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Design and structural characterization of potent and selective inhibitors of phosphatidylinositol 4 kinase III β

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Abstract

Type III Phosphatidylinositol 4-kinase (PI4KIII β) is an essential enzyme in mediating membrane trafficking, and is implicated in a variety of pathogenic processes. It is a key host factor mediating replication of RNA viruses. The design of potent and specific inhibitors of this enzyme will be essential to define its cellular roles, and may lead to novel anti-viral therapeutics. We previously reported the PI4K inhibitor PIK93, and this compound has defined key functions of PI4KIII β . However, this compound showed high cross reactivity with class I and III PI3Ks. Using structure-based drug design we have designed novel potent and selective (>1000 fold over class I and class III PI3Ks) PI4KIII β inhibitors. These compounds showed anti-viral activity against Hepatitis C Virus. The co-crystal structure of PI4KIII β bound to one of the most potent compounds reveals the molecular basis of specificity. This work will be vital in the design of novel PI4KIII β inhibitors, which may play significant roles as anti-viral therapeutics.

Graphical Abstract

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ANCILLARY INFORMATION

Supporting Information.

Supporting information including compound characterization, and two supplemental figures are found in the supplemental information, and this material is available free of charge via the *Internet* at <http://pubs.acs.org>.

PDB ID Codes: The structure factors and pdb coordinates have been deposited at the protein databank (PDB) with the coordinates 5EUQ.

Author Contributions

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Introduction

Lipid phosphoinositides are essential regulators of myriad cellular processes, including signaling, membrane trafficking, and cytokinesis¹. Phosphoinositides are generated through the phosphorylation of the inositol ring of phosphatidylinositol. Phosphatidylinositol can be phosphorylated and dephosphorylated by a diverse set of enzymes, and this results in a total of seven different mono and poly phosphorylated phosphoinositides. The lipid species phosphatidylinositol 4-phosphate (PI4P) is generated by the action of phosphatidylinositol 4 kinases (PI4Ks). PI4P is the main biosynthetic route for the multiply phosphorylated signaling lipids phosphatidylinositol 4,5-bisphosphate (PIP₂), and phosphatidylinositol 3,4,5-trisphosphate (PIP₃)². In mammals there are four different PI4K enzymes, two type II enzymes (PI4KIIα and PI4KIIβ) and two type III enzymes (PI4KIIIα and PI4KIIIβ). PI4KIIIβ is a peripheral membrane protein that is primarily localized at the Golgi and the Trans Golgi Network (TGN). This enzyme plays key roles in mediating lipid transport³, cytokinesis⁴, maintaining lysosomal identity⁵, and in tandem with Rab GTPases plays key roles in regulating membrane trafficking⁶. Interest in the development of potent small molecules of PI4KIIIβ has been driven recently by the discovery of the key role of this enzyme in both mediating viral replication⁷, as well as in mediating *Plasmodium* development⁸.

PI4KIIIβ is critical for mediating viral replication of a number of RNA viruses through the generation of PI4P enriched viral replication platforms. These membranous webs enriched in PI4P play essential roles in spatially concentrating viral replication proteins, and are key in intracellular viral replication. This process is essential for many human pathogenic viruses including Poliovirus, coxsackieviruses, Enterovirus 71, rhinovirus, and Aichi virus^{7,9–13}. There is also evidence that PI4KIIIβ together with PI4KIIIα play a key role in mediating viral replication of Hepatitis C virus¹³.

Small molecule inhibitors of PI4KIIIβ are potent anti-viral agents^{7,14,15}. We previously reported the potent PI4KIIIβ inhibitor PIK93 (compound **1**)¹⁶, and this compound has been used extensively to decipher the cellular roles of PI4KIIIβ^{3,17}, and its role in mediating viral replication of pathogenic RNA viruses^{7,10–13}. Compound **1** potently inhibits PI4KIIIβ; however, it shows cross reactivity towards a number of other lipid kinases. Compound **1** has very similar IC₅₀ values for PI4KIIIβ, class III PI3 kinase (vps34), and class IB PI3Kγ (Fig

1A). We have previously crystallized **1** in complex with PI4KIII β ¹⁸, vps34¹⁹, and with PI3K γ ¹⁶ (Fig. 1B–E).

Development of PI4KIII β as an effective drug target for anti-viral therapeutics requires the generation of highly potent and specific inhibitors. We report the development of a set of derivatives from compound **1**, and these represent some of the most potent PI4KIII β inhibitors reported to date. The selectivity profile of these compounds has been determined against vps34, PI3K δ and PI3K γ , with the most selective compounds being >1000 fold selective over the related PI3K family of lipid kinases. We have successfully determined the structure of PI4KIII β bound to one of the most potent and selective compounds, and this structure reveals the molecular basis for the increased selectivity and potency of these compounds.

Results

Design of optimized PI4KIII β inhibitors

Compound **1** is highly selective for PI4KIII β over PI4KIII α , however, it is similarly potent for a number of phosphoinositide 3-kinases (PI3Ks), specifically the class I isoforms PI3K γ (also referred to as p110 γ) and PI3K δ (also referred to as p110 δ), as well as the class III PI3K vps34 (Fig. 1A). The structures of **1** bound to vps34¹⁹, PI3K γ ¹⁶, and PI4KIII β ¹⁸ revealed that within the binding pocket there were significant opportunities to modify **1** to increase both potency and selectivity for PI4KIII β .

From examining the structures of **1** bound to each enzyme, there were three regions of the molecule that were focused on to optimize both potency and selectivity of novel PI4KIII β inhibitors. These consisted of modifying the substituent on the central phenyl ring (colored green, Fig. 1A), the substituent off the sulfonamide (colored blue, Fig. 1A), and the acetamide moiety (colored red, Fig. 1A). The chloro substituent on the central phenyl ring of **1** fits into a pocket partially composed of the activation loop of both PI3Ks and PI4KIII β (Fig. 1C). The conformation of the activation loop of PI3K γ when bound to compound **1** is positioned closer to this substituent than in either vps34 or PI4KIII β . This suggested that modifying this group to a larger substituent might increase selectivity over class I PI3Ks. Changing this group to a bromo substituent (compound **2**) caused a slight increase in potency for PI4KIII β , PI3K δ and vps34, and a slight decrease in potency for PI3K γ . Modifying this group to a methoxy substituent (compound **3**) caused both an increase in potency for PI4KIII β and a large increase in specificity over PI3K γ , PI3K δ (>40 fold selective for both) and vps34 (Fig. 2A).

The structures of **1** bound to PI4KIII β , PI3K γ , and vps34 also suggested that modification of the acetamide group derived from the central thiazole might lead to further gains in specificity. PI4KIII β has a much more open pocket around the acetamide group of **1** (Fig. 1C), compared to both PI3K γ (Fig. 1D), and vps34 (Fig. 1E) which have a number of bulky hydrophobic residues. The methyl group of **1** was replaced with either a *t*-butyl group (compound **4**) or a cyclopentyl group (compound **5**). Both compounds showed similar or slightly better potency against PI4KIII β , however, both showed a very large increase in

selectivity over PI3K γ (>600 fold for compound **4** and >90 fold for compound **5**), PI3K δ , and vps34 (Fig. 2A).

Finally the ethanolamine attached to the sulfonamide in compound **1** was also derivatized. The ethanolamine was replaced with either a para-hydroxy phenol group (compound **6**) or a para-fluoro phenyl (compound **7**). The presence of the para-hydroxy phenol group caused a large increase in potency for all lipid kinases tested, with minimal differences in both potency and specificity for the para-fluoro phenyl substituent (Fig. 2A).

Combining this set of information we generated three compound **1** derivatives (compounds **8+9+10**) that contained derivations at multiple positions of **1**. All of these compounds contained a methoxy substituent off the central phenyl, with compound **9+10** containing a para-hydroxy phenol off the sulfonamide, and either a cyclopentyl (compound **9**) or a *t*-butyl (compound **8+10**) group at the acetamide position off of the central thiazole. Both of the compounds containing the para hydroxyl phenol were extremely potent against PI4KIII β (IC₅₀s of 7 nM and 3.6 nM for compound **9+10** respectively), and were >140 and >1000 fold selective over PI3K γ , were >20 and >200 fold selective over PI3K δ and showed no inhibition of vps34 at concentrations up to 20 μ M (Fig. 2B). Compound **8** was not as potent (IC₅₀ of 36 nM), but showed an excellent selectivity profile, with no inhibition of both PI3K γ and vps34 (<20% at 50 μ M), and very little inhibition of PI3K δ (<50% inhibition at 50 μ M). Compound **10** is the most potent PI4KIII β inhibitor currently reported; with very minor off-target inhibition of PI4KIII β related lipid kinases. Both compound **9+10** were further characterized against a panel of six additional lipid kinases (Fig. S1). Compound **9** showed weak inhibition of PI3K α (IC₅₀ ~1 μ M), PI3K β (~2 μ M), and PI4KIII α (~2.6 μ M) and <50% inhibition at concentrations up to 20 μ M for PI4K2 α , PI4K2 β , and PI3K β . Compound **10** showed weak inhibition of PI3K α (IC₅₀ ~1 μ M), PI3K β (~10 μ M), and PI4KIII α (~3 μ M), and <20% inhibition at concentrations up to 20 μ M for PI4K2 α , PI4K2 β , and PI3K β .

PI4KIII β has been shown to be a key host factor for the replication of the Hepatitis C virus^{13,20}. To test the potency of the compound **1** derivative inhibitors as anti-virals we carried out viral replication assays using a luciferase reporter-linked infectious HCV clone. These results (Fig. 2, S2) generally show a trend of the most potent molecules being the most effective anti-virals, with increased specificity leading to decreased toxicity. One noticeable trend in the assay was that absence of the hydroxyl-phenyl group led to a large decrease in the anti-viral potency of the compounds. This may be due to differences in cell permeability caused by this group. The compounds with the best combination of anti-viral efficacy, and lowest toxicity were compounds **9** and **10** (Fig. S2).

Structural basis of specificity for PI4KIII β inhibitors

To determine the molecular basis for the potency and specificity of these novel PI4KIII β inhibitors we set out to crystallize them bound to PI4KIII β . We have previously crystallized a truncated construct of PI4KIII β in complex with compound **1** and the GTPase Rab11¹⁸. This structure revealed the molecular basis of its interaction with Rab11, as well as revealing the binding of compound **1** to PI4KIII β , however, this construct only crystallized in the

presence of compound **1**. To generate crystals of PI4KIII β bound to compound **9** we used a novel crystallization construct generated through the use of a hydrogen deuterium exchange mass spectrometry based approach²¹. This construct allowed us to generate crystals of PI4KIII β bound to GDP loaded Rab11 in the absence of inhibitors. These crystals were used to soak compound **9**, and allowed us to solve the co-crystal structure at 3.2 angstrom resolution. The binding mode of compound **9** was unambiguous (Fig. S3). The residues mediating contacts with compound **9** are shown in Fig. 3 A, with the shape of the inhibitor in the active site pocket shown in Fig. 3B.

Structure of PI4KIII β bound to compound **9**

Compound **9** forms a crescent shape that conforms to the active site of PI4KIII β . This molecule makes extensive contacts with PI4KIII β (Fig 3A). There are two putative hydrogen bonds formed between the thiazole and the acetamide of compound **9** with both the amide and carbonyl group of V598. The sulfonamide group also forms a hydrogen bond with Lys549. All of these hydrogen bonds are similar to those reported in the structure of **1** bound to PI4KIII β ¹⁸. Intriguingly the para-hydroxy group on the N-phenol sulfonamide also makes a putative hydrogen bond with the carbonyl of G660. The presence of this para hydroxyl group is important for potency, as molecules lacking this group are less potent against PI4KIII β (compound **6** versus compound **7**). The presence of this hydroxyl group also increases potency for both PI3K γ and vps34 suggesting that this putative hydrogen bond is most likely conserved across all three enzymes.

The structure of compound **9** also reveals the molecular mechanism for how the acetamide and methoxy substituent mediate inhibitor selectivity over PI3K γ and vps34. Aligning the structure of PI4KIII β bound to compound **9** with structures of compound **1** bound to PI3K γ and vps34 (Figure 4A–C), reveals a number of steric clashes that explain the lack of potency of compound **9** against the PI3K family kinases. The acetamide group clashes with residues W812 and M953 in PI3K γ , and residues Y746 and F673 in vps34. The methoxy group off the central phenyl group clashes with the aspartic acid from the DFG motif in the activation loop (D964 in PI3K γ and D823 in vps34).

Discussion

Phosphatidylinositol 4 kinase III β plays both key physiological and pathological roles, and the design of novel potent and selective inhibitors will be essential both in deciphering the cellular functions of this enzyme, as well as in the development of potential future therapeutic agents in diseases dependent on PI4KIII β activity. PI4KIII β mediates the replication of a variety of pathogenic RNA viruses, including members of both the *Picornaviridae* and *Flaviviridae* family of viruses⁹. These include viruses that pose significant threats to human health including SARS, MERS, Hepatitis C, and Polio. Along with this key role in mediating pathological conditions, PI4KIII β also plays key roles in a number of physiological roles including membrane trafficking²², lipid transport²³, and cytokinesis⁴ and the development of potent and selective molecules will be essential in deciphering the isoform specific functions of this enzyme.

Since PI4KIII β plays an essential role in mediating viral replication for a variety of pathogenic viruses, the development of PI4KIII β inhibitors has been an attractive target for pan anti-viral agents. A number of potent PI4KIII β inhibitors have recently been discovered^{8,11,14,20,24–27}, and experiments on a variety of RNA viruses show they are potent anti-viral agents. However, a complication of this work has been the toxic effects of some of these inhibitors on host function. There are conflicting results of the lethality of PI4KIII β inhibitors, with some showing lethality in mice²⁷, and others being well tolerated¹⁴. Deciphering if these effects are dependent on the inhibition of PI4KIII β , or through other off target effects requires the generation of novel more potent and specific PI4KIII β inhibitors. A specific goal for the development of antivirals is to avoid off target effects on both PI3K γ and PI3K δ as both of these enzymes play key roles in the immune system²⁸.

Here we describe a set of new compound **1** derivative inhibitors that are some of the most potent and specific PI4KIII β inhibitors as yet reported. Through a detailed structure based drug design approach we have designed derivatives that increased potency against PI4KIII β >5 fold while increasing selectivity >1000 fold to the related lipid kinases PI3K γ and vps34. The most potent of these molecules (compound **10**) was screened over a panel of nine related lipid kinases and was >200 fold selective for PI4KIII β across all enzymes tested. Two of the most potent and selective molecules (compound **9+10**) were also the most effective antiviral compounds in a cellular model of Hepatitis C virus replication, with the best balance of antiviral potency and low cellular toxicity.

The structure of one of the most potent molecules bound to PI4KIII β was determined, and reveals the molecular basis for specificity of the derivative molecules. The pocket that accommodates the acetamide group of the inhibitors was found to be the most important region in mediating inhibitor selectivity, and the differences in the pocket accommodating this group in PI4KIII β compared to PI3K γ and vps34 explains this observation. The acetamide group mediating specificity of PI4KIII β inhibitors strongly correlates with previous studies on compounds very similar to compound **1**. Addition of a bulky substituent in a similar position to the acetamide group in compound **1** had a limited role in potency, but greatly enhanced specificity^{26,29}. Structural and computational models of a different class of inhibitors bound to PI4KIII β suggested an important role of the pocket located near this region in mediating both potency and selectivity²⁴. The structural details of this compound bound in the active site pocket will provide a future framework for the modification of these compounds to modify the inhibitors in a way that will enhance their pharmacokinetic properties, but not lead to any decreases in their potency and specificity.

Experimental section

Protein Expression

Truncated human PI4KIII β (121–784 249–287 408–507 S294A) (plasmid JM7) and full-length human Rab11a(Q70L) (plasmid pJB88) were expressed in BL21 C41 (DE3) cells. For Rab11a(Q70L) expression, cultures were grown to an OD₆₀₀ of 0.7 and induced with 0.5 mM IPTG for 3.5 hours at 37 °C. For truncated PI4KIII β expression, cultures were induced overnight at 16 °C with 0.1 mM IPTG at an OD₆₀₀ of 0.6. Cells were harvested by

centrifugation, washed with cold phosphate-buffered saline (PBS), frozen in liquid nitrogen, and pellets were stored at -80°C .

Protein Purification

PI4KIII β (truncation) Purification—Cells were resuspended in 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM imidazole, 5% (v/v) glycerol, 2 mM β -mercaptoethanol, and a 1:1666 dilution of a protease inhibitor cocktail (Milipore Protease Inhibitor Cocktail Set III, Animal-Free), and were sonicated on ice for 5 min with cycles consisting of 10 sec on, 10 sec off. Triton X-100 was then added to a final concentration of 0.2%, and the lysate was centrifuged for 45 min at $20,000 \times g$. The supernatant was then filtered through a $0.45\ \mu\text{m}$ filter (Celltreat Scientific Products) and was loaded onto a 5 mL HisTrap FF column (GE Healthcare) equilibrated in buffer A (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM imidazole, 5% (v/v) glycerol, 2 mM β -mercaptoethanol). The column was washed with 20 mL of buffer A, followed by 20 mL of 6% buffer B (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 200 mM imidazole, 5% (v/v) glycerol, 2 mM β -mercaptoethanol), and was eluted with 100% buffer B. The His affinity tag protein was cleaved overnight at 4°C with TEV protease. The cleaved protein was then diluted to 50 mM NaCl (using 20 mM Tris-HCl pH 8.0, 10 mM imidazole, 5% (v/v) glycerol, 2 mM β -mercaptoethanol) and was loaded onto a 5 mL HiTrap Q HP column (GE Healthcare) equilibrated in buffer C (20 mM Tris-HCl pH 8.0, 50 mM NaCl, 5% (v/v) glycerol, 2 mM β -mercaptoethanol). Protein was eluted with a gradient elution using buffer D (20 mM Tris-HCl pH 8.0, 1.0 M NaCl, 5% (v/v) glycerol, 2 mM β -mercaptoethanol). Fractions containing the cleaved PI4KIII β were pooled and concentrated to 700 μL in an Amicon 50K centrifugal filter (Millipore). The protein was then loaded onto a HiPrep 16/60 Sephacryl S200 column equilibrated in buffer E (20 mM HEPES pH 7.2, 150 mM NaCl, 1 mM TCEP). The cleaved PI4KIII β was then concentrated to approximately 15 mg/mL in an Amicon 50K centrifugal filter (Millipore), and aliquots were frozen in liquid nitrogen and stored at -80°C .

Rab11a(Q70L) Purification—Cells were resuspended in 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 5% (v/v) glycerol, 2 mM β -mercaptoethanol, and a 1:1666 dilution of a protease inhibitor cocktail (Milipore Protease Inhibitor Cocktail Set III, Animal-Free). Cells were sonicated and centrifuged as described for the truncated PI4KIII β . The supernatant was filtered through a $0.45\ \mu\text{m}$ filter (Celltreat Scientific Products) and incubated for 1 hour with 4 mL of Glutathione Sepharose 4B beads (GE Healthcare) equilibrated in buffer F (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 5% (v/v) glycerol, 2 mM β -mercaptoethanol) followed by a $3 \times 15\ \text{mL}$ wash in buffer F. The GST tag was cleaved overnight on the beads with TEV protease. Anion-exchange chromatography was performed as outlined above for the truncated PI4KIII β . Cleaved Rab11a(Q70L) were then concentrated to between 5–15 mg/mL and nucleotide loaded by adding EDTA to 10 mM followed by 1 U of phosphatase (Phosphatase, Alkaline-Agarose from calf intestine, Sigma P0762-100UN) per mg of protein. Proteins were then incubated for 1.5 hours. The phosphatase was removed using a $0.2\ \mu\text{m}$ spin filter (Millipore); the flow-through was collected, and a 10-fold molar excess of GDP was added followed by MgCl_2 to a final concentration of 20 mM. Proteins were incubated for 30 min. Gel filtration was performed with cleaved GDP-loaded Rab11a(Q70L) as described above for the truncated PI4KIII β .

Crystallography

Crystals of PI4KIII β (final concentration 7.4 mg/mL) with Rab11a–GDP (final concentration 4.5 mg/mL) were obtained in 15% (w/v) PEG-4000, 100 mM sodium citrate pH 5.6, 200 mM ammonium sulfate (protein:precipitant ratio of 3:1). Refinement plates were set by gridding PEG-4000, ammonium sulfate, and glycerol. Optimized crystals were obtained by seeding using the Hampton Research Seed Bead Kit according to the manufacturer's instructions using a reservoir solution of 14.8% (w/v) PEG-4000, 100 mM sodium citrate pH 5.6, 250 mM ammonium sulfate. The best crystals were obtained in 13–15% (w/v) PEG-4000, 100 mM sodium citrate pH 5.6, 250 mM ammonium sulfate, 2% glycerol with a 1/1000 or 1/10,000 seed solution dilution, a Rab11a–GDP final concentration of 4.51 mg/mL and a PI4KIII β final concentration of 7.38 mg/mL. Crystals were frozen in liquid nitrogen using a 15% PEG-4000 (w/v), 100 mM sodium citrate pH 5.6, 250 mM ammonium sulfate, 25% (v/v) glycerol cryo solution.

Inhibitor soaks were performed by incubating crystals with 0.5 μ L of 10 μ M inhibitor stocks in cryo buffer (15% PEG-4000 (w/v), 250 mM ammonium sulfate, 100 mM sodium citrate pH 5.6, 25% glycerol (v/v)) for 30 min, followed by a 30 min incubation with 0.5 μ L of 100 μ M inhibitor stock in cryo buffer, and a final 30 min incubation in 1 mM inhibitor stock in cryo buffer. Before the final addition, 1 μ L was removed from the crystal drop and 1 μ L of the 1 mM inhibitor in cryo buffer was added.

Diffraction data were collected at 100 K at the Canadian Macromolecular Crystallography Facility (Canadian Light Source, CLS) beamline 08ID-1. Data were integrated using iMosflm 7.1.1³⁰ and scaled with AIMLESS³¹. Phases were initially obtained by molecular replacement (MR) using Phaser³², with the structure of PI4KIII β bound to compound **1** and Rab11 (pdb code: 4D0L) used as the search model. The final model of PI4KIII β bound to compound **9** in complex with Rab11 was built using iterative model building in COOT³³ and refinement using Phenix^{34,35} to $R_{\text{work}}=23.87$ and $R_{\text{free}}=26.79$. The binding mode of compound **9** was unambiguous, and ligand geometry was generated using the elbow subset of Phenix³⁶. Full crystallographic statistics are shown in Table 1.

Biochemical Assays

Lipid kinase assays were performed using recombinant enzyme, phosphoinositides purchased from Avanti Polar Lipids and $\gamma^{32}\text{P}$ -ATP (Perkin Elmer Cat #BLU502A001MC) in a membrane capture assay described previously³⁷. Each inhibitor was diluted into 10% DMSO and kinase assay buffer. Upon completion of the reaction, 4 μ L was spotted onto 0.2 μ m nitrocellulose (Bio-Rad Catalog #162-0112). The membrane was dried for 5 minutes under a heat lamp followed by 1 $\times$ 30 second wash and 6 $\times$ 5 min washes in 1M NaCl / 1% Phosphoric Acid. The membrane was dried for 20 minutes under a heat lamp followed by overnight exposure to a phosphor screen and phosphorimaging followed on a Typhoon 9500. Intensities were quantified using SPOT³⁷. Specifications for each enzyme follow.

PI4KIII β

Recombinant enzyme was purchased from Life Technologies (Cat # PV5277, Lot 943589E). L- α -Phosphatidylinositol (PI, Cat # 840024P) and DOPS:DOPC lipids (Cat # 790595P)

were sonicated in water to generate 1mg/mL PI:DOPS:DOPC. Reaction was set-up as follows 1) kinase assay buffer, PI:DOPS:DOPC, BSA and PI4KIII β , were combined in a total volume of 10 μ L (2.5x solution); 2) 5 μ L of inhibitor solution was added (5x solution) and incubated with enzyme mixture for 15 minutes; 3) 10 μ L cold ATP and γ ³²P-ATP were added (2.5x solution) to initiate the reaction which ran for 30 minutes. Final conditions were as follows: 20mM Bis-Tris Propane pH 7.5, 10mM MgCl₂, 0.075mM Triton X-100, 0.5mM EGTA, 1mM DTT, 100 μ M PI, 500ng/ μ L BSA, 2.5nM PI4KIII β , 2% DMSO, 10 μ M ATP and 1uCi γ ³²P-ATP.

PI3K γ

Recombinant enzyme was purchased from Life Technologies (Cat # PV4786, Lot # 1638926A). L- α -Phosphatidylinositol-4,5-bisphosphate (PIP₂, Cat # 840046P) and DOPS:DOPC lipids (Cat # 790595P) were sonicated in water to generate 1mg/mL PIP₂:DOPS:DOPC. Reaction was set-up as follows 1) kinase assay buffer, PIP₂:DOPS:DOPC, BSA and PI3K γ , were combined in a total volume of 10 μ L (2.5x solution); 2) 5 μ L of inhibitor solution was added (5x solution) and incubated with enzyme mixture for 15 minutes; 3) 10 μ L cold ATP and γ ³²P-ATP were added (2.5x solution) to initiate the reaction which ran for 15 minutes. Final conditions were as follows: 50mM HEPES pH 7.5, 100mM NaCl, 0.03% CHAPS, 10mM MgCl₂, 1mM EGTA, 2mM DTT, 5nM PI3K γ , 80 μ M PIP₂, 500ng/ μ L BSA, 2% DMSO, 10 μ M ATP and 1uCi γ ³²P-ATP.

PI3K β

Recombinant enzyme was purchased from Life Technologies (Cat # PV6451, Lot # 1763224). L- α -Phosphatidylinositol-4,5-bisphosphate (PIP₂, Cat # 840046P) and DOPS:DOPC lipids (Cat # 790595P) were sonicated in water to generate 1mg/mL PIP₂:DOPS:DOPC. Reaction was set-up as follows 1) kinase assay buffer, PIP₂:DOPS:DOPC, BSA and PI3K δ , were combined in a total volume of 10 μ L (2.5x solution); 2) 5 μ L of inhibitor solution was added (5x solution) and incubated with enzyme mixture for 15 minutes; 3) 10 μ L cold ATP and γ ³²P-ATP were added (2.5x solution) to initiate the reaction which ran for 20 minutes. Final conditions were as follows: 50mM HEPES pH 7.5, 100mM NaCl, 0.03% CHAPS, 10mM MgCl₂, 1mM EGTA, 2mM DTT, 2.5nM PI3K δ , 80 μ M PIP₂, 500ng/ μ L BSA, 2% DMSO, 10 μ M ATP and 2.5uCi γ ³²P-ATP.

vps34

Recombinant enzyme was purchased from Life Technologies (Cat # PV5126, Lot # 1555138A). L- α -Phosphatidylinositol (PI, Cat # 840024P) and DOPS:DOPC lipids (Cat # 790595P) were sonicated in water to generate 1mg/mL PI:DOPS:DOPC. Reaction was set-up as follows 1) kinase assay buffer, PI:DOPS:DOPC, BSA and vps34, were combined in a total volume of 10 μ L (2.5x solution); 2) 5 μ L of inhibitor solution was added (5x solution) and incubated with enzyme mixture for 15 minutes; 3) 10 μ L cold ATP and γ ³²P-ATP were added (2.5x solution) to initiate the reaction which ran for 1 hour. Final conditions were as follows: 50mM HEPES pH 7.5, 0.1% CHAPS, 2mM MnCl₂, 1mM EGTA, 2mM DTT, 10nM vps34, 100 μ M PI, 500ng/ μ L BSA, 2% DMSO, 10 μ M ATP and 2.5uCi γ ³²P-ATP.

PI4KIII α

Recombinant enzyme was purchased from EMD Millipore (Cat # 14-908, Lot # D9KN031N-B). L- α -Phosphatidylinositol (PI, Cat # 840024P) and DOPS:DOPC lipids (Cat # 790595P) were sonicated in water to generate 1mg/mL PI:DOPS:DOPC. Reaction was set-up as follows 1) kinase assay buffer, PI:DOPS:DOPC, BSA and PI4KIII α , were combined in a total volume of 10 μ L (2.5x solution); 2) 5 μ L of inhibitor solution was added (5x solution) and incubated with enzyme mixture for 15 minutes; 3) 10 μ L cold ATP and γ ³²P-ATP were added (2.5x solution) to initiate the reaction which ran for 25 minutes. Final conditions were as follows: 20mM Bis-Tris Propane pH 7.5, 10mM MgCl₂, 0.075mM Triton X-100, 0.5mM EGTA, 1mM DTT, 100 μ M PI, 500ng/ μ L BSA, 1.2nM PI4KIII α , 2% DMSO, 10 μ M ATP and 2uCi γ ³²P-ATP.

PI4KII α

PI4KII α was cloned from DNASU plasmid HsCD00003332^{38,39}. Recombinant enzyme was obtained through FLAG immunoprecipitation of FLAG-PI4KII α transfected into HEK293T cells (10ug of FLAG-PI4KII α transfected with Life Technologies Lipofectamine and Plus Reagent to a 15cm dish of HEK293Ts and 75uL elution with FLAG peptide). L- α -Phosphatidylinositol (PI, Cat # 840024P) and DOPS:DOPC lipids (Cat # 790595P) were sonicated in water to generate 1mg/mL PI:DOPS:DOPC. Reaction was set-up as follows 1) kinase assay buffer, PI:DOPS:DOPC, BSA and PI4KII α , were combined in a total volume of 10 μ L (2.5x solution); 2) 5 μ L of inhibitor solution was added (5x solution) and incubated with enzyme mixture for 15 minutes; 3) 10 μ L cold ATP and γ ³²P-ATP were added (2.5x solution) to initiate the reaction which ran for 25 minutes. Final conditions were as follows: 20mM Bis-Tris Propane pH 7.5, 10mM MgCl₂, 0.075mM Triton X-100, 0.5mM EGTA, 1mM DTT, 100 μ M PI, 500ng/ μ L BSA, 2.9uL PI4KII α , 2% DMSO, 10 μ M ATP and 2uCi γ ³²P-ATP.

PI4KII β

PI4KII β was cloned from DNASU plasmid HsCD00001592^{38,39}. Recombinant enzyme was obtained through FLAG immunoprecipitation of FLAG-PI4KII β transfected into HEK293T cells (10ug of FLAG-PI4KII β transfected with Life Technologies Lipofectamine and Plus Reagent to a 15cm dish of HEK293Ts and 75uL elution with FLAG peptide). L- α -Phosphatidylinositol (PI, Cat # 840024P) and DOPS:DOPC lipids (Cat # 790595P) were sonicated in water to generate 1mg/mL PI:DOPS:DOPC. Reaction was set-up as follows 1) kinase assay buffer, PI:DOPS:DOPC, BSA and PI4KII β , were combined in a total volume of 10 μ L (2.5x solution); 2) 5 μ L of inhibitor solution was added (5x solution) and incubated with enzyme mixture for 15 minutes; 3) 10 μ L cold ATP and γ ³²P-ATP were added (2.5x solution) to initiate the reaction which ran for 25 minutes. Final conditions were as follows: 20mM Bis-Tris Propane pH 7.5, 10mM MgCl₂, 0.075mM Triton X-100, 0.5mM EGTA, 1mM DTT, 100 μ M PI, 500ng/ μ L BSA, 2uL PI4KII β , 2% DMSO, 10 μ M ATP and 2uCi γ ³²P-ATP.

PI3K α

Recombinant enzyme was purchased from EMD Millipore (Cat # 14–602, Lot # 2150294-A). L- α -Phosphatidylinositol-4,5-bisphosphate (PIP₂, Cat # 840046P) and DOPS:DOPC lipids (Cat # 790595P) were sonicated in water to generate 1mg/mL PIP₂:DOPS:DOPC. Reaction was set-up as follows 1) kinase assay buffer, PIP₂:DOPS:DOPC, BSA and PI3K α , were combined in a total volume of 10 μ L (2.5x solution); 2) 5 μ L of inhibitor solution was added (5x solution) and incubated with enzyme mixture for 15 minutes; 3) 10 μ L cold ATP and γ ³²P-ATP were added (2.5x solution) to initiate the reaction which ran for 15 minutes. Final conditions were as follows: 50mM HEPES pH 7.5, 100mM NaCl, 0.03% CHAPS, 10mM MgCl₂, 1mM EGTA, 2mM DTT, 3.3nM PI3K α , 80 μ M PIP₂, 500ng/ μ L BSA, 2% DMSO, 10 μ M ATP and 2uCi γ ³²P-ATP.

PI3K β

Recombinant enzyme was purchased from SignalChem (Cat # P28-10H-10, Lot # F-532-3). L- α -Phosphatidylinositol-4,5-bisphosphate (PIP₂, Cat # 840046P) and DOPS:DOPC lipids (Cat # 790595P) were sonicated in water to generate 1mg/mL PIP₂:DOPS:DOPC. Reaction was set-up as follows 1) kinase assay buffer, PIP₂:DOPS:DOPC, BSA and PI3K β , were combined in a total volume of 10 μ L (2.5x solution); 2) 5 μ L of inhibitor solution was added (5x solution) and incubated with enzyme mixture for 15 minutes; 3) 10 μ L cold ATP and γ ³²P-ATP were added (2.5x solution) to initiate the reaction which ran for 15 minutes. Final conditions were as follows: 50mM HEPES pH 7.5, 100mM NaCl, 0.03% CHAPS, 10mM MgCl₂, 1mM EGTA, 2mM DTT, 0.6nM PI3K β , 80 μ M PIP₂, 500ng/ μ L BSA, 2% DMSO, 10 μ M ATP and 2uCi γ ³²P-ATP.

PI3KC2 γ

Recombinant enzyme was purchased from EMD Millipore (Cat # 14–910, Lot # 2023057-A). L- α -Phosphatidylinositol (PI, Cat # 840024P) and DOPS:DOPC lipids (Cat # 790595P) were sonicated in water to generate 1mg/mL PI:DOPS:DOPC. Reaction was set-up as follows 1) kinase assay buffer, PI:DOPS:DOPC, BSA and PI3KC2 γ , were combined in a total volume of 10 μ L (2.5x solution); 2) 5 μ L of inhibitor solution was added (5x solution) and incubated with enzyme mixture for 15 minutes; 3) 10 μ L cold ATP and γ ³²P-ATP were added (2.5x solution) to initiate the reaction which ran for 15 minutes. Final conditions were as follows: 50mM HEPES pH 7.5, 100mM NaCl, 0.03% CHAPS, 10mM MgCl₂, 1mM EGTA, 2mM DTT, 1nM PI3KC2 γ , 100 μ M PI, 500ng/ μ L BSA, 2% DMSO, 10 μ M ATP and 2uCi γ ³²P-ATP.

Organic Synthesis

Materials obtained commercially were reagent grade and were used without further purification. ¹H NMR spectra were recorded on a Varian 400 spectrometer at 400 MHz . High-resolution electron impact mass spectra were recorded on a Thermo Fisher Exactive EMR and Thermo Fisher LTQ Orbitrap Velos at the University of California-San Francisco center for Mass Spectrometry. Reactions were monitored by thin layer chromatography (TLC), using Merck silica gel 60 F254 glass plates (0.25 mm thick). Flash chromatography was conducted with Grace Reveleris flash cartridges with 40 μ m silica on an Agilent 971-FP.

All RP-HPLC were performed with a Waters 2545 binary gradient module equipped with an XBridge prep C18 column using H₂O + 0.1% formic acid and CH₃CN + 0.1% formic acid (5–95% gradient) while monitoring at 254 nm. All final compounds were >95% pure as measured by Liquid Chromatography Mass Spectrometry (LCMS). Full compound characterization details are presented in the supporting information.

Synthesis of compound **1** and derivatives was conducted similarly to methods described previously¹⁶, and is shown in scheme 1. To commercially available acetophenone chlorosulfuric acid (11eq) was added dropwise in an ice bath and the reaction was heated to 40°C for 2 hours. The reaction was stopped by dropwise transfer to ice-water. The aqueous phase was extracted 3x with ethyl acetate and the combined organic is dried with sodium sulfate, filtered and concentrated *in vacuo* to give a brown oil, the crude corresponding to sulfonyl chloride, **A**. The sulfonyl chloride was dissolved in THF (1.5M), the appropriate amino alcohol (4.5eq) was added and the reaction was allowed to stir overnight at room temperature. The reaction was concentrated *in vacuo*, water is added and the aqueous phase was extracted 3x with ethyl acetate. The combined organic phase was concentrated and intermediate **B** was purified using silica gel flash chromatography (2% - 10% methanol in dichloromethane). If pure intermediate was not obtained, the intermediate was subjected to a second purification by reverse phase HPLC using acetonitrile/water/0.1% formic acid as a solvent system.

Intermediate **B** was dissolved in THF (1.5M). (2-carboxyethyl) triphenylphosphonium bromide was dissolved in THF (1M) and intermediate **B** was added to the bromide solution dropwise at room temperature. The reaction was allowed to proceed 1 hour at room temperature and the solvent is removed *in vacuo* and the product was purified by silica gel flash chromatography (0% - 10% methanol in dichloromethane) to yield intermediate **C**.

To ethanol-recrystallized thiourea in dry toluene (0.5M) was added appropriate acyl chloride and the reaction was heated to reflux for 18 hours. Toluene was removed *in vacuo* and the reaction was diluted in ethyl acetate and filtered. The organic layer was washed 2x with water and dried with brine and sodium sulfate and reduced *in vacuo*. The resulting acetylthiourea **D** was purified by silica gel flash chromatography (25% - 100% ethyl acetate in hexanes).

Intermediate **C** was dissolved in ethanol (0.17M) and appropriate acetylthiourea **D** was added at room temperature. The reaction was heated to reflux for 30 minutes and then cooled to room temperature and compound **1** or compound **1** derivative was purified by silica gel chromatography (0% - 10% methanol in dichloromethane) and if necessary, followed by reverse phase HPLC using acetonitrile/water/0.1% formic acid as a solvent system.

Gaussia luciferase-based HCV reporter virus and stable cell line of Huh7.5 containing infectious HCV reporter virus

A fully-infectious HCVcc genotype 2a reporter virus encoding *Gaussia* luciferase between p7 and NS2 similar to Maurkian et al.⁴⁰ was generated to monitor HCV replication quantitatively. Briefly, *Gaussia* luciferase gene with an in frame foot and mouth disease virus

autoproteolytic 2A peptide sequence was inserted between HCV p7 and NS2 of a J6/JFH1 infectious HCV clone ⁴¹, resulting in the fully-infectious HCVcc reporter virus J6/JFH1-GLuc (p7-NS2-GLucFM2A). After Huh7.5 cells were infected with the Gaussia luciferase-based HCV reporter virus, the cells were maintained for three days and then passed every four days for twenty passages. A stable cell line with consistent Gaussia luciferase secretion was established.

HCV2a antiviral assay

Huh 7.5 cells stably infected with J6/JFH1-GLuc were maintained and passaged in DMEM (Mediatech, Manassas, VA) supplemented with 10% FBS (Omega Scientific, Tarzana, CA), 2 mM glutamine (Mediatech, Manassas, VA), non-essential amino acids (Mediatech, Manassas, VA), 100 IU/mL penicillin (Mediatech, Manassas, VA), and 100 g/mL streptomycin (Mediatech, Manassas, VA) in 37°C incubator with 5% CO₂ and 95% relative humidity.

Ten thousand cells were plated in 96 well plates (E&K Scientific Products, Santa Clara, CA) and supplemented with serially diluted compounds one hour after plating. After three days incubation, viability was tested using the Presto Blue cell viability reagent (Life Technologies Corporation, Grand Island, NY) per the manufacturer's protocol and replication was measured by determining the Gaussia luciferase activity in the supernatant using the luciferase reagent (Promega Corporation, Madison, WI) according to the manufacturer's protocol.

Viability and luminescence were read using a plate reader (Infinite 1000, Tecan Systems, San Jose, CA). Values were imported into Prism (GraphPad Software, La Jolla, CA) for graphing and calculations of EC₅₀ and CC₅₀ values.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PI4K	Phosphatidylinositol 4 kinase
PI3K	phosphoinositide 3 kinase

PI4P	phosphatidylinositol 4-phosphate
PIP₂	phosphatidylinositol 4,5-bisphosphate
PIP₃	phosphatidylinositol 3,4,5-trisphosphate

REFERENCES

1. Balla T. Phosphoinositides: Tiny Lipids with Giant Impact on Cell Regulation. *Physiol. Rev.* 2013; 93:1019–1137. [PubMed: 23899561]
2. Tan J, Brill JA. Cinderella Story: PI4P Goes From Precursor to Key Signaling Molecule. *Crit. Rev. Biochem. Mol. Biol.* 2014; 49:33–58. [PubMed: 24219382]
3. Tóth B, Balla A, Ma H, Knight ZA, Shokat KM, Balla T. Phosphatidylinositol 4-Kinase III β Regulates the Transport of Ceramide Between the Endoplasmic Reticulum and Golgi. *J. Biol. Chem.* 2006; 281:36369–36377. [PubMed: 17003043]
4. Polevoy G, Wei H-C, Wong R, Szentpetery Z, Kim YJ, Goldbach P, Steinbach SK, Balla T, Brill JA. Dual Roles for the Drosophila PI 4-Kinase Four Wheel Drive in Localizing Rab11 During Cytokinesis. *J. Cell Biol.* 2009; 187:847–858. [PubMed: 19995935]
5. Sridhar S, Patel B, Aphkhasava D, Macian F, Santambrogio L, Shields D, Cuervo AM. The Lipid Kinase PI4KIII β Preserves Lysosomal Identity. *EMBO J.* 2013; 32:324–339. [PubMed: 23258225]
6. Jean S, Kiger AA. Coordination Between RAB GTPase and Phosphoinositide Regulation and Functions. *Nat. Rev. Mol. Cell Biol.* 2012; 13:463–470. [PubMed: 22722608]
7. Hsu N-Y, Ilnytska O, Belov G, Santiana M, Chen Y-H, Takvorian PM, Pau C, van der Schaar H, Kaushik-Basu N, Balla T, Cameron CE, Ehrenfeld E, van Kuppeveld FJM, Altan-Bonnet N. Viral Reorganization of the Secretory Pathway Generates Distinct Organelles for RNA Replication. *Cell.* 2010; 141:799–811. [PubMed: 20510927]
8. McNamara CW, Lee MCS, Lim CS, Lim SH, Roland J, Nagle A, Simon O, Yeung BKS, Chatterjee AK, McCormack SL, Manary MJ, Zeeman AM, Dechering KJ, Kumar TRS, Henrich PP, Gagaring K, Ibanez M, Kato N, Kuhlen KL, Fischli C, Rottmann M, Plouffe DM, Bursulaya B, Meister S, Rameh L, Trappe J, Haasen D, Timmerman M, Sauerwein RW, Suwanarusk R, Russell B, Renia L, Nosten F, Tully DC, Kocken CHM, Glynn RJ, Bodenreider C, Fidock DA, Diagana TT, Winzeler EA. Targeting Plasmodium PI(4)K to Eliminate Malaria. *Nature.* 2013; 504:248–253. [PubMed: 24284631]
9. Altan-Bonnet N, Balla T. Phosphatidylinositol 4-Kinases: Hostages Harnessed to Build Panviral Replication Platforms. *Trends in Biochemical Sciences.* 2012; 37:293–302. [PubMed: 22633842]
10. Mello C, Aguayo E, Rodriguez M, Lee G, Jordan R, Cihlar T, Birkus G. Multiple Classes of Antiviral Agents Exhibit in Vitro Activity Against Human Rhinovirus Type C. *Antimicrob. Agents Chemother.* 2014; 58:1546–1555. [PubMed: 24366736]
11. van der Schaar HM, van der Linden L, Lanke KHW, Strating JRPM, Pürstinger G, de Vries E, de Haan CAM, Neyts J, van Kuppeveld FJM. Coxsackievirus Mutants That Can Bypass Host Factor PI4KIII β and the Need for High Levels of PI4P Lipids for Replication. *Cell Res.* 2012; 22:1576–1592. [PubMed: 22945356]
12. Greninger AL, Knudsen GM, Betegon M, Burlingame AL, DeRisi JL. The 3A Protein From Multiple Picornaviruses Utilizes the Golgi Adaptor Protein ACBD3 to Recruit PI4KIII β . *J. Virol.* 2012; 86:3605–3616. [PubMed: 22258260]
13. Borawski J, Troke P, Puyang X, Gibaja V, Zhao S, Mickanin C, Leighton-Davies J, Wilson CJ, Myer V, Cornellataracido I, Baryza J, Tallarico J, Joberty G, Bantscheff M, Schirle M, Bouwmeester T, Mathy JE, Lin K, Compton T, Labow M, Wiedmann B, Gaither LA. Class III Phosphatidylinositol 4-Kinase Alpha and Beta Are Novel Host Factor Regulators of Hepatitis C Virus Replication. *J. Virol.* 2009; 83:10058–10074. [PubMed: 19605471]
14. van der Schaar HM, Leyssen P, Thibaut HJ, de Palma A, van der Linden L, Lanke KHW, Lacroix C, Verbeken E, Conrath K, Macleod AM, Mitchell DR, Palmer NJ, van de Poël H, Andrews M, Neyts J, van Kuppeveld FJM. A Novel, Broad-Spectrum Inhibitor of Enterovirus Replication That

Targets Host Cell Factor Phosphatidylinositol 4-Kinase III β . *Antimicrob. Agents Chemother.* 2013; 57:4971–4981. [PubMed: 23896472]

15. Arita M, Kojima H, Nagano T, Okabe T, Wakita T, Shimizu H. Phosphatidylinositol 4-Kinase III Beta Is a Target of Enviroxime-Like Compounds for Antipoliavirus Activity. *J. Virol.* 2011; 85:2364–2372. [PubMed: 21177810]
16. Knight Z, Gonzalez B, Feldman M, Zunder E, Goldenberg D, Williams O, Loewith R, Stokoe D, Balla A, Toth B, Balla T, Weiss W, Williams R, Shokat K. A Pharmacological Map of the PI3-K Family Defines a Role for P110 α in Insulin Signaling. *Cell.* 2006; 125:733–747. [PubMed: 16647110]
17. Balla A, Kim YJ, Várnai P, Szentpetery Z, Knight Z, Shokat KM, Balla T. Maintenance of Hormone-Sensitive Phosphoinositide Pools in the Plasma Membrane Requires Phosphatidylinositol 4-Kinase III α . *Mol. Biol. Cell.* 2008; 19:711–721. [PubMed: 18077555]
18. Burke JE, Inglis AJ, Perisic O, Masson GR, McLaughlin SH, Rutaganira F, Shokat KM, Williams RL. Structures of PI4KIII β Complexes Show Simultaneous Recruitment of Rab11 and Its Effectors. *Science.* 2014; 344:1035–1038. [PubMed: 24876499]
19. Miller S, Tavshanjan B, Oleksy A, Perisic O, Houseman B, Shokat K, Williams R. Shaping Development of Autophagy Inhibitors with the Structure of the Lipid Kinase Vps34. *Science.* 2010; 327:1638–1642. [PubMed: 20339072]
20. Lamarche MJ, Borawski J, Bose A, Capacci-Daniel C, Colvin R, Dennehy M, Ding J, Dobler M, Drumm J, Gaither LA, Gao J, Jiang X, Lin K, McKeever U, Puyang X, Raman P, Thohan S, Tommasi R, Wagner K, Xiong X, Zabawa T, Zhu S, Wiedmann B. Anti-Hepatitis C Virus Activity and Toxicity of Type III Phosphatidylinositol-4-Kinase Beta Inhibitors. *Antimicrob. Agents Chemother.* 2012; 56:5149–5156. [PubMed: 22825118]
21. Fowler ML, McPhail JA, Jenkins ML, Masson GR, Rutaganira FU, Shokat KM, Williams RL, Burke JE. Using Hydrogen Deuterium Exchange Mass Spectrometry to Engineer Optimized Constructs for Crystallization of Protein Complexes: Case Study of PI4KIII β with Rab11. *Protein Sci.* Online early access. Published online: Jan 12, 2016.
22. Jovic M, Kean MJ, Szentpetery Z, Polevoy G, Gingras A-C, Brill JA, Balla T. Two Phosphatidylinositol 4-Kinases Control Lysosomal Delivery of the Gaucher Disease Enzyme, B-Glucocerebrosidase. *Mol. Biol. Cell.* 2012; 23:1533–1545. [PubMed: 22337770]
23. D'Angelo G, Vicinanza M, Wilson C, De Matteis MA. Phosphoinositides in Golgi Complex Function. *Subcell. Biochem.* 2012; 59:255–270. [PubMed: 22374093]
24. Mejdrová I, Chalupská D, Kögler M, Šála M, Plašková P, Baumlová A, Hrabáček H, Procházková E, Dejmeš M, Guillon R, Strunin D, Weber J, Lee G, Birkus G, Mertlíková-Kaiserová H, Boura E, Nencka R. Highly Selective Phosphatidylinositol 4-Kinase III β Inhibitors and Structural Insight Into Their Mode of Action. *J. Med. Chem.* 2015; 58:3767–3793. [PubMed: 25897704]
25. Arita M, Philipov S, Galabov AS. Phosphatidylinositol 4-Kinase III Beta Is the Target of Oxoglucine and Pachypodol (Ro 09-0179) for Their Anti-Poliavirus Activities, and Locates at Upstream of the Target Step of Brefeldin a. *Microbiol. Immunol.* 2015
26. Waring MJ, Andrews DM, Faulder PF, Flemington V, McKelvie JC, Maman S, Preston M, Raubo P, Robb GR, Roberts K, Rowlinson R, Smith JM, Swarbrick ME, Treinies I, Winter JGG, Wood RJ. Potent, Selective Small Molecule Inhibitors of Type III Phosphatidylinositol-4-Kinase A- but Not B-Inhibit the Phosphatidylinositol Signaling Cascade and Cancer Cell Proliferation. *Chem. Commun. (Camb.).* 2014; 50:5388–5390. [PubMed: 24366037]
27. Spickler C, Lippens J, Laberge M-K, Desmeules S, Bellavance É, Garneau M, Guo T, Huc O, Leyssen P, Neyts J, Vaillancourt FH, Décor A, O'Meara J, Franti M, Gauthier A. Phosphatidylinositol 4-Kinase III Beta Is Essential for Replication of Human Rhinovirus and Its Inhibition Causes a Lethal Phenotype in Vivo. *Antimicrob. Agents Chemother.* 2013; 57:3358–3368. [PubMed: 23650168]
28. Okkenhaug K. Signaling by the Phosphoinositide 3-Kinase Family in Immune Cells. *Annu. Rev. Immunol.* 2013; 31:675–704. [PubMed: 23330955]
29. Keaney EP, Connolly M, Dobler M, Karki R, Honda A, Sokup S, Karur S, Britt S, Patnaik A, Raman P, Hamann LG, Wiedmann B, LaMarche MJ. 2-Alkylloxazoles as Potent and Selective

- PI4KIII β Inhibitors Demonstrating Inhibition of HCV Replication. *Bioorganic & Medicinal Chemistry Letters*. 2014; 24:3714–3718. [PubMed: 25065492]
30. Battye TGG, Kontogiannis L, Johnson O, Powell HR, Leslie AGW. iMOSFLM: a New Graphical Interface for Diffraction-Image Processing with MOSFLM. *Acta Crystallogr. D Biol. Crystallogr.* 2011; 67:271–281. [PubMed: 21460445]
31. Evans PR, Murshudov GN. How Good Are My Data and What Is the Resolution? *Acta Crystallogr. D Biol. Crystallogr.* 2013; 69:1204–1214. [PubMed: 23793146]
32. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. Phaser Crystallographic Software. *J. Appl. Crystallogr.* 2007; 40:658–674. [PubMed: 19461840]
33. Emsley P, Lohkamp B, Scott WG, Cowtan K. Features and Development of Coot. *Acta Crystallogr. D Biol. Crystallogr.* 2010; 66:486–501. [PubMed: 20383002]
34. Adams PD, Afonine PV, Bunkóczi G, Chen VB, Echols N, Headd JJ, Hung L-W, Jain S, Kapral GJ, Grosse-Kunstleve RW, McCoy AJ, Moriarty NW, Oeffner RD, Read RJ, Richardson DC, Richardson JS, Terwilliger TC, Zwart PH. The Phenix Software for Automated Determination of Macromolecular Structures. *Methods*. 2011; 55:94–106. [PubMed: 21821126]
35. Afonine PV, Grosse-Kunstleve RW, Echols N, Headd JJ, Moriarty NW, Mustyakimov M, Terwilliger TC, Urzhumtsev A, Zwart PH, Adams PD. Towards Automated Crystallographic Structure Refinement with Phenix.Refine. *Acta Crystallogr. D Biol. Crystallogr.* 2012; 68:352–367. [PubMed: 22505256]
36. Moriarty NW, Grosse-Kunstleve RW, Adams PD. Electronic Ligand Builder and Optimization Workbench (eLBOW): a Tool for Ligand Coordinate and Restraint Generation. *Acta Crystallogr. D Biol. Crystallogr.* 2009; 65:1074–1080. [PubMed: 19770504]
37. Knight Z, Feldman M, Balla A, Balla T, Shokat K. A Membrane Capture Assay for Lipid Kinase Activity. *Nat Protoc.* 2007; 2:2459–2466. [PubMed: 17947987]
38. Seiler CY, Park JG, Sharma A, Hunter P, Surapaneni P, Sedillo C, Field J, Algar R, Price A, Steel J, Throop A, Fiocco M, Labaer J. DNASU Plasmid and PSI: Biology-Materials Repositories: Resources to Accelerate Biological Research. *Nucleic Acids Res.* 2014; 42:D1253–D1260. [PubMed: 24225319]
39. Park J, Hu Y, Murthy TVS, Vannberg F, Shen B, Rolfs A, Hutti JE, Cantley LC, Labaer J, Harlow E, Brizuela L. Building a Human Kinase Gene Repository: Bioinformatics, Molecular Cloning, and Functional Validation. *Proc. Natl. Acad. Sci. U.S.A.* 2005; 102:8114–8119. [PubMed: 15928075]
40. Marukian S, Jones CT, Andrus L, Evans MJ, Ritola KD, Charles ED, Rice CM, Dustin LB. Cell Culture-Produced Hepatitis C Virus Does Not Infect Peripheral Blood Mononuclear Cells. *Hepatology*. 2008; 48:1843–1850. [PubMed: 19003912]
41. Cho N-J, Lee C, Pang PS, Pham EA, Fram B, Nguyen K, Xiong A, Sklan EH, Elazar M, Koytak ES, Kersten C, Kanazawa KK, Frank CW, Glenn JS. Phosphatidylinositol 4,5-Bisphosphate Is an HCV NS5A Ligand and Mediates Replication of the Viral Genome. *Gastroenterology*. 2015; 148:616–625. [PubMed: 25479136]
42. Laskowski RA, Swindells MB. LigPlot+: Multiple Ligand-Protein Interaction Diagrams for Drug Discovery. *J Chem Inf Model*. 2011; 51:2778–2786. [PubMed: 21919503]

Figure 1. Structural basis for inhibition of PI4KIII β and PI3Ks by the inhibitor PIK93 (1)

A. Structure of compound **1**, with the ethanolamine substituent off the sulfonamide colored blue, the chloro substituent off the central phenyl colored green, and the acetamide substituent off the thiazol colored red. The potency of **1** against PI4KIII β , PI3K γ , and vps34 is graphed.

B. The structures of PI4KIII β ¹⁸ (PDB ID: 4D0L), vps34¹⁹ (PDB ID: 2 $\times$ 6J), and PI3K γ ¹⁶ (PDB ID: 2CHZ) bound to **1** aligned, showing the chloro substituent of **1** with the activation loop of each enzyme colored according to the legend.

C–E. The structures of PI4KIII β (C), PI3K γ (D), and vps34 (E) with residues within 5 angstroms of the acetamide group of **1** shown as spheres.

Figure 2. Development of novel PI4KIIIβ inhibitors

A. The structure of compound **1** is shown, with novel substituents at the R₁, R₂, and R₃ position shown in tabular form, with the IC₅₀ values for each compound against PI4KIIIβ, PI3Kγ, PI3Kδ and vps34 listed. Kinase assays were carried out in the presence of 10 μM ATP and 10mM MgCl₂ (PI4KIIIβ, PI3Kδ and PI3Kγ) or 10mM MnCl₂ (vps34). IC₅₀ values measured using MnCl₂ are 10–20 fold higher than with the physiologically relevant MgCl₂ due to increased kinase activity. Inhibitors anti-HCV antiviral activity (IC₅₀) was assessed by Gaussia luciferase assay (Gluc HCV2a activity) and cell viability (CC₅₀) by Presto Blue assay (Gluc HCV2a viability), as described in the Experimental Section.

B. Full inhibitor curves for compound **8**, **9**, and **10** are shown for PI4KIIIβ, PI3Kγ, PI3Kδ and vps34, with all measurements carried out in triplicate. For compounds **9** and **10** the curves for the related lipid kinase PI4KIIIα is shown as well, with assays against other related lipid kinases shown in Fig. S1.

Figure 3. Structural basis of inhibition of PI4KIII β by compound 9

A. Residues mediating the interaction of PI4KIII β with compound 9 are shown, with putative hydrogen bonds indicated by dotted lines. Figure generated using ligplot ⁴².

B. The fit of compound 9 in the PI4KIII β active site pocket. The kinase domain is colored with the N-lobe shown in red, and the C-lobe shown in yellow.

Figure 4. Structural basis for selectivity of compound 9

A. The structure of compound 9 bound to PI4KIIIβ, with selected residues colored in orange and shown as spheres.

B. A model of compound 9 in the active site of PI3Kγ. The model was generated by aligning the active site of the structure of compound 9 bound to PI4KIIIβ to the structure of PI3Kγ bound to compound 1 (PDB: 2CHZ). The equivalent residues shown in panel A are colored in green and shown as spheres, with steric clashes highlighted in red.

C. A model of compound 9 in the active site of vps34. The model was generated by aligning the active site of the structure of compound 9 bound to PI4KIIIβ to the structure of vps34 bound to compound 1 (PDB: 2×6J). The equivalent residues shown in panel A are colored in cyan and shown as spheres, with steric clashes highlighted in red.

Scheme 1.
Synthesis of compound **1** and derivatives.

Table 1

Data collection and refinement statistics.

Data collection	PI4K–Rab11-GDP-compound 9
Wavelength (Å)	0.9797
Space group	P 21 21 21
Unit cell	48.9 103.5 188.9, 90 90 90
Total reflections	67660 (6441)
Unique reflections	16316 (1611)
Multiplicity	4.1 (4.0)
Completeness (%)	98.63 (99.44)
Mean I/sigma(I)	8.93 (2.11)
Wilson B-factor	82.35
R-merge	0.1092 (0.7019)
R-meas	0.1249
CC1/2	0.993 (0.375)
CC*	0.998 (0.738)
Refinement	
Resolution range (Å)	47.34 – 3.2 (3.31 – 3.2)
Reflections used for R-free	5%
R-work	0.2291 (0.3328)
R-free	0.2616 (0.3660)
Number of non-hydrogen atoms	5058
macromolecules	4987
ligands	71
water	0
Protein residues	621
RMS(bonds)	0.003
RMS(angles)	0.69
Ramachandran favored (%)	96
Ramachandran outliers (%)	0.3
Clashscore	5.73
Average B-factor	96
macromolecules	96.1
ligands	93.9

Statistics for the highest-resolution shell are shown in parentheses.