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# Advances in the Development of Class I Phosphoinositide 3-Kinase (PI3K) Inhibitors

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**Abstract:** The PI3K signaling cascade is the key moderator of cell proliferation, survival, motility, and apoptosis. Class I PI3K proteins are well characterized and linked to thrombosis (PI3Kβ), rheumatoid arthritis (PI3Kδ), and cancer (PI3Kα). In this review, we explore the latest progress in the design and development of selective Class I PI3K inhibitors from the perspective of drug design and structure activity relationships.

**Keywords:** Class I PI3Ks, p100α, anticancer, drug design, mutation, LY294002, GDC-0941, NVP-BEZ235, PI3Kγ, KRAS, BRAF, EGFR, MEK, PI3K/AKT, GSK2118436, selectivity, and mTOR.

## 1. INTRODUCTION

Phosphoinositide 3-kinase (PI3Ks) enzymes are lipid kinases that phosphorylate the 3-OH group of the inositol ring, generating phosphatidylinositol 3,4,5 triphosphates ($PIP_3$), which regulate the function and localization of downstream effectors and adaptor proteins. $PIP_3$ functions as a docking site for lipid binding proteins and protein domains such as pleckstrin homology (PH), phox homology (PX), and FYVE. The PI3K signaling pathway regulates many cellular processes including cell proliferation, survival, motility, and apoptosis. PI3Ks are negatively regulated by phosophoinositide phosphatases such as phosphatase and tensin homolog (PTEN), which dephosphorylate the 3-phosphate group of $PIP_3$ [1].

There are three classes (I, II, and III) of PI3Ks, based on their substrate preference and primary structure. Class I PI3Ks are heterodimeric lipid kinases which are found in cytosol. The role of class II PI3ks (PI3K-C2) is still not well understood, but PI3K-C2α is present in pancreatic β cells, and protein expression of PI3K-C2α has been found in human insulin-producing cell lines. As opposed to the $PIP_3$ produced by Class I PI3Ks, PI3K-C2 generates a different lipid product *in vivo*, namely phosphatidylinositol-3-phosphate (PtdIns3P, PIP), therefore activating a distinct signaling pathway [2]. Reduction of PI3K-C2α mRNA levels has been observed in impaired pancreatic β cells in type 2 diabetes, leading to progressive deterioration of glycemic control and the onset of type 2 diabetes [3]. The depletion of PI3K-C2α protein levels significantly suppressed insulin- or serum-induced stimulation of ERK, leading to increased apoptotic cell death [4]. Class III PI3Ks (also known as Vps34, the yeast vacuolar protein sorting gene Vps34p**)** are heterodimers consisting of a regulatory subunit (p150) and a catalytic subunit (Vps34). Vps34 is encoded by the PIK3C3 gene and synthesizes PtdIns3P (PIP) in internal membrane compartments of the endosomal/lysosomal system. CD18-dependent activation of NADPH oxidase was found to be dependent on Vps34 and is conserved in all eukaryotes. CD18-dependent ROS (reactive oxygen species) production in response to both *S aureus* and *E coli*, in both human and mouse neutrophils, was dependent on Vps34 [5]. Blockade of PtdIns3P (PIP) synthesis by reducing Vps34 protein levels could therefore reduce phagosomal ROS response.

Class I PI3Ks are split into class IA and IB, each activated by tyrosine kinases and G-protein coupled receptors, respectively. The kinase-activated PI3K 1A family contains three isoforms: p110α, β, and δ. Each of the three isoforms are encoded by their respective genes PIK3CA, PIK3CB, and PIK3CD and denoted as PI3Kα, β, and δ. p110α and β are found in tissue, while p110δ in leukocytes. PI3K 1B only contains p110γ, encoded by PIK3CG and denoted as PI3Kγ. PI3Ks isoforms have different functions in specific cell types. PI3Kβ plays a significant role in ADP-induced platelet aggregation during thrombosis. It mediates ERK phosphorylation, $TXA_2$ generation, and platelet aggregation by $G_i$ and $G_z$- pathways [6]. PI3Kδ participates in early signaling events of leukocytes in response to stimuli. PI3Kδ is important in the development, activation and migration of T and B cells as well as NK cells. Rheumatoid arthritis is an autoimmune disease involving T and B cells. Neutrophils respond to inflammatory mediators with a transient oxidative burst. In the event of TNFα-primed neutrophils activation, it takes

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a few seconds for PI3Kγ to generate $PIP_3$, a few minutes for PI3Kδ to supply $PIP_3$. This later stage release of $PIP_3$ by PI3Kδ appears to be critical for the respiratory burst of neutrophils [7]. Therefore, inhibition of PI3Kδ and PI3Kγ activity may have therapeutic potential for the treatment of inflammatory diseases such as asthma, rhinitis, and rheumatoid arthritis.

Among different PI3K subfamily proteins, the one most important in cancer is PI3Kα, which is our current focus. The p110α contains five domains: an N-terminal adaptor (p85) binding domain (ABD, residues 31-108), a Ras binding domain (RBD, residues 173-292), C2 domain (residues 325-484) which binds to cellular membranes, a helical domain (accessory domain, residues 525-696), and a kinase catalytic domain (residues 699-1,064, containing a catalytic loop spanning from residues 912-920, and an activation loop with residues 933-957). Activation of p110α takes place while it is complexed its regulatory protein p85. Huang *et al.* reported the first apo structure of PI3Kα [8]. The ATP binding site, located in the kinase domain, and is highly conserved within class I PI3Ks isoforms. The sequence identity and homology between PI3Kα and γ are 35% and 54%, respectively.

PI3K signaling controls several cellular cascades affecting functions such as cell proliferation, survival and growth, and is regulated by endogenous PTEN. Disorders in the PI3K pathway have been found in different human cancers. For instance, hyperactivation of PI3Ks leads to tumor progression and impairments in this pathway contribute to type II diabetes. Activated Ras protein and/or activated epidermal growth factor receptors (EGFRs) phosphorylate the p110α catalytic domain, which in turn activates the downstream AKT. Phosphorylated AKT subsequently turns on the functions of downstream proteins such as MDM2, NF-κB, BAD, FOXO, mTOR, and GSK3β, leading to cell growth, proliferation, and apoptosis (Fig. **1**).

The gene encoding the catalytic subunit of PI3Kα is frequently mutated in human cancers. These mutations are clustered in exon 9 (E542K and E545K in the helical domain) and in exon 20 (H1047R in the kinase domain) [9]. Zhao and Vogt studied the effect of specific mutations and found that these three "hot spots" mutations enhanced *in vitro* kinase activity. The negatively charged glutamic acids (542 and 545) interact with the basic residues of the N- terminus SH2 domain of p85. Mutations in the helical domain (E542K, E545K, and Q546K) change the -1 charged Glu to +1 charged Lys. The increased positive charges disrupt the interaction between the helical subunit and the SH2 domain of p85, which in turn affects the PI3Kα activity [10].

In the wild-type PI3Kα, histidine 1047 forms a hydrogen bond with the main-chain carbonyl of leucine 956 in the activation loop. The structure of mutant H1047R PI3Kα/wortmannin complex (PDB code: 3HHM) shows that the H1047R mutant reorients Arg1047 away from Leu956, losing the ability to stabilize the activation loop via Leu956. However, this reorientation of positively charged Arg1047 towards negatively charged head groups of membrane phospholipids enhances the interactions between PI3Kα and cell membrane. In addition, the interaction of Arg1047 with the cell membrane makes the disordered C-terminal residues 1050-1062 (in the wt) become an ordered loop, strengthening the PI3Kα/membrane interaction, which is vital for this enzyme's lipid kinase activity [11]. The nSH2 domain of the regulatory protein p85 interacts with helical domain of p110α. The interface between these two interacting domains is governed by electrostatic interactions: the negatively charged residues Glu542 and Glu545 directly form salt bridges with Arg358 and Lys379 of nSH2, respectively. This interaction inhibits PI3Kα kinase activity. Mutations of E542K and E545K repelled the same positive charged nSH2 domain, thereby blocking the inhibitory nSH2 interactions and subsequently increasing the lipid kinase activity [11].

A crystal structure of p110α and the iSH2 domain of p85α shows that Lys948 in the p110α activation loop forms a salt bridge with Glu342 in the nSH2 domain of p85α, also forming a H-bond interaction with Gln591 (iSH2 domain,

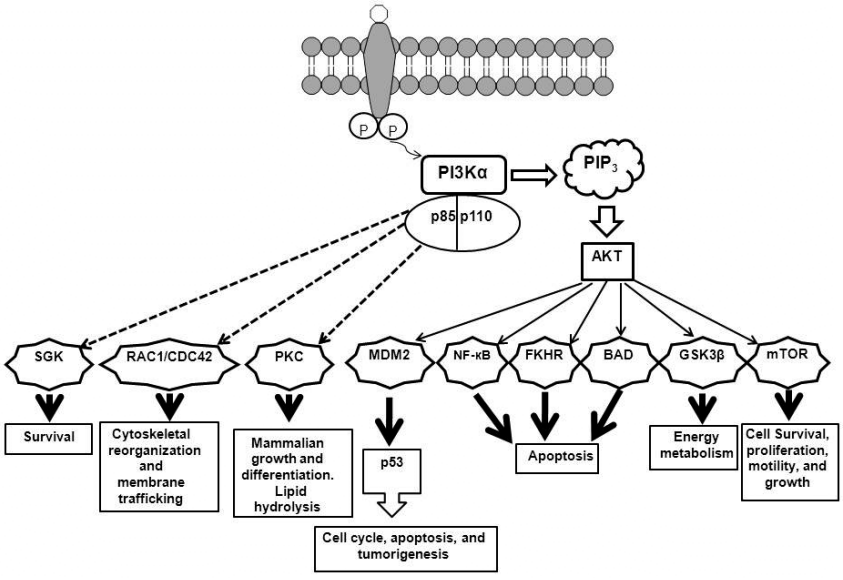



**Fig. (1)**. A simplified schematic representation of PI3Kα signaling pathway.

p85α), and thus maintaining the p110α activation loop in the inactive conformation, exerting an inhibitory effect of nSH2 domain. Another helix in the iSH2 domain (p53 residues 587-598) forms inhibitory interactions with residues Phe945 and Lys948 on the activation loop of p110α [12]. Deletion (Δ583-602) on the iSH2 domain of p85α proved to be oncogenic [13].

The H1047R mutation not only enhances lipid kinase activity of PI3Kα, it also provides a basis for selective inhibitor design. The corresponding position of H1047R in wt PI3Kγ is Arg1076. These two arginine residues are oriented 180° from each other and the Arg1076 in PI3Kγ is in a helix while Arg1047 of PI3Kα occurs in a loop structure. The residues in the ATP binding pockets of p110α and p110γ are highly conserved and these two binding pockets share similar three-dimensional structures. This is why many PI3Kα inhibitors bind to PI3Kγ as well. These two enzymes, as discussed earlier, have different functions and it is desirable for anticancer ligands to preferentially block PI3Kα. Inspection of differences in the crystal structures between these two proteins reveals that the loop between residues 771-779 (IMSSAKRPL) in PI3Kα is quite different from that of PI3Kγ (with residues 803-811, VMASKKKPL) [14]. Residue Ser774 appears to be important in isoform-specific inhibitor design, in that Ser774 in wt PI3Kα serves as a H-bond donor; whereas in the H1047R mutant, this residue can be both an H-bond donor and acceptor, while in PI3Kγ, the electrostatic map indicates that the corresponding residue Ser806 of PI3Kγ (equivalent to Ser774 of PI3Kα) is positioned as an H-bond acceptor, as suggested in our previous computational docking studies [15]. These structural differences should provide a foundation for selective inhibitor design targeting PI3Kα or PI3Kγ.

Obviously not all mutations in the kinase domain are able to induce a gain-of-function in PI3Kα. Mutations at Asp915 (Asp915Ala, D915A), and double mutations of D911A/F934A abolished almost all of the phosphoinositide phosphorylating activity. Mutants E970A and R922A led to a decrease of wt PI3Kα activity at 10% and 25%, respectively [16].

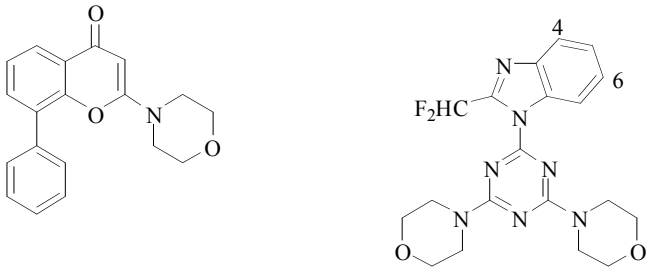
To study the role of active site residues in ligand binding and to elucidate the basis of selectivity, we have carried molecular dynamics simulations and computational alanine scanning on wild-type PI3Kα and H1047R mutant as well as PI3Kγ. Our data showed that the direct H-bond between Gln859 and wortmannin was quite unstable for both wild-type and H1047R mutant; yet the H-bond between Gln859 and Thr856 was maintained at 2.2 Å in the H1047R mutant. The corresponding distance for Gln959 to Thr856 was 3.1 Å. This distance suggests that residue Gln859 of PI3Kα, considered by many as an important residue for ligand binding, may actually only provide indirect structural support, by providing a stable hydrogen-bond network with Thr856 in the H1047R mutant. Similarly, residue Ser774 provided a stable H-bond network with wortmannin during MD simulation in the H1047R mutant model, yet such an H-bond was unstable in the wild-type model. Therefore, it seems reasonable that Gln859 and Ser774 might be used as a basis for the design of H0147R mutant specific inhibitors [17].

The importance of the PI3K signaling pathway in cancer development and prognosis has elicited a variety of reviews, focusing either on the inhibitors of PI3K alone [18-19] and/or its downstream effectors AKT [20-21] and/or mTOR [22-24]. We previously reviewed the updates of PI3Kα and mTOR selective inhibitors in clinical trials and discussed the approved drugs in cancer therapy targeting EGFR/PI3K/mTOR pathway [24]. In this review, we will mainly focus on PI3K pathway inhibitors developed in the past three years.

## 2. PAN-PI3K INHIBITORS

In our 2001 review paper [24] we discussed the pan-PI3K inhibitors such as wortmannin and staurosporine**,** myricetin, quercetin, LY294002 (**1**), and ZSTK474 (**2**). LY294002 (**1**) is a synthetic PI3Ks inhibitor which binds to the ATP binding site. It induces apoptosis and cell growth arrest *in vivo* and *in vitro* and it is frequently used as an investigational reference compound [25].

ZSTK474 (**2**) is another pan-PI3K inhibitor sharing the morpholine ring unit of LY294002. The growth inhibitory activity of ZSTK474, however is approximately 10-fold stronger than that of LY294002 [26]. It is reported that ZSTK474 induced tumor suppression in human breast cancer MCF-7 cells by arresting cells in G1 phase of the cell cycle without inducing apoptosis. Surprisingly, such an increase in G1 arrest accompanying tumor suppression was observed in a three-month long administration of ZSTK474 [27]. Structure and activity relationship (SAR) studies for ZSTK474 identified the 4 and 6 positions of the benzimidazole ring (Fig. **2**) as key points for improved inhibition. The 6-amino-4-methoxy analogue of ZSTK474 showed a strong inhibition against PI3Kα, -β, and -δ isoforms as well as in H1047R and E545K PI3Kα mutants. *In vivo*, it led to a dramatic reduction of cancer growth in U87MG human glioblastoma tumor xenograft model [28].




LY294002 (**1**) ZSTK474 (**2**)

**Fig. (2)**.The chemical structures of pan-PI3K inhibitors LY294002 and ZSTK474.

## 3. SELECTIVE PI3K INHIBITORS

A number of diversified structural scaffolds have been designed and synthesized using structure-and/or ligand-based drug design (SBDD and/or LBDD) strategies. Many of these PI3K inhibitors contain a morpholine core. Thus, we classify these PI3Ks selective inhibitors into two groups: morpholino- and non morpholino-based heterocyclic PI3Ks inhibitors.

### 3.1. Morpholino-Based Heterocyclic PI3K Inhibitors

The morpholine ring moiety in LY294002 (**1**) is essential for its activity against PI3Kα binding. The substitution of the O atom in the morpholine with S, $CH_2$, NH, or CHOH greatly decreased the affinity for PI3Kα binding [29]. This suggests that the morpholine ring is important for PI3K binding. Our docking studies of 33 PI3K inhibitors to PI3Kα also confirmed the role of this morpholine ring, in that it forms H-bonds with residues Val851 of PI3Kα, an essential interaction for PI3K binding [15]. This important morpholine ring in LY294002 has been found in a number of PI3K inhibitors including GDC-0941, GNE-493, GNE-477, PI-103, PKI-402, PKI-587, PKI-179, WJD-008, and NVP-BKM20. Readers should refer to our 2001 review paper [24] for structures and development of the above morpholino-based PI3Kα inhibitors.

CH5132799 (**3,** Fig. **3**) was successfully identified as a potent class I PI3K inhibitor using a SBDD approach. CH5132799 is metabolically stable and thus orally bioavailable. CH5132799 exhibited a strong inhibitory activity against PI3Kα with an $IC_{50}$ value of 0.014 μM and showed potent antiproliferative activity in human cancer xenotransplant models in mice [30].

The crystal structure of a PI-103/PI3Kα complex showed that functionalization at the 5' position of the phenol ring generated compound 4 with enhanced potencies against native and mutant M772A, D810A, and Y836A PI3Kα. Substitution near the phenol moiety induced a conformational change for Lys802, resulting in an additional space in the binding site due to the flexibility of Lys802. Attaching OH at 5' formed an H-bond with Lys802 and inhibited PI3Kα with an $IC_{50}$ value of 5.9 nM [31].

Replacement of the fused ring core structure in GDC-0941 while retaining the important morpholino ring yielded a new series of 8-morpholinyl-imidazo [1,2-*a*]pyrazines. This series is exemplified by compound **5**, a potent and selective inhibitory against PI3K-α ($IC_{50}$: 95 nM, $K_{iapp}$, 181 nM), -δ ($K_{iapp}$, 29 nM), -β ($K_{iapp}$, >500 nM), -γ ($K_{iapp}$, >500 nM), and mTOR ($IC_{50}$, > 10 μM), along with inhibition of AKT phosphorylation in human osteosarcoma (U2OS) cell line (the $IC_{50}$ for inhibiting the formation of pAKT was 93 nM) [32].

The selectivity of PI3Kα over PI3Kγ, or PI3Kβ over PI3Kγ appears to be easier to achieve than the selectivity within the same class IA family of PI3K, i.e., PI3Kα, β, and δ. For instance, TGX-115 (**6**) was tested against an array of lipid and protein kinases with $IC_{50s}$ of 0.13, 61, 0.63, ~100 μM against PI3K-β, –α, -δ, -γ, respectively [33]. The *R*-enantiomer of TGX-221 (**7**) is a more potent and selective PI3Kβ inhibitor than the *S*-stereoisomer, with an $IC_{50}$ value of 0.006 *vs* 0.8 μM, respectively. Modification of compound **7** using a computational docking approach and SAR study afforded compound **8** with $IC_{50s}$ of 0.001, 2.0, 0.008, 1.0 μM against PI3K-β, –α, -δ, -γ, respectively. Compound (**8**) and its structural analogues exhibited potent growth inhibition in a PTEN-deficient breast cancer cell line (MDA-MB-468) [34].

AZD6482 (**9**) is the first antithrombotic PI3Kβ inhibitor tested in humans. Similar to TGX-221 (**7**), AZD6482 is also in R configuration. A randomized, double-blind, placebo-controlled, single-dose clinical study in 40 male volunteers (18-36 years) showed that at the dose inducing a full antithrombotic effect (i.e., at the 'therapeutic' plasma concentration, 1 μM), AZD6482 did not increase bleeding time or blood loss. The plasma exposure bleeding time increased 1.6 fold when 5-6 times the therapeutic dose was used. The $IC_{50}$ values of compound **9** for inhibition of PI3K-β, –α, -δ, and -γ isoforms were 0.01, 0.87, 0.08, and 1.09 μM, respectively, corresponding to selectivity ratio (vs. PI3Kβ) of 1, 87, 8, and 109, respectively [35]. Whereas AZD6482 was well tolerated in humans, it caused a dose-dependent increase in plasma insulin; such an adverse effect could be due to its inhibition of the PI3Kα subtype ($IC_{50}$ = 0.87 μM). To improve the pharmacokinetic profile and selectivity of AZD6482, the carboxylic acid moiety was removed and the N-(2-hydroxymethyl)-N-methyl-amide moiety was introduced, which resulted in compound **10**. The $IC_{50}$ values of compound **10** against PI3K-α and –β were 2.2 and 0.058 μM, respectively, representing a 38-fold of selectivity ratio over PI3Kα. Compound **10** showed a potent antithrombotic effect *in vivo* after oral administration with minimum risk for bleeding and insulin resistance [36].

Compound 11 was identified through SBDD and SAR approaches, with an impressive potency ($IC_{50}$ of 3.8 nM) against PI3Kδ. Compound 11 was proposed to bind to the specificity pocket of PI3Kδ consisted of a tryptophan (Trp812, gamma numbering) and a methionine (Met804). This compound has selectivity of 200-400 fold against PI3Kδ over –α, -β, and -γ with fold selectivity of 340, 200, 410 for α/δ, β/δ, γ/δ, respectively [37]. The same team from Genentech replaced the core structure in compound 11 with a benzimidazole scaffold while retaining the indole ring which was important for binding to the conserved affinity pocket, yielding compound 12, with $IC_{50}$ against PI3Kδ of 1.8 nM. The fold selectivity values of 12 α/δ, β/δ, γ/δ were 129, 104, and 1444, respectively [38].

A novel series of pyrimidone anilides was discovered by a medicinal chemistry team at Sanofi as exemplified by compound (**13**). The $IC_{50}$ values of **13** against PI3K-β, –α, -δ, and -γ isoforms were 42, > 10,000, 2210, 8280, respectively, showing strong PI3Kβ isoform selectivity [39]. Continuing efforts using fragment-based scaffold replacement as a tactic generated a novel series of 2-morpholinothiazole-based PI3Kβ selective inhibitors exemplified by **14,** with $IC_{50}$ values against PI3K-β, –α, -δ, and -γ isoforms of 10, 395, 63, and 4350 nM, respectively. Compound **14** also suppressed the formation of pAKT with an $IC_{50}$ of 140 nM. This series of compounds was proposed to interact with Lys805, Asp813, Asp937, Val854, Met779, and Trp787 of a PI3Kβ homology model [40].

The design and biological tests of compounds 3-14 have demonstrated that selectivity within the same PI3K 1A family is difficult to achieve. In contrast, selectivity between class IA and IB PI3Ks is relatively easy to obtain. Structural comparison of all four isoforms (α, β, δ, γ) of class I PI3K lipid kinases showed that the ATP-binding site and affinity pocket are highly conserved among these four proteins. Therefore, ligand selectivity is hard to achieve. However it is not impossible, because several key residues are different among these four protein isoforms. For example, residue

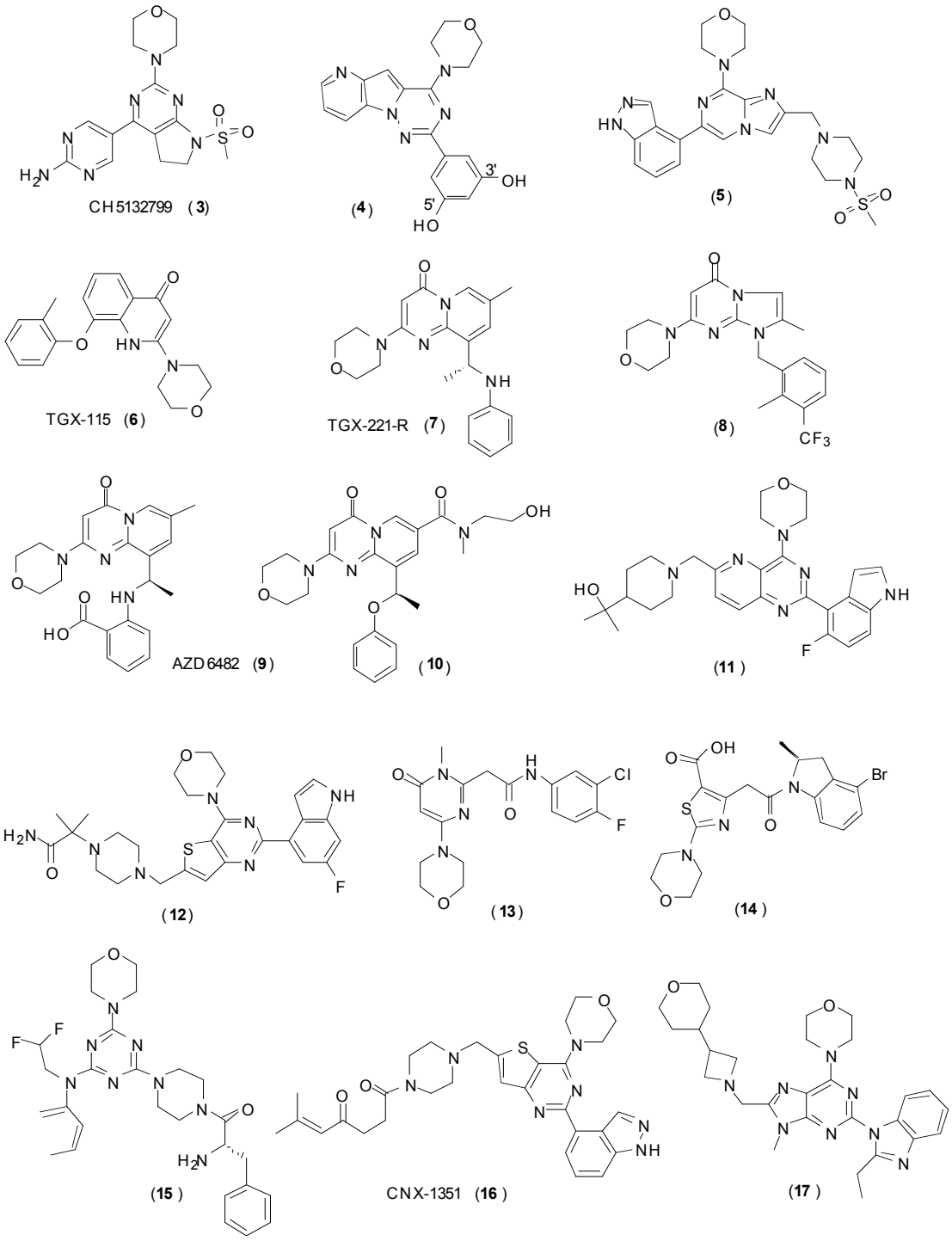



**Fig. (3)**.The chemical structures of morpholino-based heterocyclic PI3K inhibitors.

Asp862 of PI3Kβ (β numbering) can be used to develop isoform-selective inhibitors, in that this position features Gln, Lys, and Asn for PI3K-α, -γ, and –δ, respectively. Introduction of a phenylalanine unit yielded compound 15. The morpholino oxygen forms H-bonds with Val854 and the amino moiety of the phenylalanine substituent interacts with Asp862, while the aromatic ring of 15 forms aromatic π-π stack interactions with Trp787 of PI3Kβ. The $IC_{50}$s of 15 against PI3K-β, –α, -δ, and -γ were 63, 4700, 2200 nM, and >100μM, respectively, representing selectivity ratios of PI3Kβ over the –α, -δ, and -γ isoforms of 74, 35, and more than 1587 fold [41].

Similarly, examination of the ATP binding site and nearby residues identified residue Cys862 of PI3Kα (α numbering) as isoform-specific, in that the corresponding residues in the –β, -δ, and –γ isoforms are Leu, Leu, and Gln, respectively. Taking advantage of the nucleophilicity of cysteine at PI3Kα position 862, a compound named CNX-1351 (16) was designed and found to form a covalent bond with

Cys862. Due to the uniqueness of Cys862 in PI3Kα, this inhibitor is very selective. The apparent $IC_{50}$s ($IC_{50}$s_App) of 16 against PI3K-α, -β, -δ, -γ, and PI4Kα, were 6.9, 166.0, 3020.0, 240.3 nM, and > 1 μM, respectively. The impressive design of compound 16 is that it targeted a residue unique to PI3Kα (Cys862), rather than a conserved lysine residue as observed in the wortmannin covalent binding interaction. Such a covalent bond to a conserved residue resulted the lack of selectivity of wortmannin, a natural product inhibiting all PI3K family proteins [42].

Another region that can be used for isoform selective design is that spanning Typ760, Thr750 and Met752 of PI3Kδ loop (δ numbering). The reason that Thr750 can be employed for selective binding is that the residues corresponding to Thr750 of PI3Kδ are arginine in PI3Kα and lysine in both PI3Kβ and PI3Kγ. The smaller threonine creates a larger hydrophobic pocket for ligands selectively binding to PI3Kδ. This Thr750 is critical for PI3Kδ binding in that, along with Trp760, it forms a hydrophobic binding pocket.

Using a SBDD approach, a series of benzimidazoles (**17**) was designed to fit into the larger pocket of PI3Kδ. Compound **17** binds strongly to PI3Kδ with $IC_{50}$ against PI3Kδ of 2 nM, whereas the $IC_{50}$s for PI3Kα and PI3Kγ were 100 and 260 nM, respectively [43].

### 3.2. Non-Morpholino-Based PI3K Inhibitors

#### *3.2.1. Non-Morpholino-Based Heterocyclic PI3Kα Inhibitors*

Other than the morpholine ring, many different scaffolds have been introduced as PI3K inhibitors. NVP-BEZ235 (**18**) is a dual pan-PI3K/mTOR inhibitor (Fig. **4**). The imidazoquinoline core mimics the adenine moiety of ATP and thus is considered as an ATP competitive inhibitor.

NVP-BEZ235 showed an inhibitory activity against PI3Kα mutants (E545K and H1047R). A molecular modeling study suggested the formation of three H-bonds with the backbones of Val851 in PI3Kα (Val882 in γ), Asp933 in PI3Kα (Asp964 in γ), and Ser774 PI3Kα (Ser806 in γ) in the ATP-binding site of the kinase domain [15]. NVP-BEZ235 decreased the phosphorylation levels of mTOR activated kinase p70S6K. Five years of preclinical and clinical research on BEZ235 has provided evidence that NVP-BEZ235 and similar PI3K inhibitors as single-agent drugs might not

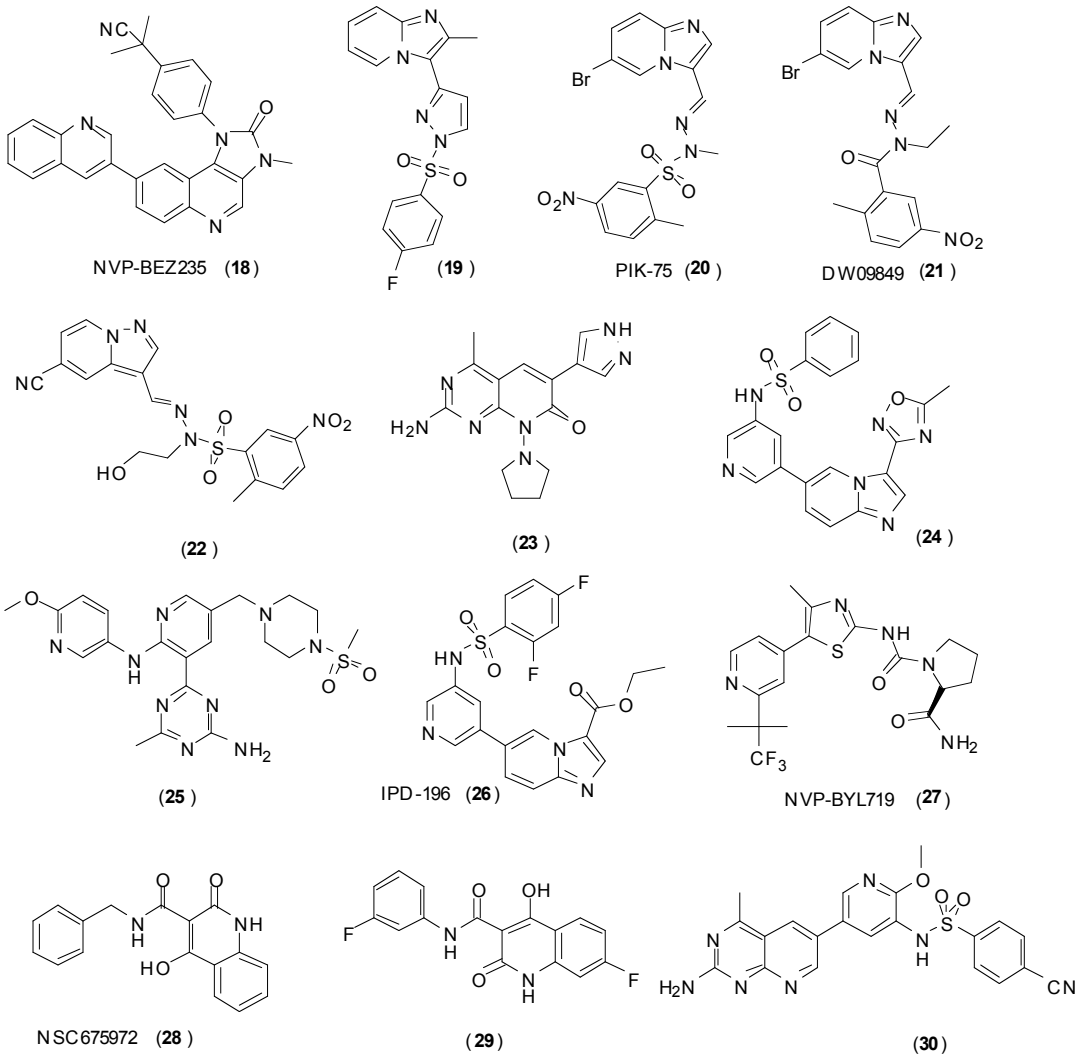



**Fig. (4)**. The chemical structures of non-morpholino-based heterocyclic PI3Kα inhibitors.

provide sufficient efficacy as anticancer drugs. A current focus is on how to identify the best combination for these PI3K inhibitors [44]. In combination with the vascular endothelial growth factor receptor (VEGFR) inhibitor RAF265, NVP-BEZ235 yielded synergistic inhibition in thyroid cancer cell lines with mutations in RAS, BRAF, PTEN, and RET. These therapies promise new approaches against medullary thyroid cancers [45].

The pyrazole-sulfone bond in the lead compound **19,** which was identified by HTS, was unstable in solution. Opening up the pyrazole ring and modification of the substituents led to a very potent compound, PIK-75 (**20**). Substitution of the -Br group on the imidazopyridine ring with –F, -Cl, $-CF_3$, $-CH_3$, $-COOC_2H_5$, and $-CONH_2$ groups reduced the PI3K inhibitory activities [46]. Compound **20** is highly selective, inhibiting PI3Kα, -β, and –γ with $IC_{50}$ values of 0.00030, 0.85, and 0.040 μM, respectively. The antiproliferative activity of **20** was evaluated in doxorubicin (ADR)-selected human breast cancer cells which were resistant to antitumor agents such as doxorubicin and paclitaxel, due to overexpression of P-glycoprotein. The *in vivo* activity of 20 was evaluated in a HeLa human cervical cancer transplant model in nude mice, which showed its anticancer effect. Compound 20 suppressed tumor growth by 62% when administered intraperitoneally at 50 mg/kg daily for two weeks without weight loss [46].

Structural modification of 20 (PIK-75) showed that removal of the bromo group decreased potency against PI3K subtypes. The interaction mode of this series in the PI3Kγ binding pocket was investigated. The imidazopyridine core moiety was deeply buried in the ATP binding pocket and interacted with Tyr867 of PI3Kγ. The bromine atom was inserted in a hydrophobic pocket. The nitrogen atom of the heterocyclic ring was hydrogen bonded to the backbone of Val882 of PI3Kγ. The less active compounds did not insert deeply in the active site, which affected their interaction with the binding residues [47].

In an effort to reduce the off-target effects of PIK-75 while retaining the subtype selectivity of **20**, the bromo-containing heterocyclic ring was preserved with replacement of the sulfonamide group with an amide moiety, leading to the discovery of DW09849 (**21**), a very potent PI3Kα inhibitor; it blocked the PI3K/protein kinase B cascade in rhabdomyosarcoma (RH30) cells and consequently induced the G1 arrest in cell cycle. In addition, it preferentially exhibited antiproliferative activity against H1047R mutant PI3Kα in human breast cancer cells [48].

Structure-activity relationship (SAR) studies of the pyrazolo[1,5-*a*] pyridines led to the discovery of **22**, a selective PI3Kα inhibitor [49] of PI3K-α, -β, and –δ with $IC_{50}$s of 1.3, 51, and 54 nM, respectively. Also, it inhibited cell proliferation and phosphorylation of AKT/PKB. *In vivo*, it exhibited inhibitory activity against H1047R HCT-116 human colon carcinoma cell lines in xenograft models. Also, it showed a good pharmacokinetic profile and water solubility *in vivo* [49-50].

By means of SBDD and physical properties-based optimization, a novel series of 4-methylpyrido pyrimidinones was designed and synthesized. The successful efforts culminated in the identification of **23** [51]. *In vivo* rat pharmacokinetic studies showed that compound **23** possessed suitable oral bioavailability. It exhibited potent inhibitory activity against PI3Kα ($K_i$: 12.5 nM) and its downstream effector mTOR ($K_i$: 10.6 nM) [51]. Through fragment-growing integration, a new series of imidazo-[1,2-*a*]pyridine derivatives was designed and synthesized. The successful efforts disclosed compound **24** as a potent PI3Kα inhibitor ($IC_{50}$: 2 nM) with 24% bioavailability. Compound **24** suppressed the HIF-1α accumulation and thus inhibited the migration of endothelial cells, an important event in angiogenesis. Not only inhibiting cell migration, the antiangiogenic effect of **24** was also exerted by inhibiting tubular network formation of the human umbilical vein endothelial cell (HUVEC) on the Matrigel beds in a dose-dependent manner. Molecular modeling using a PI3Kα homology model showed that **24** formed H-bonds with the protein via sulfonamide, pyridine nitrogen, and imidazole nitrogen atoms, indicating the importance of these three pharmacophoric points [52].

Smith *et al.* used a structure-based strategy to design and synthesize a novel scaffold targeting class I PI3Ks [53]. They successfully identified **25** as a potent class I PI3Ks inhibitor showing selectivity vs. mTOR proteins. The $K_i$ values of **25** for PI3K-α, -β, -γ, and –δ were 9, 5, 4, and 2 nM, respectively and the $IC_{50}$ for **25** against mTOR was 4800 nM. It inhibited the PI3K/AKT signaling cascade *in vivo* in a mouse model. It also showed a robust cancer inhibitory activity in a malignant glioma cell line (U-87 MG) in a xenograft model [53]. The preferred selectivity of **25** toward PI3K rather than mTOR protein is believed to be mediated via the triazin-2-amino which binds to Val882 (γ numbering, corresponding to Val851 of PI3Kα). The strong binding affinity comes from the additional binding of pyridine nitrogen to Asp841, Tyr867; and the sulfonyl group binds to Ala805 and Lys802 (γ numbering). The study suggested that residues Ala805, Lys802, Asp841, Tyr867, and Val882 of PI3Kγ are important for ligand binding. The corresponding residues for ligand binding to PI3Kα are Ser774, Ile771, Asp810, Tyr836, and Val851. Our docking studies on ligand binding to wild-type and mutant H1047R of PI3Kα have also confirmed the importance of residues Ser774, Tyr836, and Val851. We also predicted that Gln859 and Asp933 along with Ser774 are critical for mutant H1047R binding [15].

A combination of molecular docking and SAR studies identified IPD-196 (**26**) as a potent PI3Kα inhibitor with $K_d$ of 0.68 nM. It demonstrated a profound anticancer activity against human hepatocellular carcinoma cells. At 5 μM it abolished the formation of pAKT (Ser473) and p-mTOR; however, it did not suppress the levels of AKT and mTOR. Thus, compound **26** inhibited mTOR, induced apoptosis, inhibited angiogenesis, and arrested cell cycle. Molecular docking showed that, similar to compound **25**, **26** interacted with residues Lys802, Asp810, Tyr836, Val851, and Trp780 [54]. Residues Trp780 and Tyr836 were also predicted to be important for ligand binding to PI3Kα in our molecular dynamics simulation studies [55]. The 2-aminothiazole analogue NVP-BYL719 (**27**) was identified as a potent and isoform-selective PI3Kα inhibitor based on the molecular binding model. The $IC_{50}$ values of **27** for PI3K-α, -β, -γ, and –δ were 0.005, 1.2, 0.25, and 0.29 μM, respectively. The amide group of **27** formed H-bonds with Gln859 and Ser854, while

the hydrophobic pyridine end interacted with the affinity pocket consisted of residues Asp933, Pro778, Lys802, Ile848, Ile800, and Tyr836 [56].

Through pharmacophore modeling and biological testing, we have identified NSC765972 (**28**) as a novel PI3Kα inhibitor. Compound **28** inhibited the wild-type and H1047R mutant PI3Kα at $IC_{50}$s of 1.1 and 0.73 μM. It suppressed the formation of pAKT-Ser473 and induced apoptosis, as illustrated by the DNA fragmentation studies [57]. Removing the methylene group from compound **28** minimized the flexibility and led to compound **29,** with increased selectivity toward the PI3Kα H1047R mutant. Compound **29** was proposed to bind to PI3Kα with residues Gln859, Val851 and Glu849 [58]. Compound **30** was one of the latest PI3Kα-selective inhibitors. The $IC_{50}$s of **30** against PI3Kα and mTOR were 2.82 and 45.8 nM, respectively. Compound **30** interacted with residues Val882, Glu833, and Tyr867 (γ numbering) [59].

### *3.2.2. Non-Morpholino-Based Heterocyclic PI3Kγ Inhibitors*

In addition to anticancer potential, PI3K inhibitors have long been considered for the treatment of inflammatory and autoimmune disorders. However, the therapeutic benefit in treating inflammatory and autoimmune diseases is more specific to the inhibition of the γ isoform of PI3K. The observation that PIK3CG$^{-/-}$ mice are protected in mouse rheumatoid arthritis models validates PI3Kγ as a therapeutic target for inflammatory disorders (e.g., rheumatoid arthritis). Rheumatoid arthritis is a chronic systemic inflammatory disorder that affects mainly joints. Human rheumatoid arthritis is characterized by an increased level of neutrophils and mast cells in inflamed joints, inducing inflammatory response and damage. AS-605240 (**31,** Fig. **5**) was the first selective low molecular weight PI3Kγ inhibitor for rheumatoid arthritis treatment, with a $K_i$ of 0.0078 μM against PI3Kγ. Analyses of **31** binding to PI3Kγ showed that this compound bound to the ATP binding region, with the thiazolidinedione nitrogen forming an electrostatic interaction with Lys833, and the main chain peptidic bond NH of Val882 forming a H-bond with the nitrogen of the quinoxaline of **31** [60]. Treatment with the PI3Kγ inhibitor AS-605240 blocked chemoattractant-mediated protein-kinase B (PKB) phosphorylation in macrophages and primary monocytes, thus decreasing neutrophil recruitment and thereby reducing joint inflammation [60].

By means of SBDD and SAR approaches, a series of 7-substituted triazolopyridines was designed and synthesized, culminating in identification of compound **32** as a potent, selective, and orally bioavailable PI3Kγ inhibitor, with $pIC_{50}$ value of 7.9 vs 1.7 (-α), 1.3(-β), 1.3 (-δ) [61]. *In vivo*, it displayed efficacy in a collagen induced arthritis (CIA) model in mouse. This series of compounds were proposed to interact with PI3Kγ with residues Val882, Met953, Ile963, Asp964, and Tyr867 [61].

TASP0415914 (33) was identified as **a PI3K**γ inhibitor ($IC_{50}$=29 nM) through SAR investigation of a series of **2-amino-5**-oxadiazolyl thiazoles. It demonstrated *in vivo* efficacy in a collagen-induced arthritis (CIA) model in mice after oral administration [62]. The 6-aryl triazolopyridine scaffold was identified by chemoproteomic screening of a kinase library. An extension tactic accompanied with the SAR approach led to the discovery of two potent and selective PI3Kγ inhibitors (CZC19945, 34 and CZC24832, 35) [63]. Both compounds displayed a suitable pharmacokinetic profile and efficacy in a chronic inflammation model *in vivo*. Compound 34 showed $pIC_{50}$ values of 7.6 (PI3Kγ), 5.8 (-δ), 5.6 (-α), and 5.7 (pAKT), while the $pIC_{50}$ values for 35 were 7.6 (PI3Kγ), 5.1 (-δ), <5 (-α), and 5.9 (-β), showing more than 100-fold selectivity against PI3Kγ over the other three PI3K isoforms. Docking studies showed that the pyridine and sulfonamide groups of compound 34 interacted with Lys833, Asp964, and Tyr867 of PI3Kγ; while the triazole and amino group are in the affinity pocket consisting of residues Ile831, Ile881, Val882, Ile963, and Met953 [63].

The important sulfonamide group is also featured in compound **36**, a potent, selective, and orally bioavailable PI3Kγ inhibitor with an $IC_{50}$ value of 18 nM vs 435 (-α), 2059 (-β), and 690 (-δ). The crystal structure of **36**/PI3Kγ (PDB ID: 4ANU) showed that the difluorophenyl ring of **36** was present with equal population of two poses related by a

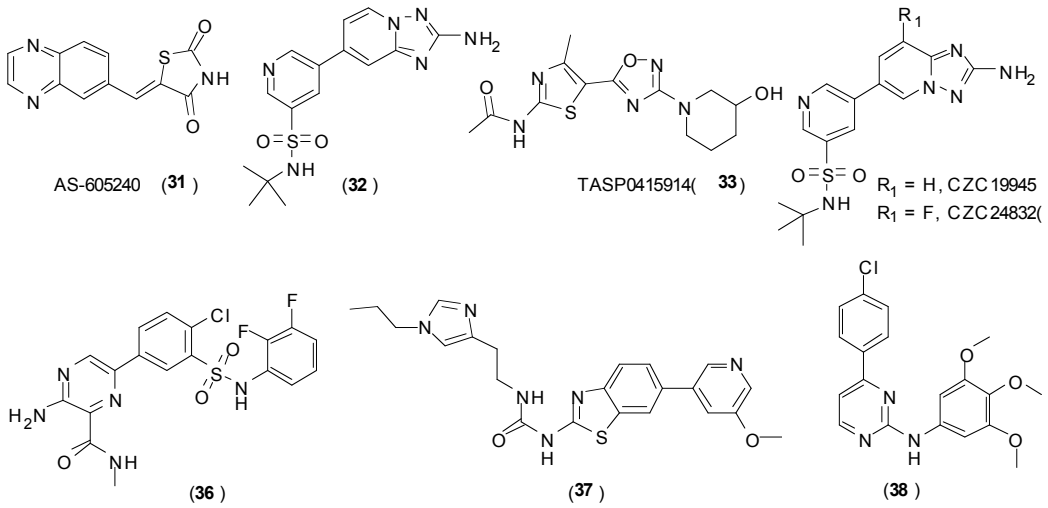



**Fig. (5)**.The chemical structures of non-morpholino-based heterocyclic PI3Kγ inhibitors.

180° flip on the phenyl plane. The PI3Kγ-selective ligands can be designed by taking advantage of residues unique to PI3Kγ such as Lys802 (Arg in –α, Lys in –β, and Thr in -δ), Ala805 of P-loop (Ser in –α, Asp in –β, and -δ), Lys890 (Glu in –α, Asp in –β, and Asn in -δ), Thr886 (His in –α, Glu in –β, and Asp in -δ), and Ala885 (Ser in all –α, –β, and -δ). Compound **36** showed impressive pharmacokinetic parameters such as bioavailability of 76.7% and a $t_{1/2}$ of 4.44 hour with no inhibition of CYP3A4, CYP2D6, and CYP2C19 [64].

In addition to residues Lys802, Ala805, Lys890, Thr886, and Ala885 that are unique to PI3Kγ, Collier *et al.* revealed residue Gly829 as another tool for PI3Kγ selective ligand design, in that this residue position is Glu in PI3Kα. X-ray crystallography demonstrated that benzothiazole analogue **37** occupies a hydrophobic binding cleft close to the ATP binding site of PI3Kγ (**37**/PI3Kγ: PDBID, 4PS3). Compound **37** is a potent and selective PI3Kγ inhibitor with high binding affinity toward PI3Kγ ($K_i$ = 0.002 μM) compared to that of –α isoform ($K_i$ = 0.132μM). Molecular modeling studies showed that there was no steric clash between the propyl group of **37** and Ala885 which may be attributed to its smaller size. However, the same position features a serine in PI3K-α, –β, and -δ is too large to accommodate the propyl group, thus contributing to very good selectivity of **37** toward PI3Kγ [65].

3D QSAR and molecular modeling studies were utilized to design and synthesize a series of *N*, 4-diphenylpyrimidin-2-amine derivatives (illustrated by **38**) as PI3Kγ selective ligands with an $IC_{50}$ of 65 nM. Compound **38** interacts with residues Ser806, Lys800, Lys807, Pro810, Ile831, Lys833, Ile879, Glu880, Val882, Asp950, and Asp964 of PI3Kγ. Quite a few residues interacting with **38** were different from those interacting with other ligands [66].

### *3.2.3. Non-Morpholino-Based Heterocyclic PI3Kδ and PI3Kβ Inhibitors*

In addition to PI3Kγ, the PI3Kδ isoform also plays a role in regulating inflammation. Impairment of PI3Kδ and PI3Kγ has been linked to immunodeficiency and inflammatory disorders. The PI3Kβ isoform, on the other hand, potentiates integrin-mediated platelet aggregation and arterial thrombosis. Hence, inhibition of PI3Kβ has the potential to be an antithrombotic agent. A pharmacophore modeling search generated some molecular templates as potential PI3Kβ inhibitors, and further optimization of these structural templates by exploiting conformational differences between PI3K isoforms led to the discovery of aminopyridine-based compound **39** (Fig. **6**), with a $K_d$ of 0.36 μM against PI3Kβ. The amino group of **39** forms an H-bond with Val854, and the sulfonyl oxygen form H-bonds with Lys805; while the aromatic phenyl group lies in the affinity pocket consisting of Asp962, Ser781, Asp923, and Lys782. Having a phenyl group on the sulfonamide favored PI3Kβ, whereas larger groups such as naphthyl enhanced PI3Kα selectivity, presumably due to differences in plasticity of the binding pocket between PI3Kα and PI3Kβ [67].

The involvement of PI3Kδ in inflammation is related to the role of PI3Kδ in B and T cell antigen receptor signaling and activation and neutrophil migration. The PI3Kδ inhibitor IC87114 (**40**) attenuated OVA-induced bronchiolar inflammation by reducing the levels of IL-4, IL-5, and IL-13 in the OVA-inflamed lungs. IC87114 blocked phosphorylation of AKT at Ser473 in lung tissue of a mouse asthma model [68]. At 2 μM concentration, compound **40** caused complete suppression of AKT phosphorylation. It also inhibited CXCL13-induced AKT phosphorylation in peritoneal B-2 and B-1 B cells, reducing chemoattractant-induced migration of splenic B-2, MZ, and B-1a B cells by 40-60%. The selectivity of **40** lies in its high affinity toward PI3Kδ ($IC_{50}$: 12 nM) with weak affinity toward other isoforms ($IC_{50}$s: 8 μM for –α, 940 nM for –β, and 800 nM for –γ isoform). It also reduced *in vivo* autoantibody responses. Thus, inhibiting PI3Kδ represents a novel approach for treating Ab-mediated autoimmune diseases [69]. Self-reactive Abs made by innate-like B cells have been associated with acute inflammatory responses. IC87114 treatment inhibited the activation of innate-like B cells, and thus reduced the production of Abs that cause acute inflammation [69]. IC87114 showed a potent inhibitory activity in systemic lupus erythematosus (SLE) in mouse and reduced the autoimmune hepatic and renal damage [70]. PI3Kδ-selective CAL-101 (Idelalisib, GS-1101) (**41**) showed promising preclinical activity in treating chronic lymphocytic leukemia (CLL), where increased PI3Kδ activity has been observed [71-73]. CAL-101 was also found to sensitize CLL cells toward cytotoxic agents such as bendamustine, fludarabine, and dexamethasone [74].

## 5. CONCLUSIONS AND FUTURE PERSPECTIVES

Class I PI3K is considered a therapeutic target for treatment of thrombosis, rheumatoid arthritis, and cancer. Selective targeting of a particular abnormal isoform, however, is still a challenge due to the similarity between structurally conserved binding regions. The advent of computer aided-drug design (CADD) software allows scientists to explore the protein binding site to identify and utilize unique residues for particular isoforms. Research from our group and others has shown: 1) that residues Lys802, Ala805, Gly829, Asp841, Tyr867, Val882, Thr886, Ala885, and Lys890 [75] of PI3Kγ might provide the γ-isoform specific binding for the treatment of inflammation in rheumatoid arthritis; 2) that residues Ser774, Asp810, Tyr836, Gln859, Val851, Glu849 and His855 [75] of PI3Kα might be used for the α-isoform specific binding; 3) that Gln859 and Asp933 along with Ser774 are critical for PI3Kα mutant H1047R binding; 4) that Typ760, Thr750 and Met752 of PI3Kδ may be exploited to design δ-isoform selective ligands, and 5) that Lys782, Ser781, Glu858, Asp862 [75], Asp923, and Asp962 of PI3Kβ should be taken into account when β-isoform selective molecules are under consideration.

In addition to designing isoform-specific inhibitors, a trend under extensive study is the combination of PI3K inhibitors with agents from other complementary pathways. One area of research is the HER2+-breast cancers, which contribute to 25% to 30% of all breast cancers. Approximately 50% of HER2+ patients develop *de novo* resistance to targeted agents such as the humanized mAb trastuzumab and the EGFR inhibitor lapatinib. Aberrant PI3K/mTOR signaling has been observed in both hormone receptor-positive and HER2+ breast cancers. Combination of trastuzumab with pan-PI3K inhibitor (NVP-BKM120), mTOR

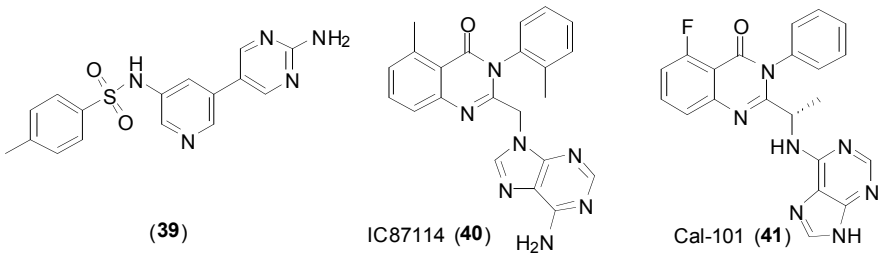



**Fig. (6)**.The chemical structures of non-morpholino-based heterocyclic PI3K inhibitors.

specific NVP-RAD001, and dual PI3K/mTOR inhibitor NVP-BEZ235 in trastuzumab-resistant xenografts led to a decrease in tumor volume of 122 $mm^3$, 62 $mm^3$, and 8 $mm^3$ in comparison to an increase of 173 $mm^3$ in mean tumor volume in the trastuzumab alone group [76]. Combination of the PI3Kα-selective inhibitor NVP-BYL719 with lapatinib induced more efficient inhibition of AKT and ERK phosphorylation than either drug alone in the lapatinib-resistant xenograft model [77].

Coexistence of PIK3CA mutations and mutations in other oncogenes such as KRAS, MEK, BRAF, and EGFR has been observed in lung adenocarcinoma [78]. In addition to aberrant PI3K/AKT signaling, 15% of all cancers exhibited RAS mutants, and activating BRAF mutations have been observed in melanoma (30-60%), thyroid cancer (30-50%), colorectal cancer (5-20%), and ovarian cancer (30%). There is evidence for cross-talk between the PI3K/AKT and RAF/MEK/ERK signaling via an MEK/EGFR/PI3K feedback loop. Hence, inhibition of one pathway can activate the other, and thus resistance occurs. For instance, resistance to epidermal growth factor receptor (EGFR) inhibition is associated with oncogenic mutations that activate MEK/ERK pathway, and resistance to MEK inhibition is complicated by PI3K pathway activation. Therefore, co-inhibition of two or more of these "semi parallel" pathway may lead to improved therapeutic outcomes. Studies have shown that dual inhibition of the PI3K and MEK/ERK pathways improved efficacy, but with greater toxicity [79]. The combination of the PI3K inhibitor GSK2126458 with the BRAF inhibitor GSK2118436 (fabrafenib) enhanced cell growth inhibition and decreased S6 ribosomal protein phosphorylation, and hence helped to overcome drug resistance in isolated GSK2118436 drug resistant clones from the A375 BRAF(V600E) and the YUSIT1 BRAF (V600K) melanoma cell [80].

PIK3CA amplification has been observed in 67% (88/131) gastric cancer patients. The overexpression of PI3Kα was positively associated with an increased level of phosphorylated AKT (pAKT) and an increased risk of gastric cancer-related death [81]. Combination of a dual PI3K/mTOR inhibitor PI-103 with a chemotherapeutic agent 5-fluorouracil (5-FU) increased 5-FU sensitivity and reduced *in vivo* tumor growth more than treatment with either single agent [82].

Considering the crucial roles of phosphatidylinositol phosphorylated derivatives (phosphoinositides) in cell proliferation and migration [83], other lipid kinases involved in phosphatidylinositol (PtdIns) metabolism are potential targets against cancer. The type III of phosphatidylinositol 4-kinase (PI4K, PI4KIIIβ), an enzyme catalyzing the generation of PI(4)P from phosphatidylinositol, is low in normal breast tissue, but has been found to exist in approximately 20% of primary human breast cancers [84]. Phosphatidylinositol 4-phosphate 5-kinase (PIP5K) is in the family of phosphatidylinositol phosphate kinase (PIPK), producing phosphatidylinositol 4,5-bisphosphate ($PIP_2$) from phosphatidylinositol 4-phosphate (PI(4)P). It has been shown that the activity of PIP5K is dramatically increased in liver cancer cells and an inverse correlation between the expression level of PIP5Kγ and the long-term survival rate of breast cancer patients has been observed [85]. Similarly, a recent study reported a correlation between overexpression of PIP5Kα and poor prognosis in prostate cancer, as well as the elevated level of the androgen receptor [86]. Importantly, the same report demonstrated that PIP5Kα inhibition by a small molecule inhibitor ISA-2011B significantly reduced tumor growth in xenograft mice with no obvious adverse effects on mice being observed, indicating PIP5Kα is a druggable target against advanced prostate cancer [86]. Another $PIP_2$ producing enzyme, phosphatidylinositol 5-phosphate 4-kinase (PIP4K), is also a member in the PIPK family and catalyzes phosphorylation on the D-4 position of phosphatidylinositol 5-phsophate (PI(5)P). A recent study showed that, in p53 knockout mice (*TP53*$^{-/-}$), partial loss of PIP4Ks (*PIP4KA*$^{-/-}$ and *PIP4KB*$^{+/-}$), which was well tolerated in the animals, led to a dramatically reduction in tumor-dependent death, strongly suggesting that PIP4K inhibition is an attractive strategy against p53 dysfunction/mutation related cancers [87]. As $PIP_2$ is primarily produced by PIP5K, the effects of PIP4K loss are thought to be associated with elevated level of PI(5)P, the substrate of PIP4K and a lipid signaling molecule mediating stress induced cell damages [88]. *PIP4KA* was also identified in a targeted knockdown screening of genes essential for acute myeloid leukemia cell proliferation and survival [89]. As PI3K inhibition may lead to activation of alternative signaling pathways which results in drug resistance, combination of PI3K inhibitors with the inhibitors of other phosphatidylinositol kinases could be a new strategy against cancer [90].

## CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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