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Isoform selective inhibition of Phosphoinositide 3-kinase: Identification of a new region of non-conserved amino acids critical for p110α inhibition.

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Short title:

Isoform selective inhibition of PI 3-kinase p110α

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Abbreviations:

Phosphoinositide 3-kinase (PI3K), phosphatidylinositol (PI), phosphatidylinositol-3,4,5-triphosphate (PIP_3)

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Abstract

The combination of molecular modelling and x-ray crystallography has failed to yield a consensus model of the mechanism for selective binding of inhibitors to the phosphoinositide 3-kinase (PI3K) p110 α-isoform. Here we have used kinetic analysis to determine that the p110α selective inhibitor, PIK-75, is a competitive inhibitor with respect to a substrate, phosphatidylinositol (PI) in contrast to most other PI3K inhibitors which bind at or near the ATP site. Using sequence analysis and the existing crystal structures of inhibitor complexes with the p110γ and δ isoforms we have identified a new region of non-conserved amino acids (region 2) which was postulated to be involved in PIK-75 p110α selectivity. Analysis of region 2, using *in vitro* mutation of identified non-conserved amino acids to alanine, showed that S773 was a critical amino acid involved in PIK-75 binding with an 8-fold-increase in the IC-50 compared to wild-type. Kinetic analysis showed that, with respect to PI, the PIK-75 Ki for the isoform mutant S773D increased 64-fold as compared to wild type enzyme. In addition, a non-conservative amino acid, H855 from the previously identified region 1 of non-conserved amino acids was found to be involved in PIK-75 binding. These results show that these two regions of non-conserved amino acids that are close to the substrate binding site could be targeted in order to produce p110α isoform selective inhibitors.

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Introduction

Phosphoinositide 3-kinase (PI3K, EC 2.7.1.153) is the member of the lipid kinase family responsible for the production of the key cellular second messenger molecule, phosphatidylinositol-3,4,5-triphosphate (PIP_3). PIP_3 is involved in regulating cellular downstream molecules directing cell growth, survival, differentiation and chemotaxis (Vanhaesebroeck et al., 2010). In addition, the PI3K gene is one of the most frequently mutated genes found in tumours which has lead to PI3K being an attractive potential drug target for cancer (Samuels et al., 2004). Class 1 PI3Ks consist of four isoforms containing either the α, β, γ, or δ catalytic subunit (p110) bound to a regulatory subunit. The catalytic subunits of the four PI3K isoforms have highly conserved amino acid sequences, particularly in the ATP binding site, in the catalytic pocket where most small molecule inhibitors have been shown to bind (Marone et al., 2008). This has made it difficult to produce small molecule inhibitors that discriminate between the isoforms. Isoform selective inhibitors have the potential to reduce toxicity by decreasing off-target effects and specifically p110α-inhibitors have the potential to target tumours which contain a mutated or amplified p110α.

There are several PI3K drugs in Phase 1 clinical trials with the majority of these being pan-PI3K inhibitors binding at the ATP binding site. The lone exception is the δ-specific inhibitor, CAL101, which has been shown to produce a conformational change in the binding site exposing a specificity pocket into which the inhibitor binds (Bowles and Jimeno, 2011; Workman et al., 2010).

One of the first potent p110α inhibitors reported was an imidazo[1,2]-pyridine, PIK-75, developed by researchers at Yamanouchi (now Astellas Pharma) and Piramed (Hayakawa et al., 2007b) (Figure 1). This molecule and its related analogues are somewhat unique in the field principally due to the impressive potency and isoform selectivity at p110α. On the other

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hand, PIK-75 exhibits cellular toxicity and inhibits numerous other kinases which has limited its clinical progression (Knight et al., 2006). With these features in mind it is important to understand the molecular basis of this potent inhibition so that second generation inhibitors might be developed.

In the absence of structural data regarding the interaction between PIK-75 and p110α information can be obtained both from SAR studies and modelling of the compound into homology models generated from known PI3K crystal structures.

From the published SAR data (Hayakawa et al., 2007a), it was shown that the potency of PIK75 and analogues was derived from the bromine substituent, the sulphonyl group and the 2-methyl-5-nitro phenyl ring (Figure 1) but no information was available on the influence of these substitutions on selectivity. Recently a further series of substituted imidazopyridines was reported where the methyl group has been replaced by amino, glycinyl and even trifluoacetylamino groups increasing p110α potency and maintaining isoform selectivity (Schmidt-Kittler et al., 2010). Four models of the PIK75/p110α complex generated by different methods have been reported (Frederick and Denny, 2008; Han and Zhang, 2009; Li et al., 2010; Sabbah et al., 2010) and each model fundamentally differs from the other with respect to the identification of non-conserved amino acids responsible for the PIK-75 p110α selectivity.

Given that the mechanism of p110α selectivity remains elusive we have taken a novel biochemical approach, in the first instance looking in detail at the kinetics of PIK-75 inhibition of p110α enzyme activity, and then *in vitro* mutagenesis of the binding site was used to ascertain which regions have the most influence on PIK-75 binding.

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To this end we have determined that PIK-75 is not a simple ATP-competitive inhibitor but rather shows mixed inhibitor kinetics versus ATP, and is in fact a competitive inhibitor of the lipid substrate PI. Secondly we have found that PIK75 binding is sensitive to mutations in specific non-conserved regions of the binding pocket. Two non-conserved amino acids, S773 and H855, from two different regions of the PI3K binding pocket have been shown to be critical for the binding of PIK-75. These results have shown that these two non conserved amino acids are responsible for the p110α selectivity of PIK-75 and that this region should be effectively targeted to produce other p110α-selective inhibitors.

Materials and Methods

Generation of baculovirus containing p110α mutant DNA

The methods used here have been described previously in Frazzetto et al, (Frazzetto et al., 2008) with the exception that the pFastBac™ system (Invitrogen, U.S.A.) rather than the pBakPak system (Clontech Labs, U.S.A.) was used to generate recombinant baculovirus. In brief, mutant plasmids were generated using the appropriate primer pair and Pfu DNA polymerase (Promega, U.S.A.) with the template DNA being either pFastBac™ wild-type p110α. The DNA sequence was then confirmed as containing the correct mutation with the remaining DNA sequence confirmed as being identical to wild-type. Mutant plasmids were then transformed into DH10Bac *E.coli* for transposition into the bacmid. Blue/white selection was used to select for colonies containing recombinant bacmids with presence of the recombinant DNA in the bacmid confirmed using PCR. Recombinant bacmid DNA was then transfected, using lipofectin (Invitrogen, U.S.A.), into Sf21 cells and supernatant containing recombinant virus was collected after 3-5 days at 27°C. High titre virus stock was then

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produced by amplification through two cycles of infection. Production of p110α protein was confirmed by western blotting of cell extracts separated by SDS-PAGE using a p110α specific antibody.

Protein expression and purification

Twenty ml p110α virus and 5 ml p85 virus were added to 200ml of SF21 cells (2 x 10^6 cells/ml) and incubated shaking at 140rpm for 48h at 27^{o}C. Following this cells were collected by centrifugation and stored at -80^{o}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 (Frazzetto et al., 2008). Fractions containing the PI3K protein were pooled and dialysed against 50mM TrisHCl pH7.5, 300mM NaCl at 4^{o}C. PI3K protein was then made 20% (v/v) glycerol and 2mM dithiothreitol and stored at -80^{o}C. .

Inhibition assays

The PI3K inhibitor, PIK75 (Figure 1) (Calbiochem, U.S.A.) was dissolved at 10mM in dimenthyl sulphoxide (DMSO) and stored at -20^{0}C. until use PI3K enzyme activity was determined in 50µl of 20mM Hepes pH 7.5, 5mM $MgCl_2$ containing 180µM phosphatidyl inositol with the reaction started by the addition of 100µM ATP (containing 2.5µCi of γ-P^{32} ATP). After a 30 min incubation at room temperature the enzyme reaction was stopped by the addition of 50µl of 1M HCl. Phospholipids were then extracted with 100µl of chloroform/methanol (1:1, v/v) and 250µl 2M KCl followed by scintillation counting. 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 analysed using GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego California USA) in order to calculate the IC-50.

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For kinetic analysis a luminescent assay measuring ATP consumption was used. PI3K enzyme activity was determined in 50µl of 20mM Hepes pH7.5, 5mM $MgCl_2$ with PI and ATP at the indicated concentrations. After a 60min incubation at room temperature the reaction was stopped by the addition of 50µl of Kinase-Glo (Promega) followed by a further 15 min incubation. Luminescence was then read using a Fluostar plate reader (BMG Labtech). Results were analysed using GraphPad.

Results

Characterisation of the kinetics of PIK-75 inhibition of p110α

The kinetics of PIK-75 inhibition of PI3K p110α was studied in more detail as shown in Figure 2. Curves of p110α enzyme activity versus ATP concentration at varying PIK-75 concentration are shown in Figure 2a and when this data was analysed using a Lineweaver-Burk plot it showed that PIK-75 was a mixed inhibitor with respect to ATP with increasing inhibitor decreasing the Vmax (y-intercept, Fig. 2b) but having some effect on the Km (x-intercept, Fig. 2b). However kinetic analysis using the mixed-inhibition mode (GraphPad Software) showed that PIK-75 inhibition was non-competitive with respect to ATP where the value of the constant α, was less than 1 (0.3) and the Ki for PIK-75 with respect to ATP was 36±6nM (n=4). This result is in contrast to the pan-PI3K inhibitor, ZSTK474, which has been previously shown to be a competitive inhibitor with respect to ATP (Kong and Yamori, 2007). Indeed, using our assay conditions here, ZSTK474 was shown to be a competitive inhibitor of p110α with respect to ATP and a mixed inhibitor with respect to PI (results not shown).

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Kinetics of PIK75 inhibition, with respect to the substrate (PI), was found to be competitive as shown by the Lineweaver-Burk plots (Fig. 2d) in which the lines intersected on the y-axis indicative of competitive inhibition. In addition, this data was analysed using the mixed-model of inhibition where the value of the constant,α, was shown to be greater than 1 (ie. 30) indicative of competitive inhibition. The K_i of PIK-75 with respect to PI was estimated to be 2.3 ± 0.6nM (n=6).

Thus both the Lineweaver-Burk plots and the mixed-inhibition model show for the first time that PIK-75 is a non-competitive inhibitor with respect to ATP and competitive with respect to the substrate, PI with a Ki value for PI considerably lower than that of ATP.

Identification of a new region of amino acid sequence heterogeneity

The first step in the identification of critical non-conserved amino acids involved in the selective binding of PIK-75 binding to p110α was a comparison of the amino acid sequences of the four class 1 p110 isoforms. This was undertaken in conjunction with an analysis of the existing crystal structures of the unliganded PI3Ks and those bound to small molecule inhibitors.

Previously we have identified Region 1 which contained two non-conserved amino acids His855 and Q859 in p110α. When mutated to the equivalent amino acid in p110β, the mutant enzymes were found to be significantly less inhibited by a p110α-selective inhibitor. From known crystal structures these amino acids were found to be located at the entrance to the enzyme catalytic cavity and also in the presence of inhibitors were found to be in different orientations (Frazzetto et al., 2008). It was postulated that exploiting the ability of these non-conserved amino acids to adopt different conformations could lead to the isolation of p110α

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isoform-selective inhibitors. Sequence analysis of Region 1 identified two additional non-conserved p110α amino acids, Arg-852 and Asn-853 which we have mutated here for inhibitor analysis (Figure 3a).

In an attempt to identify additional sequences that might confer isoform selectivity, a comparison of the five p110γ crystal structures available at the time was made. In the presence and absence of ligands, structural changes were identified in a region (designated as Region 2) of the p110α sequence that contains non-conserved amino acids as shown in Figure 3b. This sequence includes the PI3K equivalent of the p-loop or "glycine-rich" loop identified in protein kinases as being involved in ATP binding (Huse and Kuriyan, 2002; Johnson et al., 1996) which has previously been shown to be flexible in its ability to bind ATP in the presence of different peptide substrates. In contrast to the protein kinases this sequence contains no glycine residues. The binding of another PI3K inhibitor PIK-39 to p110γ (PDB no. 2CHW) and to p110δ (PDB no.: 2WXF), as observed in the crystal structure of the enzyme: inhibitor complex, induced a conformational change of Met804(γ)/758(δ) to an "open" position revealing a new "specificity" pocket in which PIK-39 bound (Figure 4b,c). By comparison Met804 was found in a "closed" conformation in the structure of ATP bound to p110γ (Figure 4a). It is important to note that Met804 is a conserved amino acid of the binding pocket and thus cannot generate selectivity *per se.* However, sequence comparisons in region 2 of the PI3K p110 isoforms show that several amino acids surrounding the conserved methionine are not conserved and would be expected to influence the conformation of this loop (Figure 3b). Also, in the superposition of p110α and p110δ crystal structures, the sequence around the methionine has a relatively high RMSD for the overlay of α-carbons (Figure 4). Thus, the heterogeneity of the residues in this region would appear to have a role in dictating the conformation of the backbone and possibly the capacity

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to expose unique binding site surfaces. In particular, the Lys802 residue, exposed in the p110γ/PIK-39 structure [pdb: 2chw] is an arginine (Arg770) in p110α and a threonine (Thr756) in p110δ. Side chains of other residues that point into the inhibitor cavity are not conserved as shown in Figure 3b and it seems plausible that this heterogeneity is a key contributor to selectivity.

In addition comparison of structures of unliganded p110α (PDB no.:2RD0 and 3HIZ) (Figure 4d) and the complex of covalently bound inhibitor, wortmannin, and p110α protein (PDB no.: 3HHM) (Figure 4e) showed that the p-loop (amino acids 772-777) shifted 3Å in the presence of the inhibitor. In contrast the equivalent p110γ complex (PDB no.:1E7U) showed no difference in the conformation of this loop upon wortmannin binding (not shown). This was postulated by the authors to be an indication that this region could be targeted in the development of p110α isoform selective inhibitors (Mandelker et al., 2009).

Analysis of Region 2 non-conserved amino acids by alanine scanning mutagenesis

Six non-conserved amino acids in Region 2, E768, R770, I771, S773, K776 and R777 were mutated to alanine in order to assess their role in binding PIK75. *In vitro* mutants were generated using the PCR reaction as described in the Methods section. The resulting six mutant pFastBac plasmids were then used to generate recombinant bacmids using DH10Bac *E.coli* competent cells. Bacmid DNA was transfected into Sf21 insect cells and the resulting recombinant virus was amplified. Protein expression was achieved by co-infection of Sf21 cells with p110α mutant or WT virus with p85 virus for 48h. Cells were then lysed and PI3K protein was purified using Ni-agarose affinity chromatography as described in the Methods. Yields of protein for all alanine mutants were comparable to the WT enzyme.

The purified alanine mutants were then tested for their ability to be inhibited by PIK-75 at a concentration of 50nM as compared to the wild-type enzyme. As shown in Figure 5a all of the mutations showed a decrease in PIK-75 inhibition indicating that this region is important in PIK-75 binding. The largest decrease in PIK-75 inhibition was seen with the S773A mutation indicating that this amino acid was critical in PIK-75 binding. S773A is an isoform specific mutation with the αS773 equivalent amino acid in p110γ being alanine. As a control for this set of assays all the alanine mutants were tested for inhibition with the pan-PI3K inhibitor, LY294002, and as seen in Figure 5b all mutants showed essentially identical inhibition to the wild-type enzyme. R770A which is not included on this graph was separately shown to have a similar IC-50 for LY294002 as the WT enzyme (0.24 vs. 0.76μM). In addition the region 2 alanine mutants showed no difference in inhibition by another pan-PI3K inhibitor, ZSTK-474 when compared to the WT enzyme (results not shown).

So, from this, S773 was identified as the important non-conserved amino acid in region 2 but mutation throughout region 2 has minor effects on inhibition perhaps pointing to a conformational role for this flexible loop region in binding to PIK-75.

Isoform specific mutations in Region 2 and its effect on PIK-75 inhibition

Following on from the results of the alanine scan of region 2 S773 was mutated to aspartate, the equivalent amino acid in the p110 β and δ isoforms. Mutant protein was expressed and purified as described above.

The amino acid, S773, is next to the conserved S774 on the p110α sequence, the equivalent S806 was shown in the P13Kγ/ATP crystal structure (1E8X) to be interacting with an oxygen

of the β-phosphate on the ATP molecule (at a distance of 2.5Å). Therefore the Km for ATP was measured to determine the effect of the mutation on the structure of the ATP binding site. Table 1A shows the Km, ATP determination for the WT, S773A and S773D mutants. S773A has a similar Km to the WT enzyme with S773D showing a Km twice that of WT but as the enzyme assay for inhibition was carried out at 100μM ATP all enzymes' activity should be at or near saturating levels of ATP. The Km ATP for all the region 2 alanine mutants described above was estimated to be not significantly different from the WT enzyme (results not shown) indicating that the ATP binding site was essentially intact and unchanged by the region 2 mutations.

Next the PIK-75 IC-50 was determined for the isoform specific mutants at S773 and it was shown that both the S773A and S773D mutants increased the IC-50 of PIK-75 by 8-fold (Table 1A) indicating that S773 was a critical amino acid in the binding of PIK75 to p110α.

Further kinetic experiments were undertaken to determine the effect of PIK-75 on the kinetics of binding of ATP and PI to the p11α, S773D mutant. Activity was estimated using a range of PI concentrations at the concentrations of 0, 50, 100 and 200nM PIK-75. Using the mixed-model of inhibition (GraphPad) it was estimated that the Km for PI was 11.2μM compared to 7.0μM for the wild-type enzyme (Table 1B). The Ki for PIK-75 was estimated to be 146nM a 64-fold increase on the value estimated for the wild-type enzyme (2.3nM). Therefore these results show that the mutant, S773D, has reduced the ability of PIK-75 to competitively inhibit the binding of PI to PI3K but does not significantly affect the direct binding of PI to the enzyme as evidenced by the unchanged PI Km of the α S773D mutant.

Despite the R770A mutation showing modest effects on PIK-75 inhibition we investigated the effect of isoform mutations at R770 due to the structural evidence of this non-conserved amino acid's mobility in several PI3K/ inhibitor complexes as demonstrated in Figure 4. As

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such the mutants R770T, p110δ equivalent amino acid, and R770K, p110β/γ equivalent amino acid were produced, expressed and purified. These were shown to have normal Km for ATP and unchanged IC-50 for the pan PI3K inhibitor, LY-294002, indicating that the ATP binding site and the inhibitor binding site were intact and functioning as in the WT enzyme. The IC-50 for PIK-75 was unchanged in comparison to WT indicating R770 was not directly involved in PIK-75 binding.

Isoform specific mutations in Region 1 and its effect on PIK-75 inhibition

The previously identified region 1 contains two non-conserved amino acids that potentially could be involved in the selective binding of PIK-75. As seen from Table 1 the isoform mutant p110α, H855E showed a 2.4-fold increase in PIK-75 IC-50. While this increase was statistically significant it was less than that observed with the region 2 S773 mutants. The other region 1 isoform mutant, p110α Q859K showed no change in PIK-75 IC-50 (results not shown). In addition replacement of the side chain of two other non-conserved amino acids, R852 and N853, with alanine had little effect on PIK-75 inhibition.

Discussion

Using a known p110α-selective inhibitor, PIK-75, we have identified a new region (Region 2) of non-conserved amino acids that should be a target for the production of p110α specific inhibitors in the future. Kinetic analysis has shown that PIK-75 is a non-competitive inhibitor of ATP binding but a competitive inhibitor of the substrate, PI. Mutation at the Region 2 amino acid, S773, increased the Ki and IC-50 for PIK-75 without significantly changing the Km for PI indicating that S773 is critical for PIK-75 inhibitor binding but not for PI binding. In addition, to a lesser extent, the mutation at the Region1 non-conserved amino acid, H855, was involved in the binding of PIK-75 but again had little direct effect on PI binding.

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Our results here are consistent with the model of (Frederick and Denny, 2008) where S773 is proposed to bind to the nitro group of the phenyl ring and H855 was proposed to bind to the sulphonyl group with the bromine substituent interacting with the hinge region invariant valine. The modelling would predict that either mutation of S773 or removal of the nitro group from the phenyl ring would cause a significant drop in potency of PIK-75 inhibition which is exactly what we have observed. It is noteworthy that three subsequent models of PIK-75 binding predicted no role for S773.

The only PI3K inhibitors previously analysed kinetically have been shown to be competitive inhibitors with respect to ATP but kinetics with respect to phospholipid substrate was not shown (Camps et al., 2005; Kong and Yamori, 2007; Workman et al., 2010). The crystal structures of these inhibitors bound to p110 showed that they interacted with conserved amino acids only (Berndt et al., 2010; Camps et al., 2005). It could be speculated that targeting the ATP binding site yields potent but not α-selective inhibitors where as targeting the phospholipid binding site produces a more α-selective inhibitor.

The crystal structure of p110γ in complex with ATP identified two key conserved amino acids that bound to the ATP phosphate groups (Walker et al., 2000). They are S806 bound to the β-phosphate and K833 to the α-phosphate which are mimicking the role of the backbone interactions between ATP and the flexible glycine loop in the classical serine/threonine protein kinases. The equivalent loop in PI3K (kβ3-kβ4) has no glycines in the sequence. The observation that a key non-conserved amino acid for the selective binding of PIK-75, S773 (equivalent of p110γ A805) is the adjacent amino acid to the conserved serine may lead to the suggestion that mutation of the adjacent amino acid would affect the binding of ATP. However the kinetics of PIK75 inhibition with respect to ATP did not show this with non-competitive inhibition observed and S773 mutants showed no significant change in Km for

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ATP compared to the WT enzyme. Examination of the crystal structures of unliganded p110α (PDB 2RD0) confirmed that the side chain of this amino acid (α-Ser & δ-Asp) was pointing away from the ATP binding serine.

The fact that PIK-75 is a competitive inhibitor of p110α with respect to the substrate was surprising but previous functional studies had pointed to the possible involvement of region 2 amino acids in the phosphoinositide substrate selectivity of the four class 1 PI3K isoforms. The serine/threonine protein kinase family contains a sequence in the active site which is involved in the substrate specificity of binding known as the activation sequence. In the PI3K family a similar loop sequence has been found which has been postulated to determine lipid substrate specificity. A study where the activation loop in the class I PI3K p110α was substituted with sequences from class II, class III and class IV PI3Ks showed that the activation loop was involved in lipid substrate specificity (Pirola et al., 2001). Further *in vitro* mutagenesis studies showed that two basic amino acids, K942 and R949, in the activation loop are responsible for the binding of PIP_2 substrate. Interestingly, although the p110α mutant containing the class 11 or class 111 activation loop was unable to phosphorylate PIP_2, PIP_2 was able to inhibit the binding of the covalent PI3K inhibitor, wortmannin, showing that the substrate was bound to the mutant enzyme but was not catalysed. This indicated that other regions of the active site may be involved in the binding of PIP_2. Subsequent modelling studies proposed that K942 bound to the 5-phosphate on the PIP_2 and R949 bound the 4-phosphate on the PIP_2. In addition it was found that the non conserved amino acid in Region 2, K776, was involved in PIP_2 binding. In all crystal structures described thus far the activation loop is disordered and not able to be seen suggesting that the loop is flexible or disordered. Gabeli et al (Gabelli et al., 2010) in their p110α structures comparing unliganded enzyme to enzyme bound to wortmannin noted that the loop containing amino acids 772-776

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(within region 2) changes conformation. Their modelling studies suggested that this loop was in the binding site for PIP_2 and that this mode of binding correctly positioned the lipid for phosphoryl transfer from ATP. However this model contradicts the mutagenesis experiments described above. This conformational change appears to be isoform-specific as it does not occur when wortmannin binds to p110γ.

A conformational basis for isoform selectivity amongst the PI3K isoforms was demonstrated by the complexes of the "propeller" inhibitors, IC-87114 and PIK-39 with p110δ (Berndt et al., 2010). It was shown that Met-752, p110δ, in a "closed" conformation in the apo enzyme underwent a conformational change to the "open" conformation when the inhibitor bound exposing a specificity pocket to which one blade of the propeller inhibitor bound. Previously it had also been shown that this conformational change occurred in p110γ (Knight et al., 2006) as well but more recently molecular simulation calculations were used to show that this conformational change was more energetically favourable in the δ isoform rather than the γ isoform explaining the selectivity in inhibition (Berndt et al., 2010). Perhaps the conformational change observed is influenced by the non-conserved amino acids of region 2 where the conserved Met-752 is located. It is feasible that given the right selective inhibitor a similar conformational change could be induced by the right p110α inhibitor.

Although there are now numerous crystal structures of PI3K inhibitor complexes few of these inhibitors are selective and few of the complexes have been shown to involve Region 2 non-conserved amino acids. (Berndt et al., 2010) have demonstrated that it is possible to develop a δ-specific inhibitor without accessing the "specificity pocket" to which the propeller-like PIK-39 and IC-87114 inhibitors bind. The tetrahydroquinazoline group of AS-15 was shown in a crystal structure with p110δ to bind closely to the conserved Region 2 hydrophobic amino acids, M772 and W780 (p110α amino acid sequence numbering) preventing the

conformational change and subsequent exposure of the specificity pocket. They postulated that the p110δ/ T756 reduced side chain size in comparison to the equivalent of R770 in α and lysine in β and γ, could explain the δ selectivity of this inhibitor. The binding surface was shown to be a small dimple to which the proximity of the larger arginine or lysine side chain may cause steric hindrance of AS15 binding. It could thus be suggested that the presence of arginine or lysine could influence selectivity. They also showed that AS15 made additional contacts with isoform-specific amino acids which could explain the specificity of this compound. The ketone oxygen of the tetrahydroquinoxoline formed a bond with the backbone amide of an aspartic acid that is the equivalent of S773 in α and alanine in γ and β and also with K708 which is also located in a region of sequence heterogeneity outside the active site. This lysine was also shown to be involved in the binding of GDC-0941 in p110δ although GDC-0941 is not a selective inhibitor. From these structures it can be seen that two region 2 amino acids were involved in the selective binding of AS15 to p110δ (Walker et al., 2000). However it is unlikely that this is the mechanism of PIK-75 binding to p110α as mutation at R770 had no effect on binding.

In conclusion, we have shown that *in vitro* mutagenesis is a valuable tool for determining non-conserved isoform selective amino acids critical in the binding of inhibitors and that kinetic analysis of enzyme inhibition provides information above and beyond the IC-50 measurement. Whilst PIK-75 has proven to be a useful *in vitro* tool its off-target reactivity and inability to target PI3K specifically *in vivo* make it far from the ideal drug (Torbett et al., 2008). As such the path to the production of a truly useful p110α selective inhibitor would appear to rely on targeting both Region 1 and Region 2 non-conserved amino acids using a different scaffold to avoid the off target reactivity.

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Author's contribution:

Participated in research design: Zheng, Thompson and Jennings.

Conducted experiments: Zheng, Amran and Jennings.

Contributed new reagents or analytic tools: Zheng, Amran and Jennings.

Performed data analysis: Zheng, Amran, Thompson and Jennings.

Wrote or contributed to the writing of the manuscript: Thompson , Jennings and Zheng.

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Footnotes:

Zheng & Jennings contributed equally to this manuscript.

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Figure legends:

Figure 1: PIK-75, p110α selective inhibitor

Chemical structure of PIK-75, 2-methyl-5-nitro-2-[(6-bromoimidazo[1,2-a]pyridin-3-yl)methylene]-1-methylhydrazide-benzenesulfonic acid.

*IC-50 for each of the four PI3K isoforms, p110α, β, γ and δ as determined previously (Knight et al., 2006)

Figure 2: Kinetic characterisation of PIK-75 inhibition of wild-type p110α

A. Kinetics of ATP binding to purified p110α in the presence of varying concentrations of inhibitor, PIK-75 (● none, ■ 20nM, ▲ 40nM and ▼ 80nM respectively) as shown by a plot of enzyme activity measured by the luminescent assay, versus ATP concentration.
B. Lineweaver-Burk plot of the kinetics of ATP binding to p110α in the presence of varying PIK-75 inhibitor concentrations (● none, ■ 20nM, ▲ 40nM and ▼ 80nM respectively).
C. Kinetics of substrate, phosphatidyl inositol (PI) binding to p110α in the presence of varying concentrations of inhibitor, PIK-75 (● none, ■ 15nM, ▲ 20nM and ▼ 25nM respectively), as shown by a plot of enzyme activity, as measured in the luminescent assay, versus PI concentration at the indicated inhibitor concentrations.
D. Lineweaver-Burk plot of the kinetics of PI binding to p110α in the presence of varying PIK-75 inhibitor concentrations (● none, ■ 15nM, ▲ 20nM and ▼ 25nM respectively).

These curves are generated using duplicates at each point and are representative of two (ATP) or three (PI) separate assays.

Figure 3: Sequence alignment of Regions 1 and 2

Amino acid sequence alignment of two regions within the catalytic subunit of the four PI3K class 1 isoforms is shown. Conserved amino acids are shaded in blue and non-conserved amino acids that have been subjected to *in vitro* mutation are shaded in green. Region 1 in p110α contains two non-conserved amino acids, H855 and Q859 previously identified ((Frazzetto et al., 2008) and two additional non-conserved amino acids, R852 and N853, which have been subjected to mutagenesis as detailed in the manuscript. Region 2 is a new region of heterogeneity identified by both sequence alignment and comparison of known crystal structures (Figure 4). PI3K isoform p110α contains five non-conserved amino acids, R770, I771 S773, K776 and R777 that have been subjected to mutagenesis.

Figure 4: Structural heterogeneity within Region 2

Common views of PI3K crystal structures highlighting the structural heterogeneity of region 2 amino acids in the presence and absence of bound ligand: ATP and small molecule inhibitors.

(A) ATP (in magenta) bound to the p110γ PI3K isoform (PDB 1E8X) – the side chain of the conserved amino acid M-804 lies along the binding pocket in the "closed" conformation masking K-802, a non-conserved amino acid in region 2.

(B) PIK39 (in magenta), a δ isoform selective inhibitor (PDB 2WXF), bound to the p110δ isoform – the δ equivalent M-758 side chain projects away from the surface in the "open" conformation generating the specificity pocket that includes the non-conserved amino acid region 2 amino acid, T-756.

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(C) PIK39 (in magenta), a δ isoform selective inhibitor, bound to the p110γ isoform (2CHW) – the side chain of the conserved amino acid, M-804 adopts the "open" conformation in the same way as in the p110δ structure generating a pocket that includes the non-conserved amino acid K-802.

(D) Structure of the p110α holoenzyme (PDB 2RD0) showing the position of an arginine, R770, lining outer edge of the binding pocket and replacing the equivalent non-conserved p110γ K-802. The positions of the conserved amino acids, M-772 and W-780 are also indicated.

(E) Wortmannin (in magenta) covalently bound to the p110α isoform (3HHM) showing that both the conserved M-772 and the non-conserved R-770 change conformation upon wortmannin binding but the conserved W780 remains essentially unchanged upon wortmannin binding.

Figure 5: Alanine scan of region 2 non-conserved amino acids and effect on PIK-75 and LY294002 inhibition.

The wild-type (WT) and five mutant PI3K, p110α enzymes were assayed in the presence and absence of the α-selective inhibitor, PIK-75 (A) and the pan-PI3K inhibitor, LY-294002 (B). The percentage inhibition was calculated using the activity determined in the absence of inhibitor as 0% inhibition and the activity in the absence of enzyme as 100% inhibition.

A: Graph of % inhibition of p110α enzyme activity by 50nM PIK-75 of wild-type and alanine mutants as indicated. Dashed line across graph is WT inhibition level. Results are shown as mean ± standard error as calculated by Prism (n= 4-6). Statistical analysis showed that the largest difference from wild-type was S773A with a mean percentage inhibition of

6% relative to the WT mean of 51% ($p<0.05$). The only other mutant that showed a statistically significant difference from the WT mean was R777A with a mean of 22% ($p<0.05$). B: Graph of % inhibition of p110α enzyme activity by 1μM LY294002 of wild-type and alanine mutants as indicated. Dashed line across graph is WT inhibition level. Results are shown as mean ± standard error as calculated by Prism (n=2). There is no statistical difference between the inhibition found for the alanine mutants and that found for the WT.

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Table 1: Kinetic properties of mutants

A: Effect of mutation on PIK-75 IC-50

Enzyme	Km,ATP (μM)#	PIK-75, IC-50 (nM)*
WT	23.6±5.0	44±11
S773A	21.7±5.3	330±76 (p=0.0002)
S773D	50.7±5.0	346±38 (p<0.0001)
H855E	23.4±5.7	107±23 (p=0.0300)

#Estimation of the Km for ATP was measured using varying ATP concentrations in the presence of 180μM PI. Km was calculated from plots of enzyme activity versus ATP concentration using the Michaelis-Menten kinetic model (GraphPad software). (n= 4)

*Estimation of inhibitor IC-50 using graphs of inhibition of enzyme activity versus inhibitor concentration where 100% activity was defined by the activity in the presence of DMSO alone. (n= 4-8)

B: Effect of S773D mutation on the kinetics of PIK-75 inhibition with respect to the substrate PI.

Kinetic analysis of the PIK-75 effect on PI binding to wild type p110α, p110α S773D and p110α H855E mutant enzymes. Kinetic constants Km for substrate and the Ki for PIK-75 were estimated using the Michaelis-Menten and competitive inhibition models respectively. (GraphPad software). (n=4)

	K_m, PI (μM)	K_i, PIK-75 (nM)
WT	7.0 ± 1.5	2.3 ± 0.6
S773D	11.2 ± 1.9	146 ± 11
H855E	3.9 ± 0.8	ND

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Figure 1

PI3K, p110	α	β	γ	δ
IC-50 (μM)*	0.0058	1.3	0.076	0.51

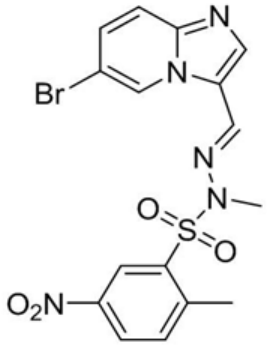

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Figure 2

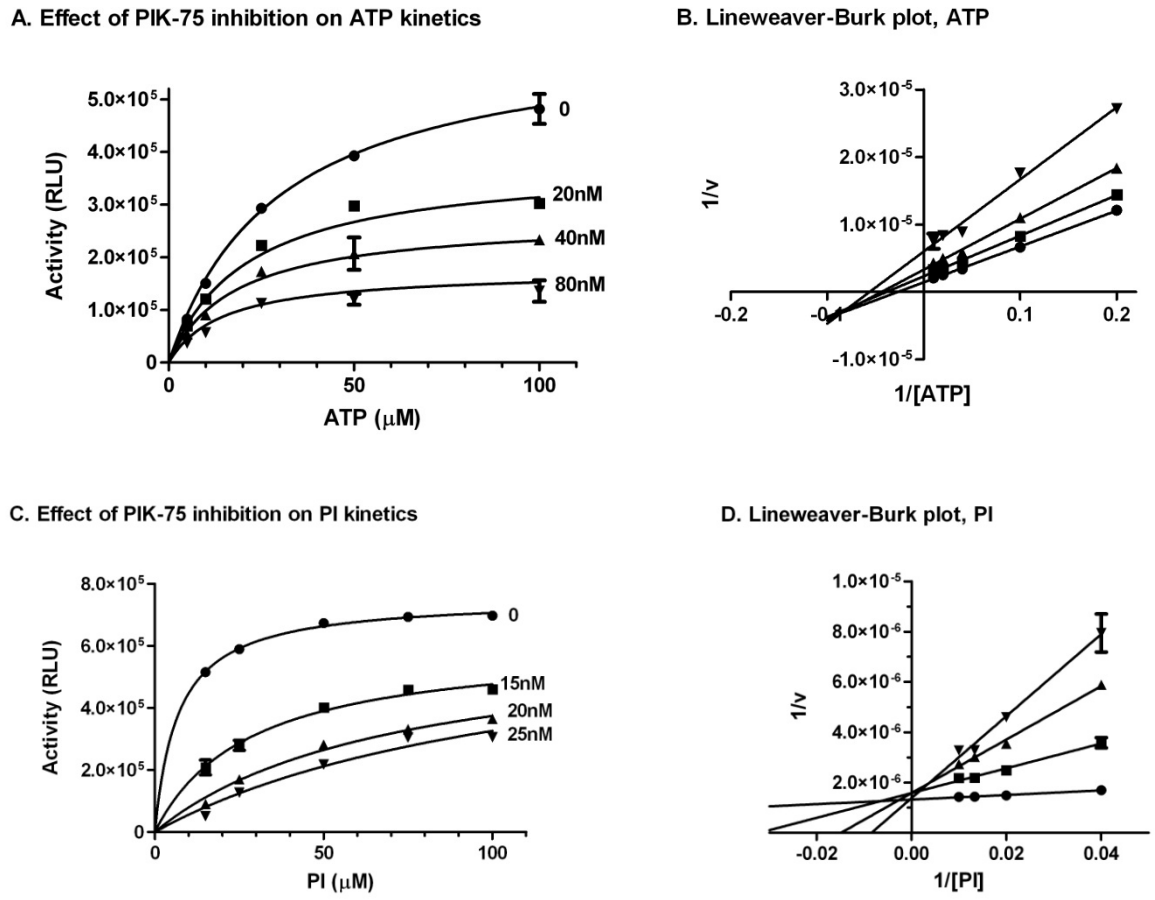

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Figure 3

Region 1

847	α	L	I	E	V	V	**R**	**N**	S	**H**	T	I	M	**Q**	I	Q	C	K	α
850	β	L	I	E	V	V	**S**	**T**	S	**E**	T	I	A	**D**	I	Q	L	N	β
878	γ	L	I	E	V	V	**K**	**D**	A	**T**	T	I	A	**K**	I	Q	Q	S	γ
818	δ	M	I	E	V	V	**L**	**R**	S	**D**	T	I	A	**N**	I	Q	L	N	δ

Region 2

764	α	L	R	L	E	E	C	**R**	**I**	M	**S**	S	A	**K**	**R**	P	L	W	L	N	W	α
771	β	L	Y	V	E	K	C	**K**	**Y**	M	**D**	S	K	**M**	**K**	P	L	W	L	V	Y	β
796	γ	L	V	I	E	K	C	**K**	**V**	M	**A**	S	K	**K**	**K**	P	L	W	L	E	F	γ
750	δ	V	C	V	E	Q	C	**T**	**F**	M	**D**	S	K	**M**	**K**	P	L	W	I	M	Y	δ

Figure 4

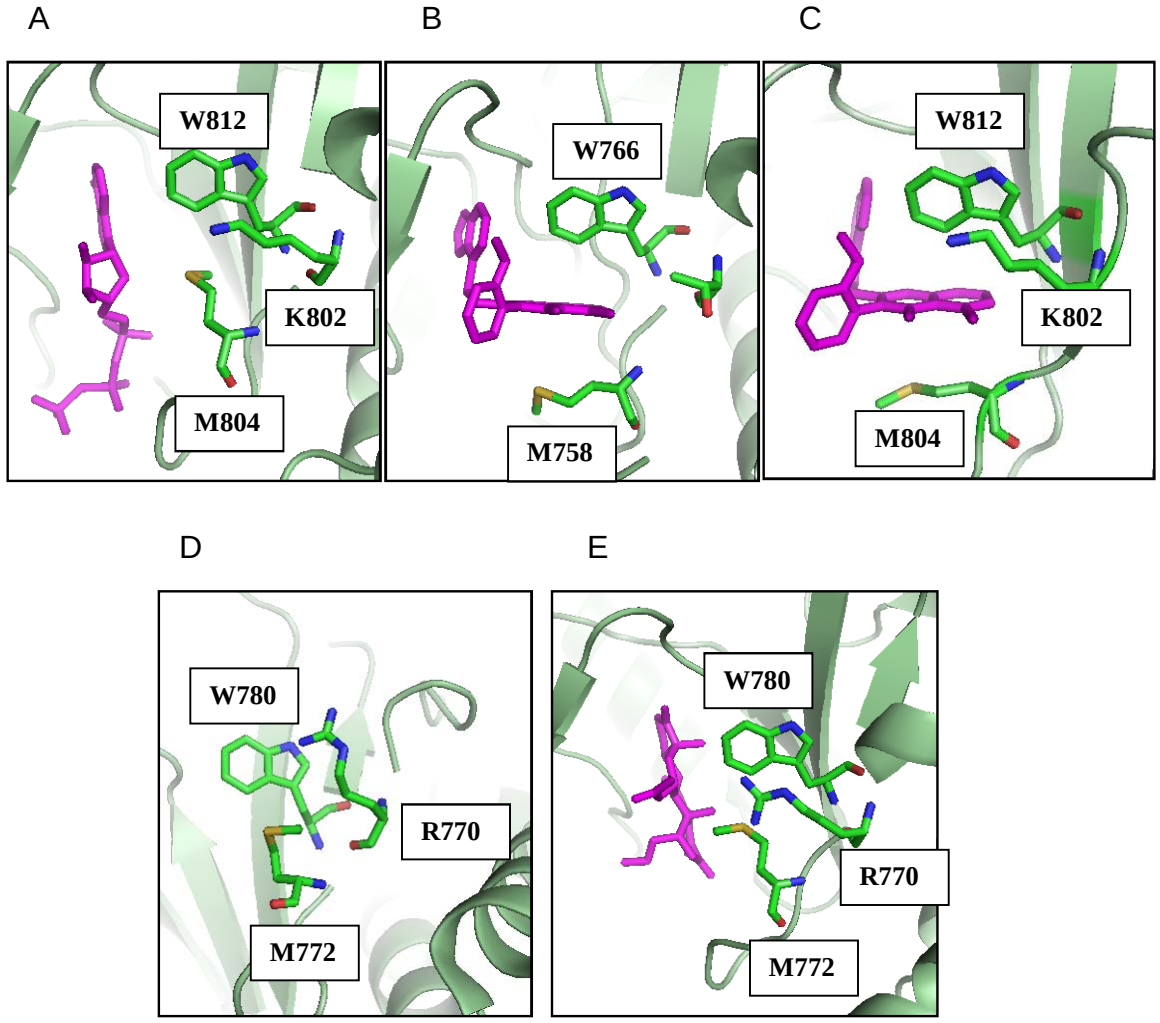

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Figure 5