A novel acridine derivative, LS-1-10 inhibits autophagic degradation and triggers apoptosis in colon cancer cells

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Autophagy promotes cancer cell survival and drug resistance by degrading harmful cellular components and maintaining cellular energy levels. Disruption of autophagy may be a promising approach to sensitize cancer cells to anticancer drugs. The combination of autophagic inhibitors, such as chloroquine (CQ) and lucanthone with conventional cancer therapeutics has been investigated in clinical trials, but adverse drug–drug interactions are a high possibility. Here we designed and synthesized a novel, small-molecule library based on an acridine skeleton and the CQ structure with various modifications and substitutions and screened the compounds for effective autophagy inhibition. We found that 9-chloro-2-(3-(dimethylamino)propyl)pyrrolo[2,3,4-kl]acridin-1(2H)-one (LS-1-10) was the most effective from our library at inhibiting autophagic-mediated degradation and could decrease the viability of multiple colon cancer cells. In addition, LS-1-10 induced DNA damage and caspase 8-mediated apoptosis. Overall, this small molecule was more efficient at reducing the viability of cancer cells than other conventional chemotherapeutic agents, such as CQ and amsacrine. The anticancer and autophagy-inhibiting activities of LS-1-10 were confirmed in vivo in a xenograft mouse model. Collectively, this study has identified a new and efficient single compound with both autophagy-inhibiting and anticancer activity, which may provide a novel approach for cancer therapy.

Cell Death and Disease (2017) 8, e3086; doi:10.1038/cddis.2017.498; published online 5 October 2017

Autophagy is an important catabolic process that is highly conserved across all eukaryotes.1–4 It is a protein degradation pathway by which cytoplasmic constituents are delivered to lysosomes for digestion.5 This process is induced in response to various stimuli, such as genotoxic chemicals, oxidative reagents and starvation, to maintain cellular metabolism and eliminate harmful damaged proteins and organelles, thus facilitate cell survival.6,7 Numerous studies have identified a complex association between autophagy and cancer development.8–10 Many cancer therapeutics, including DNA damaging agents, histone deacetylase inhibitors and ionizing radiation induce high levels of autophagy to confer cytoprotection of cancer cells.11–15 Inhibition of autophagy enhances the pro-apoptotic effects of anticancer agents and thus may be a promising strategy to augment the activity of many cancer therapeutics.16

Many combination therapies are undergoing clinical trials to verify whether adjunctive autophagy inhibitors can enhance the anticancer efficacy of small-molecule drugs.16,17 Chloroquine (CQ), lucanthone, and their analogs, are currently the only autophagic inhibitors under clinical investigation for use as cancer therapeutics.18–20 However, CQ can induce ocular toxicity and irreversible retinopathy,21 and clinical trials of lucanthone were prematurely terminated or suspended for yet unknown reasons. Additional inhibitors of autophagy are being developed with the aim of enhancing the activity of chemotherapeutic agents. Adverse drug–drug interactions may arise from these complex drug combinations, thus the development of a small, single molecule that possesses both potent anticancer and anti-autophagy activity is required.

Acridine derivatives, such as amsacrine (m-AMSA) and DACA,22–24 exhibit DNA-intercalating and topoisomerase-inhibiting activity and are prime candidates as anticancer agents.25 m-AMSA has been used to treat acute leukemia and malignant lymphoma, but is ineffective against solid tumors.22,26–29 Acridine provides an ideal scaffold as an anti-tumor drug for two reasons. First, the linear tricyclic aromatic structure of acridine ensures high DNA intercalation. Second, modifications to the chemical structure, such as the side chain on the pyridine ring, can generate numerous biologically active compounds with different activities.30

Here, we generated a novel acridine derivative (hereafter known as LS-1-10) that contains a quinoline moiety and a flexible tertiary-amine side chain similar to that of CQ and hydrochloroquine (HCQ). We verified that LS-1-10 acts as a DNA damaging agent and can simultaneously inhibit...
autophagy. We found that LS-1-10 can reduce the viability of various colon cancer cell lines with a higher efficacy than many conventional chemotherapeutic agents. Taken together, LS-1-10 possesses a dual function as a DNA damaging agent and inhibitor of autophagy. We propose that LS-1-10 may be exploited as a suitable small-molecule drug in colon cancer therapy.

Results

Screening acridine derivatives with a similar structure to CQ. Most DNA damaging agents, including m-AMSA, induce autophagy and thus promote cancer cell survival.\(^{31}\) Here, we designed and synthesized a series of small molecules based on the skeleton of acridine and the structures of CQ and HCQ (Figure 1a) with the aim of developing a drug with both anticancer and autophagy-inhibiting functions. Autophagy can be monitored by the accumulation of the autophagy marker LC3 and the degradation of p62.\(^{32}\) Inhibition of autophagic degradation usually causes accumulations and puncta formations of both LC3-I/II and p62.\(^{32}\) Thus, we analyzed the abundance and distribution of these two biological markers after treating DLD1 and LoVo human colon cancer cell lines with eight in-house generated molecules (Figures 1b and c, S1A). Among the eight molecules tested, three showed potential to effectively inhibit autophagic degradation (Figure 1b, Supplementary Figure S1A):

LS-1-10 [9-chloro-2-[(3-dimethylamino)propyl]pyrrolo[2,3,4-kl]acridin-1(2H)-one]
Among the three compounds, LS-1-10 was the most effective at inducing LC3 puncta accumulation and cleavage of PARP-1, a marker of apoptosis (Figures 1b and c, Supplementary Figure S1A). We considered, therefore, that LS-1-10 would have the highest potential as an anticancer drug, and thus focused our subsequent analyses on this compound.

**LS-1-10 inhibits autophagic degradation.** To confirm how effectively LS-1-10 can inhibit autophagic degradation, we...
Figure 2  LS-1-10 inhibits autophagic degradation. (a) DLD1 cells were treated with different concentrations of LS-1-10 (1, 2 and 5 μM) for 24 h (upper panel) or 5 μM LS-1-10 over a time course (lower panel). Immunoblotting was performed using an LC3 antibody. (b) DLD1 cells were incubated with 5 μM LS-1-10 or 10 μM chloroquine (CQ) for 24 h. Immunofluorescence was performed after staining with an LC3 antibody. Scale bars, 5 μm. (c) Quantification of the LC3-positive punctate cells shown in (b). Only cells with more than five puncta were counted. Data represent the means ± S.D. (n = 3). Student’s t-Test, ** P < 0.01. (d) DLD1 cells were transfected with ptfLC3 and then treated with 5 μM LS-1-10 or 10 μM CQ for 24 h. The distribution of GFP/RFP-LC3 was examined by confocal microscopy. (e) Quantification of the LC3 puncta shown in (d). Data represent the means ± S.D. based (n = 3). Student’s t-Test, ** P < 0.01. (f) DLD1 cells were treated with 5 μM LS-1-10, 100 nM bafilomycin A1, or both for 24 h. Whole-cell lysates were extracted for immunoblotting with the indicated antibodies. (g) Autophagic vesicles or autophagosomes in DLD1 cells were observed by electron microscopy after 24 h of 5 μM LS-1-10 treatment. Arrows in the enlargement indicate autophagic vesicles. The graph shows a statistical analysis of autophagic vesicles in DLD1 cells upon LS-1-10 treatment. Data represent the means ± S.D. (n = 3). Student’s t-Test, ** P < 0.01
at lower concentrations (when it is present in the cytosol and the nucleus; Figure 3e). AO-loaded cells manifested reduced red fluorescence and increased green fluorescence after LMP. Healthy lysosomes in control cells were stained red whereas lysosomes exposed to LS-1-10 or CQ were predominantly stained yellow (resulting from increased green fluorescence; Figure 3e). These data indicate an increase in lysosomal pH. The translocation of soluble lysosomal
components from the lysosomal lumen to the cytosol is a distinctive feature of LMP. We thus monitored the translocation of lysosomal cathepsins by immunoblotting for CTSD in various subcellular fractions (cytosol versus heavy membranes which includes mitochondria and lysosomes). Here, we observed marked reductions of both CTSD and CTSD in the lysosomes (Figure 3f). Collectively, these data demonstrate that LS-1-10 inhibits autophagosome–lysosome fusion and disrupts lysosomal function.

**LS-1-10 is cytotoxic to colon cancer cells.** Acridine has been used as an anticancer drug since 1961 and some of its derivatives can also induce death of cancer cells. Thus, we investigated the anticancer activity of LS-1-10 by CCK-8 cell viability assay. LS-1-10 reduced cell viability to a similar extent in five colon cancer cell lines (Figure 4a). Comparable results were obtained by ATP assay (Figure 4b) and trypan blue staining (Figure 4c) in two representative cell lines (DLD1 and LoVo).

**LS-1-10 induces DNA damage and caspase 8-mediated apoptosis.** As LS-1-10 exhibits efficient anticancer activity, we focused our attention on the potential mechanism underlying its cytotoxicity in cancer cells. The linear tricyclic aromatic structure of acridine permits DNA intercalation. Acridine compounds, such as m-AMSA and DACA, have also been reported to be topoisomerase II inhibitors and cause DNA double-strand breaks (DSBs). Therefore, we used a comet assay to determine whether LS-1-10 also induces DNA damage. Cells were treated with 5 μM LS-1-10 for 24 h and then harvested. The LS-1-10-treated cells exhibited a greater DNA tail area and longer DNA tail length than control cells, indicating more extensive DNA damage (Figure 5a). The degree of DNA damage was analyzed using CometScore (Sumerduck, VA, USA) software (Figure 5b) and the data suggested that LS-1-10 causes marked DNA damage in multiple colon cancer cells.

We next investigated whether LS-1-10-induced DNA damage was achieved by generating DSBs. We detected H2AX phosphorylation (γ-H2AX), a hallmark of DSBs and found that γ-H2AX increased in LS-1-10-treated cells in a dose-dependent manner compared to untreated cells (Figure 5c). We also observed activation of ATM and phosphorylation of its downstream target p53 (Figure 5c). Immunostaining for DLD1 also indicated γ-H2AX foci formation upon treatment with LS-1-10 (Supplementary Figure S3A and S3B). These results support that LS-1-10 induces DSBs in cancer cells.

DNA damaging agents cause cell cycle arrest and apoptosis in response to stress. To determine whether LS-1-10 elicits cell cycle arrest, we collected LS-1-10-treated cells and performed flow cytometry analysis by propidium iodide (PI) staining. As expected, there was notable S/G2 arrest in cells after LS-1-10 treatment (Figure 5d). We also detected an increase in the fraction of the cells with DNA content less than G0/G1 cells (region of sub-G1 in Figure 5d), which are commonly considered to be apoptotic cells. We next evaluated the cleavage of typical markers of apoptosis, namely caspase 9, caspase 8, caspase 3 and PARP1. Cleavage of caspase 8 (rather than caspase 9) and its downstream caspase 3 and PARP1 increased in a dose-dependent manner upon LS-1-10 treatment (Figure 5e and Supplementary Figure S3C). We further confirmed that LS-1-10 induces caspase 8-mediated apoptosis using a pan-caspase inhibitor (Z-VAD-FMK) and a caspase 8-specific inhibitor (Z-IETD-FMK). Both Z-VAD-FMK and Z-IETD-FMK effectively decreased the cleavage of caspase 3 and PARP1 (Figure 5f). This finding was confirmed by flow cytometric analysis of AnnexinV/PI stained cells, which showed that Z-VAD-FMK and Z-IETD-FMK blocked LS-1-10-induced apoptosis (Figures 5g and h). Collectively, these data support that LS-1-10 is a DNA-damaging agent and induces caspase 8-mediated apoptosis.

**LS-1-10 possesses more efficient anticancer activity than other conventional chemotherapeutic agents.** To further characterize the efficacy of LS-1-10, we compared the anticancer activity of LS-1-10 with CQ. LS-1-10 was significantly more potent at reducing cell viability than CQ (>50%), despite similar autophagy-inhibiting potency (Figure 6a). As previously discussed, m-AMSA is a chemotherapeutic used to treat lymphoma and leukemia. Similar to LS-1-10, m-AMSA is an acridine derivative (Figure 6b) and induces a high level of autophagy in cancer cells. We hypothesized that LS-1-10 might be more efficient at reducing the viability of cancer cells than m-AMSA as it blocks cytoprotective autophagy. Consistently, LS-1-10 was more potent than m-AMSA at reducing colon cancer cell viability (Figure 6b), as determined by CCK-8 assay. To further characterize the role of autophagy in cell death, we treated wild-type (WT) and autophagy-deficient ATG3KO cells with either m-AMSA or LS-1-10. Flow cytometric analysis of AnnexinV/PI stained cells found that compared to WT cells, ATG3KO cells had increased sensitivity to m-AMSA but not LS-1-10 (Figures 6c and d). This finding was consistent with the autophagic flux in these cells. Lucanthone is an autophagy inhibitor that is more potent than CQ at reducing breast cancer cell viability. Lucanthone is also able to disrupt topoisomerase II activity and inhibit
APE1 – an important enzyme involved in DNA base excision repair.\textsuperscript{47,48} Therefore, we assessed whether LS-1-10 is more efficient than lucanthone in reducing cancer cell viability. LS-1-10 was more effective than lucanthone at reducing the viability of multiple cancer cells (Supplementary Figure S5). Collectively, these data indicate that LS-1-10, as a single-agent, has higher anticancer efficacy than other similar drugs.

The anticancer and autophagy-inhibiting activities of LS-1-10 \textit{in vivo}. The fumarate salt of LS-1-10 was synthesized to enhance its aqueous solubility for use in \textit{in vivo} studies (‘LS-1-10’ refers to the fumarate of LS-1-10 in all the \textit{in vivo} experiments). To determine the anticancer activity of LS-1-10 \textit{in vivo}, nude mice were injected with DLD1 cells and 2 weeks later were treated with PBS, LS-1-10 40 mg/kg LS-1-10 80 mg/kg or CQ 80 mg/kg by intraperitoneal injection every 5 per 7 (5/7) days for 3 weeks. The tumor sizes and weights were significantly lower in the two LS-1-10-treated groups (Figures 7a–c). LS-1-10 was also more efficient at reducing the tumor sizes and weights when compared to the same dose of CQ treatment (Figures 7a–c). Immunohistochemical analysis of LC3 distribution found that LC3 was diffuse in the cytoplasm of PBS-treated mice, but was present in the large number of punctuates in tumor tissues of LS-1-10-treated mice (Figure 7d). These data support that LS-1-10 inhibits autophagic degradation \textit{in vivo} and confirm the anticancer and autophagy-inhibiting activities of LS-1-10.

\textbf{Discussion}

This study developed a novel derivative of acridine, LS-1-10, with a similar structure to CQ and with autophagy-inhibiting

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
\textbf{IC}_{50} (μM) & \\
\hline
LoVo & 1.25 \\
DLD1 & 1.31 \\
HT29 & 0.82 \\
HCT116 & 1.15 \\
SW480 & 1.08 \\
\hline
\end{tabular}
\caption{IC\textsubscript{50} values of LS-1-10 in various cancer cell lines.}
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{LS-1-10 efficiently reduces the viability of colon cancer cells. (a) Five colon cancer cell lines were treated with the indicated concentrations of LS-1-10 for 72 h. Cell viability was measured by CCK-8 assay (left panel). Data represent the means ± S.D. (n=3). The IC\textsubscript{50} values were calculated from the results of the CCK-8 assay (right panel). (b) DLD1 and LoVo cells were treated with LS-1-10 at the indicated concentrations for 72 h, and ATP levels were measured by ATPlite assay. Data represent the means ± S.D. (n=3). (c) Cells were treated with LS-1-10 for 72 h, and the viability was determined by trypan blue exclusion assay. Data represent the means ± S.D. (n=3).}
\end{figure}
LS-1-10 suppresses autophagy-mediated degrada-
tion by inhibiting autophagosome–lysosome fusion and
disrupting lysosome function. This molecule with autophagy-
inhibiting and DNA-damaging activity reduced the viability of
numerous tested colon cancer cell lines.

LS-1-10 shares the same acridine backbone as m-AMSA,
which was the first synthetic DNA-intercalating agent suc-
cessfully used in a clinical setting (Figure 6b). Both LS-1-
10 and m-AMSA possess the DNA-binding domain acridine
moiety and the N-appendage moiety that permits
topoisomerase II binding (Figure 6b). m-AMSA is an effective
treatment for acute leukemia and malignant lymphoma, but is
ineffective against solid tumors. The tumor microenviron-
ment has a crucial role in mediating the chemoresistance of
tumor cells. Solid tumors typically exhibit a hypoxic
environment, which results in increased metabolic stress
and thus induces autophagy. Previous studies have verified
that m-AMSA-induced autophagy has a cytoprotective role.
Consequently, we speculate that the decreased sensitivity of
solid tumors to m-AMSA may be due to high basal levels of
autophagy. As such, the effect of LS-1-10 on inhibiting autophagy may be a promising therapeutic approach for solid tumors. As expected, LS-1-10 was more effective than m-AMSA at reducing the viability of colon cancer cells (Figure 6b).

High levels of autophagy are induced in response to most DNA-damaging cancer therapeutics such as etoposide, m-AMSA or irradiation, to generate energy, maintain homeostasis and promote survival of cancer cells. Even though autophagy-mediated cell death has been reported in cancer cells, a high-content screen of ~1400 cytotoxic agents found that no single compound could induce cell death by autophagy, underscoring that autophagy is predominantly cytoprotective in cancer cells in response to anti-neoplastic therapies. Our colony formation analysis confirmed this cytoprotective function of autophagy in colon cancer cell lines (Supplementary Figures S6A and B). Specifically, Cas9 knockout of ATG3/ATG7, could disrupt autophagy flux and sensitize the cancer cells to etoposide (Supplementary Figures S6A and B). Disruption of autophagic degradation may, therefore, augment the efficacy of therapeutics used in colon cancer.

DNA damage-induced apoptosis is typically mediated by caspase 9, rather than caspase 8. Interestingly, we found that LS-1-10 triggered caspase 8-mediated DNA damage-induced apoptosis (Figure 5). It remains to be confirmed what causes caspase 8 cleavage here. Previous studies reported that cytosolic CTSD primes caspase 8 (but not caspase 9) activation by proteolysis. Consistently, we found that LS-1-10 treatment induces LMP and triggers CTSD diffusion throughout the cytosol (Figures 3e and f). We previously hypothesized that disruption of lysosomal function and the subsequent diffusion of cathepsins might be the main cause of caspase 8 activation and apoptosis. Agents that induce LMP trigger apoptosis independent of functional p53 status. In support of our hypothesis, we found the anticancer activity of

Figure 6  LS-1-10 reduces cancer cell viability. (a) DLD1 and LoVo cells were treated with varying concentrations of LS-1-10 or chloroquine (CQ) as indicated for 72 h. Cell viability was measured by CCK-8 assay. Data represent the means ± S.D. (n = 3). (b) DLD1 and LoVo cells were treated with LS-1-10 or amsacrine (chemical structures shown in the left panel) at the indicated concentrations for 72 h. Cell viability was measured by CCK-8 assay. Data represent the means ± S.D. (n = 3). (c) DLD-1 cells were treated with 5 μM LS-1-10 or 5 μM amsacrine for 48 h, and then subjected to flow cytometry to detect AnnexinV/propidium iodide staining. (d) Quantification of the apoptotic cells shown in (c). Data represent the means ± S.D. (n = 3). Student’s t-test, **P<0.01
LS-1-10 to be independent of p53 (Supplementary Figure S4). However, knockdown of CTSD only partly rescued the cleavage of caspase 8 and its substrates (Supplementary Figure S3D). Thus, we propose it is likely that other pathways are also involved in the regulation of caspase 8 activation and apoptosis and that lysosomal dysfunction may not be the main trigger of apoptosis.

Although the effects of LS-1-10 on inhibiting autophagy and inducing apoptosis are driven by different molecular mechanisms, we consider that the autophagy-inhibiting activity of LS-1-10 could augment its anticancer efficacy. Both key DNA damage related proteins, such as Sae2 (human CtIP)\textsuperscript{57}, p62\textsuperscript{54} and active caspase 8 can be degraded via autophagic pathways.\textsuperscript{58} These two aspects contribute to the cytotoxicity of LS-1-10. The specific targets of LS-1-10 in the lysosome have not been identified, thus our hypothesis and the precise contribution of autophagy inhibition to the cytotoxicity of LS-1-10 remains to be confirmed. Our cell viability analysis of LS-1-10 analogs provides some indirect support as those compounds that showed no autophagy-inhibiting activity were also less effective at reducing the viability of DLD1 cells than LS-1-10 (Supplementary Figure S5C).

CQ, HCQ and lucanthone are the only clinically relevant autophagic inhibitors currently in use as cancer therapeutics.\textsuperscript{59} However, the ocular toxicity of CQ and HCQ has severely limited their utilization. Novel inhibitors of autophagy, including many analogs of CQ, have been identified by different groups.\textsuperscript{60} Even though they may have lower toxicity and a better therapeutic index, unexpected adverse effects due to drug–drug interactions may still occur. The development of a single-agent autophagy inhibitor with anticancer activity will likely be the most effective and safe strategy. Lys05 is an autophagy inhibitor that exhibits single-agent anticancer activity without inducing toxic effects in mice.\textsuperscript{61} Lys05 is a dimeric form of CQ but exerts 10-fold higher autophagy inhibition compared to CQ.\textsuperscript{61} The Lys05 design strategy encouraged us to synthesize and test the dimeric analog of LS-1-10. Preliminary data showed that the IC\textsubscript{50} of dimeric LS-1-10 is <10 nM (unpublished data). Further investigations are now needed to clarify its biological activity.

Figure 7  LS-1-10 exhibits anticancer and autophagy-inhibiting activities in vivo. (a) Four-week-old nude mice were engrafted with DLD1 cells and randomly divided into four groups (n = 6). After 2 weeks, the tumor-bearing mice were treated with PBS, LS-1-10 40 mg/kg, LS-1-10 80 mg/kg, or CQ 80 mg/kg by intraperitoneal injection every 5 per7 (5/7) days for 3 weeks. Tumor volumes were calculated by measuring the length and width using Vernier calipers every 2 days. Data represent the means ± S.D. (n = 6). (b) Images of the tumors from a. (c) Quantification of the tumor weights from (a). Student’s t-test, *P < 0.05; **P < 0.01. (d) Immunohistochemical staining of LC3 in tumor sections treated with PBS or LS-1-10. Four-week-old nude mice were engrafted with DLD1 cells and observed until tumors reached ~100 mm\textsuperscript{3}. Tumor-bearing mice were then treated with PBS or 20 μg LS-1-10 by intra-tumoral injection once every 2 days for a total of seven injections. Mice were killed and tumors were resected 2 days after the final injection.
The anti-schistosome agent lucanthone shares the same autophagy-inhibiting and DNA-damaging activities as LS-1-10.\(^\text{46}\) However, two phase II clinical trials of lucanthone were prematurely terminated or suspended for unknown reasons. Our analysis found that LS-1-10 has higher efficacy than lucanthone at reducing the viability of multiple colon cancer cells (Supplementary Figures S5A and B). This effect may be due to the DNA-intercalating activity of LS-1-10 as lucanthone does not have a DNA-binding domain.\(^\text{47,48}\)

In summary, our study describes the first acridine derivative with autophagy-inhibiting activity in cultured colon cancer cell lines. LS-1-10 has potent in vitro anticancer activity achieved by inducing DNA damage and caspase 8-mediated apoptosis. The autophagy-inhibiting activity of LS-1-10 may augment its efficacy at reducing the viability of cancer cells. Our results demonstrate that LS-1-10 is a potent anticancer agent that may be a valuable cancer therapeutic in the future.

Materials and Methods

Reagents. All acridine derivatives, including LS-1-10 were synthesized as previously described.\(^\text{62}\) CO (C6H628) and etosipide (E1383) were purchased from Sigma-Aldrich (St. Louis, MO, USA); baflomycin A1 (S1413) was purchased from Selleck Chemicals (Houston, TX, USA); Z-VAD-fmk (KG6254) and 2-JETD-fmk (KG6260) were purchased from Keygen Biotech (Nanjing, China); Amsactine (T1326) was purchased from TargetMol (MA, USA); LcLuante (L473700) was purchased from Toronto Research Chemicals Inc (Toronto, Ontario, Canada).

Antibodies. MAP1LC3 (2775), PARP1 (9542), Rb/GX (9718), cleaved caspase 8 (9496), caspase 9 (9662), phospho-ATM (Ser188) (5883) and phospho-p53 (Ser15) (9284) were purchased from Cell Signaling Technology (Danvers, MA, USA); Atg3 (M133-3), p62/SQSTM1 (PM045) and α-tubulin (PM054) were purchased from MBL (Naka-ku, Nagoya, Japan); p3-DO-1 (sc-126) and p-Actin (sc-7210) were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA); Caspase D (ab75892), LAMP-1 (ab25630) and Caspase 8 (full-length) (ab32397) were purchased from Abcam (Cambridge, MA, USA); LcLuante (L473700) was purchased from Toronto Research Chemicals Inc (Toronto, Ontario, Canada).

Plasmids. The pH-LC3 plasmid was purchased from Addgene (Cambridge, MA, USA) (Plasmid #21074).

Cell culture and transfection. DLD1 and LoVo cell lines were purchased from the American Type Culture Collection, and grown in RPMI or DMEM medium (Invitrogen, Carlsbad, CA, USA) with manual gating using CellQuest software (Franklin Lakes, NJ, USA). Cells were treated with different compounds for 72 h. After treatment, CCK-8 was added to the cultured cell plate and incubated at 37 °C for 1 h. The absorbance of each sample was read at 450 nm.

Comet assay. The comet assay was performed as described previously.\(^\text{64}\) In brief, the samples were mixed with 0.5% normal melting agarose to form a cell suspension. After electrophoresis, the cells were transferred to 96-well plates, mixed with reaction buffer and substrate, and incubated for 30 min at 37 °C. The samples were read in a fluorometer with 400 nm excitation and 505 nm emission filters. The activity was normalized to the protein concentration.

CCK-8 assay. Equal numbers of cells (~5,000/well) were seeded into a 96-well plate 24 h before experimentation. Cells were treated with different compounds for 72 h. After treatment, CCK-8 was added to the cultured cell plate and incubated at 37 °C for 1 h. The absorbance of each sample was read at 450 nm.

Flow cytometry. Apoptotic cells were quantified using Annexin V-FITC/PI staining (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions.

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Immunoblot assay. In brief, cells were collected using a scraper and washed once with cold PBS. The cells were then lysed in lysis buffer (50 mM Tris-HCl, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1% NP-40) supplemented with 1% protease inhibitor cocktail. Equal amounts of proteins were size-fractionated by 7.5–15% SDS-PAGE. At least three independent experiments were performed.

Real-time PCR analysis of mRNA. Total RNA was isolated using TRizol reagent. The cDNA was synthesized from 2 μg of RNA using a Quantscript RT Kit (TianGen, Beijing, China, KR0109). The following primers were used for RT PCR: forward: 5'-AGCTGCTGTTGACCCACATC-3'; reverse: 5'-CACGAAAGCCCATGGACAG-3'; ACTB forward: 5'-CCAAAGCGGAGAAATGGA-3'; reverse: 5'-CACAGGCGTGACAGGATAG-3';

Immunofluorescence. Cells were cultured in confocal dishes to ~60% confluence. After transfection and treatment, cells were fixed with 4% paraformaldehyde and permeabilized with 100% methanol. The dishes were incubated in blocking solution (0.8% BSA in PBS) and exposed overnight to primary antibody (1:100 dilution for all antibodies) at 4 °C. The cells were then washed three times with blocking solution and then exposed to a secondary antibody (1:100 dilution) conjugated to FITC/TRITC. Cells were observed and analyzed under a confocal microscope (Olympus BX-51, America Inc.).

Acridine orange staining. Acridine orange (5 μg/ml; Sigma (St. Louis, MO, USA) BR-A60145) was added to cells for 10 min (37 °C, 5% CO2). After three washes with PBS, the cells were observed under a confocal microscope (Olympus BX-51, America Inc., Center Valley, PA, USA).

Generation of Cas9 ATG3/ATG7 knockout cell line. The Cas9 ATG3 knockout cell lines were generated by CRISPR-Cas9 method in DLD1 cells using the SpCas9 A-Puro vector (Addgene).\(^\text{65}\) The ATG3/ATG7 sgRNA was designed using online software (http://crispr.mit.edu). The sgRNA ATG3 sequence 5'-TGTAGGACGCA CCTAACCAC3'- and the sgRNA ATG7 sequence 5'-AACCTCCAATGTIRAAGCCAGC3'- were transfected into DLD1 cell lines and selected in 2 μg/ml puromycin.

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Statistical analysis. Three independent experiments were performed prior to statistical analysis. The data represent the means ± S.D. A P < 0.05, by unpaired Student's t-test, was considered statistically significant.

Conflict of Interest
The authors declare no conflict of interest.

Acknowledgements. This study was supported by the National Key Research and Development Program of China (Protein Machinery and Life Science Grant 2013CB911000) and the National Natural Science Foundation of China (grant numbers 31570812, 61621063, 18122208, 81621063, 81573272 and 81472581); Discipline Construction Fund of Shenzhen (2016); the Shenzhen Municipal Commission of Science and Technology Innovation (grant number JCYJ2016042710455100) and the Beijing Natural Science Foundation (7162109). We thank Dr. Jessica Tamani of E TED for language editing.. XM and SL are numbers 31570812, 81530074, 81621063, 81222028, 81621063, 81573272 and 2013CB911000) and the National Natural Science Foundation of China (grant

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