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REVIEW

Insights into the PI3-K-PKB-mTOR signalling pathway from small molecules

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Abstract This review describes the progress that has been made in understanding the PI3-K-PKB-mTOR signalling pathway by using small molecules as pharmacological probes. It briefly covers the structural characteristics, regulation of and downstream effects of several key regulators of PI3-K-PKB-mTOR signalling, then highlights the use of small molecules (by structural type) to selectively modulate specific components of the pathway.

Keywords Small molecules · Pharmacological probes · Cell signalling · PI3-K · PKB · mTOR · Rapamycin · Wortmannin

Introduction

Unravelling the many complex mechanisms of cellular signalling provides one of the greatest challenges in biological sciences, yet could provide a host of new opportunities in medicine and related fields. One signalling pathway that has a central function in regulating such varied processes as metabolism, cell growth, proliferation, apoptosis, chemotaxis and secretion is mediated by the phosphoinositide 3-kinase (PI3-K) family of enzymes predominantly through their downstream effector protein kinase B (PKB, also known as Akt) [1]. PKB acts as a central intersection in the pathway by phosphorylating a plethora of substrates and coupling PI3-K with the mammalian target of rapamycin (mTOR) pathway. Considering its key role in regulating critical cellular functions, it is not surprising that the deregulation of this signalling pathway is implicated in numerous complex disease states. Mutations of various components of the pathway are observed in the majority of human cancers, and the pathway has also been shown to play a key role in the development of type II diabetes [1, 2]. This review will discuss the progress that has been made in understanding the PI3-K-PKB-mTOR signalling pathway by using small molecules as pharmacological probes. It will briefly cover the structural characteristics, regulation and downstream effects of several key regulators of PI3-K-PKB-mTOR signalling, then highlight the use of small molecules (by structural type) to selectively perturb parts of the pathway.

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PI3-K-PKB-mTOR signalling overview

Structural characteristics of PI3-Ks

PI3-Ks are divided into three major classes based on their sequence homology [2, 3]: I, II and III. Class I PI3-Ks have attracted the majority of research interest and are heterodimeric proteins containing a catalytic and regulatory subunit. The 110-kDa catalytic subunit consists of a catalytic lipid kinase domain, a Ras binding domain (RasB), a C2 phospholipid binding domain (protein kinase C homology domain), a helical PI kinase (PIK) domain and an N-terminal domain, which forms a tight association with the regulatory subunit [3]. Class I PI3-Ks are further

subdivided into classes Ia and Ib based on their structure and mechanism of activation; class Ia are activated by growth factor receptor tyrosine kinases (RTKs) and class Ib by G-protein-coupled receptors (GPCRs) [2].

The class Ia regulatory subunit performs an adaptor function and contains two Src-homology 2 (SH2) domains. Class Ia PI3-Ks can encode five isoforms of the regulatory subunit in mammalian cells: p85α, p85β and p55γ are encoded by distinct genes, and the shorter p55α and p50α are obtained via alternative splicing of the p85α transcript [2, 3]. Additionally, three different isoforms of the catalytic subunit are produced, p110α, p110β and p110δ, which can interact with any of the regulatory subunits. The p110δ isoform appears to be largely restricted to leukocytes, whereas the other isoforms have a broad tissue distribution. A class Ib PI3-K that has been characterised consists of a p110γ catalytic subunit and a structurally distinct p101 regulatory subunit [3]. A second regulatory subunit known as p84 [4] or $p87^{PIKAP}$ [5] has also been identified. Class Ib PI3-Ks have been shown to play an important role in inflammatory processes [6].

Regulation of PI3-Ks

PI3-Ks can be activated through several mechanisms. The SH2 domains in the p85 regulatory subunit of class Ia PI3-Ks have a high affinity for phosphorylated tyrosine residues found in activated growth factor RTKs, and binding of the regulatory subunit to this motif activates PI3-K. In addition to these direct mechanisms of activation, adaptor proteins such as Grb2-associated binders (Gab1-3) and insulin receptor substrates (IRS1-4) can activate PI3-Ks when phosphorylated [7]. Grb2 can also activate Ras through prior activation of the GTPase son of sevenless. Association with the GTP-bound form of Ras via the Ras binding domain (RasB) allows direct activation of the catalytic subunit of class Ia PI3-Ks independent of the regulatory subunit [3, 8]. Due to the lack of SH2 domains on the p101 regulatory subunit of class Ib PI3-Ks, they cannot be activated by RTKs and instead are activated by binding to Gβγ subunits released upon GPCR stimulation [9].

Once activated, class I PI3-Ks are recruited to the plasma membrane and bring the protein into close proximity with its substrate, the inositol phospholipid phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2 or PIP_2]. PIP_2 is then rapidly phosphorylated at the 3-hydroxyl position of the inositol ring to produce the secondary messenger phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P_3 or PIP_3] (Fig. 1). Signalling proteins containing the Pleckstrin homology (PH) domain can bind to PIP_3 and accumulate at the membrane, facilitating the formation of signalling complexes [7].

The deactivation of PI3-K signalling is primarily regulated by the tumour suppressor protein PTEN (phos-

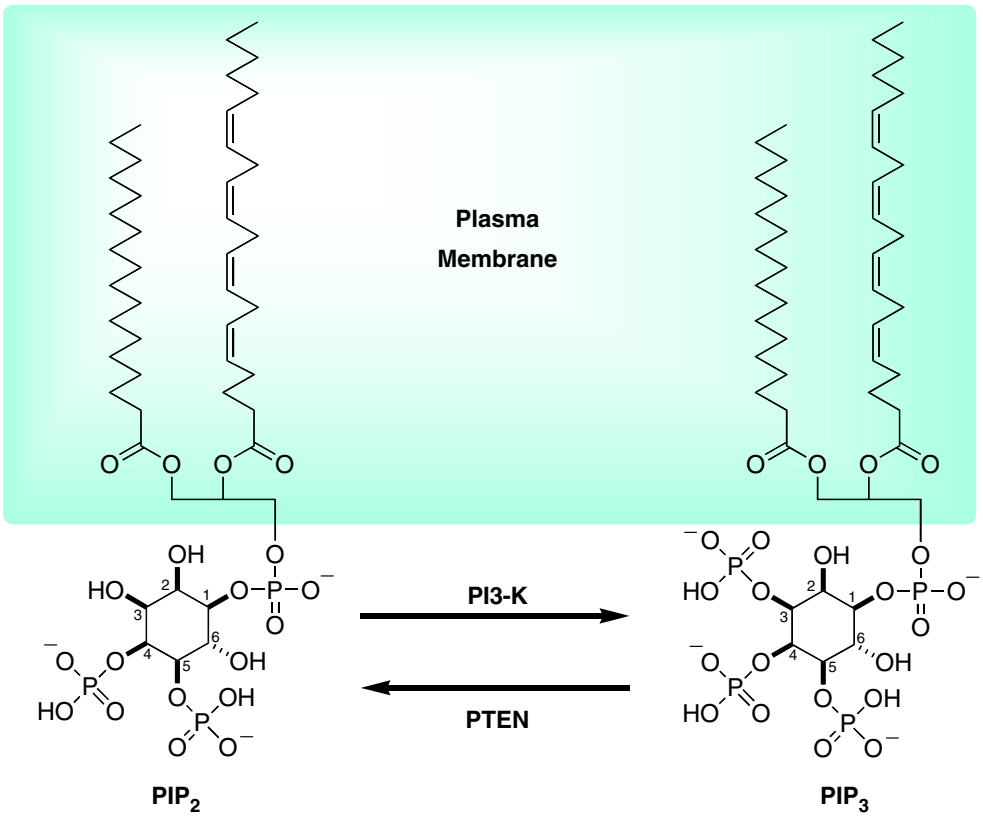


Fig. 1 Structures of PIP_2 and PIP_3 and interconversions by PI3-K and PTEN

phatase and tensin homologue deleted on chromosome 10), which specifically dephosphorylates PIP_3 at the 3-position to generate PIP_2, thereby terminating the lipid signalling. Although the SH2-containing inositol 5′-phosphatases (SHIP1 and SHIP2) are also capable of dephosphorylating PIP_3 by removing the phosphate group at the 5-position to generate phosphatidylinositol (3,4)-diphosphate, PTEN has been shown to be primarily responsible for attenuating the effects of PI3-K signalling in vivo [10, 11]. Phosphatidylinositol (3,4)-diphosphate is itself a secondary messenger that can recruit proteins containing PH domains to the membrane, which may account for these observations.

Downstream of PI3-K

Upon activation of PI3-K, the serine–threonine kinase phosphoinositide-dependent kinase 1 (PDK1) is translocated to the membrane by binding of its PH domain to the second messenger PIP_3. PDK1 can activate a variety of kinases from the AGC family including PKB, p70 ribosomal S6 kinase (S6K) and several isoforms of protein kinase C (PKC) [12]; however, only PKB phosphorylation by PDK1 is PI3-K- and PIP_3-dependent [13]. Three closely related isoforms of PKB are produced in mammals, PKBα, PKBβ and PKBγ (also known as Akt1, Akt2 and Akt3), all of which contain three domains: a PH domain at the N terminus with a module for lipid binding, a catalytic kinase domain related to other AGC family kinases and a hydrophobic motif (HM) at the C terminus, which forms a docking site for PDK1 [14]. PKB is the most significant mediator of the PI3-K signalling cascade and is localised to the membrane by interactions between its PH domain and PIP_3.

PKB is brought into proximity with PDK1 at the membrane where its activation is elegantly regulated by two independent phosphorylation events (Fig. 2). PDK1 phosphorylates PKB at threonine 308 (Thr308) located in the activation loop (T-loop) of the kinase domain [14]. The identity of the kinase responsible for phosphorylation of the HM at serine 473 (Ser473) was controversial until recently, with numerous candidate kinases identified that could replicate this event in vitro, yet no convincing in vivo data. Sarbassov et al. [15] have since provided compelling evidence that the mammalian target of rapamycin complex 2 (mTORC2) complex is the kinase responsible for Ser473 phosphorylation in vivo. Counteracting mTORC2 by dephosphorylating PKB at Ser473 are the PH domain and leucine-rich repeat protein phosphatases (PHLPPs), PHLPP1 and PHLPP2, which have different specificities for each of the three mammalian isoforms of PKB [16, 17].

The multiprotein mTORC2 complex consists of mTOR, mammalian stress-activated protein kinase interacting protein 1 (mSIN1), mammalian counterpart of yeast LST8 (mLST8, also known as GβL), rapamycin-insensitive companion of mTOR (rictor) and a protein associated with rictor (Protor; Fig. 3) [18]. mTORC2 is often referred to as the "rapamycin-insensitive" complex of mTOR; however, it has since been found that in some cell lines, prolonged exposure to rapamycin leads to a decrease in PKB phosphorylation at Ser473, apparently due to rapamycin inhibiting the formation of the mTORC2 complex [19]. Despite mTORC2's role in activating PKB, it is not essential for the successful phosphorylation of several PKB substrates in mice [18]. This may be due to the compensatory activity of other AGC kinases, or, alternatively, Ser473 phosphorylation might be unnecessary for full activation of PKB; however, the complex activity profile of mTORC2 in vivo remains uncertain at this time [18].

mTOR together with mLST8, the regulatory associated protein of mTOR (raptor), and proline-rich Akt substrate 40 kDa (PRAS40) forms another multiprotein complex known as mTORC1 which is specifically inhibited by rapamycin. PKB activates mTORC1 indirectly by phosphorylation of tuberous sclerosis complex 2 (TSC2) in the

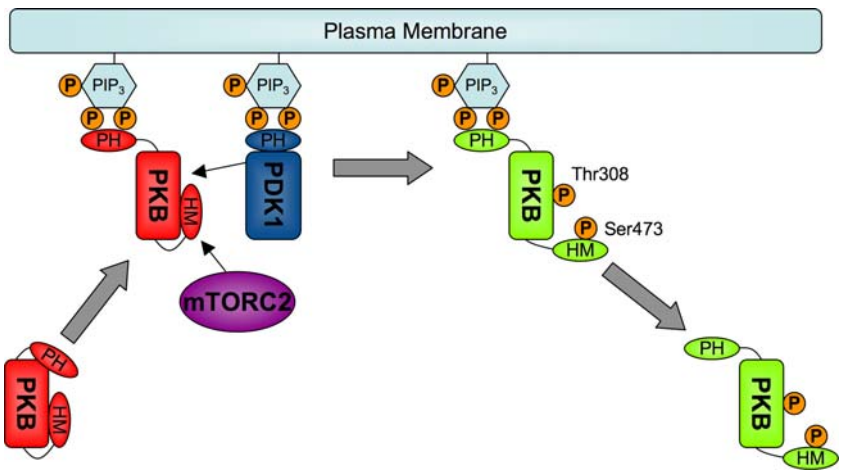


Fig. 2 Schematic diagram of PKB activation. Inactive PKB (shown in *red*) and PDK1 are recruited to the membrane via their PH domains. The kinase domain of PKB is phosphorylated at Thr308 by PDK1 and the HM at Ser473 by mTORC2 to give fully activated PKB (shown in *green*), which can remain at the membrane or migrate to the cytosol

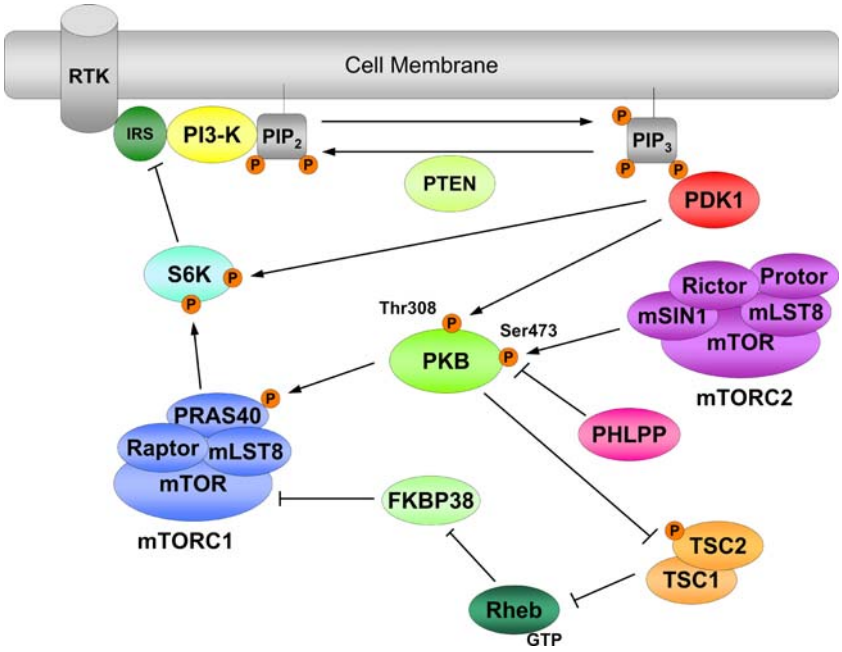


Fig. 3 The complex relationship between PI3-K, mTOR and PKB signalling

TSC1/TSC2 dimer. This phosphorylation event inhibits the guanosine triphosphatase activating protein activity of TSC2 and in turn leads to the activation of Rheb, which is active only in the guanosine triphosphate (GTP)-bound form. Rheb-GTP does not directly activate mTORC1, but binds to another protein known as FKBP38 (also known as FKBP8), a member of the FK506-binding protein (FKBP) family. The inhibited complex consists of FKBP38 bound to mTORC1; however, upon activation, Rheb-GTP binds to FKBP38, inducing its release from mTORC1 and thus activating the complex [20].

PKB also has the ability to directly phosphorylate PRAS40, a component of the inhibited mTORC1 complex. Phosphorylation of PRAS40 generates a binding site for 14-3-3 proteins, which may enable the dissociation of PRAS40 from the complex, enabling activation of mTORC1. PRAS40 therefore acts as a important intermediary between PKB and mTOR signalling [21].

Once mTORC1 is activated, it initiates a negative feedback loop that inhibits PKB by the negative regulation of IRS1 via activation of p70 ribosomal S6 kinase 1 (S6K) [21]. Together with the observation that Ser473 phosphorylation of PKB is not essential for phosphorylation of TSC1/TSC2, this model places mTORC1 upstream of mTORC2, although this assumes that mTORC2 activation is dependent on PI3-K, which has yet to be confirmed [22].

Once activated, PKB acts as a central signalling node, propagating the signal through a variety of downstream effectors (Fig. 4). Manning and Cantley [23] have published an extensive review of the literature which identified 18 PKB substrates that have been the subject of multiple independent reports, although there are almost certainly additional genuine PKB substrates yet to be comprehensively characterised. Through these numerous downstream effectors, the PI3-K-PKB-mTOR signalling network plays a fundamental role in regulation of cell survival and apoptosis, cell growth, cell cycle progression, angiogenesis, metabolic and neurological processes; therefore, loss or gain of PKB function is an underlying factor in numerous human diseases.

Investigation of PI3-K-PKB-mTOR signalling using small molecules

Our current understanding of the PI3-K-PKB-mTOR signalling cascade has been obtained using a variety of

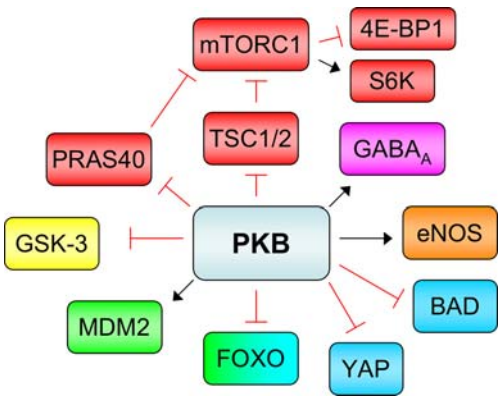


Fig. 4 An overview of the key proteins regulated by PKB that are involved in metabolism (*yellow*); cell cycle progression (*green*); cellular growth (*red*); cell survival and apoptosis (*blue*); angiogenesis (*orange*) and neurological processes (*purple*)

experimental techniques including genetic knockout and knock-in, RNA interference (RNAi), pharmacological perturbation with small molecules and more recently by combining genetic mutation with modified small-molecule inhibitors to confer selectivity, an approach referred to as chemical genetics [24]. All of these techniques are complementary and have their own unique advantages and disadvantages.

Genetic knockout and knock-in techniques have been instrumental in investigating PI3-K signalling, although due to the pathway's key role in the regulation of essential cellular functions, knockout of p110α or p110β is embryonically lethal in model organisms [25]. Mice lacking individual PKB isoforms are viable; however, knockout of more than one isoform is embryonically or neonatally lethal [26]. Significantly, knockout of one isoform of PI3-K leads to altered expression of other isoforms, and therefore, it cannot be determined if the observed phenotype can be directly ascribed to the knocked-out gene [25]. This "steady-state" effect of other components compensating for developmental defects is a general limitation of genetic approaches where there is redundancy of function in the system.

As a result of these limitations, RNAi has been extensively used; however, this technique currently has disadvantages, including slow response times, and is influenced by delivery problems in vivo. Alternatively, small molecules can be used to directly modulate the function of the protein of interest, although this reduces the potential for studying compensatory effects of other components. Small molecules are rapid acting and can be added at any given point in the experimental procedure to give effective temporal control. Furthermore, their effects are reversible due to metabolism and "washing out" of the molecule. Another benefit to this approach is its sensitivity, as varying the concentration of the small-molecule probe results in the ability to "fine tune" the phenotype, thereby allowing subtle effects to be investigated by the generation of a dose–response profile [24].

Chemical genetics expands the utility of the pharmacological approach by incorporation of a mutation into the protein of interest that allows a uniquely modified small molecule to exert unparallelled specificity in comparison to the wild-type system [24]. This is especially true for protein kinases, which have a high degree of homology in their ATP-binding sites. Although extremely powerful, such an approach is extremely time- and labour-intensive and consequently has not yet found widespread usage; however, this technique may become increasingly important in future years for interpreting the roles of specific components of the signalling pathway.

A wide variety of small-molecule modulators of PI3-K-PKB-mTOR signalling have been reported in the literature to date. Many of the compounds initially identified had low specificity. These "first-generation" compounds provided a proof of concept that the small-molecule inhibition of kinases was feasible. Due to their therapeutic potential, and to avoid anticipated toxicity problems, the pharmaceutical industry has invested considerable effort in developing a "second generation" of compounds that show higher specificity for their target kinase and, in many cases, specificity for a particular isoform. These compounds are now being joined by a "third generation" that are specific for multiple kinases in the hope that by overcoming redundancy of function in the system, their therapeutic efficacy will increase. This short review will focus on highlighting examples of specific compounds that have been successfully used to improve our understanding of the PI3-K-PKB-mTOR signalling pathway

The first inhibitors of PI3-K-PKB-mTOR signalling: natural products and derivatives

Steroidal furanoids

The fungal steroidal product wortmannin (**1**, Fig. 5) was first isolated in 1957, although PI3-K was not identified as one of its targets until 1993 [27]. Wortmannin is a potent inhibitor of PI3-K isoforms that binds irreversibly, by opening of the electrophilic furan ring at the C-20 position, to a lysine residue within the ATP binding region of PI3-K.

Wortmannin has historically been of great utility for the investigation of PI3-K-PKB-mTOR signalling, but suffers from multiple disadvantages compared to more recently developed compounds. In addition to inhibiting PI3-Ks, wortmannin has been shown to inhibit PLK1 [28] as well as other kinases such as mTOR [29]. Wortmannin is also cytotoxic and has low solubility and stability in aqueous solution. In an attempt to overcome these disadvantages, the pegylated 17-hydroxywortmannin derivative PWT-458 (**2**, Wyeth) was synthesised, which resulted in reduced toxicity and improved solubility and plasma stability while maintaining potency [30]. Similarly, the ring-opened wortmannin derivative PX-866 (**3**, Oncothyreon) was biologically stable and a broad-spectrum PI3-K inhibitor [31].

Several derivatives of wortmannin have been synthesised as fluorescent probes in order to observe the localisation of PI3-Ks in a cellular context. Addition of the fluorescent carboxylic acid NBD-sarcosinate at the C-11 position of wortmannin gave a fluorescent conjugate (**4a**) that was inhibitory towards PI3-K [32]. Similarly, C-11 derivatisation has been used to generate biotinylated (**4b**) and ^{125}I-labelled (**4c**) wortmannin derivatives [33] which inhibited PI3-K sufficiently to enable their use in in vivo systems.

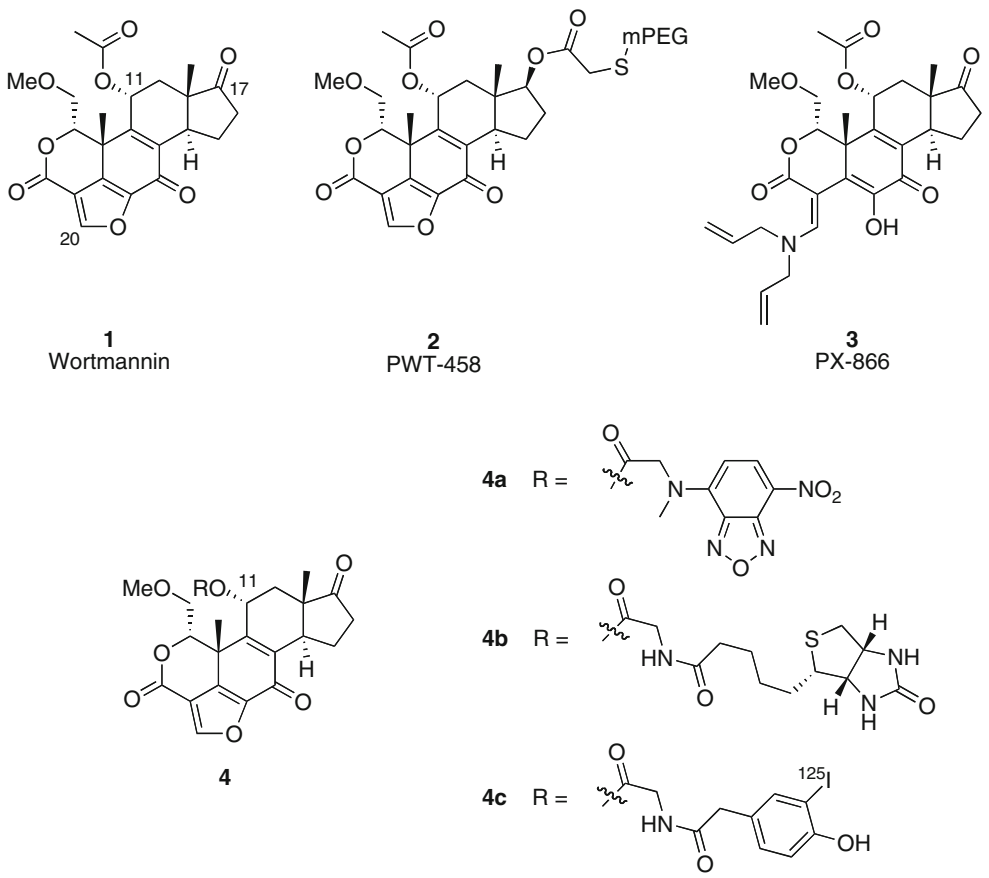


Fig. 5 Wortmannin (**1**) and derivatives (**2**–**4**)

Rapamycin and the "rapalogs"

Rapamycin (**5**) is a macrocyclic lactone (Fig. 6) isolated from a *Streptomyces hygroscopicus* strain. Rapamycin was initially developed as a potential antifungal agent; however, interest in the compound escalated after it was found to have immunosuppressive activity. Rapamycin has low solubility in aqueous media, and therefore, the closely related "rapalogs" temsirolimus (**6**), everolimus (**7**) and deferolimus (**8**) were developed with an effective water-solubilising function by pharmaceutical companies (Wyeth, Novartis and Ariad, respectively) [34].

Rapamycin forms a complex with the FK506-binding protein FKBP12. mTOR was subsequently identified as the target of the resulting rapamycin–FKBP12 complex in 1994 [35–37]. The rapamycin–FKBP12 complex binds to and inhibits the kinase activity of the mTORC1 complex (also known as the "rapamycin-sensitive" complex). Initially, it was thought that mTORC2 was insensitive to rapamycin, as the rapamycin–FKBP12 complex does not bind to mTORC2. Sarbassov et al. [19] have since shown that rapamycin is capable of inhibiting the assembly of mTORC2 in several cell types and hence act as a PKB inhibitor in these cell types. In addition, Akcakanat et al. [38] have shown that treatment with rapamycin, as well as RNAi knockdown of mTOR, causes dephosphorylation of rictor, a component of mTORC2. This provides further evidence that mTORC2 may be downstream of mTORC1; yet, despite the utility of rapamycin, study of the complex feedback loops operating in the mTOR pathway would be greatly aided by the availability of small-molecule inhibitors selective for mTORC1 and mTORC2.

Synthetic small-molecule inhibitors of PI3-K-PKB-mTOR signalling

Chromone-derived compounds

The chromone LY294002 (**9**, Fig. 7) derived from the flavonoid quercetin was first described in 1994 by Lilly Research Laboratories [39]. This importantly illustrated that the competitive inhibition of PI3-K could be achieved with a synthetic "drug-like" small molecule. X-ray crystallographic studies revealed that LY294002 binds in the ATP binding site via hydrogen bonding between the morpholino oxygen and the amide backbone of the Val882 residue and between the ketone moiety and Lys833 [40].

As with wortmannin, LY294002 is known to have significant non-specific inhibitory activity. Gharbi et al.

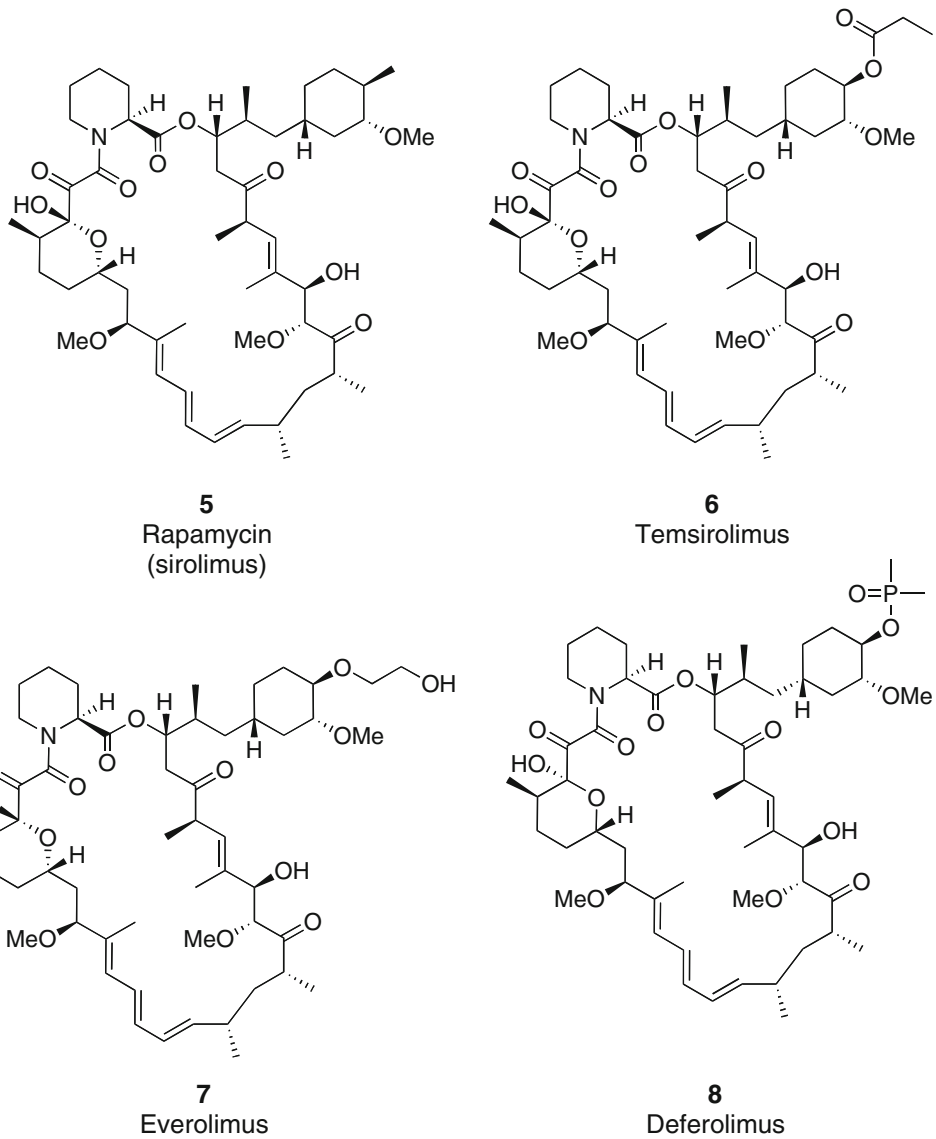


Fig. 6 The structures of rapamycin (**5**) and related "rapalogs" (**6**–**8**)

[41] have investigated the specificity of LY294002 by immobilising the compound onto Sepharose beads for affinity chromatography experiments using cellular extracts of potential protein targets. From the X-ray crystallography data, it was postulated that the exocyclic aryl group could be substituted with an aniline group to enable conjugation with the functionalised beads, as this substituent projects away from the ATP binding site. The immobilised LY294002 derivative was successfully used to affinity purify a variety of kinases including mTOR, CK2, GSK3 and PI4-K as well as PI3-K. This study indicated that the use of LY294002 for cell signalling investigation was not optimal now that compounds with an improved specificity profile are available.

Despite the limitations of LY294002, modification of the structure has yielded some promising analogues. Replacement of the chromone oxygen with nitrogen, together with modification of the C8 aryl substituent, led to improved potency and selectivity in TGX-115 (**10**, Kinacia). TGX-115 is selective for the mammalian p110β and p110δ isoforms of PI3-K, which have particularly close homology in their sequences, at nanomolar concentrations exhibiting more than 100-fold selectivity over the p110α and p110γ isoforms [42]. This specificity profile was presumed to result from interactions between the more bulky C-8 aromatic substituent and non-conserved residues that line the outside of the ATP binding site. The LY294002 derivative TGX-286 (**11**) exhibited similar potency to TGX-115; however, it has lower selectivity for the p110β and p110δ isoforms [41].

In a landmark study, Knight et al. [43] pharmacologically evaluated a structurally diverse collection of PI3-K inhibitors using a variety of in vitro and in vivo assays and X-ray crystallography. Compounds TGX-115 and TGX-286 were found to be unable to inhibit insulin-stimulated phosphorylation of any protein associated with the PI3-K

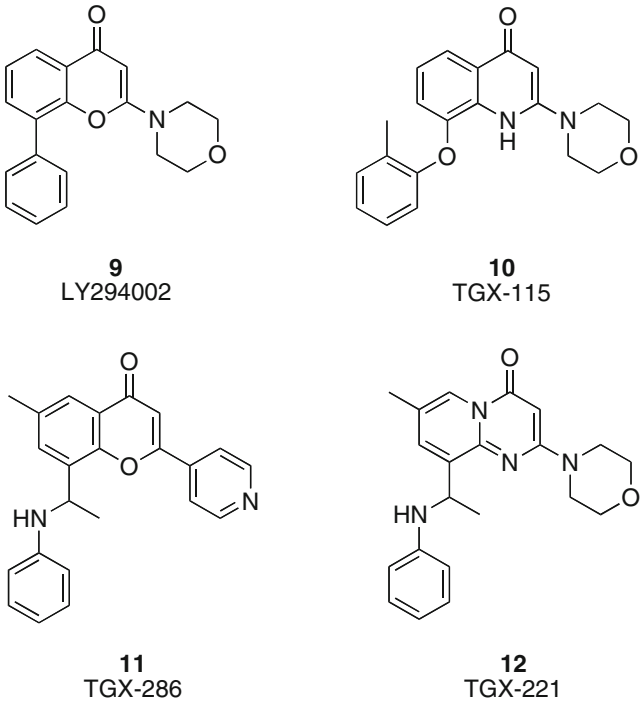


Fig. 7 From non-specific inhibitors such as LY294002 (**9**), isoform specific inhibitors (**10**–**12**) have been developed by modification of the chromone scaffold

pathway in adipocytes and myotubes despite TGX-115 lowering PtdIns(3,4)P_2 and PIP_3 levels in adipocytes by approximately 50%.

These results indicated that the p110β and p110δ isoforms were not primarily responsible for insulin signalling in adipocytes and myotubes. However, further investigation using TGX-115 revealed a secondary role for these isoforms in myotubes where they are responsible for synthesising a basal pool of PIP_3 that lowers the threshold level of p110α required for PKB phosphorylation. By contrast, addition of TGX-115 led to a partial reduction in PIP_3 levels in adipocytes, but did not inhibit p110α-induced PKB phosphorylation, suggesting that this subtle effect is not at work in this tissue [43]. In a related study Foukas et al. [44] found that TGX-221 (**12**), which is more than 1,000-fold selective for p110β over 110α, had no effect on insulin-stimulated PI3-K activity. Jackson et al. [45] have also used TGX-221 to demonstrate a role for p110β in promoting platelet activation, suggesting a potential role for p110β inhibitors as antithrombotic agents.

Pyrimidine- and quinoline-derived compounds

The pyridinylfuranopyrimidine compound PI-103 (**13**, Piramed, Fig. 8) has been of particular value for the study of PI3-K-PKB-mTOR signalling due to its unique activity profile. It is a multitarget inhibitor, though it inhibits the p110α isoform more effectively than p110β [43]. As well as being a nanomolar inhibitor of PI3-K, PI-103 is also a potent inhibitor of rapamycin-sensitive mTORC1 and rapamycin-insensitive mTORC2 (IC_{50}s of 20 and 83 nM, respectively). X-ray crystallography studies using several PI3-K inhibitors bound to p110γ were used to generate a model suggesting that PI-103 binds in the ATP binding pocket in a similar manner to LY294002 and other chromone derivatives. Its potency against PI3-K is believed to be derived from projection of the *m*-phenol moiety into a deep "affinity pocket". Due to its isoform specificity, PI-103 was used by Knight et al. [43] to show that p110α is primarily responsible for insulin signalling in adipocytes and myotubes. Chaussade et al. [46] have conducted a similar study using PI-103 and a variety of other isoform-specific inhibitors including TGX-221. In contrast to the earlier study by Knight and co-workers, they found that in several cell lines, p110α is not necessary for insulin signalling, as p110β and p110δ play a compensatory role. These results provide strong evidence that functional redundancy between PI3-K p110 isoforms occurs in vivo and is highly variable over different cell types. Small molecules such as PI-103 are particularly useful for identifying such effects, as the proteins they inhibit remain structurally intact and therefore can retain a scaffold function, whereas knockdown by RNAi can disrupt such interactions, causing a different phenotype to be observed.

The inhibitory activity of PI-103 has been further probed by Fan et al. [47]. In glioma cell lines, addition of either PI-103 (**13**) or TGX-286 (**11**, p110α and p110β inhibitors,

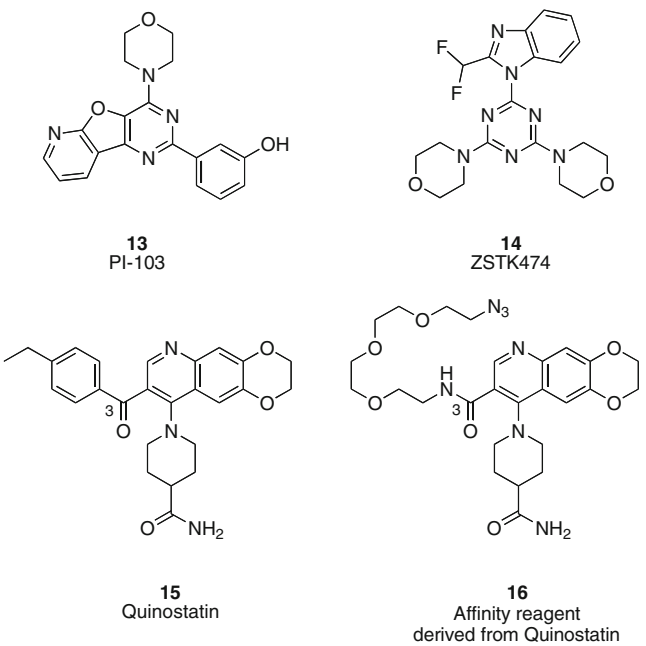


Fig. 8 Structures of pyrimidine- (**13**–**14**) and quinoline-derived (**15**–**16**) compounds

respectively) was sufficient to halt activation of PKB, although only inhibition of p110α blocked proliferation of glioma cells in vitro. Furthermore, the synergy of mTOR and PI3-K inhibition exhibited by PI-103 gave the compound significantly higher efficacy in arresting the growth of glioma cells in vivo compared to treatment with rapamycin or TGX-286 [47].

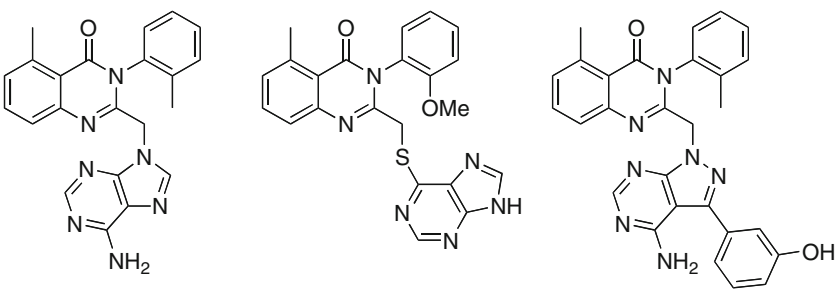
The utility of potent and specific compounds such as PI-103 has further been demonstrated by Raynaud et al. [48] who found that treatment of a variety of cell lines with PI-103 did not lead to apoptosis despite the inhibition of PKB phosphorylation being observed. While this was contrary to their expectations, they state that much of the evidence for associating inhibition of PI3-K-PKB-mTOR signalling with apoptosis arises from studies employing LY294002. This indicates that apoptosis observed upon treatment with LY294002 may result from other pathways or mechanisms. In a recent review of protein kinase inhibitor specificity, it was suggested that the use of LY294002 for inhibiting PI3-K be entirely replaced by PI-103 due to its high potency and selectivity [29].

Yaguchi et al. [49] have reported the discovery of the related compound ZSTK474 (**14**) as a result of screening a library of triazines. Compound ZSTK474 was identified as a potent inhibitor of PI3-K (IC_{50} of 37 nM), however, was non-isoform specific. Molecular modelling indicated that ZSTK474 binds in the ATP binding pocket of PI3-K in a similar manner to ATP-PI3-K, with the benzoimidazole nitrogen forming a hydrogen bond to Val882 and further hydrogen bonding to Ser806. A third hydrogen bond was noted to Lys833. Compound LY294002 by comparison has two hydrogen bonds to PI3-K, at Val882 and Lys833, which may explain why ZSTK474 is a stronger competitor for the ATP binding site [49].

Yang et al. [50] have discovered a quinoline-derived compound known as quinostatin (**15**) that was identified from a compound library of approximately 20,000 members by means of a high-throughput assay they developed to screen for inhibition of phosphorylation of ribosomal protein S6, a downstream effector of mTOR signalling. Structure–activity analyses indicated that the C-3 carbonyl group was essential for the preservation of S6K inhibition, but an ethoxy group could replace the *p*-ethylphenyl substituent without affecting inhibition. Determination of the target of quinostatin was achieved using affinity chromatography. An analogue of quinostatin (**16**) was synthesised by attachment of a polyethylene glycol chain via an amide bond to the C-3 carbonyl group, which, in turn, could be linked to agarose beads through a further amide linkage. Such a structural modification to quinostatin lead to an approximate 100-fold decrease in activity, although the conjugated compound remained sufficiently active to be used for affinity chromatography. Incubation of MCF7 cell lysate with the beads led to the identification of numerous proteins. However, a single band disappeared upon the addition of 100 μM quinostatin, and liquid chromatography–mass spectroscopy and trypsin digestion indicated that this band corresponded to the p85α and p85β subunits of PI3-K, whereas immunoblotting with a p110-specific antibody indicated that affinity purification of the catalytic subunit had also occurred. In addition, quinostatin was found to inhibit the kinase activity of p110γ. This suggests that quinostatin is targeting the catalytic subunit, as p110γ does not contain a regulatory subunit.

Quinazolinone purines

Further modification of the LY294002 scaffold led to the development of the quinazolinone purine IC87114 (**17**, ICOS, Fig. 9). Compound IC87114 is a potent inhibitor of p110δ, (IC_{50} of 0.5 μM) with more than 50-fold selectivity over p110γ, making it the most selective inhibitor of a single PI3-K isoform reported so far [51]. Interestingly, IC87114 has 100-fold selectivity over p110α and p110β compared to p110γ, in contrast to the chromones which are selective for both p110β and p110δ. IC87114 has been used to show that p110δ is primarily responsible for amplification of PIP_3 levels and the directional component of chemotaxis in neutrophils [51]. IC87114 has also subsequently been used to indicate the key role of p110δ in B cell and T cell



17 IC87114 **18** PIK-39 **19** PIK-294

Fig. 9 Further variation of the LY294002 chromone scaffold has led to the development of the isoform selective quinazolinone purine inhibitors (**17**–**19**)

development [52,53], indicating the anti-inflammatory potential of such compounds. Illustrating the phenotypic differences between pharmacological and genetic approaches, the use of IC87114 in wild-type mouse B cells led to stronger inhibition of Erk and GSK3 than observed in B cells obtained from p110$\delta^{D910A/D910A}$ knock-in mice.

Knight et al. [43] probed the impressive selectivity of the quinazolinone purines for p110δ by analysing the crystal structures of p110γ bound to PIK-39 (**18**, p110γ was the PI3-K crystal structure available at the time). PIK-39 is a closely related analogue of IC87114 containing a thiol moiety that has similar potency and specificity for p110δ. In order to be accommodated within the ATP-binding pocket, orientation of the purine differs from that of the adenine of ATP and the quinazolinone ring system projects out of the entrance to the ATP-binding pocket. This binding mode is believed to cause the Met804 residue to "flip" and induce a conformational change in the protein. According to this model, the selectivity of this class of compounds can be explained by the differing plasticity of PI3-K isoforms in the region around Met804 within the loop of the catalytic domain, and hence their ability to tolerate this induced conformational change.

The crystallographic data were used to model IC87114 bound to p110γ and show that this unique binding mode is conserved among the quinazolinone purines. Aided by this model, Knight et al. [43] designed and synthesised the IC87114 analogue PIK-294 (**19**), which incorporates a *m*-phenol group that can project into the "affinity pocket" as with PI-103. By exploiting this interaction, a 62-fold increase in potency against purified p110γ was achieved, albeit with a loss in specificity.

Thiazolidinediones

Selective ATP-competitive inhibitors of p110γ, AS-604850 (**20**) and AS-605240 (**21**, Serono, Fig. 10) based on the thiazolidinedione scaffold were reported in 2005. X-ray crystallographic studies revealed that they both bind to the ATP-binding pocket, and the thiazolidinedione nitrogen interacts, via a salt bridge, with the side chain of Lys833 and the quinoxalone nitrogen or 1,3-benzodioxole oxygen atoms forming hydrogen bonding interactions with Val882 [54]. Compounds AS-604850 and AS-605240 inhibited p110γ with more than 30-fold selectivity over p110β and p110δ. AS-604850 was more selective for p110γ over p110α than AS-605240 (18-fold and 7.5-fold, respectively); however, AS-605240 was vastly more potent (IC_{50} of 9 nM) than AS-604850 (IC_{50} of 10 μM) in vivo due to its high cell permeability [54]. The related compound PIK-124 (**22**, Warner Lambert) was also selective for p110γ over p110β and p110δ; however, it is also twice as selective for p110α over p110γ [43].

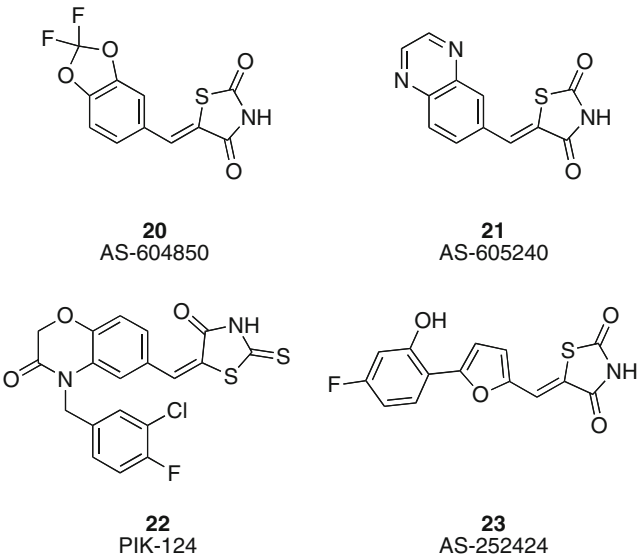


Fig. 10 Structures of inhibitors based on the thiazolidinedione scaffold (**20**–**23**)

AS-605240 and AS-604850 have proven particularly useful for probing p110γ function. In mouse macrophages, both compounds inhibited PKB phosphorylation when stimulated with C5a and chemokine MCP, cytokines that act through GPCRs [54]. In contrast, the compounds had no effect upon stimulation in the presence of a ligand that activates PI3-K by activation of RTKs. Compound AS-605240 was successfully used to block the progression of joint damage and inflammation in two different mouse models of rheumatoid arthritis [54].

Compound AS-604850 was subsequently used in conjunction with IC87114 to show that p110δ and not p110γ is the PI3-K isoform primarily responsible for the activation of signalling components downstream of B cell antigen receptors [52]. This evidence supported earlier genetic studies that indicated a role for p110δ in B and T cell activation [55], illustrating the value of isoform-selective PI3-K inhibitors for such investigations. The thiazolidinedione structure has been further modified, replacing the quinoxaline ring in AS-605240 with an aryl-substituted furan to yield AS-252424 (**23**), which had more than 20-fold selectivity for p110γ over p110α [56].

2,3-Disubstituted pyrazines and derived compounds

Several PKB inhibitors based on the 2,3-disubstitued pyrazine scaffold and known as the "Aktis" have been discovered by Merck Research Laboratories from a high-throughput screen for PKB activity (Fig. 11) [57, 58]. Characterisation of Akti-1/2a (**24**) indicated that it acted as an allosteric inhibitor, was not competitive with ATP. Akti-1/2a was eightfold selective for PKBα over PKBβ in a

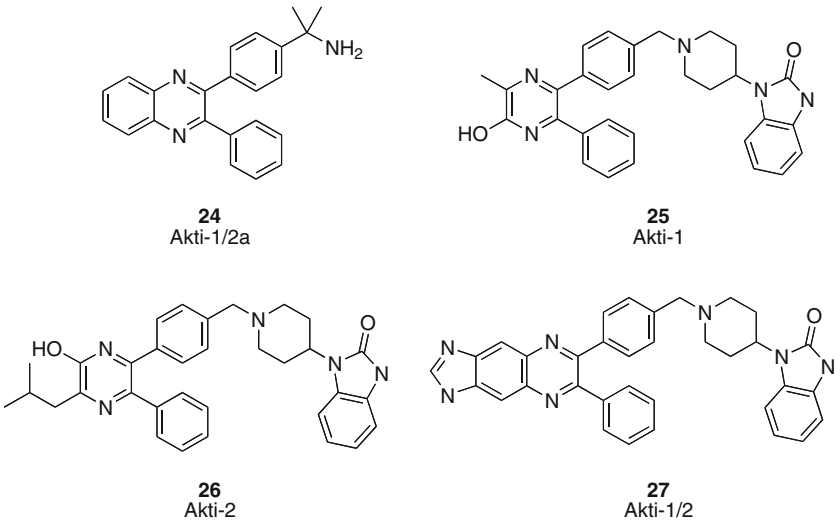


Fig. 11 Structures of the "Akti" inhibitors based on the 2,3-disubstitued pyrazine scaffold (**24**–**27**)

purified enzyme assay and twofold selective for PKBα over PKBβ in C33a cervical carcinoma cells. Further exploration around this scaffold led to compounds Akti-1 (**25**), Akti-2 (**26**) and Akti-1/2 (**27**). Akti-1 was selective for PKBα (31-fold over PKBβ), whereas Akti-2 was selective for PKBβ (58-fold over PKBα) in the purified enzyme assay; however, this selectivity was less pronounced in C33a cells. Akti-1/2 inhibited both PKBα and PKBβ, although showed low selectivity for the former. None of the compounds strongly inhibit PKBγ and were non-inhibitory towards a panel of related kinases [58].

Although binding of the Aktis was PH-domain-dependent, studies with tritium-labelled analogues indicated that they did not bind to the isolated PH domain but require intact PKB, thus suggesting that the Aktis bind to multiple domains. In order to demonstrate the therapeutic potential of small-molecule PKB inhibitors, the Aktis were used to demonstrate the induction of TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in LNCaP cells using caspase-3 induction as a readout [58]. The authors found that dual inhibition of PKBα and PKBβ was as effective at inducing apoptosis as treatment with LY294002; however, inhibition of PKBα or PKBβ alone was far less effective. Furthermore, overexpression of PKBγ was unable to rescue the LNCaP/Akt3 cells from caspase-3 activation upon treatment with Akti-1/2. In three out of four cell lines, co-treatment with Akti-1/2 was found to be more effective than treatment with rapamycin alone in inducing caspase-3 activity, illustrating the influence of signalling components downstream of PKB in inducing apoptosis.

The Aktis have also been used to demonstrate that PKB directly phosphorylates the S-phase-cyclin-dependent kinase CDK2 in vivo [59]. Epidermal-growth-factor-induced phosphorylation of CDK2 was abolished upon pretreatment with an Akti compound, yet CDK2 phosphorylation was maintained upon pretreatment with rapamycin.

Since the publication of the Aktis, Merck have published several reports of compounds with improved pharmacological properties (Fig. 12). The pyridopyrimidine compound **28** caused a threefold induction of caspase-3 activity at 0.1 μM in LnCap cells treated in combination with TRAIL [60]. By contrast, 2 μM of Akti-1/2 is required to cause a twofold increase in activity. Further derivatisation led to the

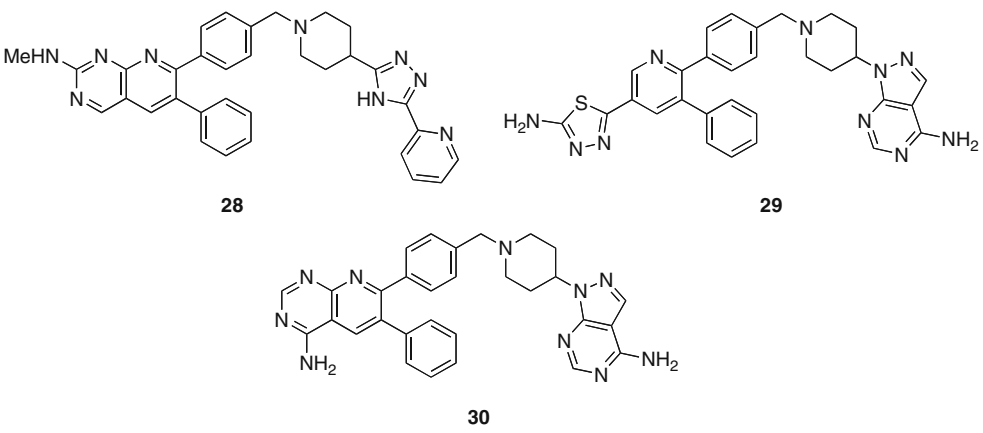


Fig. 12 Structures of inhibitors derived from the "Akti" compounds (**28**–**30**)

2,3,5-trisubstituted pyridine compound **29**, which induced an approximately sixfold increase in caspase-3 activity at 2.0 μM [61]. A related set of potent inhibitors such as the 2-unsubstituted pyridopyrimidine compound **30** have also recently been reported [62].

Deconvoluting the cell signalling pathway: future perspectives

Over the past 15 years, the use of small molecules has shed much light on the complexities of the PI3-K-PKB-mTOR signalling pathway, yet many important questions remain to be answered. The development of kinase inhibitors with high selectivity is an acutely challenging endeavour and has been the focus of extensive efforts within the academic and industrial research communities.

Due to the resource intensiveness of developing effective kinase inhibitors and their therapeutic potential, many of the compounds available for cell signalling research today are those that have been developed by the pharmaceutical companies. One particular manifestation of this is that existing compounds are all inhibitors of a small number of well-defined upstream target proteins, in particular PI3-K. While the focus on inhibition of well-defined target proteins is rational for drug discovery, there remains considerable scope for the development of small-molecule modulators of other components of the pathway that would provide useful tools for researchers exploring PI3-K-PKB-mTOR cell signalling.

If developing small-molecule kinase modulators is so resource-intensive, why continue to do so, especially given the availability of alternative techniques such as genetic knockout and knockdown and RNAi strategies? We believe that rather than taking an either/or approach, these techniques should be seen as complementary to each other. It is, however, vital that chemical biologists are aware of the advantages, disadvantages and limitations when choosing an experimental approach. In particular, it is worth highlighting that the use of RNAi and small molecules may result in a different phenotype being observed in some cases [63]. This effect is as a result of the disruption of protein–protein interactions caused by knockdown. For example, knockdown of the p110β isoform of PI3-K leads to growth arrest; small-molecule inhibition with PI-103 does not [63]. Since small molecules are the "gold standard" for the treatment of disease, they currently represent the most suitable agents for verifying the viability of a potential drug target.

A further advantage of small molecules as research tools is their versatility. Functionalities such as fluorescent labels and cross-linking dyes can be attached to an active compound to generate bespoke tools and probes for biological experimentation. It is our view that unravelling the complexities of the PI3-K-PKB-mTOR signalling pathway will require a range of experimental approaches, although small molecules will continue to be vital tools.

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