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Clathrin-mediated endocytosis is required for ANE 30-100K-induced autophagy

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Background: We identified an autophagy-inducing areca nut (AN) ingredient (AIAI) in the 30-100 kDa fraction of AN extract (ANE 30-100K). This study was to analyze the role of endocytosis in ANE 30-100K-induced autophagy.

Methods: We used benzyl alcohol, dynasore, and shRNA of clathrin and dynamin to assess whether ANE 30-100K-induced cytotoxicity and accumulation of microtubule-associated protein 1 light chain 3 (LC3)-II were affected in oral (OECM-1) and esophageal (CE81T/VGH) carcinoma cells.

Results: Both benzyl alcohol and dynasore effectively reduced ANE 30-100K-induced cytotoxicity and LC3-II accumulation in OECM-1 and CE81T/VGH cells. Downregulated protein expression of both clathrin and dynamin by their shRNA also significantly attenuated ANE 30-100K-induced elevation of LC3-II levels in CE81T/VGH cells.

Conclusions: These results indicate that AIAI may be engulfed by cells through clathrin-mediated endocytosis, which promotes the execution of the following autophagy program.

KEYWORDS
areca nut, autophagy, benzyl alcohol, clathrin, dynamin, dynasore, endocytosis

1 | INTRODUCTION

Betel quid (BQ) is a popular carcinogen used by 200-600 million people worldwide. BQ recipes vary in different areas, including areca nut (AN, *Areca catechu* L.), lime, Piper betle leaf, Piper betle inflorescence, or clove.[1] Among these diversified components, AN is the indispensable constituent of BQ, which is also regarded as a human carcinogen.[2] In addition to oral submucous fibrosis,[3] a recent systematic review and meta-analysis studies showed that AN usage is positively correlated with oral cancer.[4] Thus, mechanisms of AN-mediated cell responses are important to uncover the pathology of AN-associated diseases and worthy to be investigated.

Areca nut (AN) is firstly demonstrated to contain the apoptosis-inducing ingredients such as arecoline and oligomeric procyanidins.[5,6] In our studies, however, we found that both normal and malignant cells underwent autophagic cell death after treatment with the crude extract of AN (ANE) and the 30-100 kDa fraction of ANE (designated as ANE 30-100K).[7,8] Autophagy has been classified into macroautophagy, microautophagy, and chaperon-mediated autophagy and is a conserved lysosomal degradation pathway for cellular components, which maintains cell viability and metabolic homeostasis.[9] Among these three types of autophagy, macroautophagy (referred to as autophagy hereafter) have received the most extensive studies in the past two decades and been implicated in pathological conditions such as cancers.[10] Autophagy acts as a double-edged sword that inhibits tumor formation by preventing accumulation of damaged proteins and organelles at one hand, but facilitates the survival of established tumors by providing metabolic substrates. Thus, confirmation of the role of autophagy in individual cancer is important to design an autophagy-modulated therapy.[11]

The first three authors contributed equally to this work.
Our AN-related studies were approved by the IRB of Chi Mei Medical Center, Tainan, Taiwan, ROC

We also found that the activity of autophagy-inducing AN ingredient (AIAI) present in ANE 30-100K is sensitive to both cellulase and proteinase K, suggesting this ingredient to be a proteoglycan or glycoprotein.[12] Furthermore, ANE 30-100K-induced autophagic activity is dependent on reactive oxygen species, Beclin-1, and Atg5.[7,13] Although cytotoxic and autophagic effects of ANE and ANE 30-100K were revealed, it is thought that oral tumor cells receiving sublethal concentrations of these AN ingredients might exhibit higher but cytoprotective autophagy activities against stressed conditions in AN chewers. This speculation is further verified in our recent studies that long-term treatment of malignant cells with non-cytotoxic concentrations of ANE or ANE 30-100K (both $\leq$1.25 μg/mL) result in increased resistance against stressed conditions such as cisplatin and serum deprivation through elevated autophagic activities.[13,14] Similar increased cisplatin resistance was also observed in oral carcinoma cell lines after 6-day ANE (3 μg/mL) treatment.[15]

In addition to our studies, ANE-induced autophagy has also been demonstrated to be mediated through the activation of p38, mitogen-activated protein kinase phosphatase 1, and hypoxia-inducible factor.[16] There are also evidences demonstrating the correlations of elevated LC3 and Beclin 1 expression respectively with oral submucous fibrosis and oral cancer progression.[17,18] Collectively, the induction of autophagy by AN ingredient is a new field of AN-mediated cellular response and worthy of studying the underlying mechanisms.

Recent investigations indicate that the endocytic pathway can promote efficient autophagy by promoting phagophore formation and expansion. In addition, the early endosomes, multivesicular bodies, or late endosomes can fuse with autophagosomes before their fusion with lysosomes.[19,20] It is, therefore, speculated that endocytosis might play a role in ANE 30-100K-induced autophagy. Among different types of endocytosis, clathrin-mediated endocytosis, a dynamin-dependent process, can basically take place in all types of cells. We, therefore, focused on assessing the effects of this specific type of endocytosis on ANE 30-100K-induced autophagy in this study. We used benzyl alcohol (a membrane fluidity blocker), dynasore (a specific dynamin inhibitor),[21–23] and shRNAs of dynamin and clathrin genes to inhibit endocytosis, followed by analyzing whether ANE 30-100K-induced cytotoxicity and autophagy were interfered.

2 | MATERIALS AND METHODS

2.1 | Preparation of ANE 30-100K

We basically followed the preparation protocols of ANE as described in our previous study.[7] In brief, ANs were ground in a china bowl at room temperature, and the squeezed juice was collected and centrifuged at 12 000 *g* for 10 minute at 4°C. The supernatant was used as ANE and then subjected to lyophilization and stored at −80°C. ANE 30-100K was prepared by centrifugation of ANE at 2900 *g* for 30 minute at 4°C with 30K- and 100K-pored membranous concentration tubes to collect the 30-100 kDa fraction and stored as that of ANE. ANE 30-100K was weighed and redissolved in H_2O before usage.

2.2 | Culture and treatment of cells

Oral epidermoid carcinoma cell Meng-1 (OECM-1) and esophageal carcinoma CE81T/VGH cells were kindly provided by Dr. Dar-Bin *Shieh* (Institute of Oral Medicine, National Cheng Kung University, Tainan, Taiwan) *and Dr. Fen-Hua Wong* (Department of Life Sciences and Institute of Genome Sciences, National Yang Ming University, Taipei, Taiwan), *respectively*. They are cultured in Dulbecco's modified Eagle's medium (12800-017, Invitrogen, New York, USA) containing 10% fetal bovine serum (FBS, 26140079, Invitrogen) at 37°C in a humid atmosphere with 5% CO_2. Both cells were seeded onto each well of a 96-well plate in a 5000 cells/well manner for cell viability determination by XTT reagents (X4251, Sigma-Aldrich, Saint Louis, Missouri, USA). On the other hand, 7×10^6 cells were seeded onto a 10-cm plate for Western blot analysis. Before these analyzes, the cells were cultured in serum-free (SF) medium overnight. Cells were then pre-treated with the benzyl alcohol (9 μmol L^{-1}) or dynasore (50 μmol L^{-1}) (305197 and D7693, respectively, Sigma-Aldrich) for 10 minutes before the following treatment with or without ANE 30-100K (10 μg/mL) under SF conditions for another day and then analyzed by XTT reagents.

2.3 | Western blot analysis

Western blot analysis of lysate proteins (10 or 20 μg) from treated cells was performed as previously described.[7] Briefly, the primary antibodies of microtubule-associated protein 1 light chain 3 (LC3) (L7543, Sigma-Aldrich) were 1000-fold diluted, whereas β-actin antibody (A1978, Sigma-Aldrich) was 10 000-fold diluted. Goat anti-mouse-IgG (AP124P, CHEMICON, Billerica, USA) and goat anti-rabbit-IgG coupled with horseradish peroxidase (81-6120, Invitrogen, New York, USA) were the secondary antibodies, respectively for anti-β-actin and anti-LC3 antibodies, and 10 000-fold diluted.

2.4 | Lentivirus-mediated shRNA interference

E. coli bacteria transfected with pLKO_TRC005 plasmid containing shRNA sequences targeting human clathrin heavy chain (target sequence: CGGTTGCTCTTGTTACGGATA, Clone ID: TRCN0000342-755) and dynamin 1 (target sequence: TCCAGTGAGCCTCCTTG TCAT, Clone ID: TRCN0000380968) were bought from the National RNAi Core Facility (NRCF, Academia Sinica, Taipei, Taiwan). These sequences have been confirmed before packaging the constructed plasmids into lentivirus by NRCF, and the multiplicity of infection (MOI) was then determined. CE81T/VGH ($5\text{-}7\times10^6$ cells) were infected by plasmid-packaged virus (MOI=4), polybrene (8 μg/mL) (Sigma-Aldrich, 107689), and 1% FBS for 24 hours. Cells were then selected in medium containing puromycin (2 μg/mL) and 10% FBS for 48 hours without further cloning.

2.5 | Statistical analysis

OD450 values of triplicated wells in XTT assay and LC3-II/β-actin ratios from three independent Western Blot between two different treatments were analyzed by one-way analysis of variance (ANOVA) with the Tukey Multiple Comparison Test using IBM SPSS Statistics 20.0.0 software. $P < .05$ was considered statistically significant.

3 | RESULT

3.1 | Benzyl alcohol attenuates the cytotoxicity of ANE 30-100K

We firstly tested non-cytotoxic concentration range of benzyl alcohol in CE81T/VGH cells and found that no evident cytotoxicity was observed between 2.25 and 18 μmol L^{-1} (Figure 1A). Figure 1B showed that pre-treatment of benzyl alcohol (9 μmol L^{-1}) significantly protected CE81T/VGH cells from the following cytotoxic ANE 30-100K insult. Furthermore, OECM-1 cells also similarly responded to benzyl alcohol as those observed in CE81T/VGH (Figure 1C,D). These results suggested that inhibition of membrane fluidity, that is, endocytosis and exocytosis, may protect cells against the cytotoxicity of ANE 30-100K.

3.2 | Benzyl alcohol reduces ANE 30-100K-induced LC3-II accumulation

Our previous study showed that ANE 30-100K-stimulated LC3-II accumulation became stronger in the presence of lysosomal inhibitors,[11] a phenomena recognized as the promotion of autophagic flux.[24] Thus, the fluctuation of relative LC3-II level, that is, LC3-II/β-actin ratio was used to reflect intracellular autophagy status.

Figure 2 demonstrated that ANE 30-100K-induced LC3-II protein increase, whereas pre-treatment of benzyl alcohol did not affect LC3-II level compared to non-treated control OECM-1 and CE81T/VGH cells. In addition, benzyl alcohol significantly reduced ANE 30-100K-stimulated LC3-II increase in both cells. These data suggest that benzyl alcohol inhibits ANE 30-100K-induced autophagy.

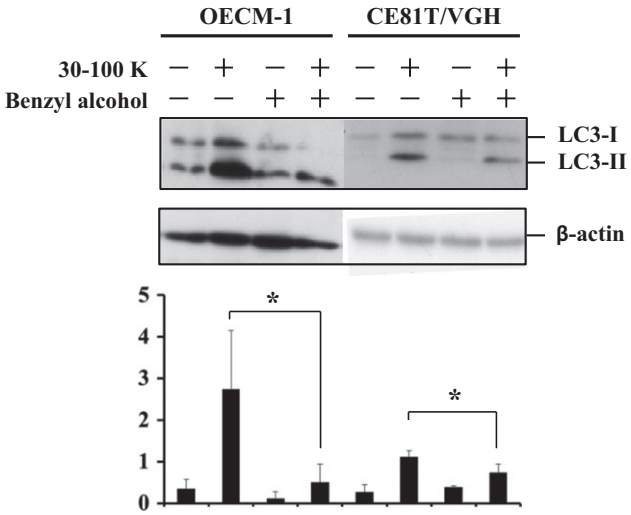

FIGURE 2 Benzyl alcohol reduces ANE 30-100K-induced LC3-II accumulation. OECM-1 (left four lanes) and CE81T/VGH (right four lanes) cells treated with or without ANE 30-100K (10 μg/mL) in the presence or absence of benzyl alcohol (9 μmol L^{-1}) were analyzed by Western blot with LC3 and β-actin antibodies. Average LC3-II/β-actin ratios (mean±SD) were plotted under each treatment. These experiments have been repeated three times with similar results. *$P < .05$

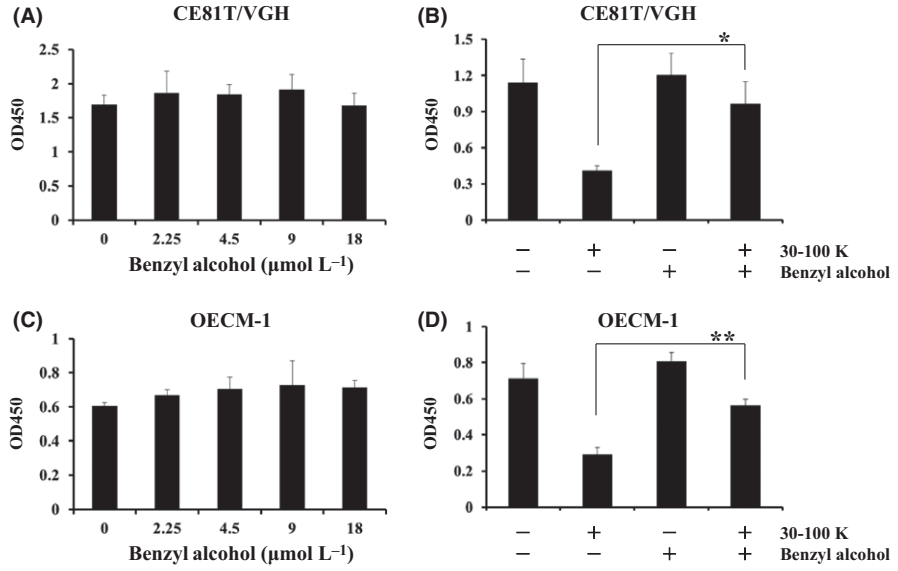

FIGURE 1 Benzyl alcohol attenuates the cytotoxicity of ANE 30-100K. (A) CE81T/VGH cells treated with the indicated concentrations of benzyl alcohol were subjected to XTT assay. Average OD450 values from triplicated wells were plotted as mean±SD. (B) CE81T/VGH cells treated with or without ANE 30-100K (10 μg/mL) in the presence or absence of benzyl alcohol (9 μmol L^{-1}) were analyzed and plotted as (A). OECM-1 cells were subjected to the same analyzes as those of CE81T/VGH in (A) and (B) and the data were respectively presented in (C) and (D). These experiments have been repeated three times with similar results. *$P < .05$, **$P < .01$

3.3 | Dynasore attenuates the cytotoxicity of ANE 30-100K

Dynamin is a GTPase protein required for membrane fission in the process of clathrin-mediated endocytosis and a cell-permeable GTPase inhibitor that inhibits dynamin activity and prevents endocytosis.[23,25]

As those analyzed with benzyl alcohol in Figure 1, there were no detectable cytotoxicity of dynasore (10-100 μmol L^{-1}) in CE81T/VGH and OECM-1 (Figure 3A,C, respectively). Meanwhile, pre-treatment of dynasore was also verified to significantly ameliorate the cytotoxic effects of ANE 30-100K on these two cells (Figure 3B,D).

3.4 | Dynasore reduces ANE 30-100K-induced LC3-II accumulation

For unknown reasons, pre-treatment of dynasore increased LC3-II levels in OECM-1 cells (Figure 4). However, this chemical significantly reduced ANE 30-100K-stimulated LC3-II increase in both OECM-1 and CE81T/VGH cells, indicating the inhibition of ANE 30-100K-induced autophagy. These results also suggest the involvement of clathrin-mediated endocytosis in ANE 30-100K-induced autophagy.

3.5 | Dynamin and clathrin knockdown inhibits ANE 30-100K-induced autophagy

As dynasore has been shown to cause cellular responses in a dynamin-independent manner,[26] we further utilized shRNA targeting human dynamin 1 (DNM1) and clathrin heavy chain to confirm whether clathrin-mediated endocytosis is required for ANE 30-100K-induced autophagy.

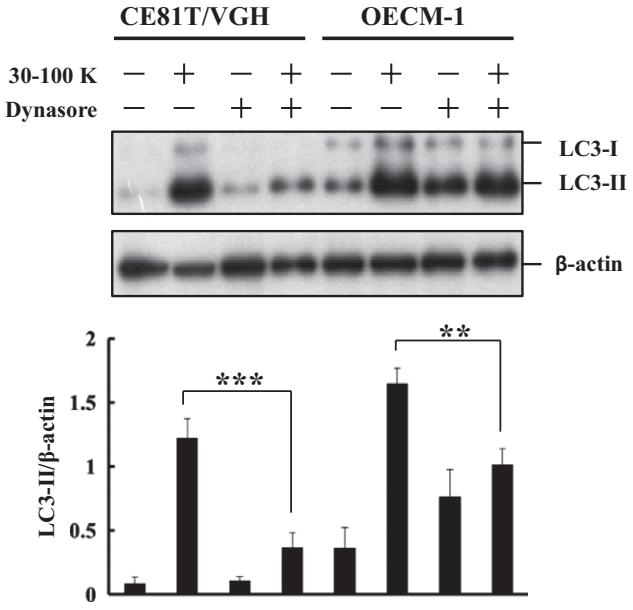

FIGURE 4 Dynasore reduces ANE 30-100K-induced LC3-II accumulation. OECM-1 (left four lanes) and CE81T/VGH (right four lanes) cells treated with or without ANE 30-100K (10 μg/mL) in the presence or absence of dynasore (50 μmol L^{-1}) were analyzed by Western blot with LC3 and β-actin antibodies. Average LC3-II/β-actin ratios (mean±SD) were plotted under each treatment. These experiments have been repeated three times with similar results. **P<.01, ***P<.001

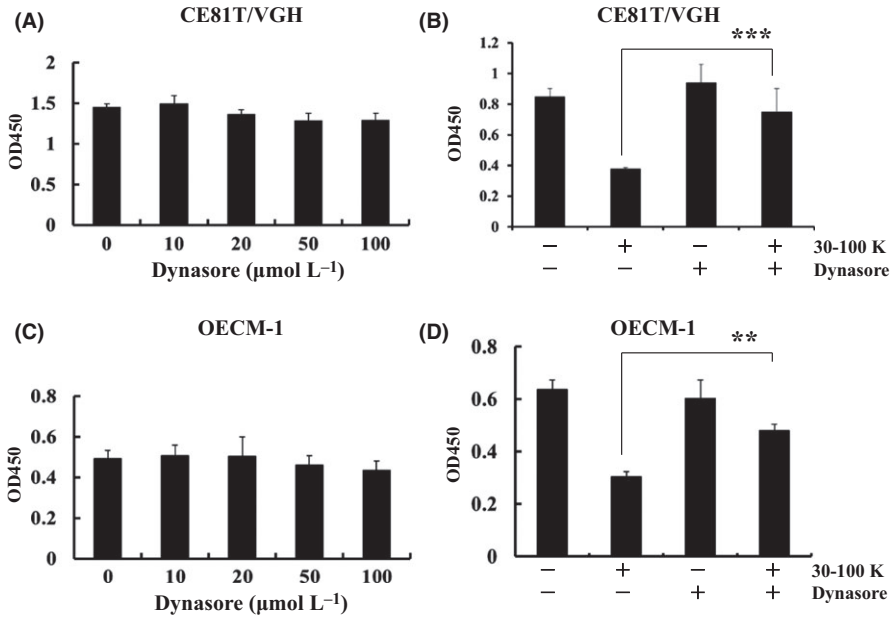

FIGURE 3 Dynasore attenuates the cytotoxicity of ANE 30-100K. (A) CE81T/VGH cells treated with the indicated concentrations of dynasore were subjected to XTT assay. Average OD450 values from triplicated wells were plotted as mean±SD. (B) CE81T/VGH cells treated with or without ANE 30-100K (10 μg/mL) in the presence or absence of dynasore (50 μmol L^{-1}) were analyzed and plotted as (A). OECM-1 cells were subjected to the same analyzes as those of CE81T/VGH in (A) and (B) and the data were respectively presented in (C) and (D). These experiments have been repeated three times with similar results. **P<.01, ***P<.001

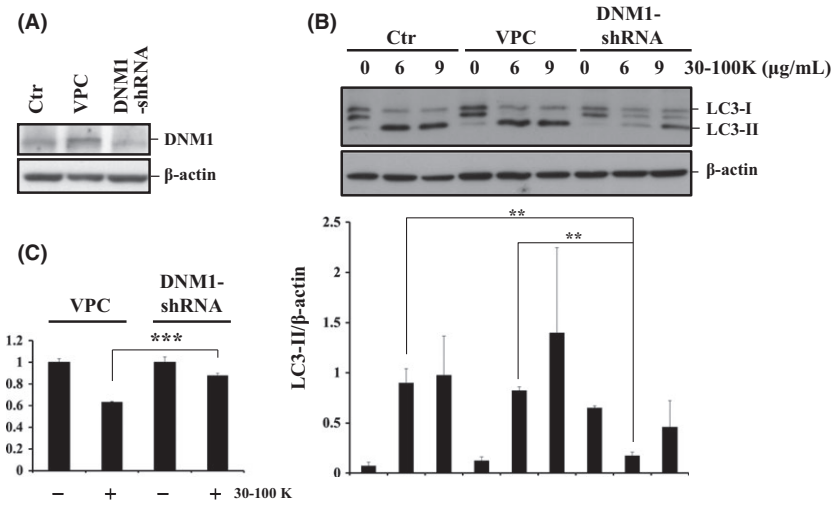

FIGURE 5 Dynamin 1 (DNM1) knockdown inhibits ANE 30-100K-induced autophagy. (A) DNM1 expression level of non-infected (control, Ctr), empty plasmid-infected (virus plasmid control, VPC), and DNM1 shRNA-infected (DNM1-shRNA) CE81T/VGH cells were analyzed by Western blot with DNM1 and β-actin antibodies. (B) Ctr, VPC, and DNM1-shRNA cells treated with indicated concentrations of ANE 30-100K were analyzed by Western blot as (A). (C) VPC and DNM1-shRNA CE81T/VGH cells treated with or without ANE 30-100K (10 μg/mL) were subjected to XTT assay. All the experiments have been repeated three times with similar results. ***P*<.01, ****P*<.001

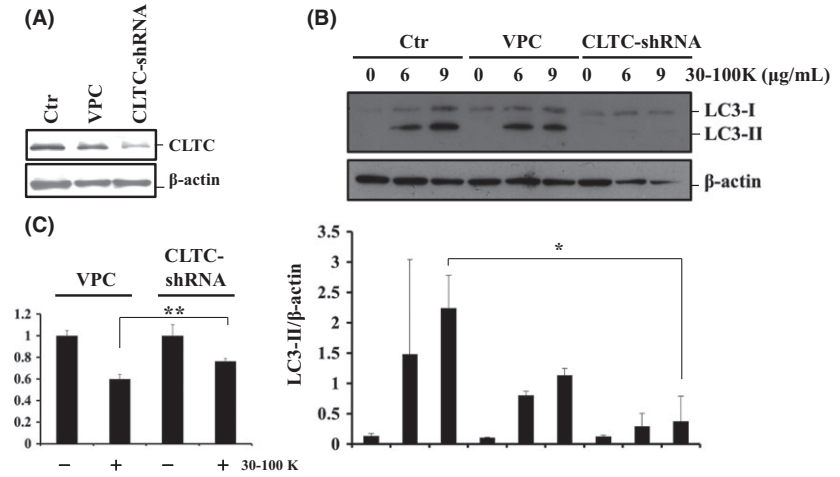

FIGURE 6 CLTC knockdown inhibits ANE 30-100K-induced autophagy. (A) CLTC expression level of Ctr, VPC, and CLTC-shRNA CE81T/VGH cells was analyzed by Western blot with CLTC and β-actin antibodies. (B) Ctr, VPC, and CLTC-shRNA cells treated with indicated concentrations of ANE 30-100K were analyzed by Western blot as (A). (C) VPC and CLTC-shRNA CE81T/VGH cells treated with or without ANE 30-100K (10 μg/mL) were subjected to XTT assay. All the experiments have been repeated three times with similar results. **P*<.05, ***P*<.01

The data showed that lentivirus-mediated shRNA interference reduced DNM1 protein expression in CE81T/VGH cells, while the expression level of DNM1 in cells infected with virus carrying empty plasmid (virus and plasmid control, VPC) was similar to non-infected control (Ctr) cells (Figure 5A). Moreover, ANE 30-100K-induced LC3-II accumulation was evidently inhibited in DNM1-knocked down cells compared to Ctr and VPC cells (Figure 5B). LC3-II level in ANE 30-100K (6 μg/mL)-treated DNM1-knocked down cells was significantly lower than Ctr and VPC cells.

Similarly, lentivirus-mediated shRNA interference also reduced clathrin heavy chain (CLTC) expression in CLTC shRNA-infected CE81T/VGH cells than Ctr and VPC cells (Figure 5C). ANE 30-100K-induced LC3-II accumulation was barely detectable in CLTC-knocked down cells compared to Ctr and VPC cells (Figure 5D). LC3-II level in ANE 30-100K (9 μg/mL)-treated CLTC-knocked down cells was significantly lower than Ctr cells.

Collectively, these results indicated the involvement of dynamin-dependent, clathrin-mediated endocytosis in ANE 30-100K-induced autophagy.

4 | DISCUSSION

In this study, we further provided evidences that ANE 30-100K-induced autophagy can be hindered by benzyl alcohol, dynasore, and shRNAs of DNM1 and CLTC, linking firstly this particular type

of autophagy with clathrin-mediated endocytosis. It is thought that the AIAI contained in ANE 30-100K may be engulfed by cells through this mechanism to stimulate autophagy. Whether there is a specific membrane receptor for AIAI remains to be elucidated.

The reason why pre-treatment of dynasore caused evident LC3-II accumulation in OECM-1 cells remains to be confirmed (Figure 4). However, if dynasore alone stimulated autophagy in OECM-1 cells, LC3-II levels induced by coculture with both dynasore and ANE 30-100K would be similar to or higher than those treated with ANE 30-100K alone. Rather, LC3-II levels in OECM-1 (as well as in CE81T/VGH) cells were significantly decreased by pre-treatment of dynasore than those treated with ANE 30-100K alone (Figure 4). It is, therefore, speculated that dynasore might somehow inhibit LC3-II degradation in OECM-1 cells. However, this speculation requires further studies.

In addition to clathrin-mediated endocytosis, there are at least three other types of endocytosis pathways: caveolin-mediated endocytosis, macropinocytosis, and phagocytosis.[27] Clathrin-mediated endocytosis forms small vesicles (~100 nm in diameter) coating with a complex of proteins that are mainly associated with clathrin, called clathrin-coated vesicles (CCVs). Caveolin-mediated endocytosis creates similar sized vesicles (50-100 nm in diameter), called caveolae. Caveolae represent the most common non-clathrin-coated vesicles, found on the surface of many cell types. Macropinocytosis often takes place in ruffled plasma membrane regions and forms larger vesicles (0.5-5 μm in diameter) containing more extracellular fluid and molecules than CCVs. Phagocytosis is used by cells to internalize particles larger than ~0.75 μm in diameter, such as cell debris and microorganisms.[28,29] The roles of caveolae, macropinocytosis, and phagocytosis in ANE 30-100K-induced autophagy are currently unknown.

When autophagy is initiated, Atg1 complex is activated, followed by autophagosome formation (vesicle nucleation). This nucleation process is in turn activated by a class III phosphatidylinositol 3-kinase complex including Vps34, Vps15, and Beclin 1 (Atg6). Then, two ubiquitin-like systems, Atg5-Atg12 and LC3 (Atg8), mediate the expansion and sealing of autophagosomal membrane. As introduced, the fate of autophagosomes can then fuse with endocytic vesicles (early endosomes, multivesicular bodies, and late endosomes) before the fusion with lysosomes.[19] It is speculated that endocytosis blockade might eliminate the source of endocytic vesicles that can fuse with autophagosomes and thus somehow attenuate the execution of ANE 30-100K-induced autophagy.

Overlapping of autophagy and endocytosis pathways seems to imply that these two pathways may act cooperatively or synergistically to degrade engulfed materials. However, their relationship may be more complicated because a recent research demonstrated that Annexin A5 promotes the fusion of autophagosome and lysosome and stimulates autophagy, but inhibits endocytosis.[30]

In conclusion, our current study suggests that the AIAI may induce autophagy through clathrin-mediated endocytosis.

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CONFLICT OF INTEREST STATEMENT

We declare no conflict of interest on this manuscript.

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