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Citation for the original published paper (version of record):

Robke, L., Laraia, L., Corrales, M A., Konstantinidis, G., Muroi, M. et al. (2017)
Phenotypic Identification of a Novel Autophagy Inhibitor Chemotype Targeting Lipid
Kinase VPS34
Angewandte Chemie International Edition, 56(28): 8153-8157
<https://doi.org/10.1002/anie.201703738>

Access to the published version may require subscription.

N.B. When citing this work, cite the original published paper.

This is the peer-reviewed version of the following article: L. Robke, L. Laraia, M. A. Carnero Corrales, G. Konstantinidis, M. Muroi, A. Richters, M. Winzker, T. Engbring, S. Tomassi, N. Watanabe, H. Osada, D. Rauh, H. Waldmann, Y.-W. Wu, J. Engel, *Angew. Chem. Int. Ed.* 2017, 56, 8153., which has been published in final form at <https://doi.org/10.1002/anie.201703738>. This article may be used for non-commercial purposes in accordance with Wiley-VCH Terms and Conditions for Self-Archiving.

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Phenotypic Identification of a Novel Autophagy Inhibitor Chemotype

Targeting Lipid Kinase VPS34

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Keywords: Autophagy, VPS34, kinase inhibitor, aminopyrimidine, privileged scaffold, phenotypic assay,

Autophagy is a critical regulator of cellular homeostasis and metabolism. Interference with this process is considered a new approach for the treatment of disease, in particular cancer and neurological disorders. Therefore, novel small molecule autophagy modulators are in high demand. We describe the discovery of autophinib, a potent autophagy inhibitor with a novel chemotype. Autophinib was identified by means of a phenotypic assay monitoring formation of autophagy-induced puncta indicating accumulation of lipidated cytosolic protein LC3 on the autophagosomal membrane. Target identification and validation revealed that autophinib inhibits autophagy induced by starvation or Rapamycin by targeting the lipid kinase VPS34.

Autophagy is a conserved eukaryotic pathway which mediates the degradation of cellular components within specialized subcellular compartments, termed autophagosomes (SI-Figure 1).^[1] Autophagy plays a crucial role in the degradation of protein aggregates involved in several neurodegenerative diseases,^[2] and promotes the survival of cancer cells.^[3] Novel small molecule autophagy modulators may offer new opportunities for drug discovery^[4–6] and to decipher the underlying molecular mechanisms of this important cellular process.^[7]

Target-agnostic cellular assays monitoring changes in phenotype followed by target identification and validation offer unbiased opportunities to discover novel chemotypes and cellular targets linked to the biological process of interest by means of phenotypic changes.^[8]

Herein we report the discovery of a potent autophagy inhibitor by means of a phenotypic screening approach monitoring small molecule induced impairment of autophagy.^[9] The compound, termed autophinib, targets the lipid kinase Vacuolar Protein Sorting 34 (VPS34)^[10] which is a promising target for selective autophagy modulation.^[11]

To identify novel autophagy inhibitors, we employed a previously described cellular assay.^[9,13] In brief, autophagy was induced in MCF7 cells stably expressing eGFP-LC3 (enhanced green fluorescent protein tagged light chain 3; MCF7-LC3) by amino acid starvation using Earle's balanced salt solution (EBSS), or by inhibition of mTOR using Rapamycin. Accumulation of eGFP-LC3 on autophagosomes is visualized and quantified as formation of puncta by means of automated microscopy and image analysis.^[13]

Screening of ca. 160.000 compounds identified a class of aminopyrimidines which dose-dependently inhibited autophagosome formation (e.g. **1**; Figure 1 and SI-Table 1). Compounds active under both starvation- and rapamycin-induced autophagy were of particular interest, since they are expected to act downstream of mTOR in canonical autophagy regulation or in non-canonical autophagy independent of mTOR.

Autophagy inhibitor **1** and analogues thereof were previously developed as inhibitors of the protein kinase TBK1.^[12] However, TBK1 inhibition would result in inhibition of autophagosome maturation, increased autophagosome and eGFP-LC3 puncta formation and, therefore, cannot explain the activity of the hit class in the screen.^[14]

Figure 1: Phenotypic screen for inhibition of LC3 accumulation. **A:** Dose-dependent inhibition of amino acid starvation induced eGFP-LC3 accumulation by **1**. **B:** Dose dependent inhibition of Rapamycin induced eGFP-LC3 accumulation by **1**. **C:** Fluorescence microscopy images of the starvation induced autophagy screen. **D:** Fluorescence microscopy images of the Rapamycin-induced autophagy screen. **E:** Structure of compound **1**. Scale bar = 50 μm . Data is mean $\pm$ SD, $n \geq 3$, representative graphs shown.

For target identification, development of a structure-activity relationship (SAR) and identification of more potent inhibitors, 46 analogues were synthesized as shown in SI-Figure 2. Introduction of small substituents lacking amino groups

at the 6-position (R^3) led to an increase in inhibitory activity as compared to compound **1** (Table 1 and SI-Tables 1-5). This finding further supported the notion that TBK1 was not the target responsible for autophagy inhibition, as the protonated piperazine moiety generates a key salt bridge with the Asp157 in TBK1 which leads to enhanced potency toward TBK1.^[12] Other smaller amine-containing inhibitors, (e.g. Table 1, entry 6) were also less potent in comparison to the chloro- or methyl-substituted analogues. Based on these observations a chloro-substituent at the 6-position was chosen for further compound improvement.

Replacement of the pyrazole in the 4-position led to loss of activity (Table 1, entries 9 - 12). By analogy to findings of Rauh et al.,^[15] this finding suggests that the pyrazole may be involved in a key hydrogen bond to the kinase hinge region. A methyl group on the pyrazole was most advantageous for activity, whilst the larger *tert*-butyl group (Table 1, entry 7) led to a significant drop in cellular activity.

At the 2-position (R^1) an ether-oxygen is favored for activity as replacement by a thioether (SI-Table 3, entry 2) or an amine (Table 1, entry 18) decreased activity. Replacement of the nitro group at R^1 led to compounds with a sulfonamide (Table 1, entry 13) and a nitrile group (Table 1, entry 15) at the *para* position respectively, for which activity was only slightly lower. Replacing the phenyl group by a pyridine, however, significantly reduced activity (SI-Table 5, entry 24).

Table 1: Inhibition of autophagy and VPS34 determined for selected analogues. Starvation = starvation induced autophagy assay; Rapamycin = Rapamycin induced autophagy assay; VPS34 = *in vitro* assay for VPS34

activity. Inactive = no inhibition at a test concentration > 10 μ M. Data is mean $\pm$ SD, $n \geq 3$ for all autophagy IC_{50} values; $n \geq 2$ for all *in vitro* VPS34 IC_{50} values.

For a full list of analogues and activities see SI-Tables 1 - 5.

entry	nr.	R ¹	R ²	R ³	starvation IC_{50} [μ M]	rapamycin IC_{50} [μ M]	VPS34 IC_{50} [μ M]
1	Autophinib (2)			Cl	0.09 $\pm$ 0.04	0.04 $\pm$ 0.01	0.019
2	3			Me	0.07 $\pm$ 0.04	0.04 $\pm$ 0.02	0.023
3	4			CF ₃	0.19 $\pm$ 0.11	0.07 $\pm$ 0.03	0.078
4	1				0.39 $\pm$ 0.07	0.13 $\pm$ 0.06	0.146
5	5				0.23 $\pm$ 0.11	0.16 $\pm$ 0.06	0.121
6	6				0.45 $\pm$ 0.16	0.28 $\pm$ 0.12	0.285
7	7			Cl	1.40 $\pm$ 0.70	2.00 $\pm$ 0.80	0.627
8	8			Cl	0.13 $\pm$ 0.03	0.10 $\pm$ 0.02	0.036
9	9			Cl	Inactive	Inactive	Inactive
10	10			Cl	Inactive	Inactive	Inactive
11	11			Cl	2.30 $\pm$ 1.20	Inactive	Inactive
12	12			Cl	Inactive	Inactive	Inactive
13	13			Cl	0.45 $\pm$ 0.19	0.42 $\pm$ 0.17	0.061

14	14			Cl	0.17 ± 0.06	0.12 ± 0.03	0.020
15	15			Cl	0.13 ± 0.08	0.09 ± 0.08	0.049
16	16			Cl	0.38 ± 0.20	0.28 ± 0.13	0.110
17	17			Cl	0.20 ± 0.09	0.47 ± 0.27	0.214
18	18			CF ₃	0.34 ± 0.16	0.44 ± 0.26	0.310

Based on the SAR investigation compound **2**, which we termed autophinib, was chosen for further in-depth characterization (SI-Figure 3). Autophinib inhibited LC3 lipidation to form LC3-II in a dose-dependent manner in starved MCF7-LC3 cells (Figure 2A). Consistent with inhibition of autophagic flux, autophinib also inhibited p62 degradation by autophagy dose-dependently in MCF7-LC3 cells (Figure 2A).^[16]

Figure 2: Phenotypic validation of Autophinibautophinib as an autophagy inhibitor. **A:** Inhibition of LC3 lipidation and p62 degradation by autophinib in MCF7-LC3 cells. $n \geq 3$, representative blot shown. **B:** Autophinib induces cell death in starved MCF7 cells. Cytotoxicity was assessed by means of the CellTox™ green dye binding to DNA due to cytotoxicity. **C:** Autophinib dose dependently induces apoptosis in starved cells. Apoptosis was assessed by using a selective caspase 3/7 probe. The experiments were performed with an Incucyte Zoom instrument. Data are presented as a ratio of green fluorescent area to cellular area as assessed by phase contrast (mean $\pm$ SD, $n \geq 3$, representative graph shown). **D:** Autophinib inhibits formation of PI3P positive vesicles in HEK293A cells stably expressing eGFP-WIPI-2. **E:** Quantification of the eGFP-WIPI-2 assay shown in **D**. Autophinib reverts the phenotype (starved, 10 μ M, $p = 0.0002$) showing a similar effect as wortmannin (Wort., 500 nM) and the opposite effect of bafilomycin A1 (Baf. A1, 50 nM). Scale bar = 10 μ m. Statistical analysis was performed using student's t-test (***) = $p \leq 0.001$). Data are shown with mean values $\pm$ 95% CI, $N \geq 25$.

As expected for an autophagy inhibitor, autophinib enhanced cell death of starved cells as compared to fed cells (Figure 2B),^[4,6] which occurred via the induction of apoptosis (Figure 2C).

Since autophinib inhibited Rapamycin induced autophagy and thus acts downstream or independently of mTOR, we performed an image based flux assay quantifying the number of autophagosomes as well as autolysosomes (SI-Figure 4). Autophinib inhibits the accumulation of autophagosomes under

fed as well as starved conditions, but does not affect the number of autolysosomes. Thus it is not a late stage autophagy inhibitor but an inhibitor of autophagosome formation. PI3P is essential to initiate autophagosome biogenesis and is mainly generated by the kinase VPS34. PI3P recruits effector protein WIPI2, which is important for the formation of autophagosomes.^[17] Therefore, eGFP-WIPI2b serves as a sensor for autophagosomal PI3P.^[18] Image analysis of HEK293A cells, stably transfected with a construct for eGFP fused to WIPI2b, showed an increasing number of fluorescent vesicles upon starvation, which was reduced upon treatment with autophinib (Figure 2D and E, see SI-Figure 5 for the full image set).

For target identification autophinib was profiled on a proteome-wide level by comparing it to compounds with known modes of action and targets (Figure 3A, SI-Tables 6-8). The ChemProteoBase method employs image based proteome analysis by means of 2-D difference gel electrophoresis (DIGE) in comparison to reference compounds.^[19] Since no compound with similarity > 0.7 was identified in this analysis, the mechanism of action of autophinib is suggested to be novel. Despite a low cosine value, several kinase inhibitors emerged at the top of the list such as the PI3K inhibitor LY294002 (SI-Table 7). This finding in combination with the potent effect of autophinib on the formation of PI3P containing puncta and structural similarity to known kinase inhibitors led us to investigate inhibition of the PI3 kinase VPS34 by autophinib.

Indeed, VPS34 was inhibited with IC₅₀ values of 19 ± 5 nM and 51 ± 8 nM at ATP concentrations of K_M (10 µM) and 100 µM respectively (Figure 3B)

suggesting an ATP competitive mode of action. Activity in the phenotypic assay correlated strongly with *in vitro* potency against VPS34 (Table 1).

Figure 3: Target identification and engagement for autophinib. **A:** Hierarchical cluster analysis of the ChemProteoBase. HeLa cells were treated with 30 μ M autophinib for 18 h, and proteomic analysis was done by the 2-D DIGE. Cluster analysis was performed using quantitative data of the 296 common spots (x-axis) derived from autophinib and those of 41 reference compounds. In the heat map, log-fold (natural base) of the normalized volume is shown on the colored scale. **B:** Dose dependent biochemical inhibition of VPS34 by autophinib at different ATP concentrations. **C:** Identification of VPS34 as target

of autophinib with ATP-biotin probes and readout via Western blot (ActiveX assay). Competition of the ATP probes with autophinib validates binding of autophinib to VPS34 in cell lysate. Data is mean $\pm$ SD, $n \geq 3$, representative graph / blot shown.

Autophinib did not inhibit other phosphatidylinositol kinases and mTOR at a concentration of 1 μ M (SI-Table 9). Remarkably, it also did not inhibit TBK1, the target of the parent compound **1**. Selectivity was further assessed in a panel of >460 kinases (SI-File 1) from which 45 were inhibited $\geq 80\%$ at a concentration of 1 μ M. However, most of them could be excluded as being not relevant for autophagy by testing known, selective inhibitors in the autophagy assay (SI-Table 10). Among 27 investigated inhibitors, the most potent autophagy inhibitory activity was observed for the two recently reported VPS34 inhibitors: SAR405 and VPS34-IN-1.^[20,21]

VPS34 engagement by autophinib in a complex biological medium was demonstrated by means of an ActiveX assay^{[22],[23]}, which employs a reactive desthiobiotin-labeled ATP-probe. Incubation of the probe with cell lysate covalently labels ATP-binding proteins, including kinases, which are then captured by streptavidin-agarose-beads, eluted and analyzed. In order to identify ATP binding targets of a small molecule the lysate is preincubated with the compound prior to incubation with the ATP-probe to block the small molecule binding site. Bound target proteins then do not bind to the ATP-probe and are not detected. Autophinib dose-dependently inhibited the covalent

binding of the ATP probe to VPS34 Figure 3C). This result further supports the notion that autophinib is an ATP competitive VPS34 inhibitor.

An isothermal dose-response fingerprint (ITDRF)^[24] confirmed target engagement of autophinib in cell lysate, which stabilized VPS34 towards heat induced aggregation (SI-Figure 6).

In summary, we report the discovery of a new autophagy inhibitor chemotype by means of phenotypic screening. Autophinib targets VPS34 but not other lipid kinases, mTOR and TBK1. Autophinib appears to be ATP-competitive, targets VPS34 in cell lysate and induces cellular phenotypes consistent with VPS34 inhibition.

Wortmannin and 3-methyladenine (3-MA) have been used widely to study the effect of PIK3 inhibition. However, both non-selectively inhibit all isoforms of the PIK3 family.^[25] In addition it has been observed that conditional knockout of VPS34 shows severe differences to 3-MA or wortmannin treatment, indicating that these inhibitors display VPS34-independent effects.^[26] Recently more potent and selective alternatives have been developed.^[20,21] However, an expanded toolkit to study autophagy and VPS34 activity would be very desirable for basic research and potential drug discovery applications. Autophinib distinguishes itself by its ready synthetic accessibility (3 steps, overall yield: 45%), combined with high *in vitro* potency, selectivity and cellular activity. It promises to be an excellent tool compound for the study of VPS34 biology and autophagy. Our results provide further proof for the utility of unbiased, target agnostic phenotypic screens as a fruitful strategy for the

discovery of potent autophagy inhibitors in particular and of new bioactive compounds in general.

Acknowledgements

This research was supported by the Max-Planck-Gesellschaft. L. R. is grateful to the Boehringer Ingelheim Fonds for a fellowship. L. L. is grateful to the Alexander von Humboldt Foundation for a fellowship. M. A. C. C. is grateful to the Swiss National Science Foundation and the Fonds der Chemischen Industrie for a fellowship. Y.-W. W. acknowledges funding from European Research Council (ChemBioAP) and Behrens-Weise-Stiftung. We thank Miho Tanaka for ChemProteoBase analyses. We thank Sharon Tooze for the gift of WIPI2 cells.

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PIK(3)C(3)ing autophagy apart: Phenotypic screening followed by target identification reveals a novel autophagy inhibiting chemotype that inhibits the lipid kinase PIK3C3 (VPS34) with high potency, disrupting autophagosome biogenesis.