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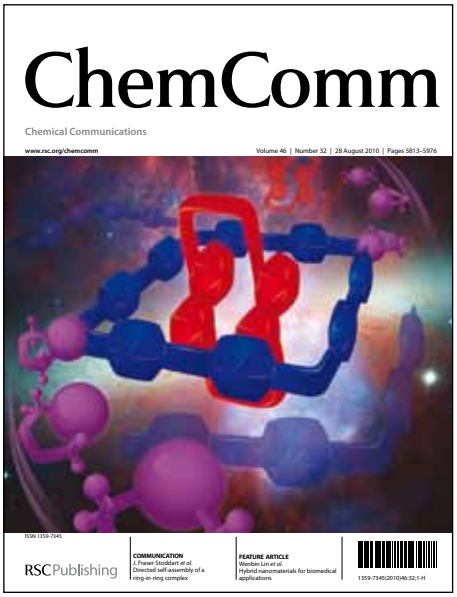

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ARTICLE TYPE

Potent, selective small molecule inhibitors of type III phosphatidylinositol-4-kinase α- but not β- inhibit the phosphatidylinostiol signaling cascade and cancer cell proliferation

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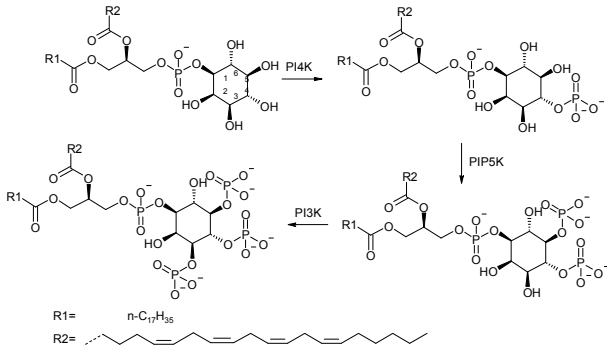
Two series of inhibitors of type III phosphatidylinositol-4-kinase were identified by high throughput screening and optimised to derive probe compounds that independently and selectively inhibit the α- and the β- isoforms with no significant activity towards related kinases in the pathway. In a cellular environment, inhibition of the α- but not the β-subtype led to a reduction in phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-phosphate concentration, causing inhibition of inositol-1-phosphate formation and inhibition of proliferation in a panel of cancer cell lines.

Phosphatidylinositol-4-kinases (PI4Ks) catalyse the phosphorylation of phosphatidylinositol (PI) at the D4 position of the inositol head-group to synthesise phosphatidylinositol-4 phosphate (PI4P). PI4P acts as a binding partner for a wide range of proteins with pleckstrin homology (PH) or related lipid-binding domains and has reported roles in membrane trafficking. PI4P is also an important signalling molecule as a precursor to other phosphoinositides such as phosphatidylinositol-4,5-diphosphate [PI(4,5)P_2] and phosphatidylinositol-3,4,5-triphosphate [PI(3,4,5)P_3] (**Scheme 1**).[1]

There are four PI4Ks in mammals, termed type II (PI4KIIα and β) and type III (PI4KIIIα and β, hereafter referred to as PI4Kα and β). Each shows differential sub-cellular distributions resulting in compartmentalised PI4P synthesis. PI4Kα is the main isoform responsible for PI4P generation at the plasma membrane and is also localised at the endoplasmic reticulum. PI4Kβ is mainly associated with the Golgi complex where it functions in the generation of Golgi-derived carriers. In addition to the Golgi apparatus, PI4Kβ has also been visualised on lysosomes where it functions to maintain lysosomal membrane integrity.[2]

PI4Ks have been implicated in human disease. There are several reports of increased mRNA expression of the different PI4K isoforms in tumours and tumour cell lines.[3,4] In addition it has been reported that over expression of the Golgi-associated PI4P-binding protein GOLPH3 results in tumour growth and transformation by activating the mTOR signalling pathway.[5] The gene encoding GOLPH3 is located in a specific region of chromosome 5p13 frequently amplified in solid tumours. There are also several reports of an important role for the PI4Ks in the replication and secretion of multiple viruses.[6,7,8]



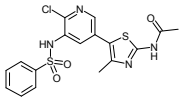
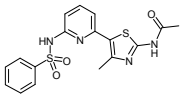
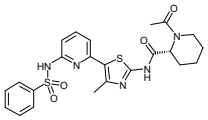
Scheme 1 The PI4K cascade

Pharmacological manipulation of cellular PI4P levels has been a challenge due to the non-specificity and lack of potency of known PI4K inhibitors such as wortmannin, LY294002 and phenylarsine oxide;[9] although more recently, reports have emerged describing PI4K inhibitors from several groups.[6,7,8] Hence, suitable chemical probes that are more potent and selective towards other enzymes in the pathway, with particular emphasis on the downstream kinases PIP5K and PI3K, were sought. These probes would elucidate the roles of PI4Kα and β in the PI signalling pathway and investigate the utility of inhibition of these enzymes in disease modification.

A set of roughly 100,000 compounds enriched in substructures likely to bind to lipid kinases was selected from the AstraZeneca compound collection and screened against recombinant PI4Kβ. Active compounds from this screen were selected for follow-up IC_{50} determination against PI4Kα and β. This process revealed acylaminothiazole **1** (**Table 1**), which was a potent inhibitor of both PI4Kα and β (pIC_{50} 8.0 and 8.3 respectively) but was more active against PI3Kα (pIC_{50} 8.5) and had some residual activity against PIP5Kγ (pIC_{50} 6.2).[10] A series of analogues based on this lead revealed that PI4Kα, PI3K and PIP5K activities could all be diminished significantly by changing the position of the nitrogen in the central pyridine ring albeit with a 1 log drop in PI4Kβ potency (illustrated by **2**). Library synthesis varying the thiazole

amide revealed that both potency and selectivity could be optimised by modifications in this region. As part of this exercise, **3** was revealed as a potent (pIC_{50} 7.8) inhibitor of PI4Kβ with good lipid kinase selectivity (pIC_{50} 5.1, 4.7 and <4.0 vs. PI4Kα, PI3Kα and PIP5Kγ respectively).

Table 1 Optimisation of PI4Kβ inhibitor probes

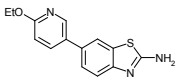
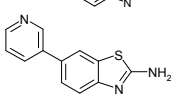
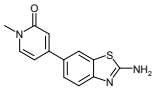
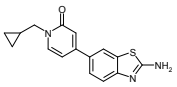
Compound	Structure	PI4Kα pIC_{50}	PI4Kβ pIC_{50}	PI3Kα pIC_{50}	PIP5Kγ pIC_{50}
1		8.0	8.3	8.5	6.2
2		5.3	7.2	6.1	6.2
3		5.1	7.8	4.7	<4.0

The same high throughput screen described above also identified **4** (**Table 2**), which was appealing as a starting point for a PI4Kα probe as it exhibited greater potency for PI4Kα (pIC_{50} 7.0) than PI4Kβ (pIC_{50} 6.4). Hence, structural modifications were explored with the aim of improving PI4Kα potency and selectivity. Truncation of the ethoxy- substituent of **4** revealed a quite exquisite structure activity relationship (SAR). Removal of the substituent as in **5** showed a loss of potency and selectivity. This lack of selectivity is perhaps unsurprising since this core motif is precedented to give rise to potent PI3K inhibitors.[11]

With the substituent removed, other replacements for the 3-pyridyl ring were investigated. Replacement with N-methylpyridone **6** led to an increase in potency (for both PI4Kα and β) without increasing PI3K activity. Introduction of an N-substituent on the pyridone showed that marked gains in potency and selectivity could be achieved once the substituent reached a certain size. Most notably, a significant increase in PI4Kα potency was achieved with the N-cyclopropylmethyl derivative **7**, which was a potent (pIC_{50} 8.2) inhibitor of PI4Kα with only weak activity against PI4Kβ, PI3Kα or PIP5Kγ (pIC_{50}s 5.9, 5.2 and <4.0 respectively).

The observed SAR was rationalised using protein structures of PI3K (pdb code 2CHZ), PI4Kα (model based on 4AOF) and PI4Kβ (model based on 2CHZ). Unselective **1** can dock reasonably into all three models (**Fig. 1 a+b**). In addition to the hinge interaction, the crucial interaction for this compound is between the anionic nitrogen of the sulfonamide (pK_a=5.6) and the nearby Lys833 residue (PI3Kγ). The equivalent Lys is also present in both PI4K isoforms but is far less accessible in the α-isoform. On altering the pyridine substitution pattern as in **2**, this nitrogen is no longer charged, the preferred ligand conformation is altered and the interaction with Lys is lost. In PI4Kβ, a new interaction is formed between the oxygen of the sulfonamide and the Lys (**Fig. 1d**) but this cannot be accommodated in PI3Kγ, hence the greater selectivity of **2**.

Table 2 Optimisation of PI4Kα inhibitor probes

Compound	Structure	PI4Kα pIC_{50}	PI4Kβ pIC_{50}	PI3Kα pIC_{50}	PIP5Kγ pIC_{50}
4		7.0	6.4	5.8	<4.0
5		5.8	6.2	5.8	4.5
6		6.4	6.5	5.6	<4.0
7		8.2	5.9	5.2	<4.0

The β-selective **3** docks well into PI4Kβ (**Fig. 1d**) but not PI4Kα under the same protocol. Comparison with the less selective **2** (**Fig. 1c**) shows that the acylpiperidine group of **3** would clash with the protein in the α-isoform while the same group fits well and makes an additional interaction with a nearby Lys residue in the β-isoform. This is likely due to the Pro residue adjacent to the hinge in PI4Kα: the restricted geometry constrains the protein conformation and thus blocks this part of the binding pocket. The equivalent residue in PI4Kβ (Val) imposes no such restrictions. Even for **2**, the α-isoform scores the pose less well, fitting with the β-bias of the whole series.

The α-selective **7** docks well in the PI4Kα model (**Fig. 1e**) but fails to dock into PI4Kβ. The similar, but less-selective compound **5** docks well in the β-isoform (**Fig. 1f**) showing that the pocket in PI4Kβ is not large enough to accommodate the cyclopropylmethyl group; this group fits well in PI4Kα. Critically, Arg1765 in PI4Kα defines the subpocket that accepts the cyclopropylmethyl group and reduces the overall volume of the site and preventing the larger compounds **2** and **3** from binding well.

Compounds **3** and **7** were tested at 1 μM in the Millipore kinase panel consisting of 259 different kinases.[12] Compound **3** showed no activity against any kinase in the panel, **7** only showed activity against three targets (FGR 98%, ZIPK 72% and STK17A 68%, for full results see ESI). Critically for subsequent experiments, neither compound showed significant activity against PDGF-receptor tyrosine kinase.

In order to assess the cellular activity of **3** and **7**, the effect of the compounds on inositol monophosphate (IP_1) was measured. PI4P is phosphorylated by the PIP5Ks to synthesise $PI(4,5)P_2$. $PI(4,5)P_2$ is hydrolysed by phospholipase Cs (PLCs) to generate inositol triphosphate and subsequently IP_1. PLCγ can be activated to hydrolyse $PI(4,5)P_2$ by stimulation of receptor tyrosine kinases. NIH3T3 cells overexpressing PDGFRβ were stimulated with PDGF resulting in a dose-dependent increase in IP_1 concentration. Treatment of the cells with **3** and **7** prior to PDGF stimulation revleaved that **7** (PI4Kα) inhibited the accumulation of IP_1 (pIC_{50} 6.3) whereas **3** (PI4Kβ) did not.

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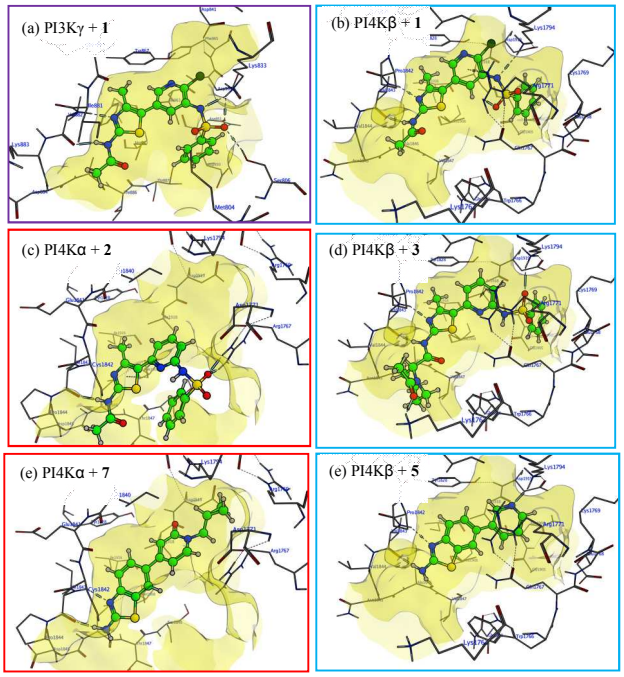

Fig. 1 Protein homology models with ligands (highlighted with green carbons) bound. Protein Van der Waals surface is shown in yellow; Positive directional interactions, including hydrogen-bonds are shown as dotted lines.

In order to examine a more proximal marker of inhibition, tandem mass spectrometry was used for the direct measurement of PIP, PIP_2 and PIP_3. NIH3T3-PDGFRβ cells were treated with **7** and **3** (30 μM for 2 hours) in serum-free media followed by subsequent stimulation with PDGF for 5 minutes. These studies revealed that the PI4Kα inhibitor **7** caused a qualitative reduction in cellular PIP, PIP_2 and PIP_3 levels (**Table 3** and ESI).[13] The PI4Kβ inhibitor **3** showed no effect.

Table 3 Effects of **3** and **7** on various endpoint in the PI pathway

Compound	IP1	Inhibition at 30 μM			
	pIC_{50}	PIP	PIP_2	PIP_3	$PI(4,5)P_2$ (basal)
3	<4.5	-	-	-	0%
7	6.3	+	+	+	19%

Since these mass spectrometric measurements are not isomer specific, effects on $PI(4,5)P_2$, were determined using a fluorescence-based method consisting of a fusion of the PH domain of PLCδ1, known to specifically bind $PI(4,5)P_2$, with red fluorescent protein (RFP) overexpressed in U2OS cells.[14] Treatment of this system with **3** and **7** at 30 μM showed that **7** (PI4Kα) inhibited the formation of basal $PI(4,5)P_2$ at the membrane (19% inhibition). The PI4Kβ inhibitor **3** showed no effect.

Both **3** and **7** were tested for growth inhibition in a panel of 183 cancer cell lines. PI4Kα inhibitor **7** inhibited cell growth with pGI_{50} values of >5.0 in 91 of the cell lines. In contrast, PI4Kβ inhibitor **3** showed a pGI_{50} >5.0 in only 18 lines. (**Fig. 2** and ESI).

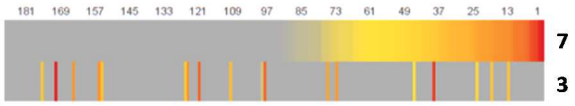

Fig. 2 Heat map of pGI_{50} values for **3** and **7** in a panel of 183 cancer cell lines. Colour shows pGI_{50} from 5.0 (yellow) to 6.1 (red), grey <5.0.

Conclusions

These studies highlight the value of high quality small molecule inhibitors as probes to interpret complex biological cascades. The exquisite selectivity of the chemical probes allows their pharmacology to be ascribed to their intended targets with a high degree of confidence and should be of further utility in the study of PI4K biology and the PI pathway in general.

Notes and references

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† Electronic Supplementary Information (ESI) available: Details of experimental procedures, assay protocols and full data for growth inhibition. See DOI: 10.1039/b000000x/

Samples of compounds **3** and **7** are available on request for further biological study.

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