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Definition of the binding mode of a new class of phosphoinositide 3-kinase α -selective inhibitors using *in vitro* mutagenesis of non-conserved amino acids and kinetic analysis

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Abstract

The binding mechanism of a new class of lipid-competitive, ATP non-competitive, p110 α isoform-selective PI3K (phosphoinositide 3-kinase) inhibitors has been elucidated. Using the novel technique of isoform reciprocal mutagenesis of non-conserved amino acids in the p110 α and p110 β isoforms, we have identified three unique binding mechanisms for the p110 α -selective inhibitors PIK-75, A-66S and J-32. Each of the inhibitor's p110 α -isoform-selective binding was found to be due to interactions with different amino acids within p110. The PIK-75 interaction bound the non-conserved region 2 amino acid p110 α Ser⁷⁷³, A-66S bound the region 1 non-conserved amino acid p110 α Gln⁸⁵⁹, and J-32 binding had an indirect interaction with Lys⁷⁷⁶ and Ile⁷⁷¹. The isoform reciprocal mutagenesis technique is shown to be an important analytical tool for the rational design of isoform-selective inhibitors.

Keywords

enzyme kinetics; *in vitro* mutagenesis; Mechanism of isoform selectivity; phosphoinositide 3-kinase (PI3K); small molecule inhibitor

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²P. R. S. has consulted for and owns stock in Pathway Therapeutics, a company developing PI3K inhibitors, although none of these compounds are used in the present study.

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AUTHOR CONTRIBUTION

Zhaohua Zheng performed the experiments; Ian Jennings, Philip Thompson and Zhaohua Zheng designed the experiments; Ian Jennings, Philip Thompson and Zhaohua Zheng analysed the data; Syazwani Amran, Jiuxiang Zhu, Oleg Schmidt-Kittler, Bert Vogelstein, Kenneth Kinzler and Peter Shepherd contributed and characterized new reagents; and Ian Jennings, Philip Thompson, Zhaohua Zheng, Bert Vogelstein, Kenneth Kinzler and Peter Shepherd wrote the paper.

INTRODUCTION

PI3K (phosphoinositide 3-kinase; EC 2.7.1.153) is the enzyme responsible for the production of PIP₃ [PI (phosphatidylinositol) 3,4,5-trisphosphate], a key second-messenger molecule involved in regulating downstream signalling pathways. The pathways PIP₃ regulates are central to cell growth, survival, differentiation and chemotaxis [1]. Class 1 PI3Ks consist of four p110 isoforms, α , β , γ and δ , each of which binds regulatory subunits. The *PIK3CA* gene, which codes for the p110 α protein, has been found to be activated in a variety of common human tumours [2]. This makes p110 α an attractive target in the development of an inhibitor that would target cancer cells [3].

As the amino acid sequences of the catalytic domains of the four class 1 PI3K isoforms are strongly conserved, it has been difficult to produce an isoform-selective inhibitor without knowledge of the mechanism of that selectivity. Most PI3K inhibitors currently in clinical trials are not isoform-selective, and indeed some target other enzymes in addition to PI3K [4]. Isoform-selective inhibitors could reduce off-target, potentially toxic, side effects and could be useful for understanding the roles for the specific isoforms in normal and disease states [5].

Previously we have identified two regions, named region 1 and region 2, of amino acids in the p110 α active site that are involved in the binding of p110 α isoform-selective inhibitors. These regions are not conserved in other PI3K isoforms. Region 1 (amino acids 852–860), particularly amino acids His⁸⁵⁵ and Gln⁸⁵⁹ were shown by *in vitro* mutagenesis to be involved in the binding of isoform-selective inhibitors [6]. Region 2 (amino acids 766–780) was identified as a region of heterogeneity by the comparison of three-dimensional structures of p110 isoforms in the presence and absence of ligands and small-molecule inhibitors. *In vitro* mutants of region 2 were tested against the p110 α -selective inhibitor PIK-75, leading to the identification of Ser⁷⁷³ as the non-conserved amino acid critical for selective inhibition by PIK-75. In addition we found that PIK-75 was a competitive inhibitor of the lipid substrate PI, in contrast with non-selective PI3K inhibitors which had previously been found to be competitive with respect to ATP [7].

Since the identification of these regions of non-conserved amino acids, p110 α inhibitors with greater selectivity over the remaining three PI3K isoforms have been developed. For example, Schmidt-Kittler et al. [8] made an extensive series of PIK-75 analogues, resulting in greater p110 α selectivity mainly due to maintaining p110 α potency while decreasing the potency towards the other isoforms. The most selective p110 α inhibitor thus far is compound A-66S, originally described in a Novartis patent [9], which was shown to be 465-, 127- and 54-fold selective for p110 α over the β , γ and δ isoforms respectively. This inhibitor was initially used as a specific p110 α inhibitor in cell transformation assays [10]. The effect on cancer cells and the isoform selectivity of A-66S inhibition was further characterized by Jamieson et al. [11]. An *in vitro* molecular model of A-66S bound to p110 α suggested that the region 1 non-conserved amino acid Gln⁸⁵⁹ was responsible for the A-66S α -isoform selectivity.

One important aspect of the selective inhibitor development process is the determination of the three-dimensional structure of the inhibitor–enzyme complex. However, in the case of p110 α , this has not been possible due to the fact that the only structure of a p110 α –inhibitor complex determined thus far is that of the covalently bound pan-PI3K inhibitor wortmannin [12]. In the present study we have used *in vitro* mutagenesis and enzyme kinetics to analyse the binding mode of these α -isoform-selective inhibitors. The three p110 α isoform-selective inhibitors have been shown to bind through three unique and different structural

mechanisms, but all exhibit competitive inhibition with respect to the lipid substrate. As such they represent a new class of PI3K inhibitors.

EXPERIMENTAL

Generation of baculovirus-containing p110 α mutant DNA

The methods used in the present study have been described previously [6,7] with the pFastBac™ system (Invitrogen) used to generate recombinant baculovirus. In brief, mutant plasmids were generated using the appropriate primer pair and Pfu DNA polymerase (Promega) with the template DNA being either pFastBac™ WT (wild-type) p110 α or WT p110 β as appropriate. The DNA sequence was then confirmed as containing the correct mutation with the remaining DNA sequence re-confirmed as being identical with WT. Mutant plasmids were then transformed into DH10Bac *Escherichia coli* cells for transposition into the bacmid. Blue/white selection was used to select for colonies containing recombinant bacmids with the presence of the recombinant DNA in the bacmid confirmed using PCR. Recombinant bacmid DNA was then transfected, using Lipofectin® (Invitrogen), into Sf21 cells and supernatant containing recombinant virus was collected after 3–5 days at 27°C. High-titre 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 an anti-p110 α - or anti-p110 β -specific antibody.

Protein expression and purification

p110 virus (20 ml) and p85 virus (5 ml) were each added to 200 ml of Sf21 cells (2×10^6 cells/ml) and incubated with shaking at 140 rev./min for 48 h at 27°C. Following this, cells were collected by centrifugation (200 *g* for 5 min at 22°C) 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 described previously [6]. Fractions containing the PI3K protein were pooled and dialysed against 50 mM Tris/HCl (pH 7.5) and 300 mM NaCl at 4°C. PI3K protein was then made up to 20% (v/v) glycerol and 2 mM DTT (dithiothreitol) and stored at –80°C.

Inhibition assays

PI3K inhibitors were dissolved at 10 mM in 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 μ l of 20 mM Hepes (pH 7.5) and 5 mM MgCl₂ with PI and ATP at the indicated concentrations. After a 60 min incubation at room temperature (22°C) the reaction was stopped by the addition of 50 μ l 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 against inhibition of enzyme activity curve which was then analysed using GraphPad Prism version 5.00 for Windows in order to calculate the IC₅₀.

RESULTS

PIK-75, J-32 and A-66S are p110 α -selective inhibitors

PIK-75 [13] (Supplementary Figure S1 at <http://www.BiochemJ.org/bj/444/bj4440529add.htm>) was the first compound described that showed a significant degree of potency and selectivity for PI3K α over other isoforms. J-32 [8] (Supplementary Figure S1) showed an increase in p110 α selectivity, yet differs from PIK-75 only by an ethyl for methyl substitution at the hydrazine group. The p110 α -selective inhibitor A-66S

(Supplementary Figure S1) was recently described by Shepherd and co-workers [11]. As a control for this compound we have used SN34452, which differs from A-66S only by lacking the carboxamide motif that was proposed to be essential for p110 α isoform selectivity.

We first confirmed the reported enzyme inhibition data in our own assay format. PIK-75, J-32 and A-66S showed an IC₅₀ for p110 α of 44, 67 and 75 nM respectively (Table 1). In addition we found that the compounds showed the reported characteristic selective inhibition of the PI3K isoforms with PIK-75, J-32 and A-66S showing, respectively, a 29-, 17- and 224-fold selectivity for inhibition of the p110 α isoform over the p110 β isoform (Table 1).

PI3K α inhibitors target distinct non-conserved regions of the PI3K-binding site

As shown in Supplementary Figure S2 (at <http://www.BiochemJ.org/bj/444/bj4440529add.htm>) we have previously identified two regions of non-conserved amino acids designated region 1 [6] and region 2 [7] that we have characterized as being involved in isoform selectivity. We have now produced a bank of *in vitro* mutant p110 α enzymes in region 1 (R852A, N853A, H855A and Q859A) as well as in region 2 (E768A, R770A, I771A, S773A, K776A and R777A). In each mutant the designated amino acid has been replaced by alanine to assess the role of the corresponding side chain in inhibitor binding. These mutant p110 α enzymes have been co-expressed with the regulatory subunit p85 in Sf21 insect cells using fully characterized recombinant baculoviruses and purified using Ni-agarose affinity chromatography. The purified mutant enzymes have been shown to exhibit a K_m for both ATP and the lipid substrate PI similar to that of the WT enzyme. In addition, the purified mutant enzymes show a similar IC₅₀ for LY294002, a pan-PI3K inhibitor, to that determined for the WT enzyme (results not shown and published previously [6,7]). This indicates that the active site of the mutant enzymes is not significantly structurally altered in comparison with the WT enzyme.

In vitro mutagenesis profiling of PI3K α shows that PIK-75, J-32 and A-66S have distinct interactions with the PI3K-binding site

The four compounds described were then screened at their approximate IC₅₀ value against our panel of PI3K α mutants to assess whether specific non-conserved residues of the binding site were involved in key interactions with the inhibitors. Results are represented graphically in Figure 1.

Effect of PI3K p110 α region 1 and 2 alanine mutants on PIK-75 and J-32 inhibition—

The first major finding was the contrasting inhibition profile of PIK-75 binding compared with that of its close homologue J-32 (Figures 1A and 1B). Although PIK-75 is much less efficacious against the S773A mutant, J-32 inhibition was unaffected by the change. In contrast, J-32 was rendered much less active by the K776A mutant (27% decrease from the inhibition of WT, $P = 0.0124$) and I771A mutant (30% decrease from WT, $P = 0.0057$), and to a lesser extent the R777A mutation (not statistically significant). The IC₅₀ of J-32 for the K776A p110 α mutant was determined to be 0.59 μ M (Table 1), representing a 9-fold loss of potency compared with that against the p110 α WT enzyme. Similarly J-32 showed a 6-fold increase in IC₅₀ against the p110 α I771A mutant compared with the p110 α WT enzyme (Table 1).

J-32 and PIK-75 showed only a modestly reduced potency against the region 1 mutants compared with the WT p110 α .

Effect of PI3K p110 α region 1 and 2 mutants on A-66S and SN34452 inhibition

—A-66S (at 50 nM) inhibition was completely abolished by the Q859A mutation (Figure

1C). None of the other mutants were shown to be important for A-66S inhibition, with no significant change in inhibition observed in comparison with the WT enzyme (Figure 1C). SN34452 at 3 μ M was most influenced by the I771A and K776A mutants, which showed decreased inhibition of 17% ($P = 0.0488$) and 14% ($P = 0.0510$) respectively relative to the 42% inhibition of WT enzyme by 3 μ M SN34452.

No significant difference in SN34452 inhibition of the Q859A mutant was observed when compared with the WT p110 α enzyme (9% difference in the means, $P > 0.05$) (Figure 1D).

Reciprocal mutagenesis shows the effect of residues upon PI3K α/β selectivity

Scanning of region 1 and 2 amino acids for their effect on p110 α inhibition has identified several critical non-conserved amino acids involved in p110 α binding. In order to confirm the critical role in selectivity played by these non-conserved amino acids we conducted a reciprocal mutagenesis analysis whereby the specific residue in p110 α was swapped for the corresponding residue in p110 β and vice versa. The reciprocal effect of *in vitro* isoform mutation on inhibition might be considered diagnostic of an isoform-selective interaction between the relevant amino acid side chain and the inhibitor as it identifies both favoured and disfavoured interactions.

The data described above showed that mutation to alanine at Ser⁷⁷³, Lys⁷⁷⁶ and Gln⁸⁵⁹ caused significant loss of inhibitory potency in the three p110 α -selective inhibitors PIK-75, J-32 and A-66S. In order to assess the importance of these residues to selectivity, the reciprocal mutant pairs were produced, namely S773D p110 α and D780S p110 β , and K776M p110 α and M783K p110 β . We have also previously constructed the mutants Q859D p110 α and D862Q p110 β , as well as H855E p110 α and E858H p110 β . The IC₅₀ determinations for PIK-75, J-32 and A-66S against specific mutant pairs are summarized in Table 1. The combined effect of the change in selectivity brought about by the switch is summarized in Table 2. We have previously suggested that shifts of an order of magnitude indicate that the residue at that position plays a significant role in the selectivity profile of the compound.

Reciprocal mutagenesis shows that Ser⁷⁷³ is critical for PIK-75 binding and isoform selectivity—As shown previously [7], when Ser⁷⁷³ is mutated to the β -isoform amino acid equivalent (alanine to aspartate) the IC₅₀ increased 8-fold relative to the WT enzyme. When the reciprocal mutant in p110 β , D780S, was tested in the present study, the reverse effect was shown, with the PIK-75 IC₅₀ decreasing from 1.27 μ M to 0.34 μ M, a 4-fold decrease. Table 2 shows that the reciprocal mutation (Ser $\leftrightarrow$ Asp) rendered PIK-75 non-selective for p110 α , and for the mutant α/β pair a 29-fold change in selectivity was observed. This demonstrates the importance of Ser⁷⁷³ as a non-conserved amino acid involved in the isoform-selectivity of PIK-75 binding.

In comparison, the region 1 mutation p110 α H885E showed a more modest increase in IC₅₀ relative to the p110 α WT enzyme (Table 1). The reciprocal mutant in p110 β , E858H, showed a 2-fold decrease in IC₅₀ relative to the WT p110 β (Table 1) and as such showed a decrease in α/β selectivity from 29-fold (WT) to 6-fold (His $\leftrightarrow$ Glu). This is a much smaller change than that observed with the Ser⁷⁷³ mutants (Table 2). This was also reflected in the reciprocal ratio with a value for Ser⁷⁷³ of 29 compared with 5 for His⁸⁵⁵ (Table 2).

Region 2 non-conserved amino acids Ile⁷⁷¹ and Arg⁷⁷⁶ are important for binding, but not for isoform selectivity, of J-32—The alanine-scanning mutagenesis experiments described above showed that Ile⁷⁷¹ and Lys⁷⁷⁶ were critical for J-32 binding, with IC₅₀ values increased by 6- and 9-fold respectively, relative to the WT enzyme (Table 1). In the reciprocal mutagenesis experiments it was found that, against the I771Y mutant,

J-32 was 10-fold less potent, consistent with a role for this position in the observed selectivity (Table 1). However, the reciprocal mutation in p110 β , Y778I, also increased the IC₅₀, albeit by just 2-fold. Overall, the change observed in selectivity for the mutant pair (Ile $\leftrightarrow$ Tyr) compared with WT was just 6-fold (Table 2). In contrast with the dramatic effect upon J-32 inhibition observed with the K776A p110 α mutant, the effects obtained with reciprocal mutants were modest. In fact, J-32 was equipotent at K776M p110 α relative to the WT enzyme. Moreover, J-32 showed just a 2-fold decrease in the IC₅₀ for the reciprocal β mutant, M783K p110 β , compared with the WT p110 β enzyme. This result was reflected in the change observed in selectivity for the mutant pair (Lys $\leftrightarrow$ Met) compared with WT being just 3-fold (Table 2).

Overall, this showed that, while the non-conserved region 2 amino acids Ile⁷⁷¹ and Lys⁷⁷⁶ are involved in the binding of J-32 to p110 α , the reciprocal mutagenesis results suggest that the non-conserved nature of the residues in those positions do not contribute greatly to the observed selectivity. The activity of J-32 against the other available reciprocal mutants was also examined, but the change was relatively marginal. The region 1 mutant p110 α H855E showed a 2.5-fold increase in IC₅₀ compared with the WT enzyme as was found for PIK-75 (Table 1). However, in contrast with PIK-75, this effect on IC₅₀ was not reciprocal as the J-32 IC₅₀ for the p110 β mutant E858H was unchanged. J-32 also showed a much smaller effect than PIK-75 when Ser⁷⁷³ was mutated to either the p110 β (aspartate) or p110 γ (alanine) equivalent. The IC₅₀ values for these mutants showed a 2-fold increase when compared with WT enzyme (results not shown).

The region 1 non-conserved amino acid p110 α Gln⁸⁵⁹ is critical for the binding and isoform selectivity of A-66S—The results for A-66S inhibition of Q859A, together with the analogue SN34422, had provided a strong indication that this residue was involved in the observed p110 α potency and selectivity. The reciprocal mutation results supported this. The p110 α mutant Q859D resulted in an 11-fold increase in the A-66S IC₅₀ compared with that of the WT enzyme (75 nM) (Figure 2 and Table 1). The reciprocal mutant, p110 β D862Q, showed a 15-fold decrease in A-66S IC₅₀ when compared with WT p110 β (16.8 μ M) (Figure 2 and Table 1).

As shown in Table 2, A-66S, which shows 224-fold selectivity towards p110 α over p110 β , loses all of that selectivity by reciprocal mutation, Gln $\leftrightarrow$ Asp. This demonstrated that Gln⁸⁵⁹ was a critical amino acid in the binding of the A-66S inhibitor, and that its binding to p110 β was significantly increased by the equivalent α -substitution. This reciprocal effect (reciprocal ratio of 224, Table 2) indicates that Gln⁸⁵⁹ is also critical in determining the isoform-selective inhibition of A-66S.

PI3K α inhibitors are competitive with respect to the lipid substrate and are mixed inhibitors with respect to ATP

Previously we have shown that PIK-75 was a competitive inhibitor of p110 α with respect to the substrate PI, but a non-competitive inhibitor with respect to ATP [7]. This is a unique property among the PI3K inhibitors thus far studied kinetically. Indeed we have previously shown that the pan-PI3K inhibitor ZSTK-474 is a competitive inhibitor with respect to ATP, but a non-competitive inhibitor with respect to PI [7]. As most current PI3K inhibitors are ATP analogues they would most probably have the same kinetic properties as ZSTK-474.

As shown in Table 3, J-32 and A-66S are competitive inhibitors with respect to PI. This was reflected in the value of the constant α as determined by the mixed model of inhibition ranging from 30 for PIK-75 to 5 and 8 for J-32 and A-66S respectively. This was indicative of competitive inhibition with respect to PI. The K_i values for PI of 6.9 nM and 25 nM was found for J-32 and A-66S respectively in comparison with the PIK-75 value of 2.3 nM.

In addition, as for PIK-75 previously, there was non-competitive inhibition of ATP. The values of the constant α , determined using the mixed-model of inhibition, were 0.3, 0.5 and 0.2 for PIK-75, J-32 and A-66S respectively, indicative of non-competitive inhibition with respect to ATP; K_i values were calculated to be 36, 46 and 78nM respectively. The kinetics of SN34452 inhibition of p110 α was also competitive with respect to PI and non-competitive with respect to ATP (results not shown). This would indicate that removal of the carboxamide while significantly decreasing the extent of inhibition does not change the nature of inhibition of PI binding to the enzyme. Therefore it also suggests that Gln⁸⁵⁹ plays little or no role in PI binding, but is a major determinant of α -isoform selectivity, a result consistent with the mutagenesis results.

These results showed that while there are different modes of binding for the three α -selective inhibitors, competitive inhibition of PI binding and non-competitive inhibition of ATP binding is a common property of all three α -selective PI3K inhibitors.

DISCUSSION

Previously we have identified two regions of non-conserved amino acids that were demonstrated to be involved in PI3K α -isoform-selective inhibitor binding. Region 1 amino acids (852–860) were shown to be involved in α/β selectivity [6] and region 2 amino acids (768–781) were identified as being involved in the α -selectivity of PIK-75 [7]. As a consequence we have produced a large bank of *in vitro* mutants of non-conserved amino acids in regions 1 and 2. In the present study we have used our bank of mutants to analyse the binding of three PI3K α -isoform-selective inhibitors and determined the mechanism of isoform selectivity. The information available from the present study shows that analysis of binding of inhibitors to non-conserved amino acids is of great value in determining the mechanism of binding both in the presence (p110 γ and p110 δ) and the absence (p110 α and p110 β) of structural information of enzyme–inhibitor complexes.

In the present study we have shown that the three α -isoform-selective inhibitors used, PIK-75, J-32 and A-66S, have three different structural mechanisms for determining their selectivity.

In the case of PIK-75 we have previously shown that the non-conserved region 2 amino acid Ser⁷⁷³ was a critical amino acid in inhibitor binding. In the present study we have extended that finding using reciprocal α/β mutagenesis to demonstrate that Ser⁷⁷³ was also a critical amino acid in the isoform-selective binding of PIK-75, in that the reciprocal mutations Ser $\leftrightarrow$ Asp in p110 α and p110 β abolished PIK-75 α -isoform selectivity. Thus a direct inhibitor-binding interaction with Ser⁷⁷³ from region 2 was responsible for p110 isoform selectivity.

J-32 is a related PIK-75 α -isoform-selective inhibitor which differs only by the substitution of 6-hydrazine from a methyl group in PIK-75 to an ethyl group in J-32. Despite this small chemical difference between PIK-75 and J-32, the mechanism through which the two compounds bind are radically different. J-32 is insensitive to Ser⁷⁷³ mutation, but two other region 2 amino acids, Ile⁷⁷¹ and Lys⁷⁷⁶, when mutated to alanine markedly decreased J-32 inhibition of p110 α enzyme activity. We have examined the reciprocal α/β mutants for these amino acids and interestingly found that they do not appear to be directly involved in isoform selectivity.

That such a small change can induce a different binding profile in mutants, but not significantly affect the observed isoform selectivity, has two important implications for drug design. First, there may be two modes of binding or conformational rearrangement of the p-loop. Secondly, one of these may be critical to broader selectivity issues in the kinome. The

model of PIK-75 bound to p110 α [14] proposed that the methyl group of PIK-75 was approaching the conserved Met⁷⁷² which is part of the flexible p-loop of PI3K. We hypothesize that the addition of the ethyl chain has shifted the orientation of J-32 through a steric clash and prevented J-32 from binding to Ser⁷⁷³.

Recent data may give clues as to what the mechanism of J-32 selectivity may be. Heffron et al. [15] showed by plotting the Poisson–Boltzmann electrostatic potential on the solvent-accessible surfaces of the PI3K α crystal structure (PDB code 3HIZ) and using a β -isoform homology model that there were distinct differences in charge that may lead to isoform selectivity. The PI3K α -isoform surface showed a lipophilic pocket in contrast with the more positive-charged region observed in the p110 β model. Our analysis of this pocket shows that it is lined on one side by region 1 and on the other side by region 2. Of the non-conserved amino acids in these regions three (Q890D, H855E and S773D) are acidic in p110 β compared with p110 α . However, we have analysed these amino acids and none had any effect on J-32 binding.

In addition Heffron et al. [15] determined a crystal structure (PDB code 3R7R) of benzoxepin (114-fold selective for p110 α over p110 β) with p110 γ which showed the inhibitor to be positioned between regions 1 and 2, but there was no direct interaction with any side chain [15]. Thus it is possible to attain this level of selectivity without any direct interaction, i.e. without hydrogen-bonding to a non-conserved amino acid side chain. However, there is a caveat to this conclusion in that this selectivity is limited to p110 β as the other two PI3K isoforms have similar potency to the α -isoform.

Although J-32 is not targeting acidic amino acids it may be selectively targeting the increased lipophilic nature of the p110 α -binding pocket. Another possible mechanism for J-32-selective binding was described by Heffron et al. [15] when they analysed a benzoxepin analogue containing a piperazineamide carbonyl moiety which was 40-fold selective for p110 α over p110 β . In a crystal structure of this inhibitor complexed with p110 γ (PDB code 3T8M) it was shown that the piperazineamide carbonyl moiety was hydrogen-bonded to a conserved amino acid in the p-loop, Ser⁸⁰⁶ (Ser⁷⁷⁴ in p110 α). J-32 could be bound to this conserved Ser⁸⁰⁶ rather than the non-conserved Ser⁷⁷³. The isoform selectivity would be produced by the non-conserved amino acids Ile⁷⁷¹ and Lys⁷⁷⁶, producing an isoform-specific p-loop conformation. Regardless, it is apparent that the role of the region 2 non-conserved amino acids in the isoform selectivity of J-32 is through an indirect interaction.

The PI3K p-loop has been shown previously to adopt several different conformations in the presence of inhibitors and ligands. Walker et al. [16] first showed that the PI3K p110 γ crystal structure (PDB code 1E8Y) shared a five-stranded β -sheet with the protein kinases. In particular the p-loop between β -sheets 3 and 4 contains a conserved Ser⁸⁰⁶ which interacts with the β -phosphate of ATP. Mandelker et al. [12] showed that, in the p110 α –wortmannin structure (PDB code 3HHM), the inhibitor induced a 3 Å (1 Å = 0.1 nm) shift in the p-loop conformation. They noted that this p-loop conformational change did not occur in the p110 γ structure in the presence of wortmannin (PDB code 1E7U). In addition, the p-loop conformation was also affected by the oncogenic mutation H1047R. The p-loop conformation has also been implicated in the different structural changes observed when the δ -specific inhibitor PIK-39 bound to p110 δ (PDB code 2WXF) and p110 γ (PDB code 2CHW) [17]. The importance of the role for conformational adaption of the target in drug–target interactions has been emphasized in a recent review [18].

A-66S is the most selective p110 α inhibitor thus far described, and in the present study we have provided the first unequivocal evidence that targeting non-conserved amino acids in

PI3K can produce an isoform-selective inhibitor. The region 1 amino acid Gln⁸⁵⁹ was critical to A-66S binding and also critical for selectivity, as shown by the reciprocal mutation in p110 β recovering the affinity of A-66S. The reciprocal ratio of 224 was considerably higher than the ratio of 29 found for PIK-75. That the carboxamide was the critical moiety in the interaction with Gln⁸⁵⁹ was confirmed by the lack of effect of the Q859A mutation on SN34452 inhibition. Thus perhaps there is a difference in the nature of the proposed interaction between PIK-75 and A-66S with the nitro oxygen atom of PIK-75 proposed by molecular modelling to form a single hydrogen bond with Ser⁷⁷³ [14] and the carboxamide forming two hydrogen bonds with the side chain of Gln⁸⁵⁹ [11]. The stronger interaction with a non-conserved amino acid leads to higher isoform selectivity. Even higher isoform selectivity could possibly be attained by substituents on A-66S binding to a non-conserved amino acid in region 2 or other non-conserved amino acids in region 1.

Although the three p110 α -selective inhibitors exhibit three different mechanisms of binding, they share the common kinetic property of being competitive inhibitors with respect to the lipid substrate. This is unique among PI3K inhibitors, whether pan- or isoform-selective, which are generally competitive with respect to ATP. This further strengthens the idea [7] that the α -selective inhibitors form a new class of PI3K inhibitors, and regions 1 and 2 form a new chemical space in which to generate further isoform-specific inhibitors. This new class of inhibitors is analogous to the situation found in protein kinases, with Type I inhibitors being ATP-competitive and Type III being ATP-non-competitive [19]. Future clinical trials will determine the utility of this new class of PI3K inhibitors in the treatment of cancers and other diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

PI	phosphatidylinositol
PI3K	phosphoinositide 3-kinase
PIP₃	phosphatidylinositol 3,4,5-trisphosphate
WT	wild-type

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Figure 1. Mutant scan of the effect of region 1 and region 2 non-conserved amino acids on the inhibition of p110 α enzyme activity

The WT enzyme and the indicated mutant enzymes were assayed in the presence and absence of the inhibitor. 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) Percentage inhibition of p110 α enzyme activity by 50 nM A-66S of WT, and region 1 and region 2 mutant enzymes as indicated. The broken line across the histogram indicates the WT inhibition level. Results are shown as the means $\pm$ S.E.M. ($n = 5-6$). (B) Percentage inhibition of p110 α enzyme activity by 3 μ M SN34452 of WT, and region 1 and region 2 mutant enzymes as indicated. The broken line across the histogram indicates the WT inhibition level. Results are shown as the means $\pm$ S.E.M. ($n = 4$). (C) Percentage inhibition of p110 α enzyme activity by 50 nM PIK-75 of WT, and region 1

and region 2 mutant enzymes as indicated. The broken line across the histogram indicates the WT inhibition level. Results are shown as the means $\pm$ S.E.M. ($n = 4$). **(D)** Percentage inhibition of p110 α enzyme activity by 60 nM J-32 of WT, and region 1 and region 2 mutant enzymes as indicated. The broken line across the histogram indicates the WT inhibition level. Results are shown as the means $\pm$ S.E.M. ($n = 4$).

Figure 2. A-66S inhibition of p110α WT and p110α Q859D along with p110β WT and p110β D862Q

Graph of the percentage of the PI3K activity remaining in the presence of various concentrations of the inhibitor A-66S. The curves shown are for the purified enzymes as follows: p110α WT (○), p110α Q859D (●), p110β WT (□) and p110β D862Q (▲).

Table 1
Analysis of isoform mutant effect on inhibitor potency and selectivity

p110 α enzymes, mutants and WT, were expressed and purified as described in the Experimental section. Mutants are of amino acids in region 1 and region 2 (Supplementary Figure S2 at <http://www.BiochemJ.org/bj/444/bj4440529add.htm>). IC₅₀ values are derived from three separate sets of duplicate determinations and are expressed as means $\pm$ S.D. The fold-change is calculated by dividing the IC₅₀ value for the mutant enzyme by that of the WT enzyme.

(a) PIK-75

Enzyme	IC ₅₀ (μ M)	Fold change (relative to WT)
p110 α WT	0.044 $\pm$ 0.011	1
p110 α S773D	0.346 $\pm$ 0.076	7.9
p110 α H855E	0.107 $\pm$ 0.023	2.4
p110 β WT	1.27 $\pm$ 0.21	1
p110 β D780S	0.341 $\pm$ 0.049	0.27
p110 β E858H	0.622 $\pm$ 0.051	0.49

(b) J-32

Enzyme	IC ₅₀ (μ M)	Fold change (relative to WT)
p110 α WT	0.067 $\pm$ 0.007	1
p110 α I771A	0.378 $\pm$ 0.092	5.5
p110 α I771Y	0.661 $\pm$ 0.042	10
p110 α K776A	0.590 $\pm$ 0.087	8.8
p110 α K776M	0.090 $\pm$ 0.008	1.3
p110 α H855E	0.166 $\pm$ 0.023	2.5
p110 β WT	1.11 $\pm$ 0.15	1
p110 β Y778I	2.00 $\pm$ 0.16	2
p110 β M783K	0.550 $\pm$ 0.062	0.50
p110 β E858H	1.07 $\pm$ 0.14	0.96

(c) A-66S

Enzyme	IC ₅₀ (μ M)	Fold change (relative to WT)
p110 α WT	0.075 $\pm$ 0.004	1
p110 α Q859D	0.853 $\pm$ 0.079	11.4
p110 β WT	16.8 $\pm$ 2.2	1
p110 β D862Q	1.1 $\pm$ 0.1	0.065

Table 2
Analysis of the reciprocal nature of isoform mutations

Results for the following mutant pairs are shown: p110 α S773D/p110 β D780S, p110 α H855E/p110 β E858H, p110 α I771Y/p110 β Y778I, p110 α K776M/p110 β M783K, and p110 α Q859KD/p110 β D862Q. Selectivity was calculated from the IC₅₀ value of the inhibitor for p110 α and the corresponding IC₅₀ for p110 β for the WT enzyme and the indicated mutant enzymes. A value of > 1 indicates a preference for p110 α . The reciprocal ratio is the fold-change of the α/β selectivity for each mutant pair from that of the WT α/β selectivity.

Compound	PI3K	Selectivity α/β	Reciprocal ratio α/β
PIK-75	WT	29	–
	Ser $\leftrightarrow$ Asp	1	29
	Glu $\leftrightarrow$ His	6	5
J-32	WT	17	–
	Ile $\leftrightarrow$ Tyr	3	6
	Lys $\leftrightarrow$ Met	6	3
	Glu $\leftrightarrow$ His	6	3
A-66S	WT	224	–
	Gln $\leftrightarrow$ Asp	1	224

Table 3
Kinetic characterization of PIK-75, J-32 and A-66S inhibition of p110α WT

Kinetic analysis of the effect of inhibitor on PI and ATP binding to the WT p110α enzyme. The kinetic constant α was estimated using the mixed inhibition model (GraphPad Prism). The constant α is indicative of competitive inhibition if > 1 and of non-competitive inhibition if < 1 . K_i and K_m ($\pm$ S.E.M., $n = 2$) were estimated using the competitive model (GraphPad Prism) for the PI kinetics and using the non-competitive model for ATP kinetics (GraphPad Prism).

Substrate Inhibitor	PI		ATP			
	PIK-75*	J-32	A-66S	PIK-75*	J-32	A-66S
α Constant	30	5	8	0.3	0.5	0.2
K_i (nM)	2.3 ± 0.6	6.9 ± 2.8	25 ± 9	36 ± 6	46 ± 5	78 ± 8
K_m (nM)	7.0 ± 1.5	1.5 ± 0.5	4.7 ± 0.7	24 ± 5	33 ± 4	51 ± 8

* Values are from previously published data [7].