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Mechanisms of PI3Kβ-Selective Inhibition Revealed by Reciprocal Mutagenesis

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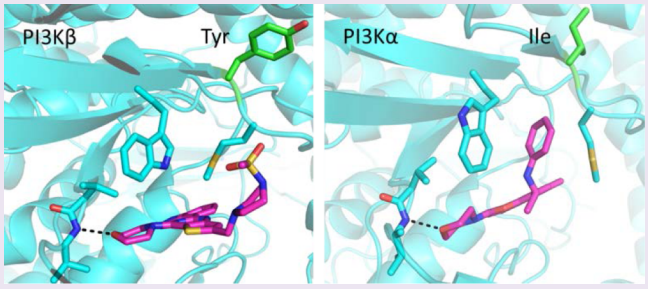
Supporting Information

ABSTRACT: The p110β isoform of PI3 kinase (PI3Kβ) has been implicated in pathological disorders such as thrombosis and cancer and a number of PI3Kβ-selective inhibitors have recently progressed into clinical studies. Although crystallography studies identify a binding site conformation favored by the inhibitors, no specific interaction explains the observed selectivity. Using site-directed mutagenesis we have identified a specific tyrosine residue of the binding site Y778 that dictates the ability of the PI3Kβ isoform to bind these inhibitors. When mutated to isoleucine, PI3Kβ has reduced ability to present a specific cryptic binding site into which a range of reported PI3Kβ inhibitors can bind, and conversely when tyrosine is introduced into the same position in PI3Kα, the same inhibitors gain potency. The results provide a cogent explanation for the selectivity profiles displayed by these PI3K inhibitors and maybe others as well.



The phosphatidylinositol 3-kinases (PI3K) are a family of lipid kinases that regulate intracellular signaling for numerous cellular events such as cell migration, growth, and survival.[1] PI3Kβ was first identified as a potential therapeutic target in thrombosis, and more recently, there has been accumulating evidence showing that PI3Kβ plays a key role in certain cancers accompanied by the loss of the tumor suppressor PTEN.[2,3] Because each of the PI3K isoforms has their own, although overlapping, physiological roles, isoform-selective PI3K inhibitors may hold some therapeutic advantages with respect to reducing off-target effects. While the majority of PI3K inhibitors currently in clinical trials are Class I pan-PI3K inhibitors,[4] some PI3Kβ isoform-selective inhibitors have emerged. These include TGX221, TGX286, and AZD6482 (aka KIN193).[5−7] Recently, a number of new PI3Kβ-selective compounds have been reported. GSK2636771 has progressed to phase I/IIa clinical trial to treat advanced solid tumors with PTEN deficiency, and other analogous series have been described by GSK.[8−10] Other compounds have also been described by Sanofi and Amgen.[11−13]

While these and other isoform selective inhibitors have been reported, the mechanistic details underpinning selectivity are poorly understood. Since the first X-ray structures of PI3K were reported, it has been apparent that much of the inner core of the binding site is highly conserved across the class 1 isoforms.[14] Two nonconserved regions of the binding site have been identified as capable of executing selective interactions with inhibitors (Supplementary Figure S1). One region encompasses a loop, which sits under the ribose pocket that is relatively large in the PI3 kinases (from PI3Kβ 855 to 862).[15] A second region encompasses PI3Kβ 772−788 and corresponds to the protein kinase P-loop that is a key contributor to substrate binding.[16,17] Here, a cryptic binding pocket has been identified, whereby a conserved methionine residue (M779 of PI3Kβ) can shift to facilitate binding by the so-called "propeller-shaped" inhibitors. This has been observed for PI3Kδ-selective compounds like PIK-39 and IC87114.[6,18] A methionine shifted conformer of PI3Kβ appears also to be accessed by PI3Kβ selective inhibitors although there is no direct evidence for this. PI3Kβ-selective inhibitors have been cocrystallized in this conformation with PI3Kα, δ (Figure 1b,c) and γ, but the only reported X-ray structure of PI3Kβ displays a conventional flat conformer (Figure 1a).[18,19]

While the conformer has been identified and can be associated with binding of PI3Kβ and PI3Kδ inhibitors, the finer details that dictate the propensity to form this pocket remain unclear. It has been speculated that the multiple differences in the P-loop sequence (Supplementary Figure S1) contribute to isoform-specific conformational behavior.[16]

Reciprocal mutation has been a useful technique to unravel the various mechanisms that underpin PI3K inhibitor selectivity. In this approach, nonconserved residues of the binding site are swapped between the isoforms of interest to determine what influence if any that residue has on inhibitor binding.[15] Recently we showed that the binding of PI3Kα selective inhibitors such as A-66, PIK75, and J-32 have been shown to be sensitive to mutation at nonconserved residues.[17] Our earlier work with TGX221 and TGX286 showed them to be insensitive to mutations in the region PI3Kβ 855 to 862.[15]

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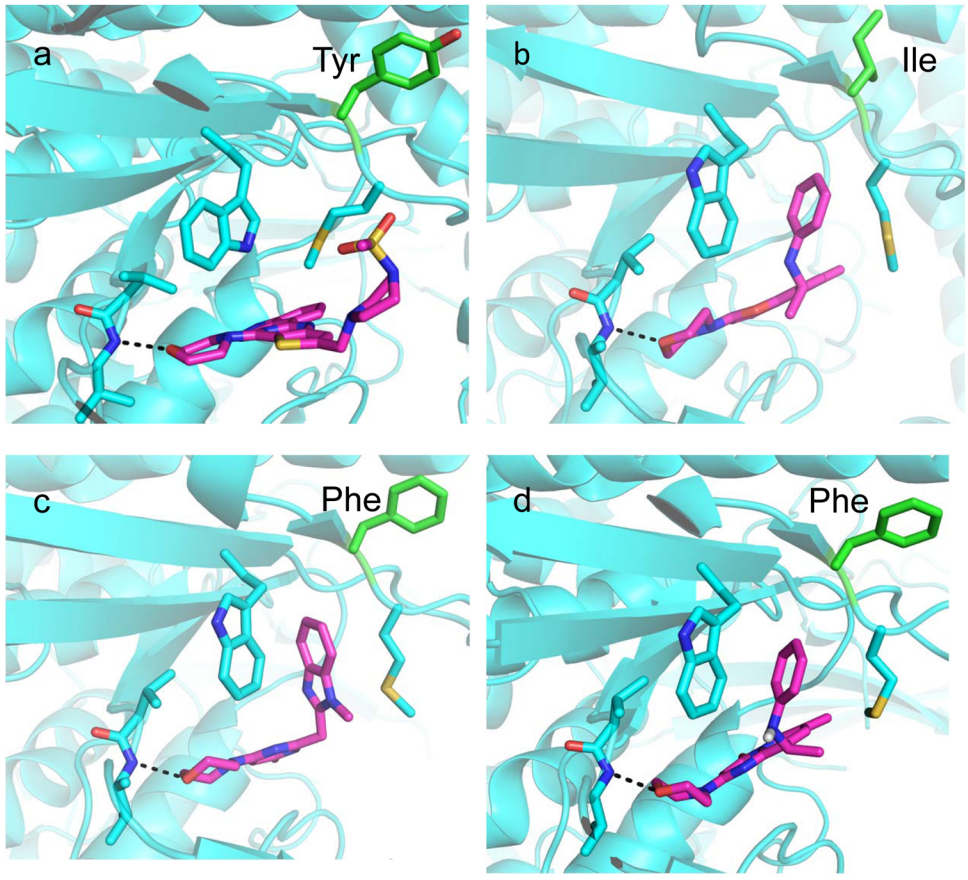

Figure 1. Structures of PI3K isoforms highlighting the active site nonconserved residues corresponding to Y778 of PI3Kβ and the conformation of the adjacent conserved methionine: (a) GDC0941 in PI3Kβ (2Y3A); (b) PIK108 in PI3Kα (4A55); (c) compound VWN in PI3Kδ (4AJW); (d) model of (R)-TGX221 in PI3Kδ. Note that the side chain projects away from the active site and is not predicted to participate directly in binding.

Here, we report on the dramatic influence of a single tyrosine residue Y778 and equally its counterpart in PI3Kα I771 in determining the potency of four chemically distinct PI3Kβ-selective inhibitors at each isoform. On the basis of crystallographic evidence, these residues play no direct part in the binding of the inhibitor, nor do they appear to change conformation (Figure 1). Critically though, the side chains appear to influence the conformers available to the loop formed by the adjacent residues of PI3Kβ 779−788. In doing so, they dictate the ability of the PI3Kβ isoform to adopt the methionine shifted conformation and thus the selectivity of these four inhibitors for PI3Kβ over PI3Kα.

Our approach exemplified in earlier work has been to screen PI3K inhibitors of interest against a range of point mutant forms of the target enzyme. In this case, we screened four PI3Kβ inhibitors TGX221, TGX286, GSK2636771, and SAN7[11] (compound 7 of Certal et al.[11]) against mutant forms of PI3Kβ where the nonconserved residues of the binding site were present as their equivalent form in PI3Kα. We screened the four compounds at or near their IC_{50} value against WT PI3Kβ (Figure 2). The results clearly show that, in each case, Y778I−PI3K was poorly inhibited by the compounds. Only modest effects were seen against PI3Kβ mutated at the other nonconserved residues.

On the basis of the screening data shown above, the IC_{50} values of the compounds were determined for both the Y778I−PI3Kβ mutant and the reciprocal mutant I771Y in PI3Kα (Table 1 and Supplementary Table 1). Each of the compounds shows a shift in IC_{50} associated with the Y778I−PI3Kβ and the reciprocal mutant I771Y−PI3Kα as compared to the corresponding wild-type isoform. Most dramatically, the IC_{50} for GSK2636771 versus PI3Kβ changes by 150-fold upon mutation. The reciprocal mutation in PI3Kα changes it from inactive to an IC_{50} of 1.6 μM, at least a 60-fold change. TGX-221 and SAN7 show lesser but still striking changes in activity. Essentially, each compound prefers to inhibit a form of each enzyme where the tyrosine residue rather than an isoleucine residue is present. Note that the mutations have no substantial effect on the catalytic properties of the enzyme (Supplementary Table 2).

The results described above clearly identify the tyrosine residue Y778 as a pivotal regulator of PI3Kβ-isoform selectivity with respect to PI3Kα, and this may extend to the relative selectivity versus the PI3Kγ and PI3Kδ isoforms where the residue at this position also differs. Inspection of the collected crystallographic data for PI3K inhibitors provides a mechanistic rationale to explain the data. First, sequence alignment shows a pivotal distinction between the four class I PI3K isoforms. At PI3Kα, β, γ, and δ, the residue at this position is isoleucine, tyrosine, valine, or phenylalanine, respectively (Supplementary Figure 1). This provides a clear distinction between the two isoforms bearing an aromatic residue (PI3Kβ and PI3Kδ) and those containing an aliphatic residue (PI3Kα and PI3Kγ). Noting that TGX221, TGX286, GSK2636771, and SAN7 all show less selectivity for PI3Kδ and the propeller-style inhibitors all prefer PI3Kβ and/or PI3Kδ, this implies that the aromatic

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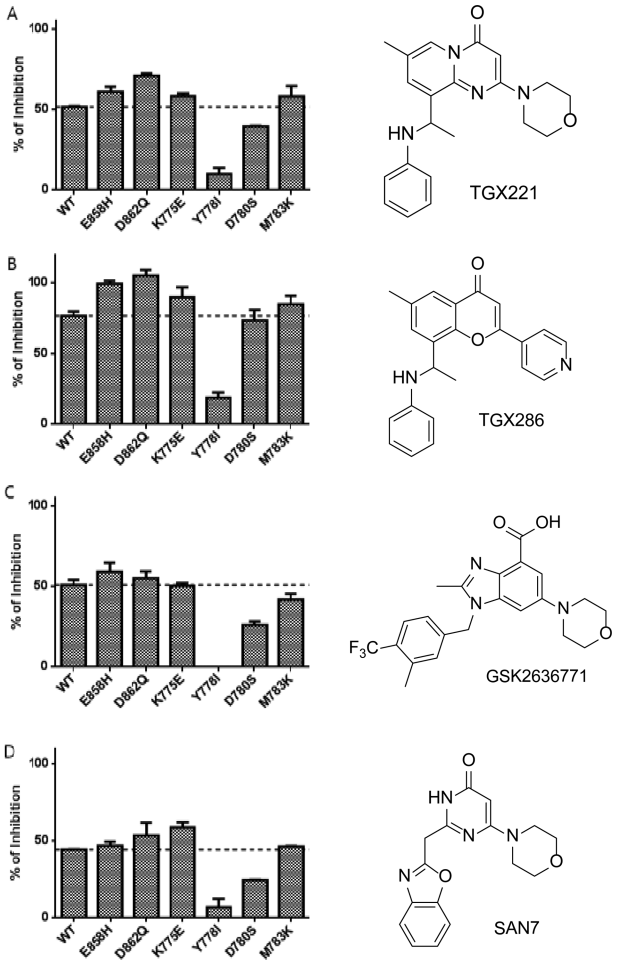

Figure 2. Effect of PI3Kβ isoform mutations on compound inhibition profiles at selected concentrations: (A) TGX221, 100 nM; (B) TGX286, 120 nM; (C) GSK2636771, 50 nM; (D) SAN7, 200 nM. Purified p110β WT and mutant enzymes were prepared as described in the Supplementary Methods section. Reciprocal mutants have the WT amino acid replaced by the equivalent amino acid in p110α. The PI3K activity assay was carried out as described in the Supplementary Methods section. Error bars represent standard deviation from two independent experiments.

residues in some way facilitate formation of the methionine-open conformer of PI3K. In addition to the striking loss of affinity resulting from the replacement of the tyrosine by isoleucine in PI3Kβ, the increase in potency when tyrosine replaces I771 in PI3Kα is also important as it shows that the tyrosine residue enables formation of an otherwise poorly accessed conformer.

So how does the residue at this position actually influence ligand binding? Two features stand out when the available crystallographic data relating to PI3K is assessed. First, in superposition of all the PI3K structures, the residue at this position is effectively fixed, irrespective of isoform or ligand binding mode, and the backbone and Cα−Cβ across all these structures basically superimpose (Figure 1). Second, this residue does not participate directly in ligand binding in any cocrystal; the side chain points away from the catalytic site. Taken together, this suggests that the residue at this position acts as a pivot point for the residues around it, directing the conformational outcomes of the solvent exposed P-loop residues to ultimately favor or disfavor the two identified conformers of the adjacent methionine. How the nature of the amino acid actually drives those conformational choices is still a challenging question to answer, particularly as the crystal structures for PI3Kβ and PI3Kα have only been solved to moderate resolution.

Another feature to be drawn from the data is the relative change in selectivity with each of the inhibitors. While the selectivity of GSK2636771 changes over 9000-fold upon reciprocal mutation, SAN7 and TGX221 show a more modest change. The likely explanation is that SAN7 and probably TGX221 can be accommodated in different binding modes, probably in the flat pose adopted by compounds such as GDC0941, while GSK2636771 cannot be.

We believe these results have multiple levels of significance for the study of PI3K inhibition. In the first instance, it provides a straightforward model for understanding inhibitor binding that can be tested experimentally, which is always of great use in ligand design. The reciprocal mutant system can also be used to identify mechanistic differences underpinning selectivity in other compound classes or other isoforms. We have recently described a novel class of PI3Kβ selective inhibitors that achieve selectivity through a distinct mechanism.[20] These compounds are unaffected by the Y778I mutation, but another residue, D862, selects for PI3Kβ via a direct interaction with an amino-acyl functional group on the inhibitor.

Second, identifying single-point changes that render the enzyme opaque to pharmacological inhibitors without an effect on catalytic activity provides for the development of exquisite systems for studying the cell based functions of PI3Kβ. As Y778I−PI3Kβ is insensitive to GSK2636771, it might be included in isogene replacement studies to characterize the specific effects of that isoform, particularly in models of cancer. The mutation has only a small influence on the catalytic activity of the enzymes (see Supplementary Table 2). It also presages a simple pathway by which a cancer cell might generate resistance to GSK2636771 or other propeller-shaped inhibitors.

Table 1. Inhibition of PI3K Isoforms and in Vitro Mutants

	PI3K isoform IC_{50} (nM)[a]						
compound	PI3Kα WT	PI3Kα I771Y	fold change αWT → α I771Y	PI3Kβ WT	PI3Kβ Y778I	fold change βWT → β Y778I	reciprocal change[b] Y ↔ I
TGX221	4040	280	↓14.5	65	1000	↑16	230
TGX286	>100000	1100	↓>90	130	2600	↑20	>1800
GSK2636771	>100000	1600	↓>60	61	9400	↑150	>9200
SAN7	9600	2200	↓4.4	240	2300	↑9.6	42

[a]IC_{50} values are means of at least three duplicate experiments (see Supplementary Table 1 for standard deviations of mean data). Assays were performed using 10 μM ATP as described in the Methods section. [b]Reciprocal change refers to the change in selectivity of inhibitor due to reciprocal mutation.

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Finally, these results show the subtlety that underpins conformational plasticity in the binding site. In the design of anticancer drugs, the concept of selectively inhibiting one of the oncogenic mutants of PI3Kα is an attractive but seemingly intractable task. We have shown in our results here that that allosteric effects can result in binding site differences between wild-type and mutant forms of PI3K. The corollary is that there might be active site conformers that are available to oncogenic PI3Kα that are not available to the wild-type enzyme. The challenge will be to find the inhibitors that can exploit those forms.

METHODS

Generation of PI3K, p110 Mutant Baculovirus. The methods used to generate the PI3K p110 mutants have been described previously.[17] Briefly, the mutant DNA was produced using a pair of oligos incorporating the appropriate mutation and PCR using Pfu DNA polymerase (Promega) with the appropriate wild-type p110 pFastBac (Life Technologies) plasmid as the DNA template. The DNA sequence was then confirmed as containing the correct mutation with the remaining DNA sequence found to be identical to the WT sequence. Mutant pFastBac plasmids were then transformed into DH10Bac *E.coli* (Life Technologies) with bacmid DNA purified and confirmed as recombinant using PCR.

Recombinant bacmid DNA was then transfected, using lipofectin (Life Technologies), into Sf21 insect cells with the medium containing recombinant virus collected after 3−5 days incubation at 27 °C. High titer virus stock was then produced by amplification through two cycles of infection. Production of p110 protein was confirmed by Western blotting of cell extracts separated by SDS-PAGE using a p110α or β specific antibody.

Protein Expression and Purification. SF21 cells (2×10^6 cells/mL, 200 mL total volume) were cotransfected with p110 and p85 recombinant baculovirus and incubated shaking at 140 rpm for 48 h at 27 °C. Following this, cells were collected by centrifugation and stored at −80 °C until ready for extraction. The p110/p85 PI3K protein complex was extracted from the cells and purified using Ni-agarose chromatography as previously described.[16] Fractions containing the PI3K protein were pooled and dialyzed against 50 mM Tris HCl, pH 7.5, 300 mM NaCl, at 4 °C. PI3K protein was then stored in 20% (v/v) glycerol and 2 mM dithiothreitol at −80 °C. Each mutant was characterized by measuring the kinetic parameters (K_m ATP, and K_m PI phosphatidyl inositol) of the PI3K enzyme reaction, and in each case, it was shown that there was no difference between the WT enzyme and the mutants.

Inhibitors. TGX221 (7-methyl-2-morpholino-9-(1-(phenylamino)-ethyl)-4*H*-pyrido[1,2-a]pyrimidin-4-one) was a generous gift of Prof. Shaun Jackson, Australian Centre for Blood Diseases, Victoria, Australia. TGX286 (6-methyl-8-(1-(phenylamino)ethyl)-2-(pyridin-4-yl)-4*H*-chromen-4-one) was synthesized according to Knight et al.[6] GSK2636771 (2-methyl-1-(3-methyl-4-(trifluoromethyl)benzyl)-6-morpholino-1*H*-benzo[*d*]imidazole-4-carboxylic acid) was obtained from Active Biochem (New Jersey, U.S.A.). SAN7 (2-(benzo[*d*]-oxazol-2-ylmethyl)-6-morpholinopyrimidin-4(3*H*)-one) was synthesized according to the method described in Certal et al.[11]

Inhibition Assays. The PI3K inhibitors were dissolved at 10 mM in dimethyl sulphoxide (DMSO) and stored at −20 °C until use. PI3K enzyme activity was determined using a luminescence assay measuring ATP consumption. PI3K enzyme activity was determined in 50 μL of 20 mM Hepes, pH 7.5, 5 mM $MgCl_2$ with PI and ATP at the indicated concentrations.[17] After a 60 min incubation at RT, the reaction was stopped by the addition of 50 μL of Kinase-Glo (Promega) followed by a further 15 min incubation. Luminescence was then read using a Fluostar plate reader (BMG Labtech). Inhibitors were diluted in 20% (v/v) DMSO at the indicated concentrations in order to generate a concentration versus inhibition of enzyme activity curve, which was then analyzed using GraphPad Prism (version 6.00 for Windows) in order to calculate the IC_{50}.

ASSOCIATED CONTENT

Supporting Information

Sequence alignment of region 1 and region 2 of nonconserved amino acids; IC_{50} determinations including standard deviations of the mean for data presented in Table 1; biochemical characterisation of WT and selected mutant recombinant isoforms. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Author Contributions

Z.Z. performed the biochemical experiments; M.S.M. performed the structural analyses and performed docking experiments; P.E.T. performed the chemical syntheses. I.G.J. and P.E.T. designed the experiments and wrote the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ATP, adenosine triphosphate; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homologue

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