Spautin-A41 Attenuates Cerulein-Induced Acute Pancreatitis through Inhibition of Dysregulated Autophagy

Kai Dong, Xia Chen, Liping Xie, Lanting Yu, Mengjun Shen, Yanping Wang, Shanshan Wu, Jiajia Wang, Junxi Lu, Gang Wei, Dongliang Xu, and Liu Yang

INTRODUCTION

Acute pancreatitis (AP) is a lethal inflammation of the pancreas. Despite most patients have a mild, self-limited disease course, around 5–10% of the patients develop severe acute necrotizing pancreatitis, which has high mortality due to complications or multiple organ failure (MOF). Most recently research showed that patients with acute pancreatitis history are associated with an increased risk of pancreatic cancer compared to the general population. AP has long been recognized as a self-digestive disorder, trypsinogen premature and disturbed Ca²⁺ signaling are early events that are known to play crucial roles in triggering acinar cell damage. Given the activation of trypsinogen plays a significant role in the progression of AP, protease inhibitor has clinically been applied as a potential treatment for AP. Aprotinin, a serine protease inhibitor, was firstly used for the treatment of AP in the 1960s. Gabexate mesylate, a synthetic protease inhibitor, was identified as a potent autophagy inhibitor. Mice treated with spautin-A41 were resistant to the cerulein-induced elevation of serum pancreatic enzyme activities and pancreatic apoptosis. Mechanistically, spautin-A41 effectively reduced the expression levels of Class III phosphatidylinositol 3 (PI3) kinase complexes and subsequently ameliorated pancreatitis by inhibiting the formation of autophagosome. Therefore, pharmacological inhibition of autophagy by spautin-A41 may serve as new target for treating or lessening the severity of AP.

Key words spautin-A41; pancreatitis; autophagy; spautin-1; Class III phosphatidylinositol 3 (PI3) kinase

Autophagy plays key roles in the development of acute pancreatitis (AP) and the regulation of impaired autophagy has therapeutic potential. The objective of the present study was to investigate whether pharmacological inhibition of autophagy could ameliorate AP in mice and examine the underlying mechanisms. In current study, by imaging-based high-throughput screening, a novel spautin-1 derivative spautin-A41 was identified as a potent autophagy inhibitor. Mice treated with spautin-A41 were resistant to the cerulein-induced elevation of serum pancreatic enzyme activities and pancreatic apoptosis. Mechanistically, spautin-A41 effectively reduced the expression levels of Class III phosphatidylinositol 3 (PI3) kinase complexes and subsequently ameliorated pancreatitis by inhibiting the formation of autophagosome. Therefore, pharmacological inhibition of autophagy by spautin-A41 may serve as new target for treating or lessening the severity of AP.

in patients with AP and a meta-analysis also showed that there is no solid evidence that the protease inhibitors prevent death due to AP. Despite substantial progress in some other promising therapeutic targets, no drugs have been authorized by Food and Drug Administration for treatment of AP. Therefore, the mechanism underlying the process of acute pancreatitis remains to be elucidated urgently.

Due to its high demand for protein synthesis and trafficking, exocrine pancreas has higher basal rate of autophagy compared with other organs, such as liver, heart, kidney or endocrine pancreas. One of the key features of pancreatitis is the accumulation of large vesicles in acinar cells, which can be observed in genetic or chemical-induced rodent models of AP and human disease. Further histologic and electron microscopy analyses reveal that the accumulation of vacuoles are predominantly large autolysosomes, indicating impaired autophagic flux in the pathogenesis of acute pancreatitis. Autophagy is an evolutionarily conserved, multistep, catabolic process whereby cells degrade long-lived proteins, misfolded protein aggregates and damaged organelles to maintain cellular homeostasis. Macroautophagy (afterwards referred to as autophagy) is the best characterized form of autophagy, begins with the generation of globular double-membrane vesicles (autophagosomes), which sequesters and wraps around organelles and biomolecules destined for degradation. Autolysosomes was developed through the fusion of autophagosomes with lysosomes, where sequestered
material is degraded. This process is mediated by a series of autophagy-related genes (Atgs), which were first characterized in yeast.\textsuperscript{54} Most of the yeast Atgs have homologs in eukaryotes, including plants, animals.\textsuperscript{35,36} Among these Atgs, around 18 of them are components of the core autophagy system.\textsuperscript{37} For example, mammalian microtubule-associated protein 1 light chain 3 (LC3), the ortholog of yeast ATG8, has an important role in the closure process of the autophagic membrane.\textsuperscript{38} Functionally, mammalian Atgs can be divided into six functional clusters, one of which is the Class III phosphatidylinositol 3-kinase (Class III PI3K) complexes consisting of VPS34, p150 and Beclin1.\textsuperscript{39} Vps34, a primordial isomorph of phosphoinositide 3-kinase (PI3K), can phosphorylate phosphatidylinositol (PtdIns) at the 3' position on the inositol ring to produce PtdIns3P.\textsuperscript{40} Accumulating evidence demonstrates a principal role of Class III PI3K complexes in orchestrating both initiation and maturation of autophagosomes.\textsuperscript{41}

Given the contributions of PI3K in autophagy process and that it is involved in the regulation of cholecystokinin-induced responses in the pancreatic acinar cells,\textsuperscript{42} pharmacologic approaches have been applied to investigate the potential of PI3K as a target for AP. Wortmannin and LY294002, inhibitors of PI3K, were shown to reduce trypsinogen activation in fractions of the core autophagy system.\textsuperscript{35,36} For example, mammalian microtubule-associated protein 1 light chain 3 (LC3), the ortholog of yeast ATG8, has an important role in the closure process of the autophagic membrane.\textsuperscript{38} Functionally, mammalian Atgs can be divided into six functional clusters, one of which is the Class III phosphatidylinositol 3-kinase (Class III PI3K) complexes consisting of VPS34, p150 and Beclin1.\textsuperscript{39} Vps34, a primordial isomorph of phosphoinositide 3-kinase (PI3K), can phosphorylate phosphatidylinositol (PtdIns) at the 3' position on the inositol ring to produce PtdIns3P.\textsuperscript{40} Accumulating evidence demonstrates a principal role of Class III PI3K complexes in orchestrating both initiation and maturation of autophagosomes.\textsuperscript{41}

Spautin-1 was reported as an autophagy inhibitor.\textsuperscript{45} In this study, we presented the derivative of spautin-1, termed spautin-A41 with higher autophagy inhibition activity and better stability compared with spautin-1. Spautin-A41 could inhibit autophagy via decreasing the abundance of Class III PI3K complexes, so it can be used as an improved autophagy inhibitor in both basic research and understanding the physiological role of autophagy in disease manifestation. As we have revealed here that spautin-A41 can attenuate cerulein-induced AP, which represented a potential therapeutic strategy for the treatment of AP by autophagy inhibition.

MATERIALS AND METHODS

Spautin-1 Derivatives, Plasmids, Reagents and Antibodies A small-molecule library based on spautin-1 with various substitutions and modifications, were kindly provided by Junjing Yuan (Interdisciplinary Research Center of Biology and Chemistry, Shanghai, China).\textsuperscript{38} Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, U.S.A.). Na pyruvate was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). BCA Protein Assay Kit, Enhanced chemiluminescence (ECL) substrate, protease and phosphatase inhibitors were from Thermo Fisher Scientific (Waltham, MA, U.S.A.). Polyvinylidene difluoride (PVDF) membrane was from Millipore (Billerica, MA, U.S.A.). Western blot were obtained from Rabbit polyclonal antibody anti-Vps34, were from Abcam (Cambridge, U.K.). Rabbit polyclonal antibody anti-LC3B was from Sigma-Aldrich. Mouse p150 antibody was from Proteintech (Chicago, IL, U.S.A.). Mouse monoclonal antibody anti-β-tubulin was from CST (Danvers, MA, U.S.A.). Rabbit polyclonal antibody anti-Beclin1 was from Santa Cruz (Santa Cruz, CA, U.S.A.). Rabbit polyclonal antibody anti-Atg14 was from MBL (Woburn, MA, U.S.A.). Cerulein was purchased from MedChemExpress (NJ, U.S.A.).

High-Throughput Image Analysis H4-LC3 or H4-FYVE cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) and stained with 3 mg/mL 4’6-diamidino-2-phenylindole (DAPI) (Sigma). Images of 1000 cells for each compound treatment were collected with an ArrayScan HCS 4.0 Reader with a 20× objective (Cellomics ArrayScan VTI) for DAPI-labeled nuclei and green fluorescent protein (GFP)/red fluorescent protein (RFP)-tagged intracellular proteins.

In Vitro Metabolic Stability Analysis In vitro metabolic stability of spautin-A41 in human liver microsome was carried out as reported.\textsuperscript{46} Briefly, a master-mix containing human liver microsomes (BD, San Jose, CA, U.S.A.), phosphate buffer, Ultra-pure water (generated from Milli-Q ultra-pure water system) and reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH) solution was made and pre-warmed in the 37°C for 2 min. After adding approximate volume of test compounds or control solution to the above master solution to initiate the reaction, aliquot of 50 µL was pipetted from the reaction solution and stopped by the addition of 3 volume of cold methanol containing an internal standard at the designated time points. The incubation solution was centrifuged at 16000 rpm for 5 min to precipitate protein. Aliquot of 100 µL of the supernatant was used for LC-MS/MS analysis.

Cell Culture Pancreatic cell line AR42J (ATCC, Rockville, MD, U.S.A.) was cultured in DMEM, supplemented with 10% FBS. H4-LC3-GFP and H4-FYVE-RFP cells (gifted from the laboratory of Jun Ying Yuan in Shanghai, China) were cultured in DMEM containing 10% FBS and 1 × Na pyruvate. Cells were cultured in an atmosphere of 5% CO₂ at 37°C.

Animals Male C57BL/6J mice (20–25 g) were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China). All applicable institutional and/or national guidelines for the care and use of animals were followed. All protocols were approved by the Medical Science Ethics Committee of Shanghai General Hospital. Mice were randomly divided into three groups (n = 12 for each group): control group, cerulein treatment group, cerulein and spautin-A41 treatment group. For the cerulein treatment group, mice were injected intraperitoneally with cerulein (50 µg/kg body weight) for 5 times at hourly intervals to induce AP models. For cerulein and spautin-A41 treatment group, mice were injected intraperitoneally with cerulein (50 µg/kg) for 5 times with spautin-A41 (5 mg/kg) every two hours for 2 times. After the last injection, all mice were anesthetized by intraperitoneal injection of 3% pentobarbital sodium (40 mg/kg body weight) and then sacrificed, with blood and pancreas samples collected for further analysis.

Western Blot Mice pancreatic tissues or cultured cells were homogenized in RIPA lysis buffer containing 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholic acid, 150 mmol/L NaCl, 50 mmol/L Tris–HCl, pH 7.4 and freshly added protease inhibitors. After centrifugation, the supernatants were quantified by a BCA Protein Assay Kit according to the manufacturer’s instructions. Whole protein extract (30 µg) was separated by SDS-polyacrylamide gel
electrophoresis (PAGE) and transferred to Polyvinylidene difluoride (PVDF) membrane. After blocking in 5% skim milk in TBST buffer (10 mmol/L Tris–HCl, pH 7.4, 0.9% NaCl, and 0.05% Tween-20), the membranes were probed with primary antibody at 4°C overnight and then using horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The membranes were detected with an ECL detection kit according to the manufacturer's instructions. In our study, an anti-β-tubulin antibody was used as a loading control.

Serum Amylase Activity
Serum amylase levels were determined using amylase detection kit (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) according to the manufacturer's instructions. Briefly, samples were acted with substrate for 7.5 min at 37°C, optical density (OD) value of each reactant was read in wavelength of 660 nm. Amylase activity was calculated with a formula suggested in the manufacturer's instruction.

Trypsin Activity Assay
Trypsin activity in AR42J cells lysates or mice serum was assayed by Trypsin Kit (Biovision, Milpitas, CA, U.S.A.) according to the manufacturer's instructions. Briefly, samples were incubated with substrate to release p-nitroanilide (pNA), which were detected at OD 405 nm. Trypsin activity was calculated with a formula suggested in the manufacturer’s instruction.

Pancreas Histology
The Pancreatic tissues were fixed overnight in 10% neutral-buffered formalin, embedded in paraffin and then sectioned. The sections were stained with hematoxyline–eosin (H&E) using standard protocols. For each section, 10 high-power fields were selected and the extent of edema, inflammation, vacuolization and necrosis was evaluated and scored using a scale of 0–4 (normal to severe) as described previously. The final score was the sum of the above four individual parameters. Slides were evaluated by two experienced pathologists unaware of the study target.

Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labeling (TUNEL) Assay
TUNEL staining of pancreatic tissue sections was performed using an in situ Apoptosis Detection Kit (Vazyme, Nanjing, China) according to the manufacturer's instructions. In short, sections were dewaxed and rehydrated. Slides were then incubated in permeabilization solution (proteinase K 20 µg/mL in phosphate buffered saline (PBS)). After incubation for 20 min at room temperature, equilibration buffer was added for 15 min. Next, the tissue was incubated with fresh TUNEL reaction mixture at 37°C for 1 h, then rinsed twice with PBS followed by incubation with DAPI for 5 min.

Statistical Analysis
All measurement data were presented as mean ± standard error of the mean (S.E.M.). Statistical analysis was conducted using unpaired Student’s t-test between two experimental groups, and one-way ANOVA or two-way ANOVA for multiple comparison by GraphPad Prism 5.0. $p < 0.05$ was considered statistically significant.
RESULTS

Screening Autophagy Inhibitors from the Spautin-1 Derivative Library  In an imaging-based high-through screening with LC3-GFP as a marker for autophagy, a small-molecule library based on spautin-1 with various substitutions and modifications was screened, we selected small molecules based on their efficacy in inhibiting autophagy. Through the analysis of two hundred and forty spautin-1 derivatives, we eventually identified spautin-A41 (6-fluoro-N-[4-(trifluoromethyl)phenethyl]quinazolin-4-amine) as an efficient autophagy inhibitor with the lowest IC50 for inhibition of autophagy (Fig. 1A). Spautin-A41 was an effective autophagy inhibitor with an IC50 of 47 nM (Fig. 1B), which was about
eighteen times less than that of spautin-1 as showed in early studies. Consistently, the quantitative analysis of LC3-GFP+ puncta utilizing high throughput microscopy revealed that the treatment of spautin-A41 reduced the basal level of spot intensity to 50% in 1 h, and rapamycin-induced LC3-GFP+ autophagic puncta were also decreased in the present of spautin-A41 (Fig. 1C). Confocal microscopy showed that GFP-LC3 level of H4-LC3 cells were dramatically decreased in spautin-A41 treated-group (Fig. 1D).

**Spautin-A41 Shows Improved Effectiveness and Stability than Spautin-1**

To investigate whether spautin-A41 had a better efficacy in inhibiting autophagy than spautin-1, we stimulated H4-LC3 cells with rapamycin, an inducer of autophagy, in the presence of increased dose (0.1–10 µM) of spautin-1 or spautin-A41. Protein level of microtubule-associated protein light chain 3 form II (LC3II), an indicator of autophagic activity, was increased when cells were treated with rapamycin as expected (Fig. 2A). 0.1 µM spautin-A41 efficiently reduced rapamycin-induced autophagy in H4-LC3 cells. In contrast, 0.1 µM spautin-1 had little effect on it (Fig. 2A). Additionally, this result showed that spautin-A41 inhibited autophagy in a dose-dependent manner (Fig. 2A). Since the approach of liver microsomes is suitable to determine the *in vitro* intrinsic clearance of a compound. To further compare the stability of spautin-A41 with spautin-1 in the biological environment, the same amount of spautin-1 and spautin-A41 were added into the parallel test system and only 20% of spautin-1 were left after one-hour incubation in liver microsome assay buffer (Fig. 2B). By contrast, the left amount of spautin-A41 reached 40%, twice than that of spautin-1 (Fig. 2B). Taken together, these results indicate that spautin-A41 is a powerful and stable autophagy inhibitor.

**Spautin-A41 Inhibits Dysregulated Autophagy and Trypsinogen Activation Induced by Cerulein in Pancreatic Acinar Cells**

We next examined the autophagy inhibition effects of spautin-A41 on pancreatic cell line AR42J, as shown in Fig. 3A, spautin-A41 had higher efficiency in inhibiting autophagy in AR42J cells with cerulein treatment.
pothesized that spautin-A41 inhibiting dysregulated autophagy could lead to the protection effect against trypsinogen activation in AR42J cells. The results showed that spautin-A41 can effectively inhibit trypsinogen activation induced by cerulein (Fig. 3B).

Spautin-A41 Ameliorates Acute Pancreatitis Induced by Cerulein in Mice

Cerulein-induced AP model was widely constructed since cerulein could act as a cholecystokinin analogue to stimulate certain smooth muscle contraction and pancreatic and gastric secretion, eventually cause acinar cell death, and pancreatic edema. Given the relationship between AP and imbalanced autophagy, we hypothesized that spautin-A41 could protect against cerulein-induced AP by autophagy inhibition. Since spautin-A41 was more effective than spautin-1, we determined whether the autophagy induced by cerulein was decreased under the condition of spautin-A41 injection. The result showed that spautin-A41 can inhibit the protein level of LC3II upon cerulein-induced AP (Fig. 4A). Moreover, the elevations of serum amylase and trypsin activity were declined in the mice with both cerulein and spautin-A41 injection compared with cerulein-injection alone (Figs. 4B, C). Morphological examination further revealed that the treatment of spautin-A41 can ameliorate the pancreatic damage induced by cerulein, such as pancreatic edema, vacuolization, and disorder of acinar cells (Figs. 4D, E). Besides, apoptotic cells in cerulein-induced AP tissue were detected by TUNEL staining, our results showed that spautin-A41 could alleviate the apoptosis induced by cerulein in pancreatic tissue. (Figs. 5A, B). Autophagy plays a key role in maintain cellular homeostasis. Dysregulated autophagy may directly trigger the activation of trypsinogen and inflammatory response in the pancreas, which lead to cellular toxicity and cell death.48) Spautin-A41 were shown here to restore efficient autophagy in pancreatic cells and ameliorate acute pancreatitis induced by cerulein.

The results showed that spautin-A41 lessens acute pancreatitis induced by cerulein in mice. Spautin-A41 Reducing the Expression of Class III PI3K Complexes

To explore the mechanism by which spautin-A41 inhibited autophagy, we first tested the effect of spautin-A41 on FYVE-RFP, which can be recruited by PI3P to regulate initiation and maturation of autophagosomes.49) Figures 6A and B showed treatment with spautin-A41 reduced the levels of FYVE-RFP puncta, suggesting that spautin-A41 reduced the levels of PI3P. Because Class III PI3 kinase complexes (Beclin1/VPS34/P150/Atg14) can phosphorylate PtdIns to generate PI3P. We hypothesized that spautin-A41 could reduce the levels of Class III PI3 kinase complexes. The results showed that the levels of Beclin1, Vps34, P150 and Atg14 were considerably lower in spautin-A41 treated cells than that of control cells (Figs. 6C, D). This downregulation by spautin-A41 occurs in a dose-dependent manner. We speculated that the degradation of Class III PI3 kinase complexes by spautin-A41 leaded to the reduction in the levels of PI3P and a consequent inhibition of autophagy.

DISCUSSION AND CONCLUSION

In this study, we found that spautin-A41, a derivative of spautin-1, served as a novel potent molecule inhibitor of autophagy, and protected mice against cerulein-induced acute pancreatitis efficiently. In comparison with spautin-1, spautin-A41 had a stronger autophagy inhibition effect and better microsomal stability than spautin-1. Mechanistically, our data suggested that the downregulation of autophagy under the treatment of spautin-A41 may partly due to the promotion of Class III PI3 kinase degradation.

Collecting evidence indicated that impaired autophagic flux played critical roles in the pathogenesis of pancreatitis,23,25,30
Accumulation of large vacuoles may due to increased autophagosome formation, declined lysosomal degradation or both. A recent study showed that acinar cells from mice stimulated with cerulein in the presence of lysosomal inhibitors have higher levels of LC-3II in compare with cells from control mice, signifying that the autophagosome formation is boosted during the progression of acute pancreatitis. Acinar cell-specific disruption of Atg5 using Cre recombinase regulated by elastase (Atg5 F/F; Ela-Cre mice), observed suppressed trypsinogen activation and less severe acute pancreatitis in Autophagy-Related 5 (Atg5) null mice. Additionally, recent research proved that impaired autophagy influx due to deficient lysosomal degradation was probably the key factor for intra-acinar trypsin activation and pancreatitis. In addition, the imbalance of the fusion between autophagosome and lysosome due to the lack of local lysosome-associated membrane protein 2 (LAMP2) also contributes to zymogen activation, suggesting that autophagy blockage and autophagosome accumulation are important factors for developing pancreatitis.

Interestingly, lots of studies showed that genetic disruption of autophagic or lysosomal pathways cause pancreas injury. Deficiency of genes encoding proteins that responsible for autophagosome formation (ATG5 or ATG7) or lysosomal function (LAMP2) caused severe damage of the pancreas. Demonstrating that the basal autophagy is pivotal for maintaining pancreatic acinar cell homeostasis. The discrepancy phenotype between pancreatic Atg5 null mice (Atg5F/F; Pdx-Cre mice, Atg5F/F; Ela-Cre mice) may due to the differences in timing of recombination works and the cell types influenced.

Spautin-1 has been reported as an autophagy inhibitor and the blockage of autophagy by spautin-1 had potential therapeutic effects in reducing cancer cell viability. Through medicinal chemistry modifications, we testified that spautin-A41 had a stronger autophagy inhibition effect. LC3II is associated with autophagosome membrane formation and could be used as an indicator of the amount of autophagosome. In our study, we found that spautin-A41 could diminish rapamycin-induced LC3-GFP autophagic puncta, LC3II level in vitro and cerulein-induced LC3II level in vivo, indicating that the protective effect of spautin-A41 on cerulein-induced pancreatitis is mediated by inhibiting autophagosome formation.

Next, we elucidated the mechanism by which spautin-A41 inhibits autophagy. Our data suggested that the downregulation of autophagy under the treatment of spautin-A41 may partially due to the degradation of Class III PI3 kinase complexes. It is reported that spautin-1 can inhibit deubiquitination activity of USP13 and USP10, two deubiquitinating enzymes, which negatively regulate the ubiquitination and degradation of Class III PI3 kinase complexes. We also observed that spautin-A41 reduced the interaction between USP13 and beclin1 as shown in supplementary Fig. 1, which may further cause the ubiquitination and degradation of beclin1. As the stabilities of different members of Class III
PI3 kinase complexes are dependent on each other, as degradation of one component often decreases the levels of other parts in the complexes,\(^{58}\) we hypothesize that, like spautin-1, spautin-A41 might inhibit deubiquitination of Class III PI3 kinase complexes, which leads to increased ubiquitination and proteasomal degradation of Class III PI3 kinase complexes.\(^{59}\) A recent study also verified that spautin-1 ameliorated the pathogenesis of AP partly through alleviating calcium overload.\(^{59}\) Although the regulation of calcium on Vps34 is still controversial,\(^{60}\) it provides us another potential causal mechanism of how spautin-A41 acts as an autophagy inhibitor.

Finally, our research focused on the effect of spautin-A41 on cerulein-induced AP, which is the most widely used rodent pancreatitis models.\(^{61}\) Cerulein, an ortholog of cholecystokinin, was administered intravenously to induce a mild and reversible form of pancreatitis in rats by Lampel and Kern date back to 1977.\(^{62}\) The Cerulein-induced AP model is widely used because of its high reproducibility and simplicity. Moreover, the premature and intrapancreatic activation of trypsinogen can be studied in this model,\(^{63}\) which was found to have parallels in human AP. However, whether cerulein-induced pancreatitis model could mimic human AP is still under exploration. There are other experimental models used to reproduce human AP, for example, L-arginine model can parallel severe, bile salt-induced pancreatitis.\(^{64}\) Alcohol abuse is a primary cause of both acute and chronic pancreatitis,\(^{65–67}\) the combination of alcohol exposure and endotoxemia is a well-established experimental protocol to trigger pancreatitis.\(^{52}\) Thus, further studies of the protective effects of spautin-A41 on different pancreatitis models are urgently needed. To date, there are a variety of potential novel targeted pharmacologic approaches that target different aspects of the pathogenesis of pancreatitis, including anti-secretory agents, protease inhibitors, anti-oxidants, anti-inflammatory agents, probiotics, tumor necrosis factor alpha inhibitor.\(^{68}\) However, most agents reported to have therapeutic benefit in experimental models did not offer promise in clinical studies.\(^{69}\) Our study indicated that spautin-A41 may provide a new angle for alleviating the process of AP, which awaits extensive assessment. In summary, the treatment of spautin-A41, a new autophagy inhibitor, appears to have beneficial effects on AP. This function was likely mediated via the degradation of Class III PI3 kinase complexes and subsequent reduction of autophagy level. We have manifested a promising therapy for reducing the severity of AP by targeting Class III PI3 kinase and autophagy.

**Acknowledgments** We thank Dr. Junjing Yuan from the Interdisciplinary Research Center of Biology and Chemistry (IRCBC) for kindly giving the small-molecule library. This work was supported by grants from Medicine and Engineering Interdisciplinary Research Fund of Shanghai Jiao Tong University (YG2017MS18) to YL.

**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

**REFERENCES**

1. Banks PA, Bollen TL, Dervenir C, Goosszen HG, Johnson CD, Sarr MG, Tsaiot G, Vege SS. Classification of acute pancreatitis—2012 revision of the Atlanta classification and definitions by international consensus. *Gut*, 62, 102–111 (2013).

2. Zhang X, An R, Tian H, Zhao J. Increased risk of pancreatic cancer after acute pancreatitis: A meta-analysis of prospective cohort studies. *Clinics and Research in Hepatology & Gastroenterology*, (2018).

3. Gukovsky I, Cheng JH, Nam KJ, Lee OT, Lugea A, Fischer L, Penninger JM, Pandol SJ, Gukovskaya AS. Phosphatidylinositol 3-kinase gamma regulates key pathologic responses to cholecystokinin in pancreatic acinar cells. *Gastroenterology*, 126, 554–566 (2004).

4. Smith M, Kocher HM, Hunt BJ. Aprotinin in severe acute pancreatitis. *Int. J. Clin. Pract.*, 64, 84–92 (2010).

5. Mayumi T, Ura H, Arata S, Kitamura N, Kiriya I, Shibuya K, Sekimoto M, Nago N, Hirota M, Yoshida M, Ito Y, Hirata K, Takada T. Evidence-based clinical practice guidelines for acute pancreatitis: proposals. *J. Hepatobiliary Pancreat. Surg.*, 9, 413–422 (2002).

6. Consensus on the diagnosis and treatment of acute pancreatitis. *Chin. J. Dig. Dis.*, 6, 47–51 (2005).

7. Famularo G, Minisola G, De Simone C. Acute pancreatitis. *N. Engl. J. Med.*, 355, 361, author reply, 361 (2006).

8. Messori A, Rampazzo R, Sereccaro G, Olivato R, Bassi C, Falconi M, Pederzoli P, Martini N. Effectiveness of gabexate mesylate in acute pancreatitis: a metaanalysis. *Dig. Dis. Sci.*, 40, 744–758 (1995).

9. Pelagatti E, Cecchi M, Messori A. Use of gabexate mesylate in Italian hospitals: a multicentre observational study. *J. Clin. Pharm. Ther.*, 28, 191–196 (2003).

10. Setia T, Noguchi Y, Shikata S, Nakayama T. Treatment of acute pancreatitis with protease inhibitors administered through intravenous infusion: an updated systematic review and meta-analysis. *BMC Gastroenterol.*, 14, 102 (2014).

11. Gukovsky I, Li N, Todoric J, Gukovskaya A, Karin M. Inflammation, autophagy, and obesity: common features in the pathogenesis of pancreatitis and pancreatic cancer. *Gastroentology*, 144, 1199–1209.e4 (2013).

12. Habtezion A. Inflammation in acute and chronic pancreatitis. *Curr. Opin. Gastroenterol.*, 31, 395–399 (2015).

13. Bhatia M, Hegde A. Treatment with antileukinate, a CXCR2 chemokine receptor antagonist, protects mice against acute pancreatitis and associated lung injury. *Regul. Pept.*, 138, 40–48 (2007).

14. Frossard JL, Lenglet S, Montecucco F, Steffens S, Galan K, Pelli G, Spahl L, Mach F, Hadengue A. Role of CCL-2, CCR-2 and CCR-4 in cerulein-induced acute pancreatitis and pancreatitis-associated lung injury. *J. Clin. Pathol.*, 64, 387–393 (2011).

15. Haque R, Farooq A, Ghani A, Gorelick F, Mehal WZ. Lactate reduces liver and pancreatic injury in toll-like receptor- and inflammation-mediated acute pancreatitis. *Toxicol. Pathol.*, 40, 191–196 (2012).

16. Huang L, Ma J, Jiang Y, Chen P, Zhang S, Zhang Y, Yuan YZ. siRNA-based targeting of fraktalkine overexpression suppresses inflammation development in a severe acute pancreatitis rat model. *Int. J. Mol. Med.*, 30, 514–520 (2012).

17. Steele CU, Karim SA, Foth M, Rish S, Leach JD, Porter RJ, Nixon C, Jeffry Evans TR, Carter CR, Nibbs RJ, Sansom OJ, Morton JP. CXCR2 inhibition suppresses acute and chronic pancreatitis inflammation. *J. Pathol.*, 237, 85–97 (2015).

18. Xue J, Habtezion A. Carbon monoxide-based therapy ameliorates acute pancreatitis via TLR4 inhibition. *J. Clin. Invest.*, 124, 437–447 (2014).

19. Zhang H, Neuhofer P, Song L, Rabe B, Lesina M, Kurkowski MU,
Treiber M, Wartmann T, Regner S, Thorlacius H, Saur D, Weirich G, Yoshinura A, Halangk W, Miziaert JP, Schmid RM, Rose-John S, Aigul H. IL-6 trans-signaling promotes pancreatitis-associated lung injury and lethality. *J. Clin. Invest.*, **123**, 1019–1031 (2013).

Mizushima N, Yamamoto A, Matsui M, Yoshimori T, Ohsumi Y. In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Mol. Biol. Cell*, **15**, 1101–1111 (2004).

Gukovskaya AS, Gukovsky I. Autophagy and pancreatitis. *Am. J. Physiol. Gastrointest. Liver Physiol.*, **303**, E993–E1003 (2012).

Helen H, Mero M, Markkula H, Helen M. Pancreatic acinar ultrastructure in structure and function of acute pancreatitis. *Virchows Arch. A Pathol. Anat. Histol.*, **387**, 259–270 (1980).

Mareninova OA, Hermann K, French SW, O’Konski MS, Pandol SJ, Webster P, Erickson AH, Katunuma N, Grellick FS, Gukovskiy I, Gukovskaya AS. Impaired autophagic flux mediates acinar cell vacuole formation and trypsinogen activation in rodent models of acute pancreatitis. *J. Clin. Invest.*, **119**, 3340–3355 (2009).

Aho HJ, Nevalainen TJ, Havia VT, Heinonen RJ, Aho AJ. Human acute pancreatitis: a light and electron microscopic study. *Acta Pathol. Microbiol. Immunol. Scand. A*, **90**, 367–373 (1982).

Diakopoulos KN, Lesina M, Wormann S, *et al.* Impaired autophagy induces chronic atrophic pancreatitis in mice. *Nat. Rev. Nephrol.*, **10**, 626–638.e17 (2014).

Antonucci L, Fagman JB, Kim JY, Todoric J, Gukovsky I, Mackey M, Ellisman MH, Karin M. Basal autophagy maintains pancreatic acinar cell homeostasis and protein synthesis and prevents ER stress. *Proc. Natl. Acad. Sci. U.S.A.*, **112**, E6166–E6174 (2015).

Mareninova OA, Sendler M, Malik MA, Suyama M, Mizushima N, Matsui M, Mizumura K. Autophagic cell death of pancreatic acinar cells in serine protease inhibitor Kazal type-3 deficient mice. *Gastroenterology*, **129**, 696–705 (2005).

Li N, Wu X, Holzer RG, *et al.* Loss of acinar cell iK, a K+ channel triggers spontaneous pancreatitis in mice. *J. Clin. Invest.*, **123**, 2231–2243 (2013).

Gukovskiy I, Gukovskaya AS. Impaired autophagy triggers chronic pancreatitis: lessons from pancreas-specific atg3 knockout mice. *Gastroenterology*, **148**, 301–305 (2015).

Reggioli P, Komatsu M, Finley K, Simonsen A. Autophagy: more than a nonspecific pathway. *Int. J. Cell Biol.*, **2012**, 219625 (2012).

Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. *Science*, **294**, 1794–1800 (2001).

Levine B, Yuan J. Autophagy in cell death: an innocent convict? *J. Clin. Invest.*, **115**, 2679–2688 (2005).

Tsukada M, Ohsumi Y. Isolation and characterization of autophagy-dependent processes. *Gastroenterology*, **148**, 773–782 (2005).

Hashimoto D, Ohmuraya M, Hirota M, Yamamoto A, Suyama K, Iida S, Okumura Y, Takahashi I, Kudo H, Araki K, Baba H, Mizushima N, Yamamura K. Involvement of autophagy in trypsinogen activation within the pancreatic acinar cells. *J. Cell Biol.*, **181**, 1065–1072 (2008).

Fortunato F, Kroeber G. Impaired autophagosome-lysosome fusion in the pathogenesis of pancreatitis. *Autoimmun. Rev.*, **5**, 850–853 (2009).

Rah RP, Dawra RK, Saluja AK. New insights into the pathogenesis of pancreatitis. *Curr. Opin. Gastroenterol.*, **29**, 523–530 (2013).

Correa RJ, Valdes YR, Peart TM, Fazio EN, Bertrand M, McGee J, Prefontaine M, Sugimoto A, DiMattia GE, Shepherd TG. Combination of AK1 inhibition with autophagy blockade effectively reduces ascites-derived ovarian cancer cell viability. *Carcinogenesis*, **35**, 1951–1961 (2014).

Shao S, Li S, Qin Y, Wang X, Yang Y, Bai H, Zhou L, Zhao C, Wang C. Spautin-1, a novel autophagy inhibitor, enhances mitophagy-induced apoptosis in chronic myeloid leukemia. *Int. J. Oncol.*, **44**, 1661–1668 (2014).

Ichumura Y, Kirisako T, Takao T, Satomi Y, Shimonishi Y, Ishii Y, Treiber M, Wartmann T, Regner S, Thorlacius H, Saur D, Weirich G, Yoshinura A, Halangk W, Miziaert JP, Schmid RM, Rose-John S, Aigul H. IL-6 trans-signaling promotes pancreatitis-associated lung injury and lethality. *J. Clin. Invest.*, **123**, 1019–1031 (2013).
hara N, Mizushima N, Tanida I, Kominami E, Ohsumi M, Noda T, Ohsumi Y. A ubiquitin-like system mediates protein lipidation. *Nature*, **408**, 488–492 (2000).

Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J.*, **19**, 5720–5728 (2000).

Itakura E, Kishi C, Inoue K, Mizushima N. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. *Mol. Biol. Cell*, **19**, 5360–5372 (2008).

Xiao J, Feng X, Huang XY, Huang Z, Huang Y, Li C, Li G, Nong S, Wu R, Huang Y, Long XD. Spautin-1 ameliorates acute pancreatitis via inhibiting impaired autophagy and alleviating calcium overload. *Mol. Med.*, **22**, 643–652 (2016).

Yan Y, Flinn RJ, Wu H, Schnur RS, Backer JM. hVps15, but not Ca\(^{2+}\)/CaM, is required for the activity and regulation of hVps34 in mammalian cells. *Biochem. J.*, **417**, 747–755 (2009).

Lerch MM, Gorelick FS. Models of acute and chronic pancreatitis. *Gastroenterology*, **144**, 1180–1193 (2013).

Lampe M, Kern HF. Acute interstitial pancreatitis in the rat induced by excessive doses of a pancreatic secretagogue. *Virchows Arch. A Pathol. Anat. Histol.*, **373**, 97–117 (1977).

Yamaguchi H, Kimura T, Mimura K, Nawata H. Activation of proteases in cerulein-induced pancreatitis. *Pancreas*, **4**, 565–571 (1989).

Rakoczay Z Jr, Hegyi P, Dosa S, Ivanyi B, Jarmay K, Biczo G, Hraescu Z, Varga IS, Karg E, Kaszaki J, Varro A, Lonovies J, Boros I, Gukovskiy I, Gukovskaya AS, Pandol SJ, Takacs T. A new severe acute necrotizing pancreatitis model induced by L-ornithine in rats. *Crit. Care Med.*, **36**, 2117–2127 (2008).

Harper SJ, Cheslyn-Curtis S. Acute pancreatitis. *Ann. Clin. Biochem.*, **48**, 23–37 (2011).

Braganza JM, Lee SH, McClay RF, McMahon MJ. Chronic pancreatitis. *Lancet*, **377**, 1184–1197 (2011).

Apte MV, Pirola RC, Wilson JS. Mechanisms of alcoholic pancreatitis. *J. Gastroenterol. Hepatol.*, **25**, 1816–1826 (2010).

Bi Y, Atwal T, Vege SS. Drug therapy for acute pancreatitis. *Curr. Treat. Options Gastroenterol.*, **13**, 354–368 (2015).

Kambhamapati S, Park W, Habtezion A. Pharmacologic therapy for acute pancreatitis. *World J. Gastroenterol.*, **20**, 16868–16880 (2014).