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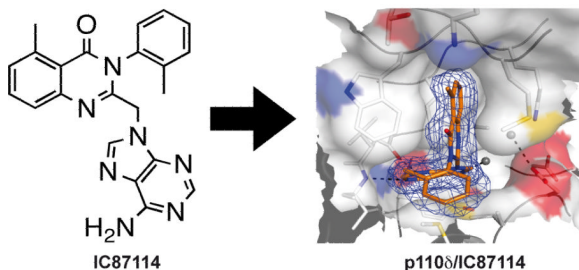
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The p110 δ crystal structure uncovers mechanisms for selectivity and potency of novel PI3K inhibitors

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Abstract



Deregulation of the phosphoinositide 3-kinase (PI3K) pathway has been implicated in numerous pathologies like cancer, diabetes, thrombosis, rheumatoid arthritis and asthma. Recently, small molecule and ATP-competitive PI3K inhibitors with a wide range of selectivities have entered clinical development. In order to understand mechanisms underlying isoform selectivity of these inhibitors, we developed a novel expression strategy that enabled us to determine the first crystal structure of the catalytic subunit of the class IA PI3K p110 δ . Structures of this enzyme in complex with a broad panel of isoform- and pan-selective class I PI3K inhibitors reveal that selectivity

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AUTHOR CONTRIBUTIONS

A.B. expressed and purified the Δ ABDp110 δ construct, crystallized the first Δ ABDp110 δ /inhibitor complexes, collected datasets, determined and refined their structures and performed the kinase activity assay. S.M. helped in the purification, crystallization and structure determination/refinement of several Δ ABDp110 δ /inhibitor complexes. O.W., D.D.L. and B.T.H. synthesized and characterized the inhibitors SW13, SW14, SW30, DL06 and DL07 with input from K.M.S. and determined their IC₅₀ values. J.I.P. performed the molecular dynamics and free energy perturbation experiments. F.G. devised and provided access to the Morpheus Screen and helped with the implementation of a microseeding protocol. W.C.H. helped with the insect cell culture and crystal data collection. Y.L. and C.R. designed and characterized the inhibitors INK654 and INK666. P.G., T.R., M.K.S. and J.P.S. synthesized and characterized the inhibitors AS5 and AS15 and helped with large scale insect cell expression. J.P.S. also provided valuable advice and support throughout the project. R.L.W. helped with the crystal data collection, structure determination/refinement and the preparation of the movies. The manuscript was written by R.L.W. and A.B.

COMPETING FINANCIAL INTEREST

Yi Liu, Ph.D. and Christian Rommel, Ph.D. are employees of Intellikine Inc, which is involved in the discovery and development of therapeutics for the prevention and treatment of human diseases.

OW and KMS are inventors on a UCSF owned patent application covering the SW series of compounds. This patent application is licensed to Intellikine Inc. KMS is a consultant and stockholder of Intellikine Inc.

towards p110 δ can be achieved by exploiting its conformational flexibility and the sequence diversity of active-site residues that do not contact ATP. We have used these observations to rationalize and synthesize highly selective inhibitors for p110 δ with greatly improved potencies.

The phosphoinositide 3-kinases are structurally closely related lipid kinases, which catalyze the ATP-dependent phosphorylation of phosphoinositide substrates^{1,2}. Together with the serine/threonine protein kinase B (PKB), PI3Ks constitute a central signalling hub that mediates many diverse and crucial cell functions like cell growth, proliferation, metabolism and survival^{1,3}. The observation that PI3Ks acting downstream of receptor tyrosine kinases (RTKs) are the most commonly mutated kinases in human cancers has spurred an immense interest in understanding the structural mechanisms how these mutations upregulate PI3K activity and in developing selective and drug-like PI3K inhibitors^{4,5}.

PI3Ks can be grouped into three classes based on their domain organisation⁶. Class I PI3Ks are heterodimers consisting of a p110 catalytic subunit and a regulatory subunit of either the 'p85'-type (associated with class IA PI3Ks with the isoforms p110 α / β / δ) or the 'p101/p84/p87'-type (associated with class IB PI3K p110 γ). The p110 catalytic subunit consists of an adaptor-binding domain (ABD), a Ras-binding domain (RBD), a C2 domain, a helical domain and the kinase domain⁷⁻¹⁰.

Mutant mice and inhibitor studies have shown less functional redundancy for the various class I PI3K isoforms than previously anticipated. While p110 α and p110 β are ubiquitously expressed, p110 γ and p110 δ are predominantly found in haematopoietic cells¹¹⁻¹³. Genetic deregulation of PI3K activity (oncogenic gain-of-function mutations, overexpression) has been implicated in cancer (all class I PI3K isoforms)¹⁴⁻¹⁷, diabetes (p110 α)¹⁸, thrombosis (p110 β)¹⁹, rheumatoid arthritis (p110 γ and p110 δ)²⁰ and asthma (p110 γ and p110 δ)^{21,22}. Consequently, the selective inhibition of individual PI3K isoforms using small molecule and ATP-competitive inhibitors is a promising therapeutic strategy²³. However, since all active-site side chains in contact with ATP are completely conserved throughout all class I PI3K family members (Supplementary Fig. 1), this is a challenging objective. Furthermore, in order to minimize undesired and often poorly understood toxic side effects, such inhibitors ideally would have to show no cross-reactivity towards off-pathway targets²⁴.

The earliest generation of small molecule and ATP-competitive PI3K inhibitors including the pan-selective LY294004²⁵ and wortmannin²⁶ were important tools to investigate PI3K-mediated cellular responses in the laboratory but their low affinities (LY294002), instability (wortmannin) as well as non-selectivity and toxicity limited their clinical use. However, further chemical modifications of some of these early inhibitors significantly helped to improve their drug-like properties. For example, PWT-458 (Wyeth) and PX-866 (Oncothyreon) are modified wortmannin-based PI3K inhibitors with improved pharmacological properties that are currently in phase I clinical trials^{27,28}.

The first crystal structures of p110 γ in complexes with pan-selective PI3K inhibitors²⁹ made it possible to begin to rationalize PI3K isoform-selective inhibitors like AS604850 (Merck-Serono) for p110 γ ³⁰. However, many of these inhibitors retained off-target activities and, partially due to the lack of crystal structures of other PI3K isoforms and PI3K related protein kinases (PIKKS), these unwanted side effects were difficult to rationalize.

Noteworthy, the development of multi- and pan-selective PI3K inhibitors as well as dual PI3K/mTOR or PI3K/tyrosine kinase³¹ rather than isoform-selective PI3K inhibitors remains a valid therapeutic strategy. XL-147 (Exelixis), which is currently evaluated in combination with other cancer therapeutics is in phase I/II clinical trials for the treatment of non-small lung cancer and GDC-0941 (Roche)³², also in phase I trials for the treatment of

breast cancer³³, are examples of pan class I selective PI3K inhibitors. NVP-BEZ235 (Novartis), currently in phase I/II trials for breast cancer³⁴ and SF1126 (Semaphore), a RGDS peptide conjugated prodrug of LY294002 in phase I trials³⁵ are examples of dual-selectivity PI3K/mTOR inhibitors.

Recently, several new class I PI3K isoform-selective inhibitors showing improved selectivities and potencies have been reported and some of them have entered clinical trials: CAL-101 (Calistoga), a derivative of the highly p110 δ -selective inhibitor IC8711436 with increased potency, entered stage I clinical trials for the treatment of acute myeloid leukaemia (AML) and B-cell chronic lymphoid leukaemia (CLL). The p110 β -selective AZD6482 (AstraZeneca) is in clinical phase I for the treatment of thrombosis. Strikingly however, despite a growing list of such isoform-selective compounds, little is known about what determines isoform-selectivity on a structural level.

Impaired PI3K δ signalling results in severe defects of innate and adaptive immune responses and suggested that targeting of this isoform would be a beneficial therapeutic strategy^{20,24}. To elucidate the molecular mechanisms of isoform-selectivity of PI3K δ inhibitors, we report the crystal structure of the catalytic core of p110 δ , both free and in complexes with a broad panel of novel and mostly p110 δ -selective PI3K inhibitors. Our study provides the first detailed structural insights into the active site of a class IA PI3K occupied by non-covalently bound inhibitors. Furthermore, our structures suggest mechanisms to achieve p110 δ selectivity and to increase potency of inhibitors without sacrificing isoform-selectivity. To obtain these structures, we developed a unique expression and purification scheme that has now been extended to all class IA PI3K isoforms.

With our new set of p110 δ crystal structures and better models of flexibility resulting from molecular dynamics simulations we are now starting to understand why p110 δ can be more easily deformed to open an allosteric pocket in which p110 δ -selective inhibitors can be accommodated.

RESULTS

Expression, purification and catalytic activity of Δ ABDp110 δ

Our initial attempts to express either the full-length or the ABD-truncated p110 δ catalytic subunit in *Sf9* cells produced only insoluble protein. However, we could readily express and purify p110 δ in complexes with only the iSH2 domain of p85 α . We devised a novel expression and purification strategy by introducing a TEV protease cleavage site in the linker region between the ABD and the RBD of p110 δ (Fig. 1a) with the objective of generating an ABD-truncated version for crystallization trials. The Δ ABDp110 δ construct showed significantly enhanced lipid kinase activity *in vitro* when compared with either the holo p110 δ /p85 α or the p110 δ /nicSH2 complex (Supplementary Fig. 2).

Overall structure of Δ ABDp110 δ

Crystallographic statistics for all p110 δ datasets are given in Supplementary Table 1. The overall fold of p110 δ is very similar to the catalytic subunits of p110 γ and p110 α (Fig. 1b)^{8,37}. The helical ABD-RBD linker packs tightly against the helical domain and bridges the Ras-binding and the C2 domain. Helices α 1 and α 2/ α 2' form a hairpin in the N-lobe that sits on top of a five-stranded β -sheet formed by β 3- β 7, and this hairpin structurally distinguishes PI3Ks from protein kinases. These helices extend the antiparallel A/B pairs of α -helices found in the helical domain. The kinase domain has an extensive, tightly packed interface with the helical domain. All of the catalytically important motifs within this domain are well ordered with the exception of residues 920-928 of a region known as the "activation" or phosphoinositide-binding loop. Remarkably, the residues within the p110 δ

893-DRH-895 motif located in the “catalytic” loop, a motif conserved in all PI3Ks and inverted (HRD) in protein kinases, adopt a different conformation from what was previously observed in the structure of p110 γ (Supplementary Fig. 3)⁸. This different conformation might be critical for the correct positioning of the DFG aspartate at the beginning of the “activation” loop.

All the domains of p110 δ superimpose closely on previously reported structures (Supplementary Fig. 4a-f). However, the most striking difference in the overall structure of p110 δ relative to p110 α or p110 γ is a change in the orientation of the N-lobe with respect to the C-lobe of the kinase domain. This shift may reflect motions characteristic of the catalytic cycle, analogous to the hinging and sliding motions of the N- and C-lobes have been described for protein kinases³⁸. Furthermore, the RBD shifts relative to the N-lobe of the kinase domain (Supplementary Fig. 4g). The RBD mediates interaction with Ras in a GTP-dependent manner for all three isoforms^{11,12,39,40}. Despite the great sequence divergence among the isoforms in the RBD, the overall RBD backbone conformation is very closely preserved among the various class I isoforms (Supplementary Fig. 4f). However, differences in the orientation of the RBD relative to the kinase domain suggest the possibility of different mechanisms of activation by Ras. The conformation of the loop connecting k β 4 and k β 5 (Tyr763 to Val774 in p110 δ) in the N-lobe is remarkably different in all the isoforms (longest in p110 α , shortest in p110 δ) and this correlates with the orientation of the RBD. Within the RBD of p110 δ residues 231-234 are disordered. The equivalent region in p110 α is an ordered helix (R α 2), whereas in p110 γ this region is ordered only in the Ras/p110 γ complex, although it has a completely different conformation than in p110 α .

Co-crystallization of p110 δ with inhibitors

We chose a set of chemically diverse inhibitors in order to understand structural mechanisms that underlie p110 δ -specific inhibition in contrast to broadly specific PI3K inhibitors. Even though we obtained crystals grown in the presence of ATP, only a weak density somewhat larger than what would be expected for an ordered water molecule was observed in the hinge region. We will refer to this structure as the apo-form of p110 δ .

ATP-binding pocket

All of the compounds presented here contact a core set of six residues in the ATP-binding pocket (Supplementary Table 2), and - apart from the hinge residue Val827 in p110 δ - these residues are invariant in all of the class I PI3K isotypes. Based on our inhibitor-bound structures of p110 δ as well as previously described PI3K complexes^{18,29,30,32,41}, we can define four regions within the ATP-binding pocket that are important for inhibitor binding (Fig. 2a): An “adenine” pocket (hinge), a “specificity” pocket, an “affinity” pocket and the hydrophobic region II located at the mouth of the active-site^{18,42}. Of the core active-site residues, only two are in contact with inhibitors in all complexes: Val828 and Ile910. Residues 825-828 line the “adenine” pocket and form a hinge between the N-lobe and C-lobe of the catalytic domain. The backbone amide of the hinge Val828 makes a characteristic hydrogen bond in all of the p110/inhibitor complexes. Additionally, the backbone carbonyl of hinge Glu826 establishes hydrogen bonds to most of the inhibitors.

Our selection of inhibitors can be organized into three types: Firstly, inhibitors that adopt a propeller-shaped conformation (two roughly orthogonal oriented aromatic ring systems) when bound to the enzyme (Fig. 2a-e **and** Supplementary Fig. 5). These are mostly p110 δ -selective inhibitors, which stabilize a conformational change that opens a hydrophobic “specificity” pocket in the active site that is not present in the *apo*-structure of the enzyme as previously reported for the p110 γ /PIK-39 crystal structure¹⁸. Secondly, we co-crystallized the p110 δ enzyme with a set of mostly flat and multi- to pan-selective class I PI3K

inhibitors that do not provoke such a conformational rearrangement. AS15, which has a distorted propeller-shape when bound to the enzyme, is the only member of a third type of inhibitor, which is highly selective for the p110 δ isoform, although it does not open the “specificity” pocket.

The propeller-shaped p110 δ -selective inhibitors IC87114 and PIK-39

The discovery of the p110 δ -selective inhibitor IC87114 (ICOS) in 200336 was a proof-of-principle that isoform-selectivity of PI3K inhibitors can be accomplished, and to date, it remains one of the most selective p110 δ inhibitors known.

The crystal structures of the p110 δ /IC87114 (**compound 1**) (Fig. 2a) and the p110 δ /PIK-39 (**compound 2**) (Fig. 2b) complexes show that the purine group of the compounds resides within the “adenine” pocket and establishes hydrogen bonds to the hinge residues Glu826 and Val828. The quinazolinone moiety is sandwiched into the induced hydrophobic “specificity” pocket between Trp760 and Ile777 on one side and two P-loop residues, Met752 and Pro758 on the other side. The “specificity” pocket is not present in the apo enzyme where the P-loop Met752 rests in its “in” position leaning against Trp760. The toluene group (IC87114) and the methoxyphenyl group (PIK-39) attached to the quinazolinone moiety project out of the ATP-binding pocket over a region that we will refer to as hydrophobic region II.

PIK-39 binding to both p110 δ and p110 γ induces a slight opening in the ATP-binding pocket. The p110 δ ATP-binding pocket accommodates the PIK-39-induced conformational change by a local change in the conformation of the P-loop (residues 752-758 in p110 δ) whereas the equivalent opening of the p110 γ pocket is accompanied by a conformational change that involves much of the N-lobe moving with respect to the C-lobe. The loop between $\alpha 1$ and $\alpha 2$ of p110 γ (residues 752-760) sits on top of the P-loop (residues 803-811) and appears to rigidify it, so that the compound-induced opening of the pocket is accompanied by a shift of the N-lobe as a unit (Supplementary Fig. 6, **Supplementary Movies 1 and 2**). In contrast to p110 γ , in p110 δ the slightly shorter $\alpha 1$ - $\alpha 2$ loop leaves the P-loop largely free and able to move independently of the rest of the N-lobe. We proposed that opening of the “specificity” pocket might be easier in p110 δ compared to p110 γ .

Molecular dynamics simulations and free energy perturbation speak to the greater flexibility of p110 δ compared with p110 γ

Perturbation analysis by molecular dynamics simulations suggests that the free energy of the “specificity” pocket closure is more favourable in p110 γ than p110 δ (Supplementary Fig. 7). To quantify the higher degree of flexibility within the p110 δ active site we performed molecular dynamics simulations of the *apo* enzymes of both isoforms (see Supplementary Methods and **Supplementary Movies 3 and 4**). The potential energy of the interaction of PIK-39 with the enzyme is more favourable for p110 δ than for p110 γ (Supplementary Fig. 8). Our results further show that the distance between Trp760 (Trp812 in p110 γ) and the P-loop Met752 (Met804 in p110 γ) does not change appreciably in p110 δ over the course of the simulation because the conformational changes observed for both residues are synchronized with each other, i.e. the tryptophan smoothly follows the methionine and *vice versa*. In contrast, in p110 γ , as the Met804 transiently assumes alternate rotamers, it briefly creates gaps between itself and Trp812. Trp812 of p110 γ is sterically constrained by a hydrogen bond to Glu814 (Met762 in p110 δ) and is therefore unable to flex in synchrony with Met804 as in p110 δ . Additionally, in p110 γ there is a more pronounced hydrophobic interaction between the Trp812 and the hinge Ile881, which might further restrain the

position of the tryptophan. The transient opening of the “specificity” pocket in p110 γ would allow water to become trapped, leading to an unfavourable entropy change.

Mechanisms to increase potencies of propeller-shaped p110 δ -selective inhibitors

The SW series (**compounds 3-5**) (Williams O. & Shokat K. M. et al., submitted) and INK series (**compounds 6-7**) of inhibitors take advantage of both the “specificity” pocket and the “affinity” pocket (synthesis details for these compounds are given in the **Supplementary Methods** section). This pocket is lined by a thin hydrophobic strip formed by Leu784, Cys815 and Ile825 at the back of the ATP-binding pocket and flanked on the top by the side chain of Pro758 and Lys779 and on the bottom by Asp787 (hydrophobic region I in protein kinases). These mostly p110 δ -selective compounds (SW14 is dual-selective for p110 γ/δ) are also propeller-shaped, but have additional decorations when compared to IC87114 and PIK-39 in the form of an ortho-fluorophenol (SW14), a para-fluorophenol (SW13) or a butynol group (SW30) attached to the central pyrazolopyrimidineamine scaffold (Fig. 2c-e). These groups explore the “affinity” pocket where they engage in hydrogen bonds with Asp787 (SW13/14/30) and Lys779 (SW13/14). Additionally, the butynol OH group of SW30 also serves as a hydrogen bond donor to the DFG Asp911 at the start of the “activation” loop, and the phenolic OH group of SW13 engages in hydrogen bonding with Tyr813. This set of novel inhibitor-enzyme interactions leads to a significant increase in the inhibitors’ potencies towards p110 δ , which is reflected in their greatly lowered IC₅₀ values (Supplementary Tables 2 and 3). The propeller-shape of a compound alone does not guarantee p110 δ specificity as shown by INK666 (Supplementary Fig. 5b).

Our structures of p110 δ in complex with SW13/14/30 also speak to a conformational flexibility for the catalytic DFG Asp911. This residue assumes two alternative conformations in the p110 δ /SW structures. One of these, the “in” conformation, coincides with its putative ATP/Mg²⁺-binding position (based on the p110 γ /ATP complex). The other conformation has the DFG Asp911 swung away (“out” conformation). In the p110 δ /SW14 and p110 δ /SW30 structures, DFG Asp911 is found in the “out” conformation, while in the p110 δ /SW13 complex it is “in”. In protein kinases, a shift of the DFG aspartate from the in-conformation (ATP-bound) to the out-conformation is characteristic of the catalytic cycle. By analogy, it may be that these inhibitors are inducing conformations characteristic of the PI3K catalytic cycle.

p110 δ in complex with flat and multi-selective class I PI3K inhibitors

ZSTK47443 (**compound 8**), DL06 (**compound 9**), DL07 (**compound 10**), AS5 (**compound 11**) and GDC-094132 (**compound 12**) are fairly flat compounds that do not open the “specificity” pocket and achieve relatively little isotype selectivity. Their binding provokes some motions of the P-loop side chains of p110 δ , and these conformational changes are coordinated with changes in conformation of the DFG Asp 911 in the C-lobe.

The DL06/07 inhibitors represent a minimalistic approach to achieve PI3K inhibition

The DL06/07 series of PI3K inhibitors (Williams O. & Shokat K. M. et al., submitted) can best be described as pan-selective p110 inhibitors, which represent a minimalistic approach to achieve PI3K inhibition (see Supplementary Methods for synthesis details). They are flat and small compounds with a minimal design just sufficient enough to span the “adenine” pocket via their pyrazolopyrimidine moiety and project into the “affinity” pocket by means of a phenol (DL07) or a pyridine (DL06) group attached to a propyne “stick” (Fig. 3a,b). The DL07 phenol group interacts with the DFG Asp911, forcing it to its “in” conformation. It also induces rotations in the side chain of P-loop Met 752, but not to its “out” conformation. Similar interactions are formed by DL06.

p110 δ /ZSTK474

Yaguchi et al. discovered and characterized the novel pan-selective triazine PI3K inhibitor ZSTK474, which strongly inhibits the growth of tumor cells in human cancer xenografts and therefore is a potential candidate for further clinical development⁴³. Its crystal structure in complex with p110 δ shows it flipped over relative to what was predicted in a computational p110 γ /ZSTK474 model⁴³ (Fig. 3c). The oxygen of one of the morpholino groups is positioned as the hinge hydrogen bond acceptor and the morpholino ring adopts a chair conformation. The benzimidazole group extends into the “affinity” pocket where its nitrogen acts as a hydrogen bond acceptor for the primary amine of Lys779. The difluoromethyl group points towards Pro758 in the upper wall of the hydrophobic “affinity” pocket. The second morpholino group adopts a somewhat twisted chair conformation and projects out of the ATP binding pocket in a same manner as the phenyl group of LY294002 where it occupies the hydrophobic region II.

AS5 reveals the potential of phosphate-mimetics as kinase inhibitors

AS5 is a relatively flat p110 α /p110 δ dual-selectivity inhibitor with only modest affinities for these two isoforms. Its dimethoxyaniline group occupies the “adenine” pocket, where it interacts with the hinge Val828, but does not project deeply into the “affinity” pocket (Fig. 3d). It is conceivable that modifications on this scaffold that target polar moieties within the “affinity” pocket could increase potencies of AS5 derivatives. Coupled to the quinoxaline group is a *p*-fluorobenzenesulfonamide, and when superimposed on the p110 γ /ATP crystal structure it becomes apparent that the sulfonyl group of AS5 co-localizes with the α -phosphate group of ATP. This compound reveals two strategies to mimic the ATP phosphates to achieve inhibition of p110 α and p110 δ . Firstly, one of the sulfonyl oxygens of AS5 is a hydrogen bond acceptor for P-loop Ser754. Secondly, the fluorophenyl group exits the active site close to the DFG Asp911, in the proximity of the space occupied by the β/γ -phosphates in the p110 γ /ATP structure.

GDC-0941 shows use of the space above hydrophobic region II for moieties that confer better drug-like properties

The identification, characterization and development of the tricyclic pyridofuopyrimidine lead PI-10344-46, a very potent dual-selective PI3K/mTOR inhibitor, has led to the pan-selective class I PI3K thienopyrimidine inhibitor GDC-0941, which has no off-target activity against mTOR³². GDC-0941 is orally bioavailable and currently in phase I trials for the treatment of solid tumors³³.

Its structure in complex with p110 δ (Fig. 3e) confirms the previously described binding mode to p110 γ ³² but also reveals interesting new features. Whereas the piperazine ring adopts a twisted chair conformation in the p110 γ structure, it is present in a distorted boat conformation in the structure of p110 δ . The terminal methanesulfonylpiperazine group is also oriented differently in both structures. In p110 δ , this group is marginally tilted with respect to the central thienopyrimidine scaffold and thereby comes closer to the P-loop. Instead of the Lys802-p110 γ (Arg770-p110 α), the Thr750 at the equivalent position in p110 δ is unable to establish a hydrogen bond to the inhibitor's sulfonyl oxygen. However, a different lysine residue (κ 2 Lys708) interacts with the sulfonyl group of GDC-0941, thereby indicating why this compound does not lose affinity for p110 δ .

AS15 is a non-propeller-shaped and highly p110 δ -selective inhibitor that exploits non-conserved residues outside of the active-site

Although AS15 (**compound 13**) is chemically related to the quinazolinone purine inhibitor PIK-39, its co-crystal structure with p110 δ reveals an unexpected mode of binding (Fig. 4).

Instead of wedging in between the Met752 and Trp760, the tetrahydroquinazolinone group presses tightly against Met752 (in its 'in' position) and Trp760. By comparing the binding modes of PIK-39 and AS15 to p110 δ , three reasons can be deduced why PIK-39, but not AS15, is able to induce the "specificity" pocket. Firstly, whereas the purine group of PIK-39 acts as a hydrogen bond donor and acceptor, the AS15 quinoxaline group interacts only with the backbone amide of hinge Val828. Secondly, the non-planar nature of the hexahydroquinazolinone may exceed the capacity of the "specificity" pocket. In its alternate location, the hexahydroquinazolinone packs into a shallow dimple formed between Met752, the small side chain of Thr750 and Trp760. In other p110 isotypes, the residue equivalent to Thr750 is a lysine or arginine. This interaction may account for the extraordinary isotype selectivity of this compound. Thirdly, compared with the shorter thiomethyl linker of PIK-39, the longer methylthioacetamide linker of AS15 might be more conformationally restrained due to the planar nature of the linker's peptide bond. This planarity might prevent the tetrahydroquinazolinone from being positioned in a way that would allow for the induction of the "specificity" pocket.

A number of additional p110 δ -specific interactions are formed in a manner whereby the ketone oxygen from the tetrahydroquinazolinone group acts as a hydrogen bond acceptor for the backbone amide of the P-loop Asp753 and for the primary amine of Lys708. The P-loop Asp753 is specific to p110 δ (the corresponding residue is Ser773 in p110 α and Ala805 in p110 γ), and Lys708, which is located outside of the active site, has an equivalent only in p110 α (Lys729) but not in p110 γ (Ser 760). Since AS15 does not occupy the "affinity" pocket, modifications of the compound exploring this pocket should result in an increased potency for p110 δ .

DISCUSSION

The p110 δ /inhibitor crystal structures presented here show that selectivity can be achieved by exploiting both differences in flexibilities among the isoforms and isotype-specific contacts beyond the first-shell of residues that interact with ATP. Flexibility-based inhibitors are generally able to utilize the inherently greater pliability of the p110 δ P-loop. All propeller-shaped inhibitors create a new "specificity" pocket not present in the apo-form of the enzyme. Small modifications of this framework (as found in AS15) can result in inhibitors that are highly selective by establishing unique p110 δ -specific interactions without the formation of the "specificity" pocket. The plasticity of p110 δ may enable this isoform to more readily accommodate even very rigid compounds. Our structures also suggest that introducing moieties interacting with the hydrophobic region II at the mouth of the active site might help to improve pharmacokinetic properties of drug-like PI3K inhibitors such as GDC-0941.

Initial molecular dynamic simulations suggest that allosteric pockets, such as the "specificity" pocket can be identified with computational approaches. A similar method that imposes stress on the ATP-binding pocket may identify new strain-prone regions that could be exploited by inhibitors.

The strategy to explore the "affinity" pocket is a very powerful approach to augment potency of inhibitors while maintaining selectivity. Further development of selective inhibitors for other isotypes and for overcoming potential resistance mutations that frequently accompany treatment with inhibitors will require a broader range of PI3K and PIKK structures.

METHODS

Construct design, expression and purification of Δ ABDp110 δ

Briefly, the TEV-insertion construct was generated using the overlapping PCR method, digested with *Bgl*III and *Xho*I at sites encoded by the primers and ligated into pFastBac-HTa (Invitrogen) cut with the *Bam*HI and *Xho*I restriction enzymes (NEB). The correct insertion of the TEV-site was confirmed by DNA-sequencing (amino acid sequence: 101-LVARE-(105)-ENLYFQG-(106)-GDRVKK-111). The construct has an N-terminal extension encoded by the vector (MSYHHHHHHHDYDIPTTENLYFQGAMDL) preceding the first residue of p110 δ . This extension has a His₆-tag and an additional vector-encoded TEV-cleavage site. Recombinant baculovirus was generated and propagated according to standard protocols. For expression, *Sf9* insect cells at a density of 1×10^6 /ml were co-infected with an optimised ratio of viruses encoding the catalytic and regulatory subunit. As a regulatory subunit, we used the iSH2 fragment of the human p85 α (residues 431-600), tagged with an N-terminal, non-cleavable His₆-tag. The culture was incubated for 48 h after infection, cells harvested and washed with ice-cold PBS, flash-frozen in liquid N₂ and stored at -20°C . For purification, cell pellets corresponding to typically 8 litres of culture were defrosted and resuspended in 250 ml of buffer A (20 mM Tris pH 8, 100 mM NaCl, 5% (v/v) glycerol and 2 mM β -ME). After addition of 2 tablets of Complete EDTA-free Proteinase Inhibitors (Boehringer) the suspension was sonicated and the lysate spun at 42000 rpm for 45 min. The supernatant was filtered through 0.45 μm filter units (Sartorius) and loaded onto a 5 ml HisTrap column (GE Healthcare). After a wash step with buffer A the column was eluted using a gradient from 0-100% buffer B (buffer A + 500 mM imidazole). The p110 δ /iSH2 fractions were pooled and loaded onto a 5 ml heparin column equilibrated with heparin A buffer (20 mM Tris pH 8, 100 mM NaCl, 2 mM β -ME). The column was washed and eluted with a gradient from 0-100% heparin B buffer (heparin A + 1 M NaCl). This chromatography step resulted in a separation of excess His₆-tagged iSH2 (earlier peak) from the p110 δ /iSH2 complex (later peak). The p110 δ /iSH2 fractions were pooled and adjusted to 5 mM β -ME. TEV proteinase at a w/w ratio of 1:10 was added and the mixture was incubated overnight at 4°C . After verifying that the cleavage reaction was complete, the solution was adjusted to 30 mM imidazole, passed over a second 5 ml HisTrap column to remove the ABD/His₆-iSH2, and Δ ABDp110 δ was collected in the flow-through. Following a concentration step using Vivaspin 20 concentrators with a 50 kDa MWCO (Vivascience), the protein was subjected to gel filtration on an S200 16/60 HiLoad column (GE Healthcare) and eluted in 20 mM Tris pH 7.2, 50 mM (NH₄)₂SO₄, 1% (v/v) ethylene glycol, 1% (w/v) betaine, 0.02% (w/v) CHAPS and 5 mM DTT. Finally, fractions were pooled and concentrated to 4.5-5 mg/ml as determined spectrophotometrically using the extinction coefficient $129,810 \text{ M}^{-1}\text{cm}^{-1}$ at 280 nm, flash frozen in liquid N₂ and stored at -80°C . We have applied this strategy to all other class IA isoforms (not shown).

Synthesis and characterization of SW13/14/30 and DL06/07

A detailed description for the synthesis and characterization of these compounds can be found in the **Supplementary Methods** section.

X-ray crystallography

High-quality diffraction data of Δ ABDp110 δ crystals grown in the presence of inhibitors were obtained using a microseeding protocol implemented on our robotic setup. All crystal structures were solved by molecular replacement. See Supplementary Methods for additional details.

Lipid Kinase Activity Assay

To compare of the PI3K lipid kinase activity of the crystallized murine Δ ABDp110 δ construct with the full-length murine p110 δ /murine p85 α complex and the murine p110 δ /human p85 α nicSH2 construct, a Transcreeper ADP Assay (Bellbrook Labs) was performed according to the manufacturer's instruction. Briefly, for the generation of the ADP/ATP standard curve, 10 μ l of a 60 μ M ADP/ATP (2x) mixture of various ADP:ATP concentrations were mixed with 5 μ l of anti-ADP antibody at 80 μ g/ml (4x) and 5 μ l of ADP Alexa633 tracer at 40 nM (4x) in a low-volume, black and round bottom Corning 384-well plate (Corning). The plate was protected from light and shaken at 500 rpm for one hour prior to polarization measurements using a PHERAstar (BMG Labtech) fluorescence polarization microplate reader (λ_{exc} =612 nm, λ_{em} =670 nm). For the kinase reaction, 10 nM of enzymes were incubated for 1 hour at 25°C in a buffer consisting of 50 mM HEPES (pH 7.5), 4 mM MgCl₂, 2mM EGTA, 30 μ M diC₈-PIP₂ (Echelon) and started by the addition of 30 μ M ATP (Sigma-Aldrich, neutralized). The control included the same components with the exception of the diC₈PIP₂ substrate. The reaction was stopped by mixing 10 μ l of the kinase reaction with 10 μ l of the Stop & Detect buffer (20 mM HEPES pH 7.5, 40 mM EDTA, 0.2% Brij-35) containing 20 nM ADP Alexa633 tracer (2x) and 40 μ g/ml ADP antibody (2x). To allow for signal stabilization, the plate was shaken at 500 rpm for 1 hr prior to fluorescence polarization measurements. The data were plotted and fitted in Kaleidagraph (Synergy Software) using an exponential decay function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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PDB Accession codes. Coordinates of all p110 δ structures and accompanying structure factors have been deposited under the following accession codes: **2wxe** (p110 δ /IC87114), **2wxf** (p110 δ /PIK-39), **2wxg** (p110 δ /SW13), **2wxh** (p110 δ /SW14), **2wxi** (p110 δ /SW30), **2wxj** (p110 δ /INK654), **2wxk** (p110 δ /INK666), **2wxl** (p110 δ /ZSTK474), **2wxm** (p110 δ /DL06), **2wxn** (p110 δ /DL07), **2wxo** (p110 δ /AS5), **2wxp** (p110 δ /GDC-0941), **2wxq** (p110 δ /AS15) and **2wxr** (apo-p110 δ).

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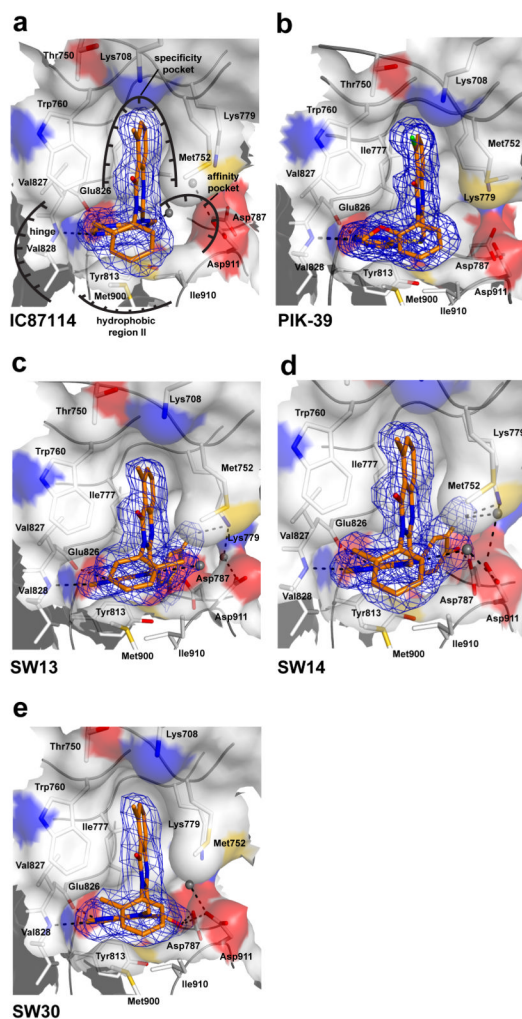
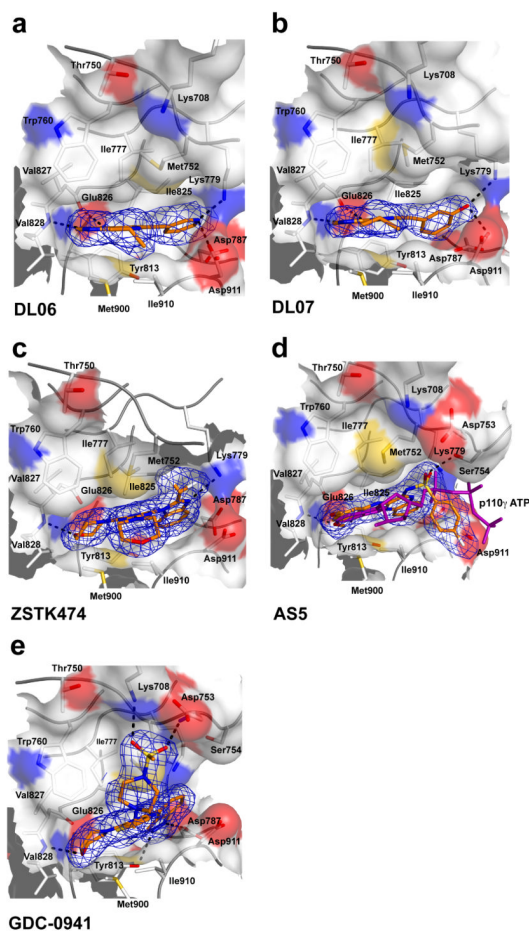


Figure 2. The propeller-shaped p110 δ -selective inhibitors induce the formation of the “specificity” pocket. Shown are the active sites of p110 δ in complex with the inhibitors IC87114 (a), PIK-39 (b), SW13 (c), SW14 (d) and SW30 (e). Key residues that outline the active site and interact with the compounds and the $2mF_o-DF_c$ electron densities (contouring level 1σ) are presented. Selected water molecules in the active sites are shown as gray spheres. Note, that IC87114 and PIK-39 do not fill the “affinity” pocket, whereas SW13, SW14 and SW30 do. Dashed black lines represent hydrogen bonds.

**Figure 3.**

The flat inhibitors DL06, DL07, ZSTK474, AS5 and GDC-0941 are multi- to pan-selective class I PI3K inhibitors that do not induce the opening of the “specificity” pocket. Shown are the binding modes of DL06 (a), DL07 (b), ZSTK474 (c), AS5 (d) and GDC-0941 (e) in the active site of p110 δ . Met752 is in its “in” position for all these compounds. For panel (d), the structure of the p110 γ /ATP complex (PDB entry 1e8x) was superimposed on the C α -backbone of p110 δ to show the proximity of the sulfonyl group of AS5 to the alpha phosphate group of ATP (purple). This sulfonyl group is a hydrogen bond acceptor to Ser754 located in the P-loop of p110 δ . (e) GDC-0941 is a pan-class IA PI3K inhibitor that (like AS15) interacts with residues outside the active site. GDC-0941 occupies the “adenine” pocket and the “affinity” pocket within the active site of p110 δ and engages there in hydrogen bonds with Val828, Tyr813 and Asp787. Additionally, the substituted piperazine group of GDC-0941 extends out of the ATP-binding site where its methylsulfonyl moiety acts as a hydrogen bond acceptor for Asp753 of the P-loop and Lys708 at the beginning of α 2. The contouring level of the 2mF $_o$ -DF $_c$ electron densities is 1 σ for each compound.

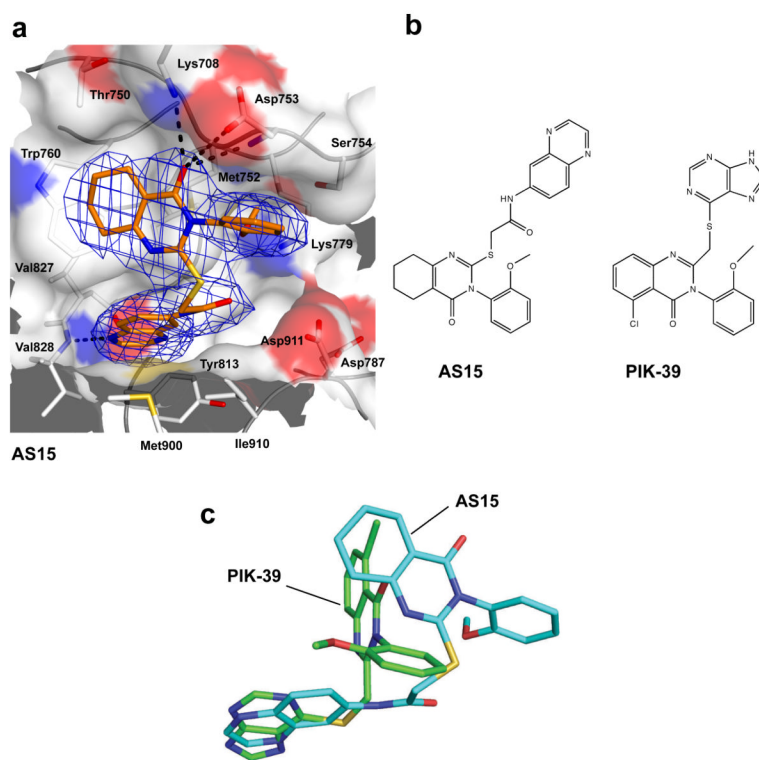


Figure 4. Binding mode of the p110 δ -selective PI3K inhibitor AS15 and comparison of AS15 with the propeller-shaped inhibitor PIK-39 (2mF_o-DF_c contouring level 1 σ). **(a)** The highly p110 δ -selective compound AS15 does not open the “specificity” pocket and makes extensive use of a hydrophobic patch between Trp760, Thr750 and Met752 adjacent to the adenine-binding pocket. **(b)** Chemical structures of the highly p110 δ -selective inhibitors AS15 and PIK-39. **(d)** Superposition of the AS15 and PIK-39 to demonstrate their different mode of binding within the active site of p110 δ .