed for their potential effects on metabolic signaling.

7.5 Approaches to Target PtdIns(3,4,5)P₃ Signaling

The importance of PtdIns(3,4,5)P₃ regulation has been described in detail above. Dysregulation of the pathway is implicated in a wide variety of disorders and, therefore, targeting the pathway pharmacologically represents a great promise. Thus, it is not surprising that the development of small-molecule inhibitors of various components in the PtdIns(3,4,5)P₃ signaling cascade is a hot topic, especially in the fields of cancer and inflammation. At present there are over 100 clinical trials underway of PI3K inhibitors alone (<http://www.clinicaltrials.gov>). This does not include clinical trials on drugs targeting other proteins in the PtdIns(3,4,5)P₃ pathway, such as mTOR, Akt, or PDK1, or drugs inhibiting upstream targets, such as activity of RTKs. Discussion of upstream inhibition of PtdIns(3,4,5)P₃ pathway by targeting RTKs such as EGFR or IGFR has been extensively reviewed elsewhere and will not be covered here [237, 238].

7.5.1 PI3K Inhibitors

The most straightforward method to inhibit PtdIns(3,4,5)P₃ signaling is to prevent the PI3K mediated conversion of PtdIns(4,5)P₂ to PtdIns(3,4,5)P₃. Many small molecule PI3K inhibitors have been developed with several advancing to clinical trials (Fig. 7.4) [96, 239]. Wortmannin, a sterol like fungal metabolite, was the first PI3K inhibitor and one of the most extensively studied. It was initially discovered to have an inhibitory effect on respiratory burst in neutrophils [240]. Subsequent studies illustrated wortmannin functions as an irreversible inhibitor of PI3K [241]. Nonspecific inhibition of other protein kinases, poor stability and high toxicity were all limiting factors preventing its use in the clinic [242, 243]. The first synthetic PI3K inhibitor was

developed by Eli Lilly in 1994 [244]. LY294002 has been most widely utilized as a research tool delineating PI3K signaling in cells. The clinical development of LY294002 has been limited by low potency and poor aqueous solubility [35, 245].

Second generation PI3K inhibitors based on the structures of wortmannin and LY294002 have been developed and are currently undergoing clinical trials. PX-866, in development by Oncothyreon, is an irreversible PI3K inhibitor based on the structure of wortmannin [243]. It has shown to have good oral bioavailability and an improved toxicity profile in a phase I trial. SF-1126 developed by Semaphore Pharmaceuticals is a LY294002 pro-drug with improved pharmacological properties and tumor targeting [245]. This compound is currently in clinical trials as well.

An emerging strategy in PI3K inhibition is to design isoform specific inhibitors. Previous discussion has demonstrated the importance of individual isoforms in various disorders. By selectively inhibiting the isoform implicated in disease, an improved therapeutic index is possible [142]. Initial efforts focused on specific inhibition of p110 α and p110 β due to their role in tumorigenesis. However, these isoforms are ubiquitously expressed and play critical roles in normal homeostasis, such as glucose metabolism. Therefore, development of p110 δ and p110 γ inhibitors has attracted increasing attention [246]. p110 δ is an important isoform present in immune cells and is commonly overexpressed in inflammatory disorders and lymphomas. IC-87114 (ICOS Corporation), CAL-101 and CAL-263 (Calistoga Pharmaceuticals) are all specific p110 δ inhibitors with 40–300 fold increased potency versus other isoforms [148, 247, 248]. CAL-101 in particular has shown promising clinical results in patients with various hematologic malignancies [249]. CAL-101 and CAL-263 have both entered clinical trials for the treatment of allergic rhinitis, potentially indicating utility in inflammatory diseases. p110 γ specific inhibitors were initially thought to be effective against several inflammatory disorders [250]. AS-605240 (Merck Serono) was tested for isoform specific

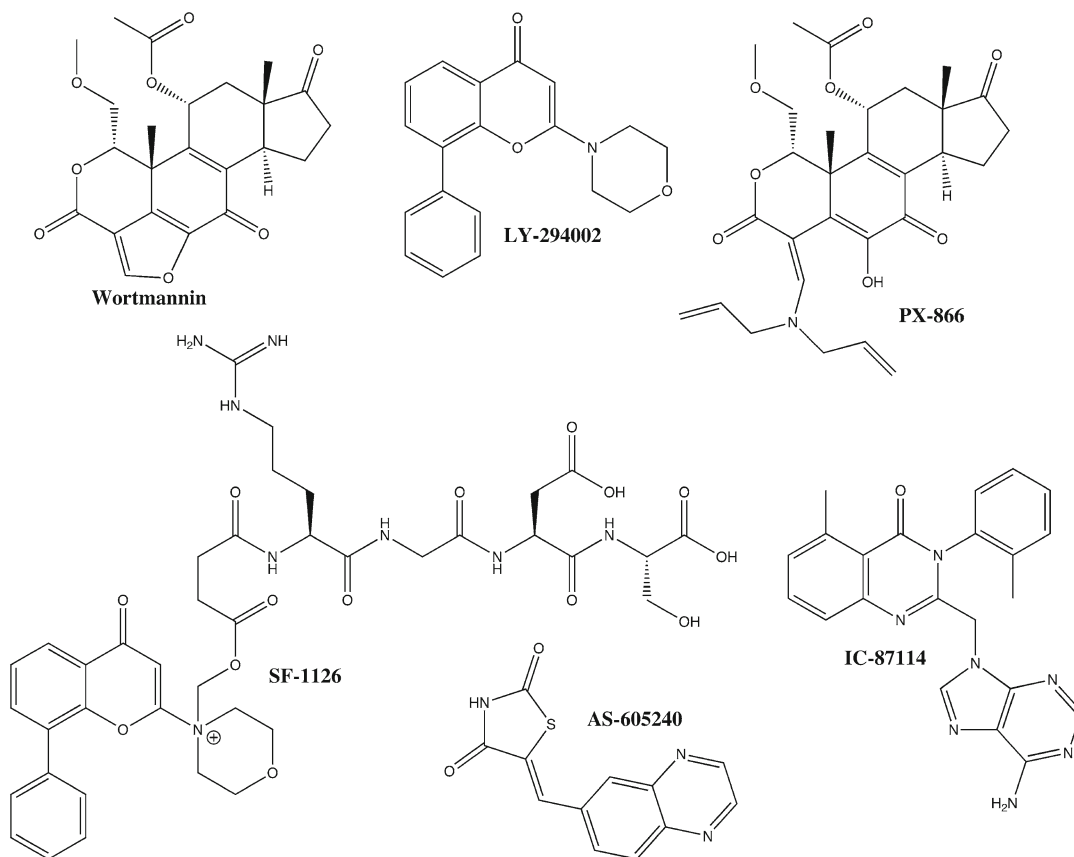


Fig. 7.4 Structures of several PI3K inhibitors

inhibition of p110 γ with some success [251]. However, subsequent studies have been disappointing as selectivity to p110 γ is insufficient to overcome off-target effects [142].

7.5.2 Inhibitors of Downstream Targets of PtdIns(3,4,5)P₃

Targeting specific isoforms of PI3K is only one way to improve therapeutic index and tolerability. Several downstream activators of PtdIns(3,4,5)P₃ signaling have also been targeted for inhibition. It is thought that targeting the critical components specifically contributing to particular pathologies could lead to improved efficacy and reduced toxicity compared broad PI3K inhibition. Akt and mTOR are the two most commonly targeted proteins downstream of PtdIns(3,4,5)P₃ [252],

although other proteins, such as PDK1, have been targeted as well.

7.5.2.1 Akt Inhibitors

Akt is one of the primary downstream effectors of PtdIns(3,4,5)P₃ signaling contributing towards increased cell survival, proliferation and metabolic alterations in cancer cells. Several inhibitors have been developed that target all three isoforms of Akt (Fig. 7.5). Both Perifosine and triciribine inhibit Akt activation by binding to its PH domain, thus preventing the membrane localization and activation by PDK1 [253, 254]. Both of these agents have seen limited success in the clinic due to poor response rates and adverse events, though there remains potential for use as part of combination therapies [252]. The allosteric inhibitor MK-2206 (Merck) showed promising results in combination therapy in xenograft

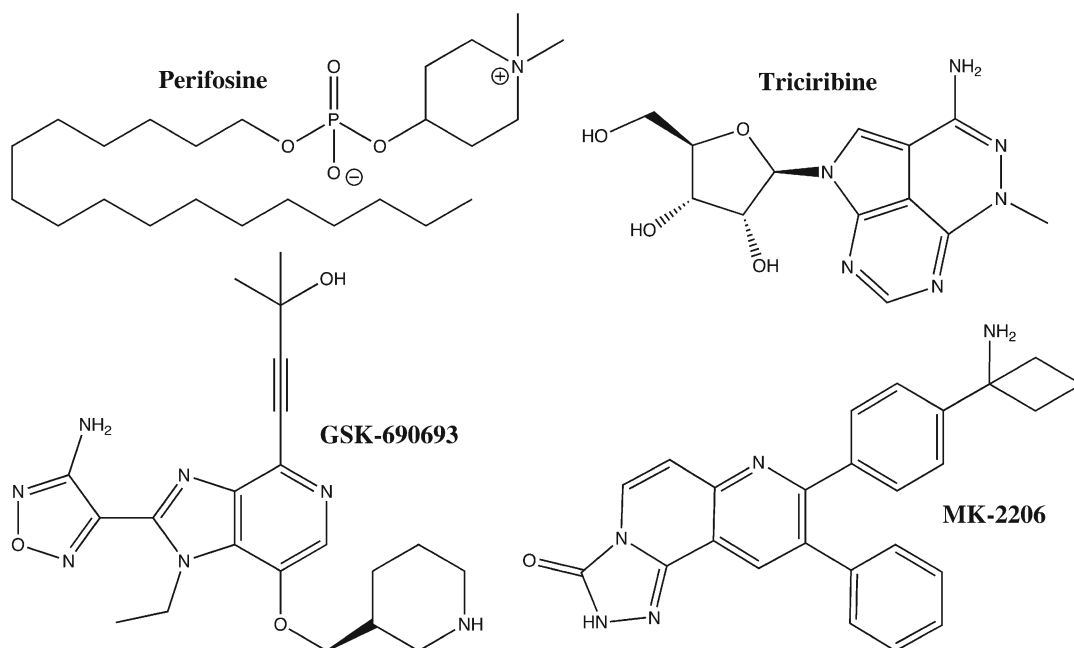


Fig. 7.5 Structures of several Akt inhibitors

models and is currently in Phase I trials [239, 255]. GlaxoSmithKlein has also developed two ATP competitive inhibitors of Akt, GSK-690693 and GSK-2141795 [256–258]. GSK-2141795 in particular has shown promising results in several phase I trials.

7.5.2.2 mTOR Inhibitors

Rapamycin (Wyeth) is a relatively selective mTORC1 inhibitor originally investigated as an antifungal agent. It has now been clinically approved as an immunosuppressant used to prevent rejection in organ transplantation. Analogs of rapamycin (known as rapalogs) such as temsirolimus (Wyeth) and everolimus (Novartis) have been approved for the treatment of renal cell carcinoma (Fig. 7.6) [259, 260]. Other cancers have lower response rates following treatment of rapalogs, thus indicating the heterogeneity of mTOR signaling in human cancers [252, 260]. mTORC1 is responsible for several but not all of mTOR-dependent functions. It has therefore been proposed that targeting the kinase activity of mTOR is a more effective means of PtdIns(3,4,5)P₃ signal inhibition [261, 262]. Very potent class

ATP-competitive mTOR inhibitors (Torin-1 and Torin-2) have shown promise in pre-clinical development [263, 264]. Other ATP-competitive inhibitors, AZD-8055 (AstraZeneca), INK-128 (Intellikine), and OSI-027 (OSI Pharmaceuticals) are also under development and currently undergoing clinical trials [252, 265].

7.5.2.3 Dual PI3K/mTOR Inhibitors

Many PI3K inhibitors under development are also active against structurally similar proteins, including mTOR [265]. mTOR is activated by both nutrient signaling and PI3K dependent growth factor signaling. Therefore, inhibition of PI3K does not completely abolish mTOR activity. Additionally, mTOR inhibition can increase PI3K activity through suppression of p70S6K-dependent negative feedback loop [96]. Therefore, strategies targeting both PI3K and mTOR have been pursued and several dual PI3K/mTOR inhibitors have been described (Fig. 7.7) [266]. PI-103 in particular was developed as a dual inhibitor with much preclinical success [267, 268]. Despite its potency, PI-103 has poor solubility and rapid clearance. Therefore several

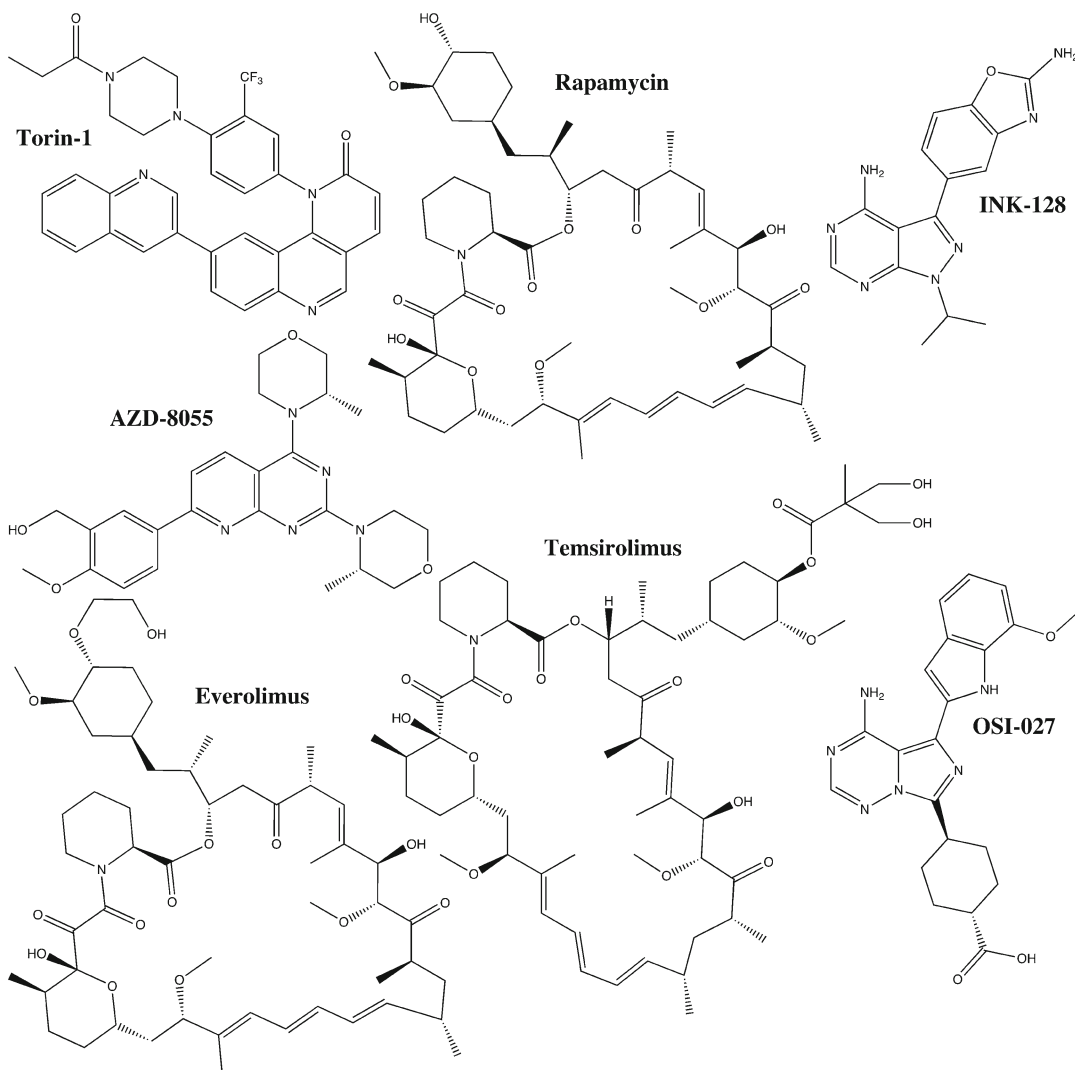


Fig. 7.6 Structures of several mTOR inhibitors

analogs, GDC-0980 and GDC-0941, have been developed by Genentech and are currently in clinical trials [252, 265]. Two other PI3K/mTOR inhibitors are being developed by Novartis, BEZ-235 and BGT-226. BEZ-235 is an orally available ATP-competitive inhibitor with low nanomolar IC_{50} values against both PI3K and mTOR [269, 270]. Phase I trials have indicated this compound is well tolerated with significant activity against breast cancer cells with high levels of PI3K signaling [271]. BGT-226 has also showed promising results both *in vitro* and *in vivo*

and the clinical development of this compound is also underway [272, 273].

7.5.2.4 Btk Inhibitors

Another important target in the treatment of B-cell malignancies and autoimmune arthritis is the PtdIns(3,4,5) P_3 -dependent Btk. As discussed above, Btk is a member of the Tec family of cytosolic tyrosine kinases and is activated upon recruitment to the inner leaflet of the membrane through interaction with PtdIns(3,4,5) P_3 and its PH domain. Two inhibitors of note

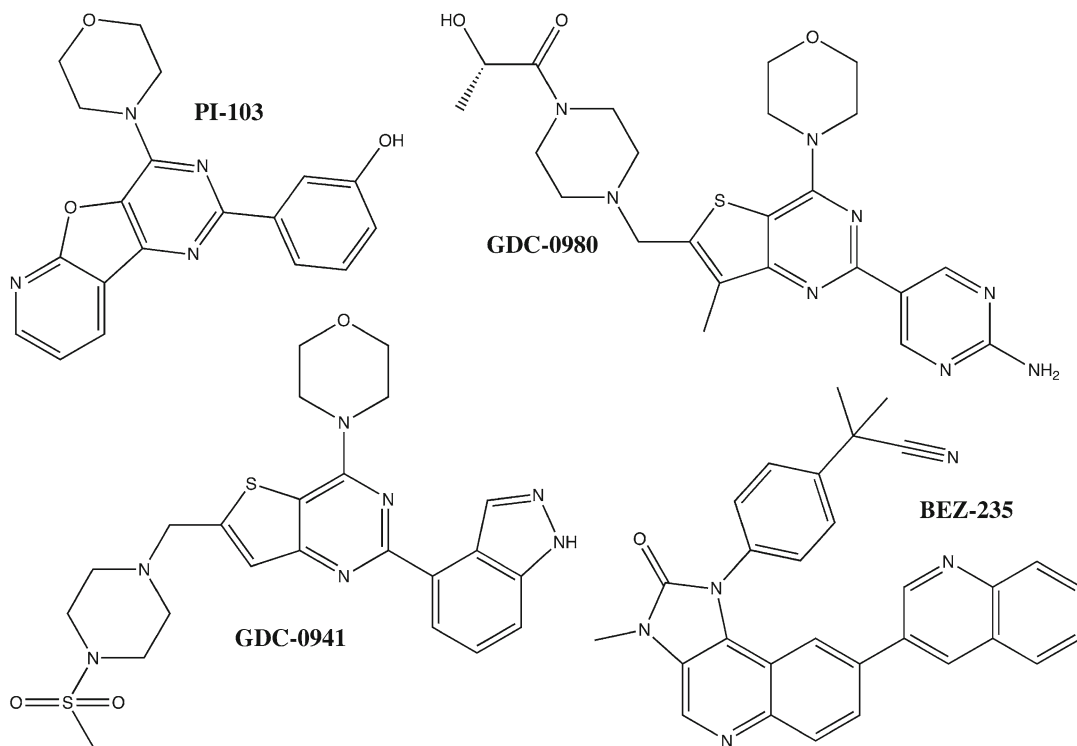


Fig. 7.7 Structures of several dual PI3K/mTOR inhibitors

have been developed targeting Btk, primarily for the treatment of B-cell malignancies, Pharmacyclics' PCI-32765 and Avila's AVL-292 (recently purchased by Celgene) [274]. PCI-32765 in particular has advanced through the clinic with positive Phase II results and is also being investigated as a treatment in autoimmune arthritis [275].

7.5.3 PH Domain Inhibitors

Another approach to inhibit PtdIns(3,4,5)P₃ signaling involves preventing the interaction of various proteins with membrane associated PtdIns(3,4,5)P₃. Blocking this interaction prevents the proper localization and activation of downstream effectors. Research into the structure and binding properties of the PH domain of Akt has provided insights into PH-PtdIns(3,4,5)P₃ interaction and development of inhibitors [17, 24, 276]. Inhibiting PtdIns(3,4,5)P₃ signaling

at this step should universally block downstream activation of PI3K mediators.

PtdIns(3,4,5)P₃-PH inhibition can be achieved either by lipid-based antagonists or by non-lipid small molecule antagonists (Fig. 7.8) [265]. Initially, modified water-soluble head groups of PIs, inositol polyphosphates, were investigated for their PtdIns(3,4,5)P₃-PH inhibition [277, 278]. Later optimization revealed a compound capable of increased specificity for PDK1 and inhibition of mTOR [279]. The previously discussed perifosine is another lipid based PtdIns(3,4,5)P₃-PH inhibitor with specific activity against Akt, preventing recruitment to the membrane. Non-lipid antagonists under investigation include metabolite of tricirbine (tricirbine-phosphate), NSC-348900, PHT-427 and API-1 [265]. Additionally, a novel class of compounds known as PITENINs has been developed [280, 281]. Chemical modification of the lead compound DM-PIT-1 has resulted in more potent and selective inhibition of Akt-PtdIns(3,4,5)P₃ interaction [281, 282].

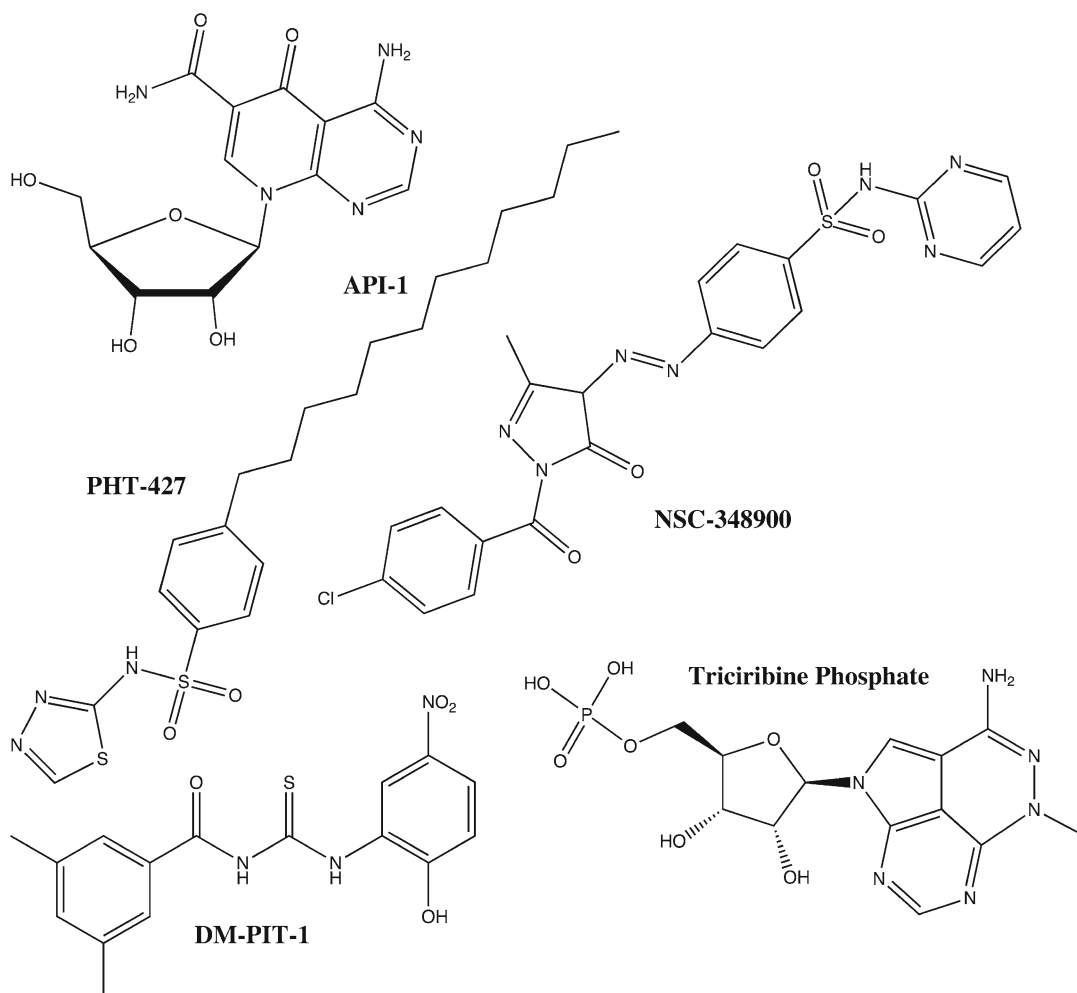


Fig. 7.8 Structures of several inhibitors of PtdIns(3,4,5)P₃/PH interactions

7.5.4 Drug Resistance

Many *in vitro* studies have demonstrated that PtdIns(3,4,5)P₃ signaling is associated with decreased response to therapy and drug resistance. Constitutively active Akt signaling is implicated in both paclitaxel and TNF α -related apoptosis inducing ligand resistance [140, 283]. Additionally, breast cancer cell lines demonstrating activated HER2/PI3K/Akt signaling were less susceptible to chemotherapeutic apoptosis and inhibiting either PI3K or Akt restored drug sensitivity [284]. Furthermore, clinical evidence

implicates PI3K and PTEN mutations with Herceptin resistance in breast cancer [285, 286]. Increased PtdIns(3,4,5)P₃ signaling has also been linked to colon cancer resistance to EGFR inhibition [287]. As a result of the increasing evidence implicating PtdIns(3,4,5)P₃ signaling in drug resistance, combination therapy strategies involving the targeted inhibition of the PtdIns(3,4,5)P₃ pathway and the Raf/MEK/ERK and BRAF pathways are being pursued [288, 289]. Several clinical trials are underway investigating the utility of PtdIns(3,4,5)P₃ pathway inhibition in avoiding drug resistance.

7.6 Conclusion

PtdIns(3,4,5)P₃ has emerged as an important second messenger in many cellular processes with implications for a wide range of diseases. Genetic studies in mice have illustrated the complexity of PtdIns(3,4,5)P₃ formation with various PI3K isoforms uniquely contributing to cellular signal propagation. PTEN expression and regulation has also emerged as an important mediator of PtdIns(3,4,5)P₃ activity. Following the formation of PtdIns(3,4,5)P₃, effector proteins such as Akt, PDK1, Btk, and others are able to bind to PtdIns(3,4,5)P₃ via PH domains and assemble at the inner plasma membrane where they are able to exert their effects resulting in cell survival, proliferation, cytoskeletal rearrangement, and gene expression. To better understand the effects of PtdIns(3,4,5)P₃ signaling in human diseases, additional elucidation of PI3K isoform activities needs to be performed. The ubiquitous expression of PI3K in healthy tissue necessitates the use of therapies targeting specific isoforms to reduce off target effects. Additionally, while a lot of progress has been made in understanding the regulation of key PtdIns(3,4,5)P₃ effectors, the contribution of many of the putative targets remains to be fully characterized.

As we begin to understand the complex relationships between upstream receptors, PI3K and downstream effectors, new small molecule inhibitors targeted at specific components are being developed with several successfully advancing through the clinic. Development of key biomarkers indicating which elements of the pathway to target will be a key step in the advancement of PtdIns(3,4,5)P₃ pathway inhibitors.

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