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Abstract Areca nut (AN) is recognized as a human carcinogen; however, few studies of the cytotoxic effects of AN ingredients on cells have been reported. In Taiwan, AN, lime and inflorescence of *Piper betle* are the common

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Keywords (separated by '-') Arecoline - Areca nut - Apoptosis - Autophagy - Mammalian target of rapamycin (mTOR)

Footnote Information Shyun-Yeu Liu, Mei-Huei Lin, Yu-Rung Hsu and Ya-Yun Shih contributed equally to this work.
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Arecoline and the 30–100 kDa fraction of areca nut extract differentially regulate mTOR and respectively induce apoptosis and autophagy: a pilot study

Shyun-Yeu Liu · Mei-Huei Lin · Yu-Rung Hsu · Ya-Yun Shih · Wei-Fan Chiang · Chin-Hai Lee · Da-Shiung Chou · Young-Chau Liu

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Keywords Arecoline · Areca nut · Apoptosis · Autophagy · Mammalian target of rapamycin (mTOR)

Shyun-Yeu Liu, Mei-Huei Lin, Yu-Rung Hsu and Ya-Yun Shih contributed equally to this work.

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Introduction

Betel quid (BQ) chewing may represent one of the most popular oral habits with about 600 million users worldwide, and is thought to cause oral leukoplakia, oral submucous fibrosis, and oral cancer [1]. In Taiwan, BQ is usually composed of areca nut (AN, *Areca catechu* L.) and lime, with or without a piece of inflorescence of *Piper betle* (IPB) or *Piper betle* leaf. Around 10% of the Taiwanese population (2–2.8 million people) has this oral habit. Clinical observations support that habitual BQ chewing raises the risk of oral [1], pharynx [2, 3], esophagus [4, 5], liver [6, 7], and stomach [8] cancers. Therefore, mechanisms implicated in the cytotoxic impact of AN ingredients on cells are worthy of study.

Some ingredients of AN and IPB, such as arecoline (Are) and hydroxychavicol, have been shown to induce growth arrest, necrosis, and apoptosis [9–11]. To our knowledge, there is no evidence so far demonstrating the existence of an autophagy-inducing activity in AN constituents. Autophagy is a regulated self-cannibalism, classified as type II programmed cell death, and is preceded by the inhibition of the mammalian target of rapamycin (mTOR) [12]. Numerous hallmarks used for demonstrating autophagy have been developed: for example, the cleavage of the precursor form of microtubule-associated protein 1 light chain 3 (LC3-I) (18 kDa) to the active form LC3-II (16 kDa) and the emergence of autophagic vacuoles (AVs) and acidic vesicles [13].

In our previous study, we found that some matrix metalloproteinases are upregulated by arecoline (Are) and/or extracts of lime, AN, and IPB [14]. Surprisingly, we have recently noticed a unique death pattern induced by the AN extract (ANE) in several types of carcinoma cells from mouth (OECM-1 and SCC-9), bladder (UB-09 and T24), and esophagus (CE81T/VGH) origins. These dying cells exhibit rounding morphology with a shrunken nucleus and empty cytoplasm. Using OECM-1 cells as the experimental model, we partially isolated these activities. The rounding cell-inducing activity in ANE was first traced to the ≥10 kDa fraction (ANE ≥ 10 K) and subsequently the 30–100 kDa fraction (ANE 30–100 K). Only the cell shrinkage activity without rounded morphology was induced by cisplatin, Are, and extracts of lime and IPB. Thus, Are may be a potential apoptosis inducer whereas the ANE, ANE ≥ 10 K, and ANE ≥ 30–100 K fractions may induce an unknown death pathway in OECM-1 cells (data are presented in this study).

We have further tried to verify the different death pathways mediated by these reagents by analyzing morphological change, activation status of caspase-3, cleavage of LC3-I, and emergence of AVs and acidic vesicles in OECM-1 cells. Finally, regulation of the phosphorylation status of mTOR-Ser^{2448} by Are and ANE 30–100 K was also examined.

Materials and methods

Preparation of ANE, ANE ≥ 10 K, lime, and IPB extract

The preparation of ANE was different from other researchers and was similar to that of BQ extract in our previous study [14]. Briefly, ANE was prepared by grinding tender AN (3.6–4.0 g/nut) at RT, and the squeezed juice was centrifuged at 12,000 × *g* for 10 min. The supernatant was then regarded as the ANE. ANE was further centrifuged at 2,900 × *g* for 30 min at 4°C with 10, 30, or 100 kDa-pored membranous concentration tubes. Thus, ANE and fractions of ANE ≤ 10 K, ANE ≥ 10 K, and ANE 30–100 K used in this study were obtained. They were lyophilized, weighed and re-dissolved in H_2O.

The lime and IPB were separated from a commercial BQ and ground in 1 ml H_2O. Both extracts were then prepared by the same method as ANE.

Culture and treatment of cells

The carcinoma cell lines from mouth (OECM-1 and SCC-9), bladder (UB-09 and T24), and esophagus (CE81T/VGH) origins were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Rockville, MD, USA) as described earlier [14]. They were seeded into each well of a 96-well plate (1×10^4 cells/well) for the observation of morphological change, or into each well of a slide chamber for the nuclear staining, immunofluorescent analysis, and acridine orange staining, each observed in triplicate. Alternatively, 6×10^6 cells were seeded onto a 10-cm plate for caspase-3 activity, Western blot, electron transmission microscopy (TEM), and flow cytometrical analysis and subjected to a 24-h treatment with each reagent under serum-free conditions. When analyzing the phosphorylation level of mTOR-Ser^{2448}, cells were cultured in DMEM supplemented with 10% fetal bovine serum (HyClone, South Logan, VT, USA).

To determine the 50% inhibitory concentration (IC_{50}) of each reagent, cells treated with various concentrations of each reagent were analyzed by XTT reagents (Roche Molecular Biochemicals, Basel, Switzerland) as instructed by the manufacturer.

Measurement of nuclear diameter

Treated samples on chamber slides were fixed with 3% paraformaldehyde for 30 min, and treated with 0.1% Triton X-100 for 15 min. The slides were coated with 3% BSA for 1 h and stained with 0.01% Hoechst 33258 for 10 min. The stained cells were photographed under a fluorescent microscope (OLYMPUS DP70), and the

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diameters of 15 randomly chosen nuclei were measured using a micrometer.

Measurement of caspase-3 activation status

Treated OECM-1 cells were incubated with 100-fold diluted anti-active caspase-3 antibody (Sigma-Aldrich, USA) for 1.5 h, followed by further incubation with goat anti-rabbit-IgG antibody coupled with fluorescein isothiocyanate (Sigma-Aldrich) for 40 min. Cells were then incubated with 0.1% propidium iodide for 10 min, and photographed under a fluorescent microscope (OLYMPUS DP70).

For the enzyme activity assay, treated OECM-1 cells (as in the immunofluorescent assay) were lysed in lysis buffer (0.5% NP-40, 50 mM Tris–HCl, pH 7.4). Lysate proteins (100 μg) from each treatment were subjected to caspase-3 activity assay with the caspase-3 Cellular Assay Kit (Biomol International, PA, USA) as instructed by the manufacturer.

Observation of morphological change, AVs, and acidic vesicles

Treated samples were observed and photographed using light microscopy or TEM. For TEM analysis, samples were fixed in 2.5% glutaraldehyde at 4°C for 1 h and soaked in 1% OsO_4 for 20 min. After dehydration in an ethanol gradient [50% (10 min), 75% (10 min), 85% (10 min), and 95% alcohol (2 × 10 min)], samples were treated with propylene oxide (2 × 10 min), impregnated with a 1:1 mixture of propylene oxide/EA (Epon 812:Araldite 502:DDSA:DMP-30 = 1.34:0.93:2.60:0.14, Electron Microscopy Sciences, PA, USA), and embedded in 100% EA. Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were examined by TEM (JEOL JEM-1200EX) at 80 kV. Photographs were made with electron microscopy film 4489 Estar Thick Base (Kodak, NY, USA).

For the observation of acidic vesicles, treated samples were stained with acridine orange (1 μg/ml) for 10 min and photographed under a fluorescent microscope, or flow cytometrically analyzed by 525 nm (FL1) and 575 nm (FL2) with FACSCalibur (BD Biosciences, NJ, USA).

Western blot analysis

Lysate proteins (60 μg) from treated OECM-1 cells were subjected to Western blot analysis following the protocols previously described [14]. Briefly, the first antibodies used against LC3 (MBL International Corporation, MA, USA), mTOR, and phosphor-mTOR-Ser^{2448} (Cell Signaling Technology, Inc., MA, USA), as well as β-actin proteins (Sigma-Aldrich) were 1,000- and 10,000-fold diluted, respectively. The secondary antibody was goat anti-mouse-IgG or goat anti-rabbit-IgG coupled with horseradish peroxidase (Sigma-Aldrich) and 10,000-fold diluted.

Statistical analysis

Two groups of data presented as mean ± sd were analyzed by Student's t-test. A value of $P < 0.05$ was regarded as statistically significant.

Results

Characterization of the unique cytotoxic effect of ANE

Extracts of the three BQ components (lime, AN, and IPB), Are, and cisplatin exhibited cytotoxic effects on OECM-1 cells after 24 h of treatment. The average IC_{50} of these reagents under serum-free conditions is listed in Table 1. OECM-1 cells treated with the IC_{50} of these reagents showed a shrunken phenotype, except that the ANE-treated cells exhibited rounding membrane and condensed nucleus-like spheres (Fig. 1a). The rounding cell-inducing activity of ANE was also observed in other carcinoma cells, including SCC-9, UB-09, T24, and CE81T/VGH (Fig. 1b). To address the molecular weight of this activity, the ANE was separated into two fractions by passing through a centrifugal concentration tube with 10 kDa pores. The results showed that this activity was in the ANE ≥ 10 K fraction (1,120 μg/ml), whereas fractions of ANE ≤ 10 K (1,120 μg/ml), lime ≥ 10 K (400 μg/ml), lime ≤ 10 K (400 μg/ml), IPB ≥ 10 K (220 μg/ml), and IPB ≤ 10 K (220 μg/ml) only induced cell shrinkage (Fig. 1c).

After treatment with the IC_{100} of ANE and ANE ≥ 10 K for 24 h, most OECM-1 cells exhibited rounding morphology, and each of them contained a shrunk nucleus-like sphere (data not shown). We further subjected these cells to Hoechst 33258 staining, and found that the

Table 1 The average IC_{50} of each reagent against OECM-1 under serum-free conditions

Reagent	IC_{50} (mean ± sd)
Lime extract	396 ± 56 μg/ml
IPB extract	226 ± 48 μg/ml
ANE	37 ± 13 μg/ml
ANE ≥ 10 K	590 ± 459 μg/ml
ANE ≤ 10 K	2412 ± 1342 μg/ml
ANE 30-100 K	15 ± 5 μg/ml
Are	205 ± 51 μg/ml
Cis	43 ± 5 μM

IC_{50}, 50% inhibitory concentration; ANE, areca nut extract; Are, arecoline; Cis, cisplatin

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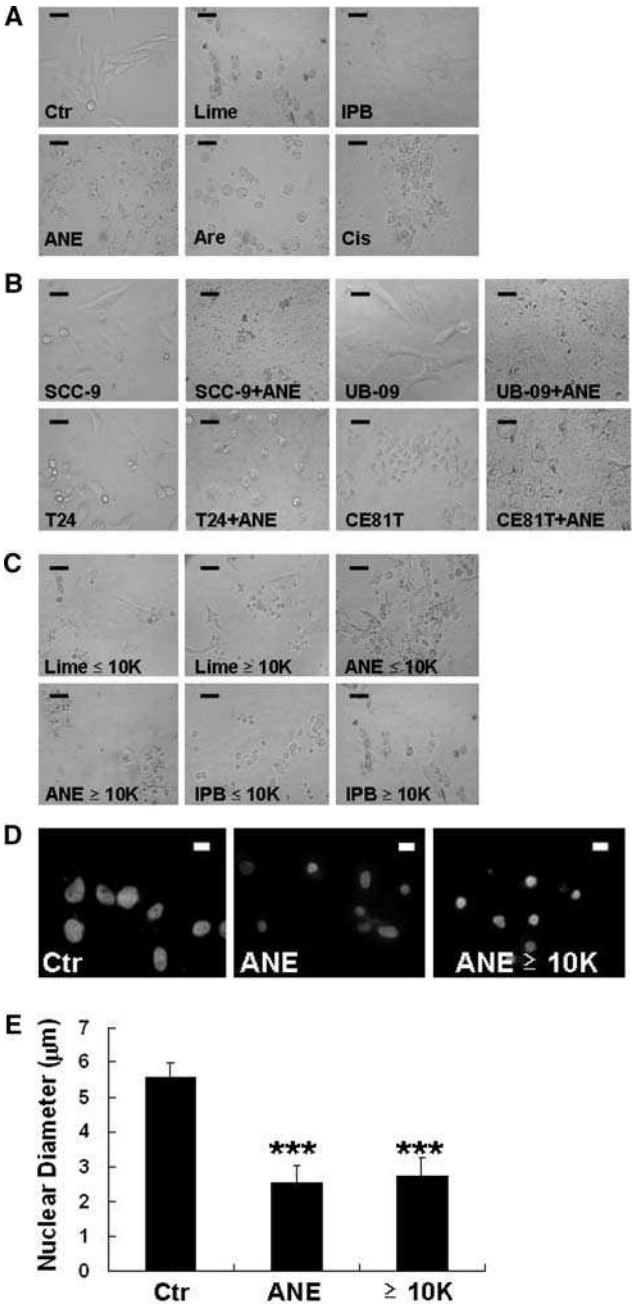

Fig. 1 Unique death pattern induced by 24-h treatment of ANE and ANE ≥ 10 K. (**a**–**c**) Phase contrast images of control (Ctr) or treated cells; bar = 10 μm. (**a**) OECM-1 cells versus IC_{50} of lime, IPB, ANE, Are, or cisplatin (Cis). (**b**) ANE (20 μg/ml) versus SCC-9, UB-09, T24, or CE81T/VGH (CE81T) cell lines. (**c**) OECM-1 cells versus lime ≤10 K (400 μg/ml) or lime ≥ 10 K (400 μg/ml), ANE ≤ 10 K (1120 μg/ml) or ANE ≥ 10 K (1,120 μg/ml), and IPB ≤ 10 K (220 μg/ml) or IPB ≥ 10 K (220 μg/ml) fractions. (**d**) Hoechst 33258 staining of OECM-1 cells versus IC_{100} of ANE or ANE ≥ 10 K fraction; bar = 5 μm. (**e**) Nuclear diameter of OECM-1 cells treated with ANE or ANE ≥ 10 K; bar: mean ± sd of nuclear diameter from three or more experiments; *** $P < 0.001$

suspected nucleus-like spheres were stainable by this dye (Fig. 1d). Compared to the non-treated control cells, the nuclei were significantly smaller in ANE- and ANE ≥ 10 K-treated cells (Fig. 1e; $P < 0.001$ in both cases, $n = 15$).

Induction of caspase-3 activation by ANE and Are

Caspase-3 is known to be one of the down-stream effector caspases of apoptosis [15]. Although Are has been shown to induce apoptosis in KB cells [10], it remains uncertain whether it induces apoptosis in OECM-1 cells. Furthermore, it is not known whether this protease plays a role in ANE- and ANE ≥ 10 K-mediated signals. Thus, the induction of caspase-3 activation by Are, ANE, ANE ≥ 10 K, and cisplatin (used as the positive control) was analyzed. First, immunofluorescent analysis with anti-active caspase-3 antibody revealed that cells treated with the IC_{50} of ANE, Are, and cisplatin for 24 h exhibited stronger staining than those of control and ANE ≥ 10 K (IC_{50})-treated cells (Fig. 2a). Secondly, protease activity assessed with a commercial kit also demonstrated a significant elevation of caspase-3 activity in the lysates of ANE-, Are-, and cisplatin-treated cells compared to that of ANE ≥ 10 K-treated cells (Fig. 2b; $P < 0.05$, 0.01, and 0.05, respectively).

Induction of cellular autophagy by ANE, ANE ≥ 10 K, and ANE 30–100 K

To verify whether ANE ≥ 10 K induces autophagy, the ratio of LC3-II/LC3-I, a specific index of autophagy, was analyzed by Western blot. The results showed that the IC_{50} of ANE (Fig. 3a) and ANE ≥ 10 K treatment significantly enhanced LC3-I cleavage in OECM-1 cells after 24 h (Fig. 4c, d; $P < 0.05$ and 0.01, respectively). Moreover, ANE ≥ 10 K was further shown to dose-dependently increase the LC3-II/LC3-I ratio (Fig. 3a).

Further, OECM-1 cells were subjected to TEM analysis after 24-h treatment of ANE (IC_{50}) and ANE ≥ 10 K (IC_{50}). The results showed that numerous AVs appeared in ANE [Fig. 3b; ANE (1)]- and ANE ≥ 10 K [Fig. 3b; ≥10 K (1)]-treated cells, whereas the Are (IC_{50})-treated cells exhibited peri-nuclear chromatin condensation [Fig. 3b; Are (1)] and micronucleation [Fig. 3b; Are (2)]. Interestingly, in some ANE- and ANE ≥ 10 K-treated cells, the cytoplasmic substances, including nucleus, were massively degraded before the disintegration of the cell membrane [Fig. 3b; ANE (2) and ≥10 K (2)].

After treating OECM-1 cells for 24 h, the IC_{50} of ANE ≥ 10 K is about 30-fold greater than ANE under serum-free conditions (Table 1), meaning that the autophagy-inducing component was not enriched in the ANE ≥ 10 kDa

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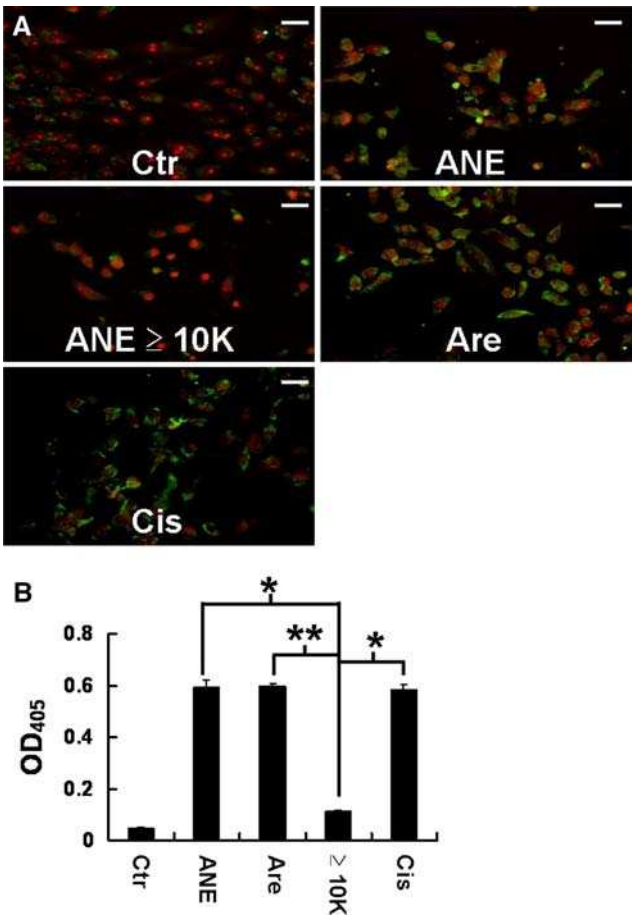

Fig. 2 Activation of caspase-3 after 24-h treatment. (**a**) Anti-active caspase-3 immunofluorescent staining of OECM-1 cells versus control (Ctr) or versus IC_{50} of ANE, ANE $\geq$ 10 K, Are, or cisplatin (Cis); bar = 20 μm. (**b**) Measured caspase-3 activity from protein lysates (100 μg) of cells treated as in (**a**); Bar: mean ± sd of OD_{405} absorbance from three or more experiments; * $P < 0.05$, ** $P < 0.01$

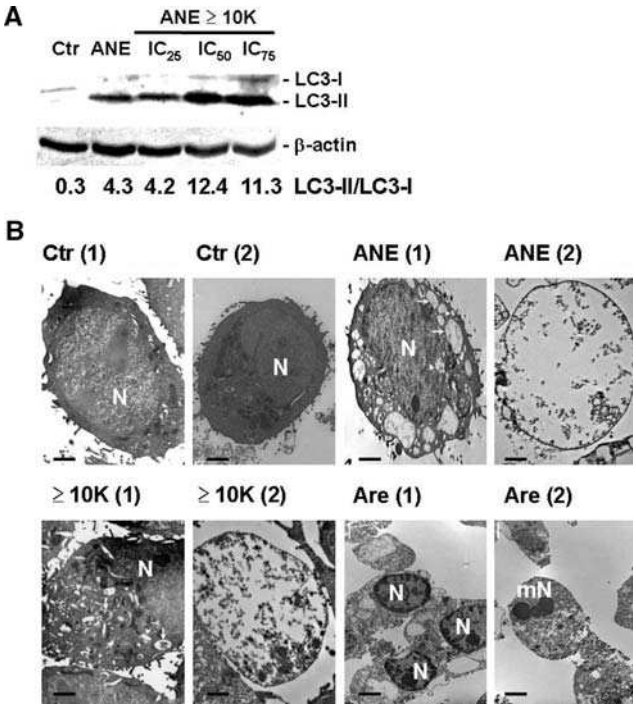

Fig. 3 Induction of cellular autophagy after 24-h treatment. (**a**) Western blot of LC3 proteins from treated OECM-1 cells. The LC-3-II/LC3-I ratio is listed under each treatment. (**b**) TEM micrographs of OECM-1 cells versus IC_{50} of ANE, ANE $\geq$ 10 K, or Are. Ctr: control; N: nucleus; Arrows and arrowheads: AVs, including residual digested material and empty vacuoles, respectively; mN: micronucleated nucleus; bar = 2 μm

fraction. We further used concentration tubes with 30 and 100 kDa pores to separate ANE, and found that the rounding cell-inducing activity was localized in the 30–100 kDa fraction (ANE 30–100 K; Fig. 4a). The IC_{50} of ANE 30–100 K against OECM-1 cells was 15 ± 5 μg/ml (Table 1), about 2.5-fold lower than that of ANE. Thus, the effectiveness of ANE $\geq$ 10 K in autophagy induction may be interfered with by 10–30 kDa molecules in ANE. ANE 30–100 K (IC_{50}) also induced numerous AVs in OECM-1 cells (Fig. 4b), and significant LC3-I cleavage (Fig. 4c, d; $P < 0.05$).

Induction of acidic vesicles by ANE, ANE $\geq$ 10 K, and ANE 30–100 K

Acidic vesicles are also one the autophagy hallmarks. We found that cells treated with the IC_{50} of ANE, ANE $\geq$ 10 K, and ANE 30–100 K for 24 h, as well as those cultured in non-glucose medium (used as the positive control), exhibited numerous acidic vesicles after acridine orange staining. In contrast, the acidic vesicles were barely detected in non-treated control and Are (IC_{50})-treated cells (Fig. 5a). The percentages of acidic vesicle-containing cells were 1.0%, 88.0%, 90.2%, 88.9%, and 16.3% in control, glucose-starved, ANE-, ANE 30–100 K-, and Are-treated cells as analyzed by flow cytometry, respectively (Fig. 5b).

Differential regulation of the mTOR-Ser^{2448} phosphorylation by Are and ANE 30–100 K

The phosphorylation status of mTOR-Ser^{2448} was recently shown to be correlated with the extent of autophagy [16]. We were thus interested to assess whether it is differentially regulated by Are and ANE 30–100 K. However, the phosphorylated mTOR-Ser^{2448} could only be detected when OECM-1 cells were cultured with 10% serum (Fig. 6a, b, d; lane 1). Under this conditions and to achieve 50% cytotoxicity, the concentrations of Are, ANE, ANE 30–100 K, ANE $\geq$ 10 K, and ANE $\leq$ 10 K were raised to 297, 200, 313, 1,760, and 2,000 μg/ml, respectively. Note that ANE $\leq$ 10 K, up to 8 mg/ml, was not cytotoxic to OECM-1 in the presence of 10% serum. The results showed that the

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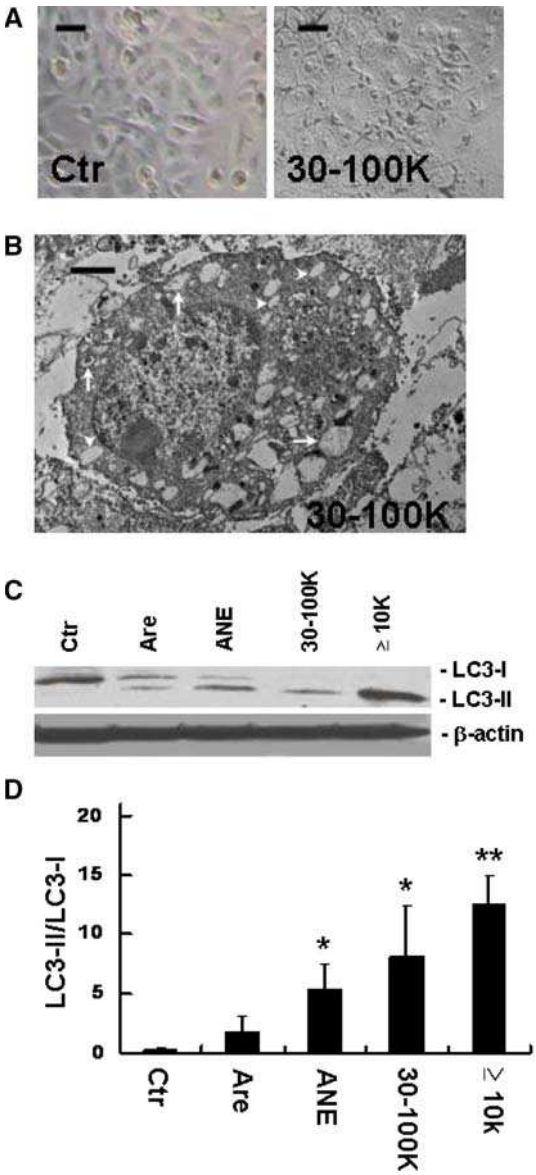

Fig. 4 Induction of cellular autophagy by ANE 30-100 K after 24-h treatment. (**a**) Phase contrast images of control (Ctr) and ANE 30–100 K (IC_{50})-treated OECM-1 cells. bar = 10 μm. (**b**) Electron micrograph of an ANE 30–100 K (IC_{50})-treated OECM-1 cell. Arrows and arrowheads: AVs, including residual digested material and empty vacuoles, respectively. bar = 2 μm. (**c**) Western blot of LC3 proteins from OECM-1 cells treated with IC_{50} of Are, ANE, ANE 30–100 K or ANE ≥ 10 K. (**d**) Average LC3-II/LC3-I ratios (mean ± sd) from three experiments. * $P < 0.05$, ** $P < 0.01$

phosphorylation level of mTOR-Ser^{2448} was significantly inhibited after 24-h treatment with ANE 30–100 K (Fig. 6a, c; $P < 0.05$). In contrast, Are treatment did not attenuate the phosphorylation level at this site within 24 h (Fig. 6b, c). ANE ≥ 10 K, but not Are, ANE and ANE ≤ 10 K, decreased the phosphorylation level of mTOR-Ser^{2448} to a similar extent as that of ANE 30–100 K (0.09/0.16) (Fig. 6d).

Discussion

In addition to the autophagic markers presented above, the typical double-layered structure of autophagosome was occasionally observed after the 24-h ANE (IC_{50}) treatment (Supplement Figure). Taken together, we have demonstrated that ANE, ANE ≥ 10 K, and ANE 30–100 K are capable of triggering cellular autophagy. To our knowledge, this study has provided the first evidences of an autophagy-inducing AN ingredient (AIAI). In addition to KB cells [10], Are was also shown to induce apoptosis in OECM-1 cells (Figs. 2 and 3b) indicating the existence of both apoptosis- and autophagy-inducing ingredients in AN. However, when cells treated with ANE, they die in an autophagy manner despite the activation of apoptosis program like caspase-3 cleavage (Fig. 2). Thus, AIAI may induce a dominant autophagic death in the presence of apoptosis-inducing factors.

The shrunken nuclei in OECM-1 cells may represent the unique autophagic death pattern induced by AIAI. It might be thought that the degradation of nuclear contents is strictly "off-limits", given the importance of maintaining the integrity of genes, but it has been reported that partial degradation of the nucleus may occur in yeast [17]. In *Saccharomyces cerevisiae*, a process called "piecemeal microautophagy of the nucleus" (PMN) pinches off and degrades nonessential portions of the nucleus. PMN is a constitutive process induced to high levels by starvation or rapamycin and occurs at nucleus–vacuole junctions [18]. It might be reasonable to speculate that nuclear contents may be degraded progressively, which reduces nuclear size in the process of autophagic death. Clarke et al. observed that during the autophagic death of isthmo-optic neurons in chick embryos, nuclei of the dying neurons lost more than half of their DNA content and became more electron dense and shrank [19]. Here, we have illustrated that ANE and ANE ≥ 10 K also induced significant nuclear shrinkage (Fig. 1d, e). Taken together, it is suggested that the nucleus may be shrunk before its disintegration in different modes of autophagy.

It is speculated that the rounding morphology of SCC-9, UB-09, T24, and CE81T/VGH cells induced by ANE treatment reflects the initiation of an autophagic pathway in these different cancer cells. We have recently observed the same morphological change and the emergence of acidic vacuoles in two normal human fibroblasts (WI-38 and MCR-5) after ANE 30–100 K treatment (data not shown). Thus, AIAI may have the potential to induce autophagy in both malignant and normal cells.

Although various modes of autophagy conspire to degrade virtually every compartment of the eukaryotic cell, the plasma membrane and nucleus may be the most resistant compartments during autophagic degradation. Szende et al.

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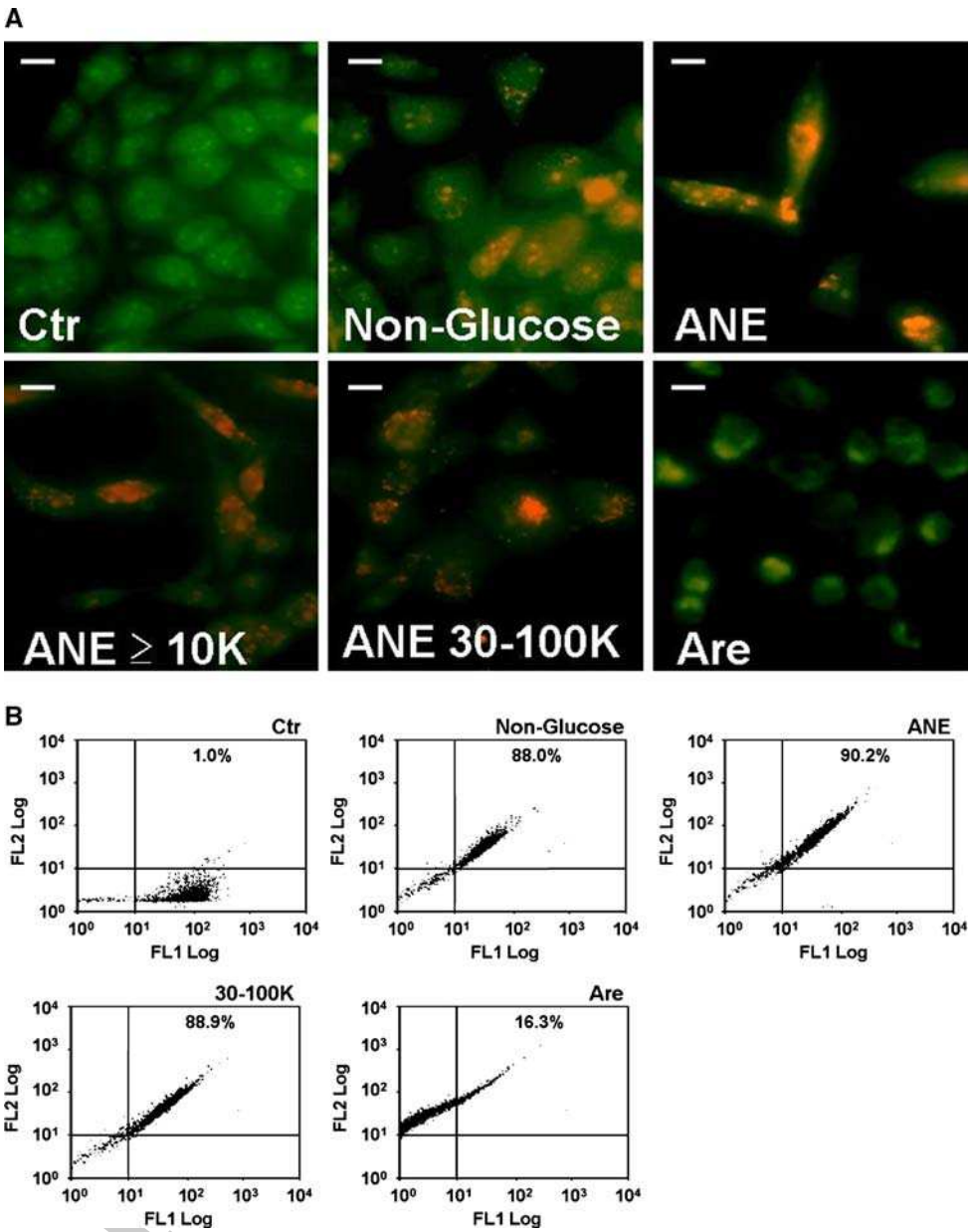

Fig. 5 Induction of acidic vesicles after 24-h treatment. (**a**) Acridine orange staining of non-treated negative control (Ctr), glucose-starved positive control (Non-glucose), and IC_{50} of ANE-, ANE ≥ 10 K-, ANE 30–100 K-, and Are- treated OECM-1 cells; bar = 5 μm. (**b**) Flow cytometry of the cell populations in (**a**)

observed that these two compartments were resistant to L-leucine methyl ester-induced autophagic digestion in mouse peritoneal macrophages [20]. Gómez-Santos et al. also demonstrated that the plasma membrane and nucleus maintained their integrity in the dopamine-induced autophagy in human neuroblastoma cells [21]. In our study, the cell membrane and nucleus also seemed to be the dominant remnants in the AIAI-induced autophagy as revealed by light microscopy (Figs. 1a–c and 4a). Furthermore, the degradation of the nucleus before the rupture of the plasma membrane after ANE and ANE ≥ 10 K treatments [Fig. 3b, ANE (2) and ≥10 K (2)] suggests that in the AIAI-induced autophagy, the cell membrane may be the most stable compartment in some OECM-1 cells.

Increase of caspase-3 activity may not necessarily result in cellular apoptosis. Sadasivan et al. recently showed that withdrawing amino acids from the culture medium of PC-12 cells activated this protease but induced autophagic cell death [22]. Similarly, we have also demonstrated that although caspase-3 was evidently activated by ANE (Fig. 2), meaning that the apoptotic signals might have been turned on by the apoptosis-inducing ingredients, the cells underwent autophagy. The AIAI (most likely in the ANE 30–100 K fraction) may thus trigger the dominant autophagic phenotype in the presence of apoptosis-inducing molecules, like hydroxychavicol and Are.

Artificial inhibition of mTOR by rapamycin, for example, often results in autophagy [23]. We have recently

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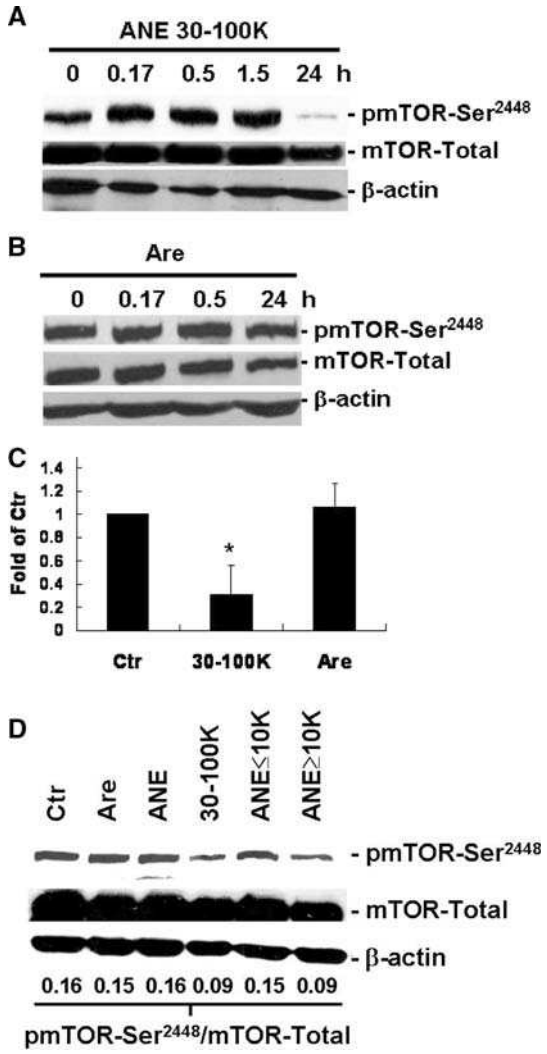

Fig. 6 Effects of Are and ANE 30–100 K on mTOR-Ser^{2448} phosphorylation. Western blots of phosphorylated mTOR-Ser^{2448} (pmTOR-Ser^{2448}), total mTOR (mTOR-Total), and β-actin in OECM-1 cells treated with IC_{50} of ANE-30–100 K (**a**) or Are (**b**) for the indicated periods under 10% serum supplement. (**c**) Average pmTOR-Ser^{2448}/mTOR-Total ratios (means ± SD) from three experiments after treatment with IC_{50} of ANE 30–100 K or Are for 24 h. (**d**) Proteins analyzed as in (**a**) in cells treated with Are (297 μg/ml), ANE (200 μg/ml), ANE 30–100 K (313 μg/ml), or ANE ≥ 10 K (1,760 μg/ml), as well as ANE ≤ 10 K (2,000 μg/ml). Ctr: control; * $P < 0.05$

found that the IC_{50} of 30–100 K against OECM-1 is significantly reduced to 8.07 ± 1.73 μg/ml ($P < 0.05$) in the presence of the mTOR specific inhibitor, rapamycin (10 nM) (data not shown). Therefore, together with the ability to inhibit mTOR-Ser^{2448} phosphorylation, ANE 30–100 K might probably induce autophagy in part through the inhibition of mTOR. On the other hand, although Are does not affect the phosphorylation level of mTOR-Ser^{2448}, whether it interferes with other regulators along the mTOR pathway remains unknown.

With 10% serum supplement, ANE 30–100 K mildly increases the level of phosphorylated mTOR-Ser^{2448} (Fig. 6a). Our unpublished data revealed that under serum-free conditions, the phosphorylated mTOR-Ser^{2448} molecule is barely detectable and can be significantly induced by ANE 30–100 K within 1 h. Furthermore, short-term (<2 h) treatment of ANE (IC_{50}) and ANE 30–100 K (IC_{50}) stimulates ERK1/2 phosphorylation, and 24-h treatment of low concentrations (<IC_{10}) of ANE and ANE 30–100 K exhibit a mild mitogenic effect on cultured cells. Thus, ANE and ANE 30–100 K, depending on their dosage and treating duration, may be endowed with a biphasic capacity to induce both cell growth and death. It might be possible that although ANE 30–100 K, due to its mitogenic effect, initially upregulates the phosphorylation of mTOR-Ser^{2448}, it interferes the responsible kinase/phosphatase system causing the dephosphorylation of mTOR-Ser^{2448} in the AIAI-induced autophagy program after prolonged treatment (24 h).

For an unknown reason, ANE treatment failed to inhibit the phosphorylation of mTOR-Ser^{2448} (Fig. 6d), but still triggered autophagic cell death (Figs. 3 and 5). Although more studies are required, it is suggested that inhibition of mTOR-Ser^{2448} phosphorylation by the AIAI molecules (30–100 kDa) might be interfered by the small molecules (≤10 kDa) of ANE. Still, the AIAI might trigger the autophagic pathway downstream of mTOR resulting in the autophagic cell death.

The ANE 30–100 K contains mainly carbohydrates as analyzed by the Molish's Test and Seliwanoff's Test, and trace amount of proteins (1.2 ± 0.2%). The exact percentage of the containing carbohydrates has been difficult to be determined for the interference of OD_{405} absorbance by the yellow-to-brown pigment (depending on its concentration) (data not shown). Furthermore, the cytotoxicity of ANE 30–100 K is significantly attenuated after cellulase digestion (data not shown). Thus, the AIAI in this fraction is speculated to be polysaccharide(s).

In conclusion, the identification of apoptosis and autophagy induced by Are and ANE ≥ 10 K (and ANE 30–100 K) respectively may serve as an ideal model to further delineate the difference and interaction between these two death pathways in our future studies.

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