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Isoform-Selective Inhibition of Phosphoinositide 3-Kinase: Identification of a New Region of Nonconserved Amino Acids Critical for p110α Inhibition

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ABSTRACT

The combination of molecular modeling 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 2-methyl-5-nitro-2-[(6-bromoimidazo[1,2-α]pyridin-3-yl)methylene]-1-methylhydrazide-benzenesulfonic acid (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 nonconserved amino acids (region 2) that was postulated to be involved in PIK-75 p110α selectivity. Analysis of region 2, using in vitro mutation of identified nonconserved amino acids to alanine, showed that Ser773 was a critical amino acid involved in PIK-75 binding, with an 8-fold-increase in the IC_{50} compared with wild-type. Kinetic analysis showed that, with respect to PI, the PIK-75 K_i for the isoform mutant S773D increased 64-fold compared with wild-type enzyme. In addition, a nonconserved amino acid, His855, from the previously identified region 1 of nonconserved amino acids, was found to be involved in PIK-75 binding. These results show that these two regions of nonconserved amino acids that are close to the substrate binding site could be targeted to produce p110α isoform-selective inhibitors.

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. It is involved in regulating cellular downstream signaling 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 tumors, which has led to PI3K being regarded as 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; specifically, p110α inhibitors have the potential to target tumors that contain a mutated or amplified p110α.

Several PI3K drugs are in phase 1 clinical trials, the majority of these being pan-PI3K inhibitors binding at the ATP binding site. The lone exception is the δ-specific inhibitor 5-fluoro-3-phenyl-2-[(1*S*)-1-(1*H*-purin-6-ylamino)propyl]-4(3*H*)-quinazolinone (CAL101), which has been shown to produce a conformational change in the binding site exposing a specificity pocket

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ABBREVIATIONS: PI3K, phosphoinositide 3-kinase; CAL101, 5-fluoro-3-phenyl-2-[(1*S*)-1-(1*H*-purin-6-ylamino)propyl]-4(3*H*)-quinazolinone; PIK-75, 2-methyl-5-nitro-2-[(6-bromoimidazo[1,2-α]pyridin-3-yl)methylene]-1-methylhydrazide-benzenesulfonic acid; PI, phosphatidylinositol; ZSTK474, 2-(2-difluoromethylbenzimidazol-1-yl)-4,6-dimorpholino-1,3,5-triazine; PIK-39, 5-chloro-3-(2-methoxyphenyl)-2-(7*H*-purin-6-ylsulfanylmethyl)quinazolin-4-one; PDB, Protein Data Bank; WT, wild type; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; PIP_2, phosphatidylinositol 4,5-bisphosphate; IC-87114, 2-[6-amino-9*H*-purin-9-yl)methyl]-5-methyl-3-(2-methylphenyl)-4(3*H*)-quinazolinone; AS15, 2-[[3-(2-methoxyphenyl)-4-oxo-5,6,7,8-tetrahydroquinazolin-2-yl]sulfanyl]-*N*-quinoxalin-6-ylacetamide; GDC-0941, 2-(1*H*-indazol-4-yl)-6-[[4-(methylsulfonyl)-1-piperazinyl]methyl]-4-(4-morpholinyl)thieno[3,2-*d*]pyrimidine.

into which the inhibitor binds (Workman et al., 2010; Bowles and Jimeno, 2011).

One of the first potent p110α inhibitors reported was an imidazo[1,2]-pyridine, 2-methyl-5-nitro-2-[(6-bromoimidazo[1,2-α]pyridin-3-yl)methylene]-1-methylhydrazide-benzenesulfonic acid (PIK-75), developed by researchers at Yamanouchi (now Astellas Pharma, Tokyo, Japan) and Piramed (now Roche, Basel, Switzerland) (Hayakawa et al., 2007b) (Fig. 1). This molecule and its related analogs are unique in the field principally because of the impressive potency and isoform selectivity at p110α. On the other 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 modeling of the compound into homology models generated from known PI3K crystal structures. The published SAR data (Hayakawa et al., 2007a) has shown that the potency of PIK75 and analogs was derived from the bromine substituent, the sulfonyl group, and the 2-methyl-5-nitrophenyl ring (Fig. 1), but no information was available on the influence of these substitutions on selectivity. A further series of substituted imidazopyridines has been reported in which the methyl group was 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 is fundamentally different from the other with respect to the identification of nonconserved 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. To this end, we have determined that PIK-75 is not a simple ATP-competitive inhibitor; rather, it shows mixed inhibitor kinetics versus ATP and is in fact a competitive inhibitor of the lipid substrate phosphatidylinositol (PI). Second, we have found that PIK75 binding is sensitive to mutations in specific nonconserved regions of the binding pocket. Two nonconserved amino acids, Ser773 and His855, from two different regions of the PI3K binding pocket, have been shown to be critical for the binding of PIK-75. These results show that these two nonconserved 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.

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

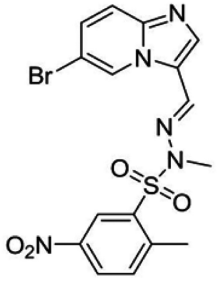

Fig. 1. Chemical structure of PIK-75, a p110α-selective inhibitor. *, IC_{50} for each of the four PI3K isoforms, p110α, -β, -γ, and -δ as determined previously (Knight et al., 2006).

Materials and Methods

Generation of Baculovirus Containing p110α Mutant DNA. The methods used here have been described previously in Frazzetto et al. (2008), except that the pFastBac system (Invitrogen, Carlsbad, CA) rather than the pBakPak system (Clontech, Mountain View, CA) was used to generate recombinant baculovirus. In brief, mutant plasmids were generated using the appropriate primer pair and *Pfu* DNA polymerase (Promega, Madison, WI), with the template DNA being pFastBac wild-type p110α. The DNA sequence was then confirmed to contain the correct mutation, and the remaining DNA sequence was confirmed to be identical to wild type. Mutant plasmids were then transformed into DH10Bac *Escherichia coli* for transposition into the bacmid. Blue/white selection was used to select for colonies containing recombinant bacmids; the presence of the recombinant DNA in the bacmid was confirmed by polymerase chain reaction. Recombinant bacmid DNA was then transfected, using lipofectin (Invitrogen), into Sf21 cells, and supernatant containing recombinant virus was collected after 3 to 5 days at 27°C. High-titer virus stock was then produced by amplification through two cycles of infection. Production of p110α protein was confirmed by Western blot analysis of cell extracts separated by SDS-polyacrylamide gel electrophoresis using a p110α-specific antibody.

Protein Expression and Purification. Twenty milliliters of p110α virus and 5 ml of p85 virus were added to 200 ml of Sf21 cells (2×10^6 cells/ml) and incubated shaking at 140 rpm for 48 h at 27°C. After 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 nickel-agarose chromatography as described previously (Frazzetto et al., 2008). Fractions containing the PI3K protein were pooled and dialyzed against 50 mM Tris-HCl, pH 7.5, and 300 mM NaCl at 4°C. PI3K protein was then made 20% (v/v) with respect to glycerol and 2 mM with respect to dithiothreitol and stored at −80°C.

Inhibition Assays. The PI3K inhibitor PIK75 (Fig. 1) (Calbiochem, San Diego, CA) was dissolved at 10 mM in dimethyl sulfoxide and stored at −20°C until use. PI3K enzyme activity was determined in 50 μl of 20 mM HEPES, pH 7.5, and 5 mM $MgCl_2$ containing 180 μM phosphatidyl inositol, with the reaction started by the addition of 100 μM ATP (containing 2.5 μCi of [γ-^{32}P]ATP). After a 30-min incubation at room temperature, the enzyme reaction was stopped by the addition of 50 μl of 1 M HCl. Phospholipids were then extracted with 100 μl of chloroform/methanol [1:1 (v/v)] and 250 μl of 2 M KCl followed by liquid scintillation counting. Inhibitors were diluted in 20% (v/v) dimethyl sulfoxide to generate a concentration versus inhibition of enzyme activity curve, which was then analyzed with the use of Prism version 5.00 for Windows (GraphPad Software, San Diego, CA) to calculate the IC_{50}.

For kinetic analysis, a luminescent assay measuring ATP consumption was used. PI3K enzyme activity was determined in 50 μl of 20 mM HEPES, pH 7.5, and 5 mM $MgCl_2$ with PI and ATP at various concentrations as indicated in Fig. 2. After a 60-min incubation at room temperature, 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, Durham, NC). Results were analyzed using Prism.

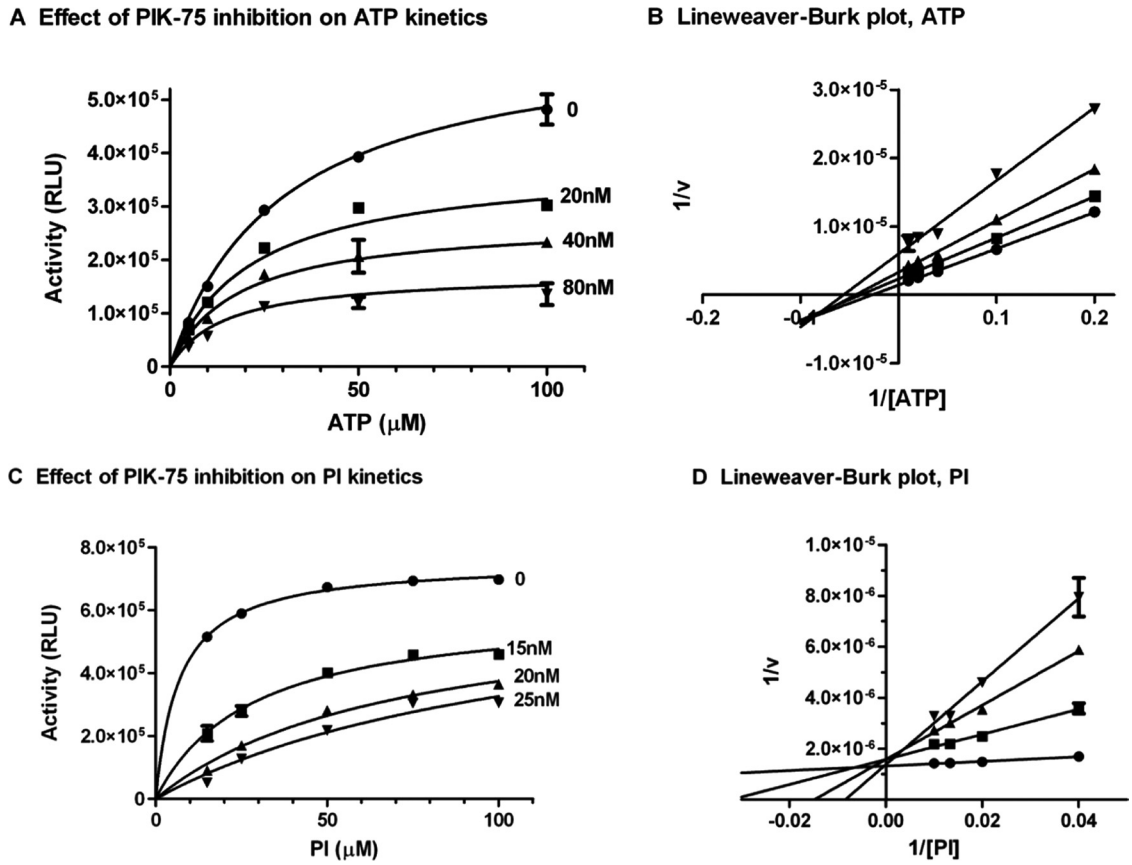

Fig. 2. Kinetic characterization of PIK-75 inhibition of wild-type p110α. A, kinetics of ATP binding to purified p110α in the presence of various concentrations of the inhibitor PIK-75 (●, none; ■, 20 nM; ▲, 40 nM; and ▼, 80 nM) as shown by a plot of enzyme activity measured by the luminescent assay versus ATP concentration. B, Lineweaver-Burk plot of the kinetics of ATP binding to p110α in the presence of various concentrations of the inhibitor PIK-75 (●, none, ■; 20 nM; ▲, 40 nM; and ▼, 80 nM). C, kinetics of substrate PI binding to p110α in the presence of various concentrations of the inhibitor PIK-75 (●, none; ■, 15 nM; ▲, 20 nM; and ▼, 25 nM), 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 various concentrations of the inhibitor PIK-75 (●, none; ■, 15 nM; ▲, 20 nM; and ▼, 25 nM). These curves are generated using duplicates at each point and are representative of two (ATP) or three (PI) separate assays.

Results

Characterization of the Kinetics of PIK-75 Inhibition of p110α. The kinetics of PIK-75 inhibition of PI3K p110α was studied in detail, as shown in Fig. 2. Curves of p110α enzyme activity versus ATP concentration at various PIK-75 concentrations are shown in Fig. 2A; when these data were analyzed using a Lineweaver-Burk plot, it showed that PIK-75 was a mixed inhibitor with respect to ATP, increasing inhibitor decreasing the V_{max} (Fig. 2B, y intercept) but having some effect on the K_m (Fig. 2B, x intercept). However, kinetic analysis using the mixed-inhibition mode (Prism) showed that PIK-75 inhibition was noncompetitive with respect to ATP, where the value of the constant α was less than 1 (0.3) and the K_i for PIK-75 with respect to ATP was 36 ± 6 nM (n = 4). This result is in contrast to the pan-PI3K inhibitor 2-(2-difluoromethylbenzimidazol-1-yl)-4,6-dimorpholino-1,3,5-triazine (ZSTK474), which has been shown previously 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).

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 in a manner indicative of competitive inhibition. In addition, these data were analyzed using the mixed-model of inhibition, where the value of the constant α was shown to be greater than 1 (i.e., 30), indicative of competitive inhibition. The K_i of PIK-75 with respect to PI was estimated to be 2.3 ± 0.6 nM (n = 6). Thus, both the Lineweaver-Burk plots and the mixed-inhibition model show for the first time that PIK-75 is a noncompetitive inhibitor with respect to ATP and competitive with respect to the substrate PI, with a K_i 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 nonconserved 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 I 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.

We have previously identified region 1, which contained two nonconserved amino acids, His855 and Gln859, 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. On the basis of 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 nonconserved amino acids to adopt different conformations could lead to the isolation of p110α isoform-selective inhibitors. Sequence analysis of region 1 identified two additional nonconserved p110α amino acids, Arg852 and Asn853, which we have mutated here for inhibitor analysis (Fig. 3).

In an attempt to identify additional sequences that might confer isoform selectivity, we compared the five p110γ crystal structures available at the time. In the presence and absence of ligands, structural changes were identified in a region (designated region 2) of the p110α sequence that contains nonconserved amino acids, as shown in Fig. 3. This sequence includes the PI3K equivalent of the p-loop or "glycine-rich" loop identified in protein kinases as being involved in ATP binding (Johnson et al., 1996; Huse and Kuriyan, 2002), 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, 5-chloro-3-(2-methoxyphenyl)-2-(7*H*-purin-6-ylsulfanylmethyl)quinazolin-4-one (PIK-39), to p110γ (PDB id 2CHW) and to p110δ (PDB id 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 (Fig. 4, B and C). By comparison, Met804 was found in a "closed" conformation in the structure of ATP bound to p110γ (Fig. 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 (Fig. 3). In addition, in the superposition of p110α and p110δ crystal structures, the sequence around the methionine has a relatively high root-mean-square deviation for the overlay of α-carbons (Fig. 4). Thus, the heterogeneity of the residues in this region seems to have a role in dictating the conformation of the backbone and possibly the capacity to expose unique binding site surfaces. In particular, the Lys802 residue, exposed in the p110γ/PIK-39 structure (PDB id 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 Fig. 3, and it seems plausible that this heterogeneity is a key contributor to selectivity.

In addition, comparison of structures of unliganded p110α (PDB ids 2RD0 and 3HIZ) (Fig. 4D) and the complex of the covalently bound inhibitor wortmannin and p110α protein (PDB id 3HHM) (Fig. 4E) showed that the p-loop (amino acids 772–777) shifted 3 Å in the presence of the inhibitor. In contrast, the equivalent p110γ complex (PDB id 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).

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

Fig. 3. Sequence alignment of regions 1 and 2. Amino acid sequence alignment of two regions within the catalytic subunit of the four PI3K class I isoforms is shown. Conserved amino acids are shaded in blue and nonconserved amino acids that have been subjected to in vitro mutation are shaded in green. Region 1 in p110α contains two nonconserved amino acids, His855 and Gln859, identified previously (Frazzetto et al., 2008) and two additional nonconserved amino acids, Arg852 and Asn853, which have been subjected to mutagenesis. Region 2 is a new region of heterogeneity identified by both sequence alignment and comparison of known crystal structures (Fig. 4). PI3K isoform p110α contains five nonconserved amino acids, Arg770, Ile771 Ser773, Lys776, and Arg777, that have been subjected to mutagenesis.

Analysis of Region 2 Nonconserved Amino Acids by Alanine Scanning Mutagenesis. Six nonconserved amino acids in region 2, Glu768, Arg770, Ile771, Ser773, Lys776, and Arg777, were mutated to alanine to assess their role in binding PIK75. In vitro mutants were generated using polymerase chain reaction as described under *Materials and Methods*. 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 coinfection of Sf21 cells with p110α mutant or WT virus with p85 virus for 48 h. Cells were then lysed, and PI3K protein was purified using nickel agarose affinity chromatography as described under *Materials and Methods*. Yields of protein for all alanine mutants were comparable with that of the WT enzyme.

The purified alanine mutants were then tested for their ability to be inhibited by PIK-75 at a concentration of 50 nM compared with the wild-type enzyme. As shown in Fig. 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; the αSer773 equivalent amino acid in p110γ is alanine. As a control for this set of assays, all the alanine mutants were tested for inhibition with the pan-PI3K inhibitor 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002), and as seen in Fig. 5B, all mutants showed inhibition essentially identical to that of the wild-type enzyme. R770A, which is not included on this graph, was separately shown to have an IC_{50} for LY294002 similar to that of the WT enzyme (0.24 versus 0.76 μM). In addition, the region 2 alanine mutants showed no difference in inhibition by another pan-PI3K inhibitor, ZSTK474 compared with the WT enzyme (results not shown). So, on the basis of these results, Ser773 was identified as the important nonconserved 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, Ser773 was mutated to aspar-

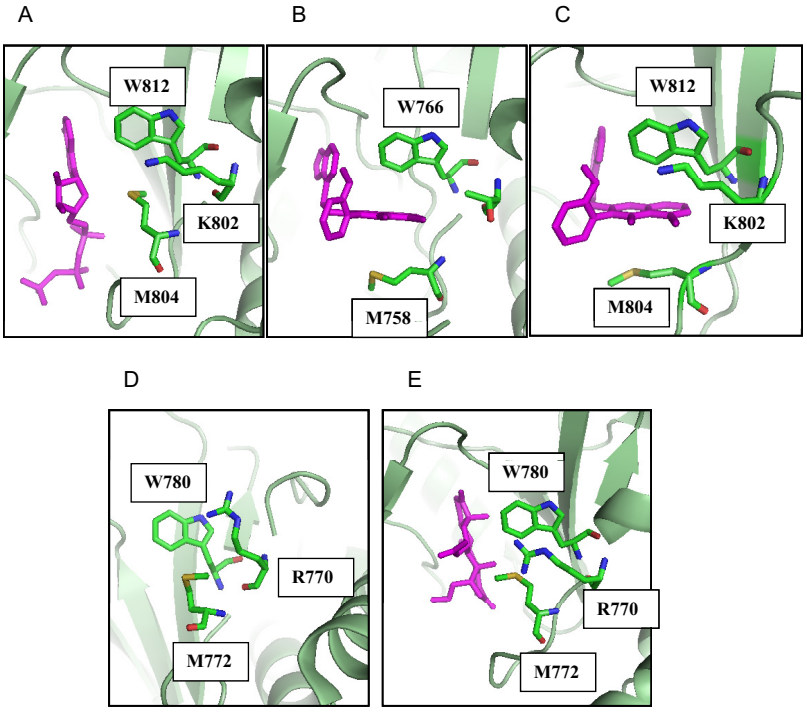

Fig. 4. Structural heterogeneity within region 2. Common views of PI3K crystal structures highlighting the structural heterogeneity of region 2 amino acids in the presence and absence of bound ligand-ATP and small-molecule inhibitors. A, ATP (in magenta) bound to the p110γ PI3K isoform (PDB id 1E8X); the side chain of the conserved amino acid Met804 lies along the binding pocket in the "closed" conformation, masking Lys802, a nonconserved amino acid in region 2. B, PIK39 (in magenta), a δ isoform-selective inhibitor (PDB id 2WXF), bound to the p110δ isoform; the δ equivalent Met758 side chain projects away from the surface in the "open" conformation, generating the specificity pocket that includes the nonconserved region 2 amino acid Thr756. C, PIK39 (in magenta), a δ isoform-selective inhibitor, bound to the p110γ isoform (PDB id 2CHW); the side chain of the conserved amino acid Met804 adopts the "open" conformation in the same way as in the p110δ structure, generating a pocket that includes the nonconserved amino acid Lys802. D, structure of the p110α holoenzyme (PDB id 2RD0) showing the position of an arginine, Arg770, lining the outer edge of the binding pocket and replacing the equivalent nonconserved p110γ Lys802. The positions of the conserved amino acids Met772 and Trp780 are also indicated. E, wortmannin (in magenta) covalently bound to the p110α isoform (PDB id 3HHM), showing that both the conserved Met772 and the nonconserved Arg770 change conformation upon wortmannin binding, but the conserved Trp780 remains essentially unchanged.

tate, the equivalent amino acid in the p110 β and δ isoforms. Mutant protein was expressed and purified as described in the previous section.

The amino acid Ser773 is next to the conserved Ser774 on the p110α sequence; the equivalent Ser806 was shown in the PI3Kγ/ATP crystal structure (PDB id 1E8X) to be interacting with an oxygen of the β-phosphate on the ATP molecule (at a distance of 2.5 Å). Therefore, the K_m for ATP was measured to determine the effect of the mutation on the structure of the ATP binding site. Table 1 shows the K_m ATP determination for the WT, S773A, and S773D mutants. S773A has a K_m value similar to that of the WT enzyme, and S773D has a K_m value twice that of WT. Because the enzyme assay for inhibition was carried out at 100 μM ATP, however, all enzyme activity should be at or near saturating levels of ATP. The K_m 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 Ser773, and it was shown that both the S773A and S773D mutants increased the IC_{50} of PIK-75 by 8-fold (Table 1), indicating that Ser773 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 200 nM PIK-75. Using the mixed model of inhibition (Prism) it was estimated that the K_m for PI was 11.2 μM compared with 7.0 μM for the wild-type enzyme (Table 2). The K_i for PIK-75 was estimated to be 146 nM, a 64-fold increase on the value estimated for the wild-type enzyme (2.3 nM). 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 K_m of the αS773D mutant.

Despite the fact that the R770A mutation showed modest effects on PIK-75 inhibition, we investigated the effect of isoform mutations at Arg770 because of the structural evidence of this nonconserved amino acid's mobility in several PI3K-inhibitor complexes, as demonstrated in Fig. 4. Therefore, 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 K_m for ATP and unchanged IC_{50} for the pan-PI3K inhibitor, LY294002, 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 compared with WT, indicating that Arg770 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 nonconserved amino acids that potentially could be involved in the selective binding of PIK-75. As seen

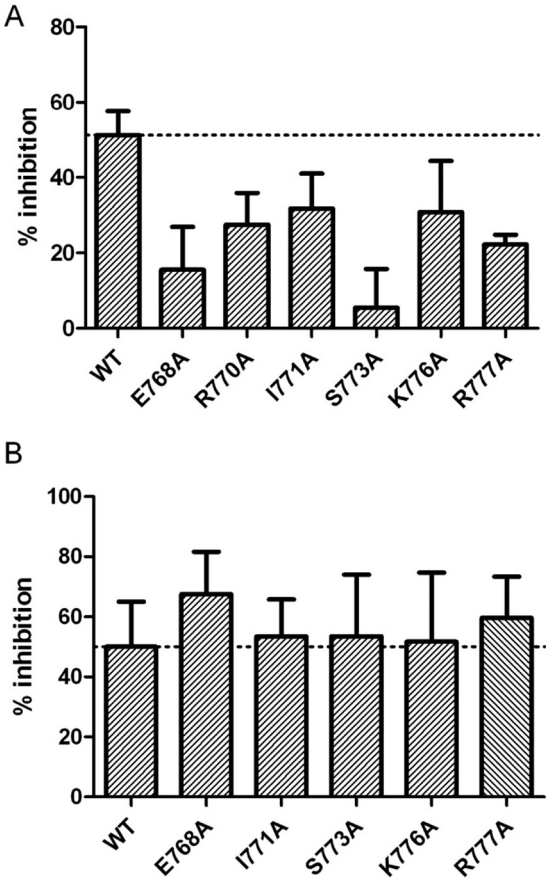

Fig. 5. Alanine scan of region 2 nonconserved amino acids and effect on PIK-75 and LY294002 inhibition. The WT and five mutant PI3K p110α enzymes were assayed in the presence and absence of the α-selective inhibitor, PIK-75 (A) and the pan-PI3K inhibitor LY-294002 (B). The percentage inhibition was calculated 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 percentage inhibition of p110α enzyme activity by 50 nM PIK-75 of wild-type and alanine mutants as indicated. Dashed line across graph is the WT inhibition level. Results are shown as mean ± S.E. as calculated by Prism (n = 4–6). Statistical analysis showed that the largest difference from wild-type was S773A, with a mean 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 percentage inhibition of p110α enzyme activity by 1 μM LY294002 of wild-type and alanine mutants as indicated. Dashed line across graph is the WT inhibition level. Results are shown as mean ± S.E. as calculated by Prism (n = 2). There was no statistical difference between the inhibition found for the alanine mutants and that found for the WT.

from Tables 1 and 2, the isoform mutant p110α H855E showed a 2.4-fold increase in PIK-75 IC_{50}. Although this increase was statistically significant, it was less than that observed with the region 2 Ser773 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 nonconserved amino acids, Arg852 and Asn853, 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 nonconserved 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 noncompetitive inhibitor of ATP binding but

TABLE 1

Kinetic properties of mutants: effect of mutation on PIK-75 IC_{50}

Estimation of the K_m for ATP was measured using various ATP concentrations in the presence of 180 μM PI. K_m was calculated from plots of enzyme activity versus ATP concentration using the Michaelis-Menten kinetic model (GraphPad Prism) (n = 4). Estimation of inhibitor IC_{50} was made using graphs of inhibition of enzyme activity versus inhibitor concentration, where 100% activity was defined by the activity in the presence of dimethyl sulfoxide alone (n = 4–8).

Enzyme	K_m ATP	PIK-75 IC_{50}
	μM	*nM*
WT	23.6 ± 5.0	44 ± 11
S773A	21.7 ± 5.3	330 ± 76*
S773D	50.7 ± 5.0	346 ± 38**
H855E	23.4 ± 5.7	107 ± 23***

* P = 0.0002.
** $P < 0.0001$.
*** P = 0.0300.

TABLE 2

Kinetic properties of mutants: 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 K_m for substrate and the K_i for PIK-75 were estimated using the Michaelis-Menten and competitive inhibition models, respectively (GraphPad Prism) (n = 4).

	K_m PI	K_i PIK-75
	μM	*nM*
WT	7.0 ± 1.5	2.3 ± 0.6
S773D	11.2 ± 1.9	146 ± 11
H855E	3.9 ± 0.8	N.D.

N.D., not determined.

a competitive inhibitor of the substrate PI. Mutation at the region 2 amino acid Ser773 increased the K_i and IC_{50} for PIK-75 without significantly changing the K_m for PI, indicating that Ser773 is critical for PIK-75 inhibitor binding but not for PI binding. In addition, to a lesser extent, the mutation at the region 1 nonconserved amino acid His855 was involved in the binding of PIK-75 but again had little direct effect on PI binding.

Our results here are consistent with the model of Frederick and Denny (2008), in which Ser773 is proposed to bind to the nitro group of the phenyl ring, His855 was proposed to bind to the sulfonyl group, and the bromine substituent interacted with the hinge region invariant valine. The modeling would predict that either mutation of Ser773 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 Ser773.

The only PI3K inhibitors previously analyzed 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 (Camps et al., 2005; Berndt et al., 2010). It could be speculated that targeting the ATP binding site yields potent but not α-selective inhibitors, whereas 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 Ser806, bound to the β-phosphate, and Lys833, bound to the α-phosphate, which are mimicking the role of the backbone inter-

actions between ATP and the flexible glycine loop in the classic serine/threonine protein kinases. The equivalent loop in PI3K (kβ3-kβ4) has no glycines in the sequence. The observation that a key nonconserved amino acid for the selective binding of PIK-75, Ser773 (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; noncompetitive inhibition was observed, and Ser773 mutants showed no significant change in K_m for ATP compared with the WT enzyme. Examination of the crystal structures of unliganded p110α (PDB id 2RD0) confirmed that the side chain of this amino acid (α-Ser and 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 I PI3K isoforms. The serine/threonine protein kinase family contains a sequence in the active site that is involved in the substrate specificity of binding known as the activation sequence. In the PI3K family, a similar loop sequence has been found that has been postulated to determine lipid substrate specificity. A study in which the activation loop in the class I PI3K p110α was substituted with sequences from class II, III, and IV PI3Ks showed that the activation loop was involved in lipid substrate specificity (Pirola et al., 2001). Furthermore, in vitro mutagenesis studies showed that two basic amino acids in the activation loop, Lys942 and Arg949, are responsible for the binding of PIP_2 substrate. It is noteworthy that although the p110α mutant containing the class II or III 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 catalyzed. This indicated that other regions of the active site might be involved in the binding of PIP_2. Subsequent modeling studies proposed that Lys942 bound to the 5-phosphate on the PIP_2 and that Arg949 bound the 4-phosphate on the PIP_2. In addition, it was found that a nonconserved amino acid in region 2, Lys776, 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. Gabelli et al. (2010), in their p110α structures comparing unliganded enzyme with enzyme bound to wortmannin, noted that the loop containing amino acids 772 to 776 (within region 2) changes conformation. Their modeling 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 seems to be isoform-specific in that it does not occur when wortmannin binds to p110γ.

A conformational basis for isoform selectivity among the PI3K isoforms was demonstrated by the complexes of the "propeller" inhibitors, 2-[6-amino-9*H*-purin-9-yl)methyl]-5-methyl-3-(2-methylphenyl)-4(3*H*)-quinazolinone (IC-87114) and PIK-39 with p110δ (Berndt et al., 2010). It was shown that Met752 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. It had also been shown previously that this conformational change occurred in p110γ (Knight et al., 2006), but more recent molecular simulation calculations were used to show that this conformational change was more energetically favorable 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 nonconserved amino acids of region 2 where the conserved Met752 is located. It is feasible that the right p110α inhibitor could induce a similar conformational change.

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 nonconserved 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 2-[[3-(2-methoxyphenyl)-4-oxo-5,6,7,8-tetrahydroquinazolin-2-yl]sulfanyl]-*N*-quinoxalin-6-ylacetamide (AS15) was shown in a crystal structure with p110δ to bind closely to the conserved region 2 hydrophobic amino acids Met772 and Trp780 (p110α amino acid sequence numbering), preventing the conformational change and subsequent exposure of the specificity pocket. They postulated that the p110δ/Thr756 reduced side chain size compared with the equivalent of Arg770 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 Ser773 in α and alanine in γ and β and also with Lys708, 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 2-(1*H*-indazol-4-yl)-6-[[4-(methylsulfonyl)-1-piperazinyl]methyl]-4-(4-morpholinyl)thieno[3,2-*d*]pyrimidine (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, because mutation at Arg770 had no effect on binding.

In conclusion, we have shown that in vitro mutagenesis is a valuable tool for determining nonconserved 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. Although 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). Therefore, the path to the production of a truly useful p110α-selective inhibitor would seem to rely on targeting both region 1 and region 2 nonconserved amino acids using a different scaffold to avoid the off-target reactivity.

Authorship Contributions

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: Zheng, Thompson, and Jennings.

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