cdc-Like/Dual-Specificity Tyrosine Phosphorylation–Regulated Kinases Inhibitor Leucettine L41 Induces mTOR-Dependent Autophagy: Implication for Alzheimer’s Disease

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ABSTRACT

Leucettines, a family of pharmacological inhibitors of dual-specificity tyrosine phosphorylation regulated kinases and cdc-like kinases (CLKs), are currently under investigation for their potential therapeutic application to Down syndrome and Alzheimer’s disease. We here report that leucettine L41 triggers bona fide autophagy in osteosarcoma U-2 OS cells and immortalized mouse hippocampal HT22 cells, characterized by microtubule-associated protein light chain 3 membrane translocation and foci formation. Leucettine L41–triggered autophagy requires the Unc-51–like kinase and is sensitive to the phosphatidylinositol 3-kinase (PI3K) inhibitors wortmannin and 3-methyladenine, suggesting that it acts through the mammalian target of rapamycin (mTOR)/PI3K-dependent pathway. Leucettine L41 does not act by modifying the autophagic flux of vesicles. Leucettine L41–induced autophagy correlates best with inhibition of CLKs. Leucettine L41 modestly inhibited phosphatidylinositol-3-phosphate 5-kinase, FYVE domain–containing activity as tested both in vitro and in vivo, which may also contribute to autophagy induction. Altogether these results demonstrate that leucettines can activate the autophagic mTOR/PI3K pathway, a characteristic that may turn advantageous in the context of Alzheimer’s disease treatment.

Introduction

Macroautophagy (hereafter referred to as autophagy) is a mechanism conserved among eukaryotes with essential roles in homeostasis and development (Mizushima and Levine, 2010). Defects in autophagy have been associated with many human diseases, including cancers, myopathy, and neurodegenerative, infectious, and cardiovascular diseases (see Yang and Klionsky, 2010, for review). Autophagy is a “self-digesting” process by which the cell engulfs cytoplasmic components in vesicles (autophagosomes) that fuse with lysosomes into autolysosomes and have their content degraded by lysosomal hydrolases (Yang and Klionsky, 2010). Autophagy allows cells to degrade long-lived, aggregated, or damaged proteins and organelles, catabolizing existing cytoplasmic components to generate nutrients and energy to maintain essential cellular activities. Many protein kinases are involved in the control of autophagy, including Unc-51–like kinases 1 and 2 (ULK1 and ULK2). This article has supplemental material available at molpharm.aspetjournals.org.

ABBREVIATIONS: BIP, immunoglobulin heavy chain binding protein; CLKs, cdc-like kinases; DMSO, dimethylsulfoxide; DYRKs, dual-specificity tyrosine phosphorylation regulated kinases; EBSS, Earle’s balanced salt solution; ER, endoplasmic reticulum; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; GroPIns, glycerophosphorylinositol; HPLC, high-performance liquid chromatography; IP3, inositol-1,4,5-trisphosphate; LC3, microtubule-associated protein light chain 3; mRFP, monomeric red fluorescent protein; mTOR, mammalian target of rapamycin; PI, phosphatidylinositol; PI(3,5)P2, phosphatidylinositol-3,5-bisphosphate; PI3K, phosphatidylinositol-3-kinase; PI(3,5)P2, phosphatidylinositol-3-phosphate 5-kinase; FYVE domain–containing; PtdIns(3,5)P2, phosphatidylinositol-3,5-bisphosphate; PtdIns3P, phosphatidylinositol-3-phosphate; PtdIns4P, phosphatidylinositol-4-phosphate; PtdIns5P, phosphatidylinositol-5-phosphate; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; SB600125, 2-[N-(3-chloro-4-methylphenyl)-2-morpholinoethyl]-N'-((4R,4'S)-3H-[1,2,4]oxadiazol-3-one); SRC, src protein tyrosine kinase; siRNA, small interfering RNA; SR protein, serine-arginine rich proteins; U0126, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenyl]thio]butadiene; ULK, Unc-51–like kinase; YM201636, 6-amino-N-[3-[4-(4-morpholinyl)pyrrolo[3′,2′:4,5]furo[3,2-d]pyrimidin-2-yl]phenyl]-3-pyridinecarboxamide; Z-PP-CHO, prolyl endopeptidase inhibitor II.
ULK2), AMP-activated protein kinase, and mammalian target of rapamycin (mTOR), which monitors cellular nutrient status and negatively regulates autophagy (Wullschlegler et al., 2006; Chan et al., 2007; Hoyer-Hansen and Jaattela, 2007). Phosphatidylinositol 3-kinase (PI3K) (3p34), which leads to the formation of phosphatidylinositol-3-monophosphate (PtIns3P), is a key inducer of the mTOR autophagic pathway (He and Klionsky, 2009). A few years ago, it emerged that autophagy can also be induced through an mTOR-independent pathway by a decrease in intracellular levels of inositol and inositol-1,4,5-trisphosphate (IP3) through inhibition of inositol monophosphatase by lithium (Sarkar et al., 2005).

Splicing involves the removal of introns from pre-mRNA and the joining of exons. Almost all human genes undergo alternative splicing. Alternative splicing is a highly regulated process that produces multiple mRNA variants from a single pre-mRNA. This influences gene expression, mRNA localization or stability, and generates several protein isoforms from a single pre-mRNA (Blencowe, 2006). The phosphorylation state of serine-arginine–rich (SR) proteins, a family of splicing factors, plays an important role in splicing regulation (Long and Caceres, 2009). SR proteins are phosphorylated by several protein kinases, including SR protein kinases (Gwi et al., 1994) and the dual-specificity tyrosine phosphorylation–regulated kinases (DYRKs; de Graaf et al., 2004) and cdk-like kinase 1 (CLK1; Duncan et al., 1997), the activity of which is required to maintain the phosphorylation state of SR proteins (Yomoda et al., 2008). Given the growing number of examples where splicing is abnormally regulated in human disease (review in Singh and Cooper, 2012), it is not surprising that there is increasing interest in pharmacological inhibitors of SR protein kinases, DYRKs, and CLKs as potential therapeutic drugs (review in Hagiwara, 2005; Smith et al., 2012). In this context we recently described leucettines, a family of low molecular weight inhibitors of DYRKs and CLKs derived from the marine natural product leucettamine B (Debab et al., 2011; Tahtouh et al., 2012).

Leucettines were recently found to interact with phosphatidylinositol-3-phosphate 5-kinase, FYVE domain–containing (PIKFyve), the lipid kinase that catalyzes the phosphorylation of phosphatidylinositol-3-monophosphate (PtIns3P) to phosphatidylinositol-3,5-bisphosphate (PI(3,5)P2) and the phosphorylation of phosphatidylinositol (PI) to phosphatidylinositol-5-monophosphate (PtIns5P) (Tahtouh et al., 2012). PIKFyve is part of the PtIns(3,5)P2 regulatory complex, comprising the scaffolding protein ArPIKfyve and the lipid phosphatase Sac3 (reviewed in Shisheva, 2012). It was demonstrated that PIKFyve is involved in autophagy, although its mechanism of action is still unclear (Jefferies et al., 2008; de Lartigue et al., 2009).

We show here that leucettines trigger autophagy in cultured HT22 and U-2 OS cells due to their ability to inhibit CLKs and PIKFyve rather than DYRKs. Moreover we show that reduction of CLK1 activity is sufficient to induce autophagy in U-2 OS cells. Leucettine–induced autophagy occurs through the mTOR-,ULK-, and PI3K-dependent pathway. This unexpected property of leucettines may turn out to be of particular relevance in view of their therapeutic potential for the treatment of cancers and Alzheimer’s disease.

Materials and Methods

Reagents. Rapamycin, wortmannin, and 3-methyladenine were purchased from Sigma-Aldrich (St. Louis, MO). SB600125 (antra[1-9-cd]pyrazole-6(2H)-one), SB230580 [4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)1H-imidazole], tunicamycin, and prolyl endopeptidase inhibitor II (Z-PP-CHO) were from Calbiochem (EMD Millipore, Billerica, MA); UO126 (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene) was purchased from Cell Signaling Technology, Inc. (Danvers, MA). Gleevec was from Cayman Chemical (Ann Arbor, MI). YM201636 (6-amino-N-3-[4-(4-morpholino)pyrrolo[3′,2′;4,5]furo[3,2-d]pyrindin-2-y]lphenyl)-3-pyrindinecarboxamide) was purchased from Synmaxis (Timaru, New Zealand); Olomoucine, rocsovitine, S-CRB [2-(5S)-1-ethyl-2-hydroxyethylamino)-6-(4-2-pyridyl)benyl-9-isopropylpurine], aloiseine, alsterpaullone, leucettines L41, L14, L33, and L28 were synthesized in the laboratory as previously described (Leost et al., 2000; Mettey et al., 2003; Bettayeb et al., 2008; Debad et al., 2011).

Cell Culture Experiments. U-2 OS cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, and 50 IU of penicillin and streptomycin. In the cases of cotreatment with wortmannin, 3-methyladenine, or Z-PP-CHO, cells were pretreated for 1 hour before addition of leucettine L41, rapamycin, or LiCl depending on the experiments. Cytotoxicity was assayed using the CytoTox 96 Non-Radioactive Cytotoxicity Test from Promega (Madison, WI) according to manufacturer’s instructions. For Earle’s balanced salt solution (EBSS)–induced autophagy, cells were washed four times with prewarmed EBSS then allowed to grow in EBSS for another 6 hours.

Cloning and Transfection Procedures. Human microtubule–associated protein light chain 3 (LC3B) si image clone (IOH103677) was purchased from ImaGene (Source Bioscience Lifesciences, Nottingham, UK). Monomeric red fluorescent protein (mRFP)–enhanced green fluorescent protein (EGFP):LC3 were generated as previously described (Kimura et al., 2007). Green fluorescent protein (GFP) constructs were transfected using FuGene-6 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. Stably expressing mRFP:EGFP:LC3 and EGFP:LC3 clones were selected with G418 treatment. Double-stranded small interfering RNA (siRNA) oligomers were transfected into U-2 OS cells using Dharmafect 1 (Dharmacon/ThermoScientific, Pittsburgh, PA), according to the manufacturer’s instructions. Validated siRNAs targeting DYRK1A (S00605241), CLK1 (S10005932), PIKFyve (S00155120), and ULK1 (S0222327) were purchased from Qiagen (Valencia, CA). Control depletion was carried out using All Star Negative Control siRNA oligomers from Qiagen.

Antibodies, Immunoblotting, and Indirect Immunofluorescence. Gel electrophoresis and immunoblotting were performed using standard protocols. For immunofluorescence, cells were grown on glass coverslips, fixed with 4% paraformaldehyde in phosphate-buffered saline, permeabilized by 50 μM digitonin, and processed using standard protocols. Images were acquired with a Leica DFC360 FX CCD camera on a Leica AF6000 microscope (Leica Microsystems, Wetzlar, Germany) using a 40× numerical aperture 0.85 objective. Image processing was performed by ImageJ (NIH, Bethesda, MD). Quantification of LC3 foci was performed using ImageJ threshold function and automated counting. Primary antibodies used in this study included anti-α-tubulin (DM1A; Sigma-Aldrich), anti-LC3 (4E12 (MBL Internationalm, Nagoya, Japan) for immunofluorescence, or 2G6 (Enzlo Life Sciences, Farmingdale, NY) for immunoblot), anti-DYRKIA (Abnova, Taipei City, Taiwan), anti–phospho-Thr704E-BP1 (Cell Signalling Technology), anti–immunoglobulin heavy chain binding protein (Bip) (Cell Signalling Technology), and anti-CLK1 (ab74044; Abcam, Cambridge, MA).

Reverse-Transformation Polymerase Chain Reaction and Polymerase Chain Reaction. Total cellular RNAs were extracted using Trizol (Gibco/Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. One microgram was used to perform cDNA synthesis using SuperScript Reverse Transcriptase III (Invitrogen/Life Technologies, Carlsbad, CA) and random decamers (Ambion/Life Technologies, Austin, TX). cDNAs were amplified using Taq polymerase (Roche Diagnostics) and appropriate primers for CLK1 (GCGTCAAATAAACTCCTG and GCCCTTAGCATGAC GACT). Products were separated by agarose gel electrophoresis and visualized using ethidium bromide.

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Protein Kinase Assays. Recombinant DYRK1A, B, 2, 3, and CLK1, 2, 3, 4 were expressed in Escherichia coli, purified, and assayed with appropriate substrates with 15 μM radiolabeled [γ-32P]ATP in the presence of a range of concentrations of drugs as described previously (Debdab et al., 2011; Tahtouh et al., 2012). IC50 values were determined from the dose-response curves.

Recombinant PIKfyve Activity Assay. Silica gel G60 thin-layer chromatography plates were from Merck (Darmstadt, Germany), [γ-32P]ATP from PerkinElmer (Waltham, MA), brain L-α-phosphatidyl ethanolamine (PE) from Avanti Polar Lipids (Alabaster, AL), and C16:0/C16:0-PtdIns3P from Echelon (Salt Lake City, UT). All other compounds used were from Sigma-Aldrich. Recombinant GST-PIKfyve protein corresponding to the short form of murine PIKfyve was produced in SF9 infected cells, purified, and its activity was measured as described previously (Chicanne et al., 2012). Briefly, increasing amounts of L41 or YM201636 were added to the samples containing recombinant PIKfyve (0.2 μg), and PtdIns3P (50 pmol)/PE (20 nmol) vesicles, and lipid kinase buffer. After 10 minutes at room temperature, the phosphorylation reactions were initiated by addition of a mixture of ATP (50 μM) and [γ-32P]ATP (20 μCi). After incubation at 37°C for 30 minutes, phosphorylated lipids were extracted, separated by thin-layer chromatography, and the amount of [32P]-PtdIns3P (3,5,5′)2 generated was quantified. To assess the effect of L41 on both PtdIns(3,5)P2- and PtdIns5P-generating activity of PIKfyve, we conducted a similar in vitro lipid kinase activity assay using the native PtdIns substrate from Avanti Polar Lipids (100 μM PtdIns, sonicated prior to use in 20 mM HEPEs, pH 7.5, 1 mM EDTA), and adenosinylated produced PIKfyve as an enzyme source, which was immunopurified on anti-PIKfyve antibodies as we described elsewhere (Sbrissa et al., 1999, 2002).

In Vivo PIKfyve Activity Inhibition Assay: myo-[2-3H]inositol Labeling, Lipid Extraction, and High-Performance Liquid Chromatography. The effect of L41 on phosphoinositide steady-state levels was assessed in 3T3 L1 adipocytes labeled with myo-[2-3H]inositol (PerkinElmer) following our protocols for adipocytes in vivo labeling (Sbrissa et al., 2012) with a slight modification. Briefly, cells (35-mm dishes) maintained for 18 hours in glucose- and inositol-free Dulbecco’s modified Eagle’s medium (DMEM) containing 5% dialyzed fetal bovine serum, 0.5% bovine serum albumin, 5 mM pyruvate, 25 mM HEPEs, pH 7.4, 100 μg/ml penicillin, and 100 μg/ml streptomycin were labeled for 40 hours with 22.5 μCi/ml myo-[2-3H]inositol in glucose- and inositol-free DMEM containing 5 μg/ml insulin, 5 μg/ml transferrin, 2 mM pyruvate, 25 mM HEPEs, pH 7.4, at 37°C in a 5% CO2/95% air humidified incubator. Treatment with 0 (vehicle, final dimethylsulfoxide (DMSO), 0.2% v/v), 1, 5, or 20 μM L41 inhibitor was for the last 75 minutes of the labeling period. Lipids were extracted in the presence of EDTA and tetrabutylammonium sulphate (5 mM each), deacylated, and analyzed by high-performance liquid chromatography (HPLC) (Waters Corporation, Milford, MA) on a 5-μm Partisphere SAX column (4.6 mm × 250 mm; Whatman/GE Healthcare Life Sciences, Pittsburgh, PA) as detailed previously (Sbrissa et al., 2012). [32P]Glycerophosphorylinositol 5P ([32P]GroPIns5P), [32P]GroPIns3P, [32P]GroPIns(4,5)P2, and [32P]GroPIns(3,5)P2 prepared by enzymatic synthesis with [γ-32P]ATP as described elsewhere (Sbrissa et al., 1999) were coinjected as internal HPLC standards. Fractions were collected every 0.25 minutes and analyzed for [3H] and [32P] radioactivity after addition of the scintillation mixture. Data evaluation and documentation were performed by Microsoft Excel. Individual peak radioactivity was quantified by area integration and presented as a percentage of the summed radioactivity from the 3H[GroPIns5P, 4P, -5P, -3,5P] and -4,5(P) peaks (“total radioactivity”).

Results

Leucettine L41 Triggers Accumulation of LC3 Foci. We first tested the ability of a variety of protein kinases inhibitors to induce autophagy. U-2 OS, a human osteosarcoma cell line, and HT22, a mouse immortalized hippocampal cell line, were treated with a small panel of inhibitors at 10 μM and autophagy was assayed after 24 hours by counting LC3 foci, a hallmark of autophagy. With the exception of alsterpaullone, a glycogen synthase kinase-3β inhibitor, similar results were obtained for both cell lines, and U-2 OS results are described below (Fig. 1; Supplemental Fig. 1). Rapamycin, an mTOR inhibitor, and Gleevec, a c-Abi tyrosine kinase inhibitor, were used as positive controls (10 ± 0.3 and 17 ± 1 LC3 foci per cell, respectively; Ertmer et al., 2007). Inhibition of cyclin-dependent kinases with olomoucine, roscovitine, or S-CR8 did not induce an increase of autophagy compared with cells treated with DMSO (5.1 ± 0.4, 5.9 ± 0.3, and 6.6 ± 0.24, respectively, versus 4.1 ± 0.5 LC3 foci per cell; Fig. 1A). Inhibition of glycogen synthase kinase-3β with aloïsine (4.7 ± 0.2 foci per cell) or alsterpaullone (6.6 ± 0.2 foci per cell) or inhibition of c-Jun N-terminal kinases (4.3 ± 0.4 foci per cell) and p38 (5.9 ± 0.5 foci per cell) with SB600125 and SB203580, respectively, did not induce an accumulation of LC3 foci in cells (Fig. 1A). On the other hand, treatment with 10 μM leucettine L41 (L41), an inhibitor of both DYRKs and CLKs recently developed in our laboratory (Debdab et al., 2011; Tahtouh et al., 2012; Fig. 1D), induced LC3 foci formation (11 ± 1 foci per cell) in a dose-dependent manner (Figs. 1A and 2A), suggesting that inhibition of DYRKs, CLKs, and/or other targets could induce autophagy. A low level of toxicity of L41 was confirmed by measuring amounts of lactate dehydrogenase released in the medium after 24 hours of treatment with 10 μM L41 (Fig. 1B). Autophagy and LC3 foci accumulation can result from endoplasmic reticulum (ER) stress (see Deegan et al., 2013, for review). To determine whether L41 treatment was triggering ER stress, the expression levels of BiP, a protein upregulated upon ER stress, was estimated. Tunicamycin was used as a positive control. As shown in Fig. 1C, BiP protein levels were unaffected by L41 treatment, demonstrating that L41 does not induce ER stress. Because under certain circumstances LC3 foci can be dissociated from autophagy (Kuma et al., 2007), we further confirmed autophagy induction upon L41 treatment by detecting the conversion of the soluble LC3-I form to the membrane-bound LC3-II form by immunoblotting (Fig. 2B). Moreover, treatment with 3-methyladenine, an inhibitor of autophagy, prevented L41-induced autophagy in U-2 OS cells (3.2 ± 0.6 foci per cell; Fig. 2C), further confirming that the observed accumulation of LC3 foci upon L41 treatment is genuine autophagy. Taken together our data show that L41 is a new inducer of autophagy.

Leucettine L41 Triggers Autophagy through a ULK1- and mTOR-Dependent Pathway. Several signaling pathways are known to regulate autophagy. One is controlled by mTOR and PI3K and requires the activation of ULK1 (Chan et al., 2007). Another pathway is mTOR-independent that depends on IP3 levels and can be activated by lithium (Sarkar et al., 2005). To determine whether leucettine L41 is inducing autophagy through the mTOR-dependent or mTOR-independent pathway we used siRNA and chemical inhibition approaches. As shown in Fig. 2D, ULK1-depleted cells treated with L41 were unable to undergo autophagy (6.0 ± 0.3 foci per cell versus 16.2 ± 4.9 in L41 control siRNA-treated cells) suggesting that ULK1 is required for L41 to induce autophagy (Fig. 2D). The PI3K inhibitor wortmannin is a well known inhibitor of mTOR-dependent autophagy. Loss of autophagy (5.3 ± 0.6 foci per cell) upon cotreatment with leucettine L41 and wortmannin further confirmed that L41 induces autophagy
through the mTOR and PI3K-dependent pathway (Fig. 2C). U-2 OS cells treated with leucettine L41, rapamycin or DMSO were analyzed for mTOR activation. mTOR phosphorylates 4E-BP1 at Thr70 (see Tchevkina and Komelkov, 2012, for review). 4E-BP1 phosphorylation was inhibited by rapamycin, as shown by the complete phospho-Thr70 signal disappearance (Fig. 2E). Leucettine L41 treatment caused significant reduction of 4E-BP1 phosphorylation further confirming that L41-induced autophagy is mediated by the mTOR-dependent pathway (Fig. 2E). Next, we assessed if L41-induced autophagy was also dependent on IP3 levels. Intracellular levels of inositol were raised using an inhibitor of prolyl-endopeptidase (Z-PP-CHO) or myo-inositol (Sarkar et al., 2005) and cells were cotreated with either rapamycin, LiCl, or L41. As shown in Fig. 2F, Z-PP-CHO was able to reduce autophagic response to LiCl (5.0 ± 0.1 foci per cell versus 11.8 ± 0.7) and had no effect on mTOR-dependent autophagy (10.8 ± 0.7 foci per cell versus 11.0 ± 0.3). Cells treated with L41 and Z-PP-CHO or LiCl and myo-inositol (Fig. 2F; Supplemental Fig. 2) for 24 hours still underwent autophagy as evidenced by accumulation of LC3 foci (12.1 ± 0.9 and 11.5 foci per cell respectively). Taken together these results strongly suggest that autophagy observed upon L41 treatment is mediated solely through the mTOR/PI3K-dependent pathway.

Accumulation of LC3 foci in L41-treated cells could result from the perturbation of autophagic flux rather than from induction of autophagy per se. To investigate this possibility we established a stable U-2 OS cell line expressing the human inclusion of exon 4 in the mRNA, thereby increasing the level of the full-length kinase isoform. Changes in abundance of this splice variant thus acts as a marker of CLK1 activity (Duncan et al., 1997; Muraki et al., 2004). Reverse-transcription polymerase chain reaction analysis of mRNA from L41-treated U-2 OS cells shows that L41 treatment induces a 150-fold increase of the exon 4-containing CLK1 mRNA (Supplemental Fig. 3B; Tahtouh et al., 2012). It has been reported that CLK1 regulates its own splicing and that CLK1 inhibition promotes inclusion of exon 4 in the mRNA, thereby increasing the level of the full-length kinase isoform. Changes in abundance of this splice variant thus acts as a marker of CLK1 activity (Duncan et al., 1997; Muraki et al., 2004). Reverse-transcription polymerase chain reaction analysis of mRNA from L41-treated U-2 OS cells shows that L41 treatment induces a 150-fold increase of the exon 4-containing CLK1 mRNA (Supplemental Fig. 3B; Muraki et al., 2004). Similarly to L41, treatment with TG003, a widely used CLK inhibitor (Supplemental Fig. 3A; Muraki et al., 2004) also had inhibitory effects on DYRK1A and CLKs and induced autophagy in U-2 OS cells, further confirming that inhibition of either DYRKs or CLKs leads to autophagy (Supplemental Fig. 3C). We used leucettines that are more specific to either one or the other kinase family (Fig. 4A; Debdab et al., 2011) to determine which of the two kinase families is involved in autophagy. Treatment of U-2 OS cells with leucettine L38, an inhibitor showing more potency toward DYRKs, did not induce autophagy (Fig. 4B; 4.5 ± 0.2 LC3 foci per cell compared with 4.9 ± 1.9 in DMSO-treated cells), whereas treatment with leucettine L14, an inhibitor more specific to CLKs (Fig. 4B), induced autophagy to levels similar to those of rapamycin-induced autophagy (15.4 ± 1.5 LC3 foci per cell compared with 14.2 ± 4.2 in rapamycin-treated cells). Leucettine L33, inactive on both families of kinases, was unable to trigger autophagy (4.6 ± 0.4 LC3 foci per cell). These results suggest that inhibition of CLKs contributes more to autophagy induction by leucettines than inhibition of DYRKs. To further confirm these results, we used siRNA against either
A partial depletion of CLK1 was sufficient to trigger autophagy (50% depletion, 14.4 ± 1.3 foci per cell versus 6.4 ± 0.4 in control siRNA-treated cells; Fig. 4, C and D), whereas depletion of DYRK1A was not (~95% depletion; 5.9 ± 0.7 foci per cell). Taken together our data suggest that induction of autophagy by leucettines likely results from inhibition of CLKs, rather than from inhibition of DYRKs. This is further confirmed by the lack of an additive effect upon combined depletion of CLK1 and DYRK1A (Fig. 4D).

**PIKfyve Kinase Is Inhibited by Leucettine L41 and Is Implicated in Leucettine L41–Induced Autophagy.** Surprisingly, we noticed that addition of L41 to CLK1 siRNA increased autophagy even higher (30.2 ± 3.3 LC3 foci per cells versus 14.4 ± 1.3 in CLK1 siRNA-treated cells), suggesting that L41 could also affect other proteins involved in autophagy. Other CLKs are obvious candidates, but PIKfyve (Fig. 5D) is also interesting in this context. Indeed a recent study demonstrated that L41 could bind the lipid kinase PIKfyve from HT22...
cell line extracts and from mouse brain tissue (Tahtouh et al., 2012). Moreover, treatment with L41 induces vacuole formation in U-2 OS cells (18.1% ± 0.8 versus 21.1% ± 0.2 in untreated cells; Fig. 5A), a phenotype reminiscent of PIKfyve inhibition by the YM201636 compound (Jefferies et al., 2008; Ikonomov et al., 2009; Fig. 5A; 30.8% ± 0.6). Additionally, inhibition of PIKfyve by YM201636 triggers autophagy (de Lartigue et al., 2009; Martin et al., 2013; Fig. 5B). Taken together these data made PIKfyve a strong potential candidate that could explain the increased autophagy observed upon CLK1 siRNA and L41 cotreatment, prompting us to test whether L41 could inhibit PIKfyve activity. As shown in Fig. 5C, L41 is able to reduce recombinant PIKfyve in vitro activity for PtdIns(3,5)P2 synthesis (IC50 = 33.3 μM). We also verified the effect of L41 on PtdIns5P generation with adenosinovally produced PIKfyve and native PtdIns substrate preparation (Shisheva, 2013), which data demonstrated that both PtdIns5P and PtdIns(3,5)P2 synthesis were similarly inhibited, with an IC50 value of 0.7–1 μM (data not shown). These data suggest that the synergy observed upon CLK siRNA and L41 treatment could result from PIKfyve inhibition. The reference PIKfyve inhibitor, YM201636, efficiently inhibited both the PtdIns(3,5)P2- and PtdIns-5P–producing activities of recombinant PIKfyve, with an IC50 of 0.04 μM, very close to the previously reported value (Fig. 5C and data not shown; Jefferies et al., 2008; Ikonomov et al., 2009). L41 also modestly inhibited PIKfyve in vivo as evidenced by HPLC analyses of steady-state levels of phosphoinositides in differentiated 3T3L1 adipocytes (Fig. 5, D and E). We observed a decline in PtdIns5P levels and an increase in PtdIns3P levels upon exposure of 3T3L1 adipocytes to the L41 compound (Fig. 5E). The degree of autophagy observed in CLK1 knocked-down cells treated with L41 (38.9 ± 0.7 foci per cells), PIKfyve knocked-down cells treated with L41 (44.2 ± 0.3 foci per cells), or cells knocked down for both CLK1 and PIKfyve (43.1 ± 5.9 foci per cells) were all similar, further underscoring the potential involvement of PIKfyve in L41-induced autophagy (Fig. 5F).

In this latter case addition of leucettine L41 did not increase autophagy levels (38.9 ± 3.5 foci per cells; Fig. 5F) suggesting that other potential L41 targets (Tahtouh et al., 2012) are not involved in autophagy. Taken together our results suggest that autophagy induced by L41 treatment likely results from combined inhibition of both CLK1 and PIKfyve kinases, albeit single reduction of the activity of any of these two kinases is sufficient to trigger autophagy. Thus leucettines are new, nontoxic autophagy inducers acting through the mTOR-dependent pathway at least partly through PIKfyve and CLK inhibition.

Discussion

CLKs/DYRKs Inhibitory Leucettines Induce Autophagy. Leucettines have been described as a novel class of low molecular weight pharmacological inhibitors essentially selective for the DYRK and CLK kinases (Debdab et al., 2011; Tahtouh et al., 2012). The discovery of their ability to induce autophagy was rather unexpected. We here indeed demonstrate that leucettine L41 induces autophagy in HT22 and U-2 OS cells in a dose-dependent manner without affecting the autophagic flux. This autophagy is characterized by the classic appearance of LC3 foci associated with a shift of its electrophoretic mobility linked to its conversion from the LC3-I to the LC3-II form. Leucettine-induced autophagy reaches levels similar to those seen by classic autophagy inducers, rapamycin and Gleevec. It is also sensitive to classic inhibitors of autophagy such as the PI3K inhibitor wortmannin and 3-methyladenine. We thus establish leucettines as a new class of autophagy-inducing drugs.

Molecular Mechanisms of Leucettines in Their Autophagy-Inducing Activity. By employing siRNA-mediated protein silencing (ULK1) and pharmacological inhibition (rapamycin, wortmannin, 3-methyladenine, Z-PP-CHO, myo-inositol, LiCl) (Fig. 2), we demonstrate herein that L41-induced autophagy is mediated through the mTOR/PI3K, ULK1-requiring pathway rather than through the mTOR-independent, IP3-requiring pathway. Besides the well described autophagy inducers, rapamycin (mTOR), Gleevec (c-Abl), and staurosporine (unselective kinase inhibitor), all other kinase inhibitors tested were unable to trigger autophagy in U-2 OS cells (Fig. 1); therefore, we suggest that inhibition of the specific kinase targets of leucettines might be involved in triggering autophagy. Our data from protein silencing and pharmacological inhibition (Figs. 4 and 5) support the implication of CLK1 inhibition in leucettine-induced autophagy. Furthermore, we show that interaction with PIKfyve may contribute to autophagy induced by leucettines. Although L41 is not a potent inhibitor of PIKfyve catalytic activity, our results indicate that PIKfyve may contribute to autophagy induced by leucettines.
activity, binding of PIKfyve to L41 may also alter its intracellular distribution and ability to interact with other regulators. Interference with PIKfyve activity indeed appears to be sufficient to induce autophagy. L41 potentiates CLK1 siRNA-induced autophagy to levels reached by CLK1/PIKfyve double siRNA treatment, suggesting the coexistence of two independent pathways implicating CLK1 and PIKfyve. Inducing autophagy by inhibiting these two kinases could, therefore, be additive.

**Downstream Events of Leucettines’ Action Leading to Autophagy.** CLKs and DYRKs are key regulators of pre-mRNA splicing as they phosphorylate serine-arginine–rich splicing factors (see Debdab et al., 2011, and references therein). Splicing is a major source of gene diversification, allowing the generation of several proteins from a single gene. It is estimated that, on average, more than 100,000 proteins are generated from the 25,000 human genes. We have shown that, indeed, leucettines modify the splicing pattern of a number of pre-mRNAs, thus leading to changes in the proportion of splice variants (Debdab et al., 2011; Tahtouh et al., 2012; T. Tahtouh, personal communication). Global transcriptomics studies confirm this alternative splicing induced by leucettines (T. Tahtouh, personal communication). It is thus quite likely that splicing of pre-mRNAs encoding regulators of autophagy is altered by leucettines. There are a few examples linking splicing and autophagy. For instance, siRNA-mediated depletion of core spliceosome components reduces mTOR mRNA and protein levels leading to autophagy (Quidville et al., 2013). Splicing of X-box–binding protein 1 mRNA leads to an autophagic response in endothelial cells through transcriptional activation of Beclin-1 (Margariti et al., 2013). We can thus envisage that inhibition of CLKs may trigger autophagy by modulating the splicing of pre-mRNAs of specific autophagy regulators. These remain to be identified.

CLK1 inhibition-induced autophagy could also be mediated by p53, a transcription factor critical to cellular stress responses. It was recently demonstrated that p53 plays an important and complex role in autophagy (Maiuri et al., 2010, for review) and is activated upon disruption of the splicing machinery (Allende-Vega et al., 2013). The p53 pre-mRNA itself undergoes different splicing events, leading to the generation of at least 11 isoforms (review by Marcel et al., 2013). Transcriptomic studies could help us to understand the transcriptional events leading to autophagy induced by CLK1 knockdown and L41 treatment.

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**Fig. 4.** Inhibition of CLKs rather than DYRKs is sufficient to induce autophagy. (A) Structure of leucettines used in this study. (B) LC3 foci induction by various leucettines correlates with their ability to inhibit CLKs. (Left) Average number of LC3 foci per U-2 OS cell after 24-hour treatment at 0.5 μM of rapamycin or 20 μM of the indicated drugs. (Right) IC50 (μM) on DYRK1A and CLK1 of leucettines L14, L33, L38, and L41. (C) siRNA depletion of CLK1, but not DYRK1A, triggers autophagy. (Top) Whole lysates of cells treated for 68 hours with control siRNA (ctntrl) or siRNA directed against DYRK1A or CLK1 were analyzed by immunoblotting with an anti-DYRK1A or CLK1 antibody; α-tubulin was used as a loading control. (Bottom) Immunofluorescence detection of LC3 (green) and DNA (blue) in U-2 OS cells following treatment with the indicated siRNA. Scale bar, 10 μm. Right, average number of LC3 foci in treated cells. (D) L41 potentiates CLK1 siRNA effects. Cells were treated with the indicated siRNA and rapamycin (Rapa) or leucettine L41 as indicated, and the number of LC3 foci was assayed. (Mean of at least three independent experiments; more than 150 cells were analyzed per experiment; error bars show S.E.M.; *P < 0.05; **P < 0.01.)

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Leucettine L41 Induces Autophagy 447

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For instance are p53- or autophagy-specific genes upregulated? This could be of importance for cancer and neurodegenerative diseases treatment.

### Induction of Autophagy, a Contribution to Neurodegeneration Therapeutic Properties of Leucettines.

Splicing is regulated by CLK1 through phosphorylation of SR proteins and it is influenced by environmental stresses (Biamonti and Caceres, 2009). Aberrant splicing is implicated in the development of many pathologies (Faustino and Cooper, 2003), including cancer (Kaida et al., 2012) and type 1 myotonic dystrophy (Romeo, 2012) and neurodegenerative diseases (Mills and Janitz, 2012). New drugs targeting splicing are currently under development (Bonnal et al., 2012) and could be of great benefit to fight these diseases.

Defective autophagy is linked to neurodegenerative diseases. Restoring defective autophagy provides protection against neurodegeneration (see Marino et al., 2011, and Hochfeld et al., 2013, for reviews on autophagy and neuroprotection). For instance, defective clearance of autophagosomes induces their accumulation in brain samples from patients suffering from Alzheimer’s or Parkinson’s diseases and is linked to neurodegeneration (Boland et al., 2008; Dehay et al., 2010; Vila et al., 2011). Moreover, lowering mTOR activity (thereby inducing autophagy) reduced amyloid-β and tau hyperphosphorylation, rescuing cognitive defects through autophagy in mice models of Alzheimer’s disease (Caccamo et al., 2010; Spilman et al., 2010). Rapamycin reduces cognitive impairments linked to plaques and tangles (Majumder et al., 2011). Autophagy downregulates amyloid-β levels (Tian et al., 2011). Recently, latrepirdine, a potential Alzheimer’s disease drug, was shown to trigger autophagy, to reduce amyloid-β neuropathology, and to prevent behavioral deficits in Alzheimer’s disease mouse models (Steele and Gandy, 2013). Stimulation of autophagy by trehalose in P301S tau transgenic mouse improves the tau pathology (Schaeffer and Goedert, 2012). Methylene blue also activates autophagy and reduces taupathy (Congdon et al., 2012). Altogether these data support the idea that increasing the basal level of autophagy should be beneficial in the treatment of neurodegenerative diseases like Alzheimer’s disease.

By inducing autophagy through the mTOR/PI3K pathway leucettines could be used to target neurodegenerative diseases, the low toxicity of leucettine L41 being an asset. Our laboratory recently showed that leucettines protect against glutamate-induced neurotoxicity in HT22 cells (Tahtouh et al., 2012). Glutamate-mediated excitotoxicity contributes to acute and chronic central nervous system diseases. Leucettines’ protective effects could thus be mediated by their proautophagic properties. Inhibition of Dyrk1A is also seen as a promising approach for the treatment of Alzheimer’s disease.
disease. Thus, the ability of leucettines to inhibit both DYRK1A and CLKs and to trigger autophagy without inducing cell death promotes this class of molecules as promising therapeutical drug candidates for Alzheimer’s disease that deserve more investigation.

Authorship Contributions

Participated in research design: Fant, Meijer.
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Wrote or contributed to the writing of the manuscript: Fant, Meijer, Payratste, Shisheva, Meijer.

References

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