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Potent, Selective, and Orally Bioavailable Inhibitors of VPS34 Provide Chemical Tools to Modulate Autophagy *in Vivo*

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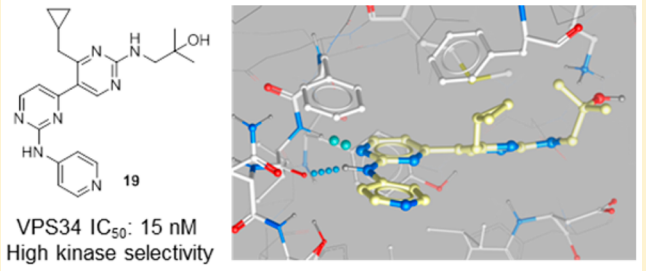
Supporting Information

ABSTRACT: Autophagy is a dynamic process that regulates lysosomal-dependent degradation of cellular components. Until recently the study of autophagy has been hampered by the lack of reliable pharmacological tools, but selective inhibitors are now available to modulate the PI 3-kinase VPS34, which is required for autophagy. Here we describe the discovery of potent and selective VPS34 inhibitors, their pharmacokinetic (PK) properties, and ability to inhibit autophagy in cellular and mouse models.



KEYWORDS: *VPS34, autophagy, phosphoinositide 3-kinase*

The autophagy pathway is important for the transport of cytoplasmic constituents such as damaged mitochondria and protein aggregates to lysosomes where they are degraded and recycled back into the cytoplasm.[1] Progress over the last 10 years supports an essential role for autophagy in cellular homeostasis and in some human diseases where mutations in core pathway components have been identified.[2,3] Given the role that autophagy plays in normal physiology and disease, there is interest in identifying new pharmacological agents to modulate pathway activity. Several steps regulate the formation of new autophagosomes and subsequent fusion with lysosomes. The initiation step takes place following redistribution of the ULK1-ATG13-FIP200 protein complex to the endoplasmic reticulum (ER) surface, which triggers recruitment of the vacuolar protein sorting 34 (VPS34)-VPS15-Beclin1-ATG14 complex. VPS34 is a phosphatidylinositol-3-kinase (PI3K) and its presence at the ER generates local production of phosphatidylinositol-3-phosphate (PtdIns(3)P), which provides a high affinity binding site to recruit additional factors that contain PtdIns(3)P-binding domains.[4−6] Subsequently, the ATG5-ATG12-ATG16L1 complex redistributes from the cytoplasm to the autophagosome formation site, which promotes expansion of the organelle structure. The LC3 protein is then directly conjugated to phosphatidylethanolamine on the forming membrane to help recruit cytoplasmic cargos and eventual autophagosome maturation.

The catalytic pocket of VPS34 is highly homologous to other PI3K enzymes such as PI3K alpha for which highly selective small molecule inhibitors have been developed.[7−9] The small molecule 3-methyladenine is widely used as an inhibitor of VPS34 and autophagy, but this also inhibits PI3Kα.[10] We recently described a highly selective inhibitor of VPS34, PIK-III, and its use to discover NCOA4 as an autophagy substrate.[8] Herein, we describe our efforts leading to the identification of PIK-III and novel derivatives that are orally bioavailable and inhibit autophagy in mice.[11]

Novel bisaminopyrimidine compound **1** (Table 1) was identified in a high-throughput screen as a potent inhibitor of VPS34. In addition to enhancing potency against VPS34, our efforts focused on developing selectivity against the other lipid kinases (PI3Kα, β, δ, and γ) as well as identifying a compound with suitable properties for *in vivo* studies. Initial metabolic identification (met ID) and *in vivo* pharmacokinetic (PK) studies also highlighted a number of metabolic liabilities within **1** that required further optimization (CL *in vitro* mouse = 82.6 mL min^{-1} kg^{-1}).

Prior to obtaining a crystal structure, a homology model of VPS34 was generated based on an available crystal structure of PI3Kγ (PDB code: 2CHZ) and a published protein sequence alignment using the WHAT IF program.[12,13] Utilizing this model, the binding mode of **1** in the ATP binding site was predicted (Figure 1) and was used as a guide for our medicinal

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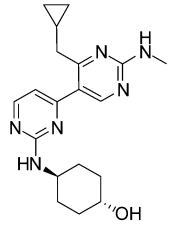
Table 1. VPS34 Inhibitory Activity and Lipid Kinase Selectivity Profile for HTS Hit 1



1

enzyme	IC_{50} (nM)
VPS34	110
PI3Kα	6200
PI3Kβ	>9100
PI3Kγ	8500
PI3Kδ	7000
PI4Kβ	3800

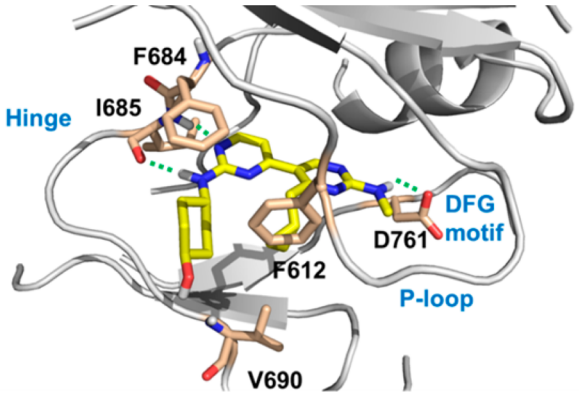


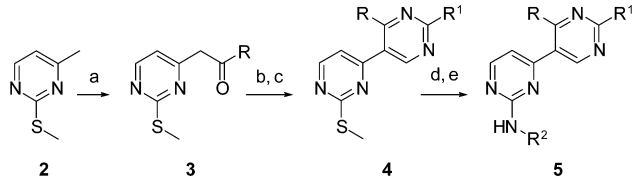
Figure 1. Compound **1** docked into the VPS34 homology model.

chemistry efforts. Given the presence of two aminopyrimidine moieties, one could envision either to act as the canonical hinge binding group. However, the compound could only be docked without steric clash with the cavity, when the aminopyrimidine bearing the cyclohexanol group was used as the hinge binding fragment. Thus, in this binding mode, the cyclohexanol moiety is predicted to sit in the solvent exposed area of the pocket, while the aminopyrimidine to which it is attached interacts with residues I685 and F684 of the hinge region. A possible interaction of the secondary amine with D761 of the DFG motif was also noted.

Our chemistry optimization efforts initially centered on replacement of the cyclohexanol portion of **1**, which was indicated to be partially responsible for high clearance *in vivo* due to a glucuronidation mechanism. Compound **1** and analogues **5** were synthesized as described in Scheme 1, commencing with alkylation of 4-methyl-2-(methylthio)-pyrimidine (**2**) with R-substituted Weinreb amides or alternatively R-substituted benzyl esters to afford ketones **3**.[14] Further reaction of **3** with DMF-DMA and subsequent cyclization led to bispyrimidine **4**. Conversion to final compounds **5** was accomplished by oxidation of the sulfide to either the sulfoxide or sulfone, followed by amine displacement.

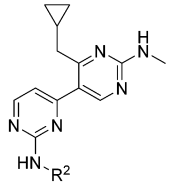
The biochemical inhibition of VPS34 (IC_{50}) was measured using a luminescence-based ATP detection assay (Table 2). Initial results indicated that while measurable changes in IC_{50} values were observed, no modifications to the R^2 moiety led to

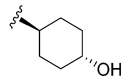
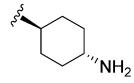
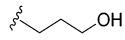
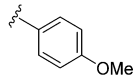
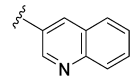
Scheme 1. Synthesis of VPS34 Inhibitors[a]



[a]Conditions: (a) LHMDS, −10 °C to rt, then RCO_2Bn or RCONMeOMe, 3 h. (b) DMF-DMA, 80 °C, 2 h. (c) K_2CO_3, amidine or guanidine, DMF, 120 °C, 2 h then rt, 18 h. (d) *m*CPBA, DCM, rt 2 h. (e) DMSO, amine, microwave, 160 °C, 10 min or THF, LHMDS, −78 °C, 30 min, then aniline, −78 °C to rt, 1 h.

Table 2. SAR of Cyclohexanol Replacement Analogues



Compound	R^2	VPS34 IC_{50} (nM)
1		110
6		410
7		400
8		3400
9		6300
10		290
11		120
12		130
13		37

complete loss of activity. This tolerability supports the binding hypothesis (later confirmed by X-ray cocrystal, Figure 2) that this portion of the molecule is solvent exposed. Within this solvent exposed area, some structural features do confer more potent activity. In general, unsaturated ring systems provide additional potency over the corresponding saturated counterparts (**10** and **8**). A possible pi-stacking interaction between the aromatic ring of **10** and Phe684 may lead to this enhancement in activity. Also of note, the introduction of heteroatoms provided an incremental enhancement in potency (**7** cf. **8**, and **10** cf. **13**), while maintaining a similar lipid kinase selectivity profile to compound **1** (Compound **13**: PI3Kα = 6800 nM,

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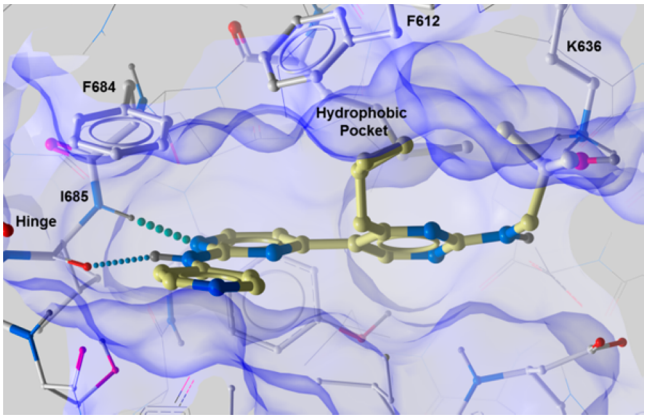


Figure 2. Cocrystal structure of compound **19** in the human VPS34 active site (PDB: 5ENN) with the active site surface of PI3Kα overlaid.

PI3Kβ = >9100 nM, PI3Kδ = 2900 nM, and PI3Kγ = 1900 nM). It was hypothesized that a water mediated hydrogen bond between the heteroatom and the backbone NH group of Val690 could be the reason for this gain in potency, which was later confirmed by X-ray crystallography.

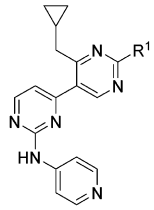
Given the potent VPS34 inhibition of compounds in the biochemical assay and the attractive lipid kinase selectivity profile (Table 1 and compound **13** above), we further evaluated the ability of compounds to inhibit VPS34 in cells. We employed a GFP-FYVE reporter assay before and after treatment of cells with compounds as reported previously.[8] IC_{50} values generated in the GFP-FYVE cellular assay correlated well with those generated in the VPS34 biochemical assay (Table 3), indicating potent on target inhibition of VPS34 in cells, with no apparent diminishment of activity when moving to a whole-cell context.

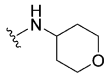
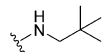
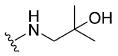
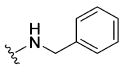
While compound **13** proved to be a potent inhibitor of VPS34 *in vitro*, the progression of this compound was limited by its metabolic stability profile (*in vitro* CL mouse = 87 mL min^{-1} kg^{-1}). Met ID studies indicated *N*-demethylation and cyclopropyl oxidation as major pathways of metabolism. We first set out to investigate the amine substitution R^1 in order to identify more tractable analogues for *in vivo* studies.

Unmethylated analogue **PIK-III** (Table 3) does retain activity, however, and showed only a modest improvement in *in vitro* metabolic stability (*in vitro* CL mouse =73 mL min^{-1} kg^{-1}), likely due to the presence of the cyclopropyl group. *In vivo* PK studies confirmed high clearance and short elimination half-life (data not shown).

Initial SAR exploration of the R^1 moiety (Table 3) identified a number of modifications tolerated in this area of the molecule and prior to obtaining an X-ray cocrystal structure also provided us with insight into the potential binding mode. The majority of the compounds prepared contained a bisaminopyrimidine core and thus two possible bidentate hinge binding sites. The modest loss in inhibitory potency with replacement of one of the amino groups (R^1), as in compounds **14** and **15**, suggests that this amine makes important contacts within the active site of VPS34 but is not essential for activity, and therefore supports the binding mode hypothesized in the docking model (Figure 1). As described in our recent publication, the VPS34 X-ray cocrystal structure with **PIK-III** confirms that the predicted aminopyrimidine is acting as the hinge binding group in this class of compound.[8] In addition, we

Table 3. SAR of Methylamine Replacement Analogues



Cmpd	R^1	VPS34 IC_{50} (nM)	GFP-FYVE IC_{50} (nM)	CL *in vitro* mouse (mL min^{-1} kg^{-1})	PI3Kδ IC_{50}[a] (nM)
13		40	14	86.7	2900
PIK-III		18	12	72.8	1200
14		210	330	69.9	>9100
15		91	ND	84.7	8500
16		240	270	65.8	4200
17		35	ND	85.5	740
18		85	ND	67.0	500
19		15	25	63.2	820
20		76	ND	74.8	3700

[a]IC_{50} values were measured against PI3K α, β, γ, and δ. Values for PI3K α, β, and γ are higher than those shown for δ.

have solved the X-ray cocrystal structure of Vps34 with compound **19**, confirming the predicted binding mode and providing support for the high selectivity observed (described in detail below in Figure 2).

While removing this amine all together (compound **14**) does result in a drop off in activity, the consequent small but measurable improvement in *in vitro* metabolic stability provides a path forward for further modification to this purpose. Benzyl and *t*-butyl analogues **20** and **17** both maintained potency against VPS34, however, and showed no substantial improvement in clearance.

Conversely amino-pyran derivative **16** showed an improvement in clearance, but this modification led to a loss in potency. A balanced solution was identified with branched analogue **18**, which exhibited much improved microsomal stability while maintaining modest binding affinity for VPS34. Further incorporation of a hydroxyl group (**19**) led to an additional enhancement in both potency and metabolic stability providing an excellent candidate for *in vivo* PK evaluation. In addition to its high potency against VPS34, **19** is extraordinarily selective over other lipid and protein kinases (>100-fold against >280

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kinases evaluated, except TAK1 and PI3Kδ, 10-fold and 40-fold, respectively).

This selectivity can be rationalized by the structure of VPS34 in complex with compound **19** (Figure 2), which was solved near the end of the medchem campaign. As is characteristic for lipid and protein kinases, the active site of VPS34 is rather compact and hydrophobic in nature, preferentially binding coplanar aromatic molecules. As was predicted from the homology model and SAR analysis, the aminopyrimidine moiety attached to the pyridine acts as the hinge binder, forming two hydrogen bonds with the backbone amide and carbonyl oxygen of I685. Additionally, the aminopyridine moiety forms an extended solvent-mediated hydrogen-bonding motif with the side chains of Asp671 and Asp644. Interestingly, the cyclopropyl group of **19** fits nicely into a hydrophobic pocket formed by the side chains of Phe612, Pro618, and Phe684 and appears to be the basis of selectivity. An overlay with the PI3Kα active site surface shows this pocket in VPS34 to be closer in proximity to the hinge region compared to the PI3Kα pocket. This shift is due to the presence of the Phe612 within VPS34, which allows the cyclopropyl group to fit into the hydrophobic pocket while maintaining optimal interaction of the donor/acceptor moiety of **19** with I685. Within PI3Kα, the Phe612 of VPS34 corresponds to a methionine residue, which does not allow for binding of the cyclopropyl group. Additionally, the tertiary alcohol moiety of **19** would be obstructed by the PI3Kα P-loop, which descends to the space occupied by this moiety.

Such selectivity is ideal for a tool compound to unambiguously assess the pharmacological consequences of VPS34 inhibition *in vivo*. Moreover, the ability of **19** to prevent the degradation of autophagy substrates p62, NCOA4, NBR1, NDP52, and FTH1 was similar to PIK-III (Figure 3). In addition, treatment of cells with compound **19** leads to an increase in the lipidated and nonlipidated forms of LC3 (Figure 3) similar to previous reports using PIK-III.[8]

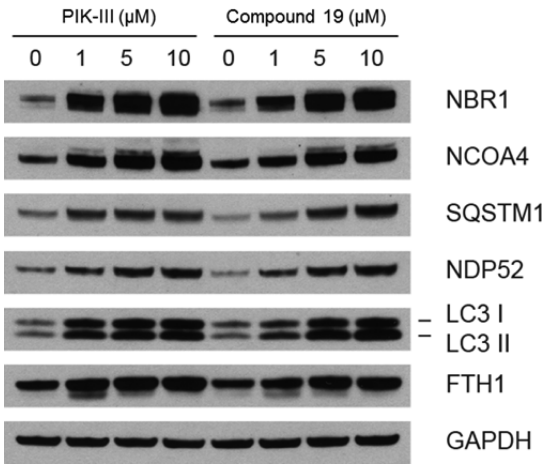

Figure 3. DLD1 cells treated for 24 h with **PIK-III** and compound **19**.

The pharmacokinetic profile of analogue **19** was determined in C57BL/6 mice (Table 4). After oral administration at 10 mg/kg, the compound was rapidly absorbed and showed moderate mean systemic clearance (30 mL/min/kg, approximately 33% of hepatic blood flow), with good oral bioavailability (F% = 47). Based on these PK parameters and the cellular activity described above, compound **19** constitutes a suitable candidate for *in vivo* studies.

Table 4. Pharmacokinetic Profile of Compound 19 in C57BL/6 Mice Following a Single IV or Oral Dose

PK parameter	mean IV 2 mg/kg	mean PO 10 mg/kg
AUC_{inf} (nM·h)	2855	6725
CL (mL/min/kg)	30	
V_{dss} (L/kg)	1.5	
$t_{1/2}$ (h)	1.2	
T_{max} (h)		0.7
C_{max} (nM)		2994
F (%)		47

We next investigated the ability of compound **19** to inhibit autophagy *in vivo* by using a PK/PD model in RKO colon cancer tumor bearing nude mice. Upon oral administration of **19** at 50 mg/kg twice a day (BID) for 7 days, we observed time-dependent accumulation of LC3-II consistent with reduced autophagic capacity (Figure 4). Although no reduction in tumor volume was observed, a clear effect on autophagy was evident. Additional studies with chronic dosing may be required to see an effect on tumor size.

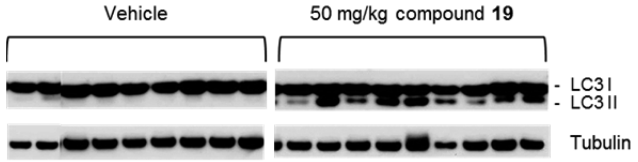

Figure 4. Seven-day BID treatment of compound **19** in tumor bearing mice. Each lane represents tumor samples from individual mice.

Through medicinal chemistry optimization of an HTS hit, we have been able to develop highly potent and selective VPS34 inhibitors exhibiting nanomolar potency biochemically and in cellular assays. These inhibitors of VPS34 display high selectivity against both lipid kinases and protein kinases and in cellular assays are capable of robustly inhibiting autophagy. Further optimization of the pharmacokinetic properties of this class of molecules led to the development of an orally bioavailable compound that is the first disclosed selective inhibitor of the autophagy process *in vivo*. While Ronan and co-workers have recently reported a compound shown to inhibit VPS34 *in vivo*, compound **19** constitutes the first example of a VPS34 inhibitor shown to inhibit autophagy *in vivo*.[15] Thus, compound **19** provides a selective *in vivo* tool to probe this process further and ultimately may provide therapeutically relevant treatments for a number of indications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.5b00335.

Biological assays, *in vivo* methods, and experimental procedures (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

PI3K, phosphoinositide 3-kinase; PK, pharmacokinetic; ND, not determined

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