Ampelopsin-induced autophagy protects breast cancer cells from apoptosis through Akt-mTOR pathway via endoplasmic reticulum stress

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Our previous study has shown that ampelopsin (AMP), a flavonol mainly found in *Ampelopsis grossedentata*, could induce cell death in human breast cancer cells via reactive oxygen species generation and endoplasmic reticulum (ER) stress pathway. Here, we examined whether autophagy is activated in AMP-treated breast cancer cells and, if so, sought to find the exact role and underlying molecular profile of autophagy in AMP-induced cell death. Our results showed that AMP treatment activated autophagy in MDA-MB-231 and MCF-7 breast cancer cells, as evidenced by the accumulation of autophagosomes, an increase of microtubule-associated protein 1 light chain 3 beta-2 (LC3B-II) and the conversion of LC3B-I to LC3B-II, the degradation of the selective autophagic target p62 ∕SQSTM1, and the formation of green fluorescent protein (GFP)-LC3 puncta. Blockage of autophagy augmented AMP-induced cell death, suggesting that autophagy has cytoprotective effects. Meanwhile, AMP treatment suppressed Akt-mammalian target of rapamycin (mTOR) pathway as evidenced by dose- and time-dependent decrease of the phosphorylation of Akt, mTOR and ribosomal protein S6 kinase (p70S6K), whereas Akt activator insulin-like growth factor-1 (IGF-1) pretreatment partially restored Akt-mTOR pathway inhibited by AMP and decreased AMP-inuduced autophagy, signifying that AMP activated autophagy via inhibition of the Akt-mTOR pathway. Additionally, blocking ER stress not only reduced autophagy induction, but also alleviated inhibition of the Akt-mTOR pathway induced by AMP, suggesting that activation of ER stress was involved in induction of autophagy and inhibition of the Akt-mTOR pathway. Taken together, these findings indicate that AMP induces protective autophagy in human breast cancer cells through Akt-mTOR pathway via ER stress.

A number of studies have suggested that chemotherapeutic drugs can induce both apoptosis and autophagy, and autophagy is a prospective target for cancer therapy.[1,2] However, these studies report conflicting roles of autophagy in drug-induced cancer cell death, with some studies concluding that it protects cells from apoptosis, and others reporting that it promotes cell death.[3–5] Therefore, it is necessary to clarify the exact role of autophagy induced by anti-cancer agents.[6,7]

Breast cancer has become the second leading cause of cancer-related death among women worldwide. Although concerted efforts have been made in breast cancer therapy, for the year 2013, it is estimated that there were over 230 000 new cases and over 39 000 deaths from breast cancer in the USA.[8] New therapeutic drugs and strategies need to be explored urgently. Natural compounds have been considered as major resources of attractive chemotherapeutic candidates for cancer.[9–11] Ampelopsin (AMP) belongs to flavonids and is the major bioactive component extracted from the Chinese medicinal herb *Ampelopsis grossedentata*, which is widely grown in South China and its

tender leaves and stems are used as a healthy tea product.[12–14] Our previous study found that AMP treatment inhibits cell growth and induces cell apoptosis in MCF-7 and MDA-MB-231 human breast cancer cells, but not in human non-malignant breast epithelial cells MCF-10A.[15] It is not known whether AMP can induce autophagy. If it could induce autophagy, we seek to understand autophagy's role in AMP-induced breast cancer cell apoptosis and the underlying molecular mechanism of AMP-induced autophagy.

Accumulating studies indicated that autophagy is negatively controlled by the mammalian target of rapamycin (mTOR) kinase,[16,17] and inhibition of the Akt-mTOR pathway has been recently confirmed to lead cell growth inhibition along with activation of autophagy in cancer cells.[18–20] Endoplasmic reticulum (ER) stress is considered to be an important regulator of several cellular pathological processes, including cancer cell death pathways in response to anticancer drugs.[21] Autophagy has been shown to be an adaptive response during ER stress, consequently helping cells to restore ER homeostasis, or contrib-

uting to ER stress-induced cell death.[22,23] Numerous experiments have reported that the Akt-mTOR pathway is associated with ER stress-induced apoptosis and also implicated in the induction of autophagy, and the Akt-mTOR pathway can be negatively regulated in cancer cells in response to anticancer drugs.[24,25] Our pervious study has shown that anti-tumor effects of AMP on breast cancer are partially mediated by the ER stress pathway,[15] but whether ER stress induced by AMP could lead to the activation of autophagy remains unclear.

In this study, we found that AMP treatment induced autophagy along with induction of cell apoptosis, and blockage of autophagy significantly augmented AMP-induced cell death. Inhibition of the Akt-mTOR signaling pathway has also been found in both breast cancer cell lines after treatment with AMP. Moreover, ER stress was involved in activation of autophagy and inhibition of the Akt-mTOR signaling pathway induced by AMP. Our results indicate that AMP induces protective autophagy in human breast cancer cells through the Akt-mTOR pathway via ER stress, and that the combination of AMP with autophagy inhibitor may be a useful strategy in enhancing the anti-tumor efficacy of AMP in breast cancer.

Materials and Methods

Antibodies and reagents. The GFP-MAP1 LC3B plasmid was kindly provided by Dr Tamotsu Yoshimori (Department of Cell Biology, National Institute for Basic Biology, Presto, Japan). Ampelopsin (AMP) was bought form Chengdu Must Bio-technology (Sichuan, China) (HPLC ≥ 98%). Rapamycin (Rapa), bafilomycin A1 (Baf A1), 4-phenylbutyric acid (4-PBA), thapsigargin (Thap), 3-methyladenine (3-MA) and antibody against MAP1LC3B were purchased from Sigma-Aldrich (St Louis, MO, USA). Insulin-like growth factor (IGF)-1 was purchased from Peprotech (Peprotech, USA). The LysoTracker Green (LTG) was purchased from Invitrogen (Carlsbad, CA). Antibody against PERK was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against p62/SQSTM1, Beclin-1, phospho-p70S6K (Thr389), p70S6K, phospho-mTOR (ser2448), mTOR were obtained from Cell Signaling Technology (Danvers, Massachusetts, USA).

Cell culture and treatment. Human breast cancer cell lines MCF-7 and MDA-MB-231 were cultured as described by us previously.[15] Stock solution of AMP was prepared in DMSO and an equal volume of DMSO (final concentration 0.1%) was added to the control. When indicated, 4-PBA (2 mM), 3-MA (5 mM), Baf A1 (5 nM), Rapa (100 nM) and Thap (150 nM) were added 2-h before AMP administration, and IGF-1 (100 ng/mL) was given to cells for 1 h before AMP administration.

Cell viability and cell apoptosis measurement. The Cell Counting Kit-8 (CCK-8) (BestBio, Shanghai, China) was used for measure cell viability, and Annexin V-FITC Apoptosis Detection kit (Dojindo Laboratories, Kumamoto, Japan) was used for measure cell apoptosis as described by us previously.[15]

Transmission electron microscopy. Cells were harvested and fixed in a solution containing 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium cacodylate for 2 h, postfixed with 1% OsO_4 for 1.5 h, washed and stained in 3% aqueous uranyl acetate for 1 h. The samples were then washed again, dehydrated with a graded alcohol series, and embedded in Epon-Araldite resin. Ultra-thin sections were cut on a Reichert ultramicrotome, counter-stained with 0.3% lead citrate and examined on a Philips EM420 electron microscope.

siRNA assay. siRNAs for Beclin-1, ATG5 and PERK were purchased from Santa Cruz Biotechnology. According to the manufacturer's protocol, cells were treated as described by us previously.[15]

Western blot. Cell lysates were prepared for western blot analysis as described previously.[15] About 30–50 μg of protein were separated using SDS-PAGE and transferred to PVDF membranes. The membranes were blocked in 5% skim dry milk (2 h), rinsed, incubated with primary antibodies overnight at 4°C, followed by secondary antibodies for 1.5 h at room temperature. The proteins were visualized by ECL exposure to X-ray film.

Confocal microscopy. *Laser scanning confocal microscopy of breast cancer cells transfected with GFP-MAP1LC3B-expressing plasmid.* Cells were transfected with the GFP-MAP1LC3B-expressing plasmid and cultured for 24 h. After being treated with various drugs as indicated for 24 h, cells were washed with PBS, fixed by incubation for 20 min at 37°C in 4% paraformaldehyde, permeabilized with 0.1% (vol/vol) Triton X-100 and washed with PBS containing 2% fetal bovine serum albumin. A Radiance 2000 laser scanning confocal microscope was used for confocal microscopy.

Laser scanning confocal microscopy of cells loaded with LTG. Cells were cultured overnight in cell culture dish, and were then exposed to various treatments as specifically indicated. After washing with fresh medium three times, cells were loaded with green-fluorescing LTG (0.5 μM) for 15 min in humidified air at 37°C. Afterwards, cells were washed with PBS three times and measured by a Radiance 2000 laser scanning confocal microscope.

Statistical analyses. All of the experimental data are expressed as the mean ± SEM, and each experiment was performed at least three times. The statistical analysis was performed by *t*-text and one-way analysis of variance, using SPSS 13.0 (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant, and Turkey–Kramer was applied as *post-hoc* test if $P < 0.05$.

Results

AMP induces autophagy in human breast cancer cells. To investigate whether AMP could induce autophagy in MCF-7 and MDA-MB-231 cells, the autophagic activity was measured by the formation of autophagosomes under transmission electron microscopy (TEM), which is one of the most widely accepted methods for monitoring autophagy.[26] The formation of double-membraned autophagic vacuoles, which is indicative of autophagy induction, was frequently observed in cells treated with 60 μM AMP, but not in control cells (Fig. 1a). To confirm these observations, we further tested the expressions of MAP1LC3B-II (LC3B-II), which is a marker of autophagy,[27] and p62/SQSTM1, which acts as a cargo receptor for autophagic degradation of ubiquitinated targets, by western blotting. AMP treatment induced LC3B activation and time-dependently increased the expression of LC3B, enhanced the conversion of LC3B-I to autophagosome-associated LC3B-II in both breast cancer cell lines, along with downregulation of p62/SQSTM1 (Fig. 1b,d). The formation of punctate spots (puncta) with green fluorescent protein (GFP)-MAPLC3B reporter is a well-characterized marker for visualizing autophagosomes formation and represents the accumulation of LC3B-II on autophagic vesicles.[27] Moreover, we assessed the formation of GFP-MAPLC3B puncta as an autophagic marker under fluorescence microscopy. MCF-7 and MDA-MB-231 cells after treatment with 60 μM AMP for 24 h showed a significant increase in the percentage of cells containing GFP-MAP1LC3B puncta, and autophagy inhibitor 3-methy-
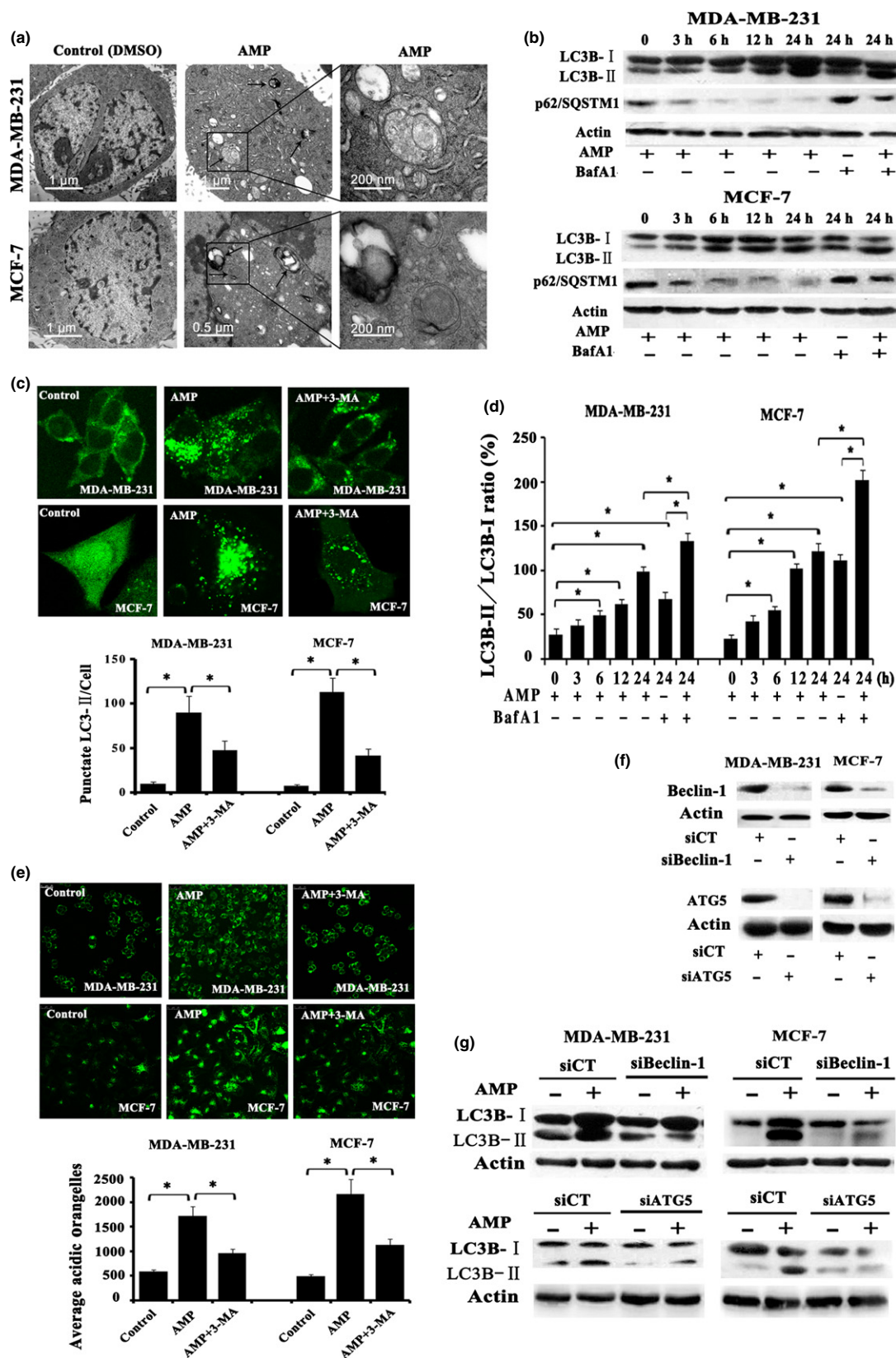
Fig. 1. Ampelopsin activates autophagic flux in human breast cancer cells. (a) Representative transmission electron micrographs demonstrating the ultrastructure of breast cancer cells. Arrow indicates the autophagosomes. (b) Time-dependent effects of Ampelopsin (AMP) on LC3B-II and p62/SQSTM1 expressions of breast cancer cells. (c) Representative images of breast cancer cells by confocal microscopy and the number of GFP-MAP1LC3B puncta were counted. (d) Bar charts show the conversion of LC3B-I to LC3B-II in both breast cancer cells. (e) Representative image of confocal microscopy and the average green LTG fluorescence detected by MFI using the IPP 6.0 software. (f) Efficiency of Beclin-1 and ATG5 knockdown. (g) Effects of silencing of Beclin-1 or ATG5 on AMP-induced autophagy. The results of c, d and e represent the mean ± SEM of three independent experiments. *$P < 0.05$. The results of b, f and g are representative Western blot of three independent experiments.

ladenine (3-MA, 5 mM) partially blocked the increase of GFP-MAP1LC3B puncta induced by AMP ($P < 0.05$) (Fig. 1c).

Since changes in LC3B-II levels could be caused by either autophagosome formation or degradation in lysosomes, it is necessary to clarify whether the increase in LC3B-II levels induced by AMP was due to the increased autophagosome formation or the decreased autophagosome degradation. The levels of LC3B-II and p62/SQSTM1 in both breast cancer cell lines were measured in the presence or absence of the late-stage autophagy inhibitor bafilomycin A1 (Baf A, 5 nM). The data revealed that Baf A1 challenge further increased the expressions of LC3B-II and p62/SQSTM1 in both cell lines (Fig. 1b,d), suggesting that the AMP-induced increase in LC3B-II levels was mainly attributed to the increased autophagosome formation. To further confirm these observations, we inhibited the initiation of autophagasome formation with Beclin-1 or ATG5 siRNA. As expected, AMP failed to induce the accumulation of LC3B-II in cells transfected with siRNA targeting Beclin-1 or ATG5 (Fig. 1f,g). In addition, LysoTracker Green (LTG) was used to assess autophagosome degradation in response to AMP treatment. Interestingly, we found that treatment with AMP led to significantly increased green fluorescence signal compared with control cells, and these changes induced by AMP were partially alleviated by pretreatment with 3-MA ($P < 0.05$) (Fig. 1e). Collectively, these observations provide strong evidence that autophagic activity (autophagic flux) is upregulated in MCF-7 and MDA-MB-231 cells treated with AMP.

Autophagy protects breast cancer cells from AMP-induced apoptotic cell death. In our previous study, we have reported that AMP dose-dependently induced cell death in MCF-7 and MDA-MB-231 cells without in MCF-10A.[15] Many studies revealed that autophagy is involved in the promotion or inhibition of cancer cell survival in response to chemotherapeutic drugs.[28,29] We therefore clarified the exact role of autophagy in the anticancer action of AMP in breast cancer cells. After MCF-7 and MDA-MB-231 cells were pre-treated with the autophagy inhibitor Baf A1 (5 nM) or 3-MA (5 mM), or the autophagy activator rapamycin (Rapa, 100 nM) for 2 h, following treated with 60 μM AMP for 24 h, then cell viability and apoptosis were examined. A significant increase of cell growth inhibition induced by AMP was observed in both breast cancer cell lines after autophagy was inhibited by Baf A1 or 3-MA treatments, in contrast to Rapa treatment (Fig. 2a). In agreement with cell viability data, similar results were found in cell apoptosis. Autophagy inhibitor Baf A1 or
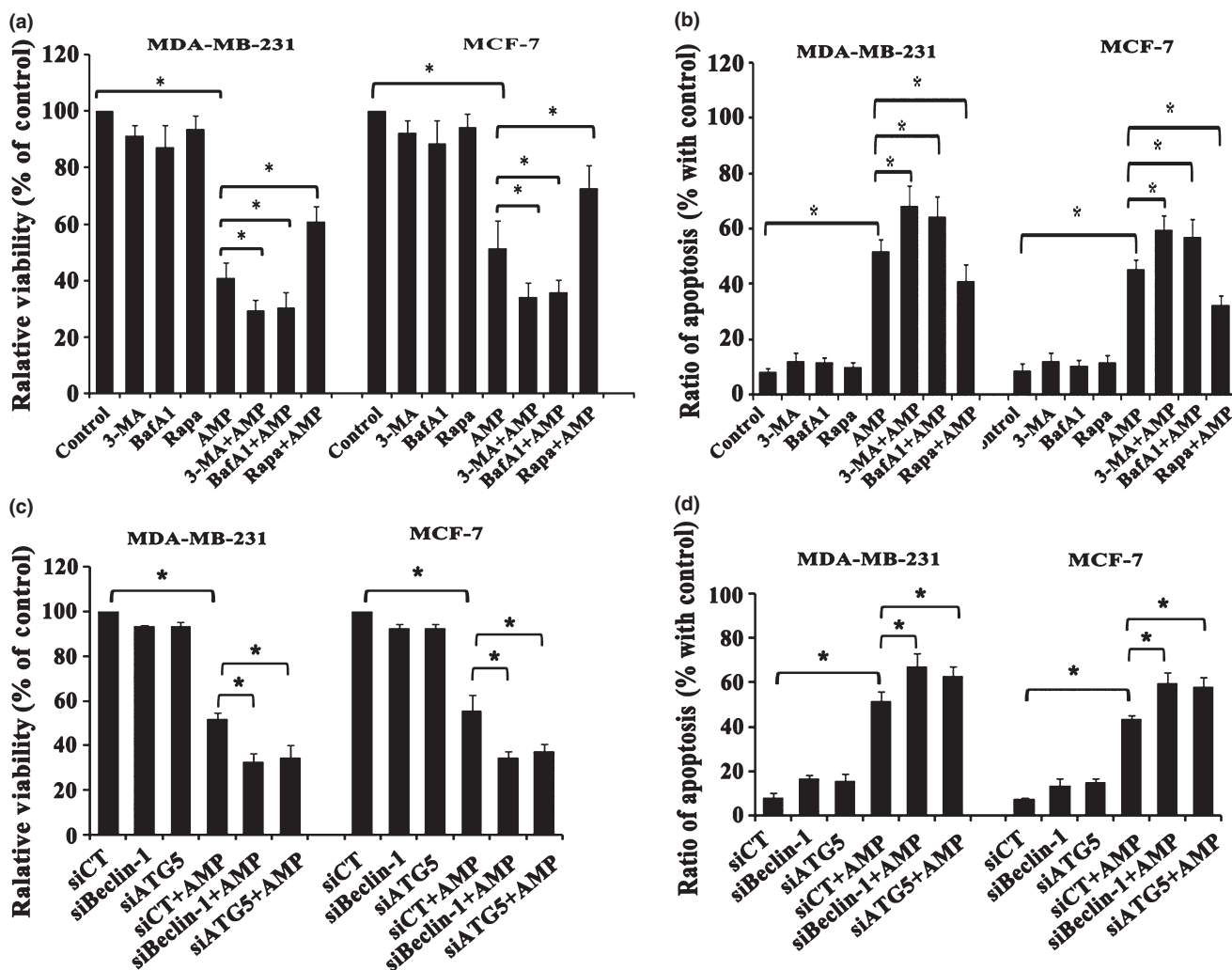

Fig. 2. Autophagy protects against Ampelopsin-induced cell death. (a and b) Effects of autophagy inhibitors or activators on Ampelopsin (AMP)-induced cell growth inhibition and cell apoptosis. (c and d) Effects of silencing of Beclin-1 or ATG5 on AMP-induced cell growth inhibition and cell apoptosis. All results represent the mean ± SEM of three independent experiments *$P < 0.05$.

3-MA treatment significantly enhanced AMP-induced cell apoptosis, in contrast to Rapa treatment (Fig. 2b). To further confirm these data, we next abrogated autophagy by genetic approach using Beclin-1 or ATG5 siRNA. The siRNA-mediated knockdown of Beclin-1 or ATG5 led to increased AMP-induced apoptotic cell death, consistent with the results obtained from studies with the pharmacological inhibitors of autophagy (Fig. 2c,d). These results suggest that autophagy protects against AMP- induced breast cancer cell death.

AMP induces autophagy through inhibition of the AKT-mTOR pathway. Recent reports have suggested that the Akt-mTOR signaling pathway is a major negative regulator of autophagy.[20,30] The suppression of mTOR activity by rapamycin can induce autophagy and also reduce cell growth in different cancer cells.[19,31] Now, we further determined whether the Akt-mTOR pathway is involved in AMP-induced autophagy.

Western blot analysis showed that, along with upregulation of LC3B-II, Beclin-1 and ATG5, AMP dose-dependently and time-dependently decreased the phosphorylation of Akt (Ser473), mTOR (Ser2448) and p70S6K (Thr389) in MCF-7 and MDA-MB-231 cells (Fig. 3a,b). Conversely, pretreatment with Akt activator insulin-like growth factor 1 (IGF-1) markedly attenuated AMP-induced inhibition of phosphorylation of Akt, mTOR and p70S6K in both breast cancer cells (Fig. 3c). Moreover, IGF-1 pretreatment obviously decreased AMP-induced expressions of LC3B-II, Beclin-1 and ATG5 in both breast cancer cells, suggesting that activation of Akt-mTOR pathway could reduce autophagy induced by AMP (Fig. 3d). Additionally, in our study, although IGF-1 treatment alone could promote cell viability, there were no significant changes of cell viability during AMP-induced cell death in both breast cancer cells (data not shown). Together, these results indicate
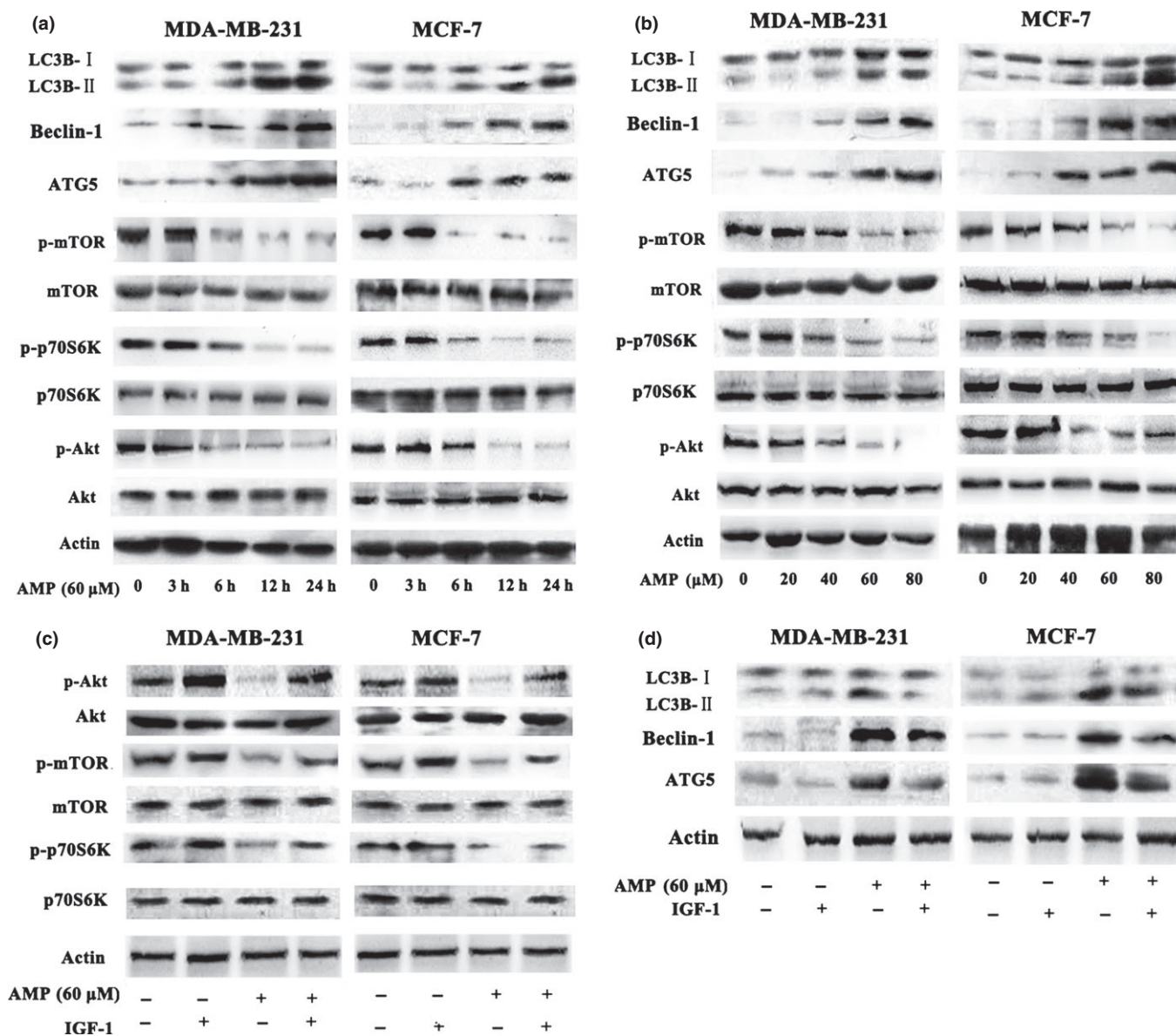

Fig. 3. The suppression of Akt-mTOR pathway is involved in Ampelopsin– induced autophagy. AMP time-dependently (a) and dose-dependently (b) inhibited Akt-mTOR pathway in breast cancer cells, along with autophagy induction. (c) Effects of IGF-1 on AMP-induced suppression of Akt-mTOR pathway. (d) Effects of IGF-1 on AMP-induced autophagy. All blots representative of three independent experiments with similar results are shown.

that AMP-induced autophagy is attributed to the inhibition of Akt-mTOR pathway in human breast cancer cells.

AMP-induced ER stress results in autophagy induction. Autophagy and ER stress have been considered two essential mechanisms to promote cell survival, and activation of ER stress along with induction of autophagy have been observed in cancer cells in response to chemotherapeutic drugs.[30,32,33] Several reports have demonstrated that there is a bidirectional regulation pattern between ER stress and autophagy.[34,35] Our previous study has shown that AMP can trigger ER stress through two UPR branches (PERK and ATF6), and PERK-CHOP pathway may be mainly involved in AMP-induced apoptosis in MCF-7 and MDA-MB-231 cells.[15] Thus, we next explored whether ER stress was involved in AMP-induced autophagy. It was found that blockage of ER stress by chemical inhibitor 4-phenylbutyric acid (4-PBA) decreased the conversion of LC3B, and inhibited the expressions of Beclin-1 and ATG5 induced by AMP, whereas ER stress inducer thapsigargin (Thap) played opposing effects (Fig. 4a,b). To further confirm that AMP-induced ER stress would result in autophagy, seeing as PERK-CHOP pathway plays a major role in AMP-induced apoptosis in our previous study,[15] we next abrogated ER stress by silencing of PERK. As anticipated, similar to chemical ER stress inhibitor 4-PBA, blockage of ER stress by PERK siRNA strongly abolished the expressions of LC3B-II, Beclin-1 and ATG5 in both breast cancer cells in response to AMP (Fig. 4c). The observations above suggest that AMP-induced autophagy in MCF-7 and MDA-MB-231 cells can, at least in part, be attributed to the activation of ER stress.

AMP-induced inhibition of Akt-mTOR pathway is attributed to ER stress. It was shown that ER stress negatively regulates the Akt-mTOR signaling pathway, resulting in the induction of autophagy.[36,37] We next investigated if AMP-induced inhibition of Akt-mTOR pathway was mediated by ER stress. As shown in Figure 5(a,b), accompanied with downregulating AMP-induced expressions of ER stress-associated proteins including PERK, p-elF2α and cleaved ATF6α, ER stress inhibitor 4-PBA partially restored AMP- induced phosphorylation of Akt, mTOR and p70S6K in MCF-7 and MDA-MB-231 cells. Similar results were also found in both cell lines in response to blockage of ER stress by silencing of PERK (Fig. 5c). On the contrary, the activation of ER stress by thapsigargin (Thap) exacerbated the inhibition of Akt-mTOR signaling pathway by AMP (Fig. 5a,b). These results demonstrate that AMP-induced ER stress is involved in the inhibition of Akt-mTOR pathway, resulting in autophagy induction.

Discussion

Here, for the first time we reveal that AMP can induce protective autophagy through Akt-mTOR signaling pathway via ER stress in human breast cancer MCF-7 and MDA-MB-231 cells. This novel finding is approved by the following evidence: (i) AMP simultaneously induced autophagy along with inhibition of cell viability and induction of apoptosis; (ii) blockage of autophagy using pharmacological or genetic methods further increased AMP-induced apoptotic cell death; (iii) AMP time- and dose-dependently suppressed Akt-mTOR signaling pathway as evidenced by the decreased phosphorylation levels of Akt, mTOR and p70S6K; and (iv) blocking ER stress alleviated AMP-induced inhibition of Akt-mTOR pathway and decreased AMP-induced autophagy.

Recently, many chemotherapeutic candidates have been reported to induce autophagy, but the roles of altered autophagy are inconsistent, because it can either protect cells from apoptosis, or promote cell death.[2,38] As one of the flavonoids, AMP was isolated from *Ampelopsis grossedentata*, which has
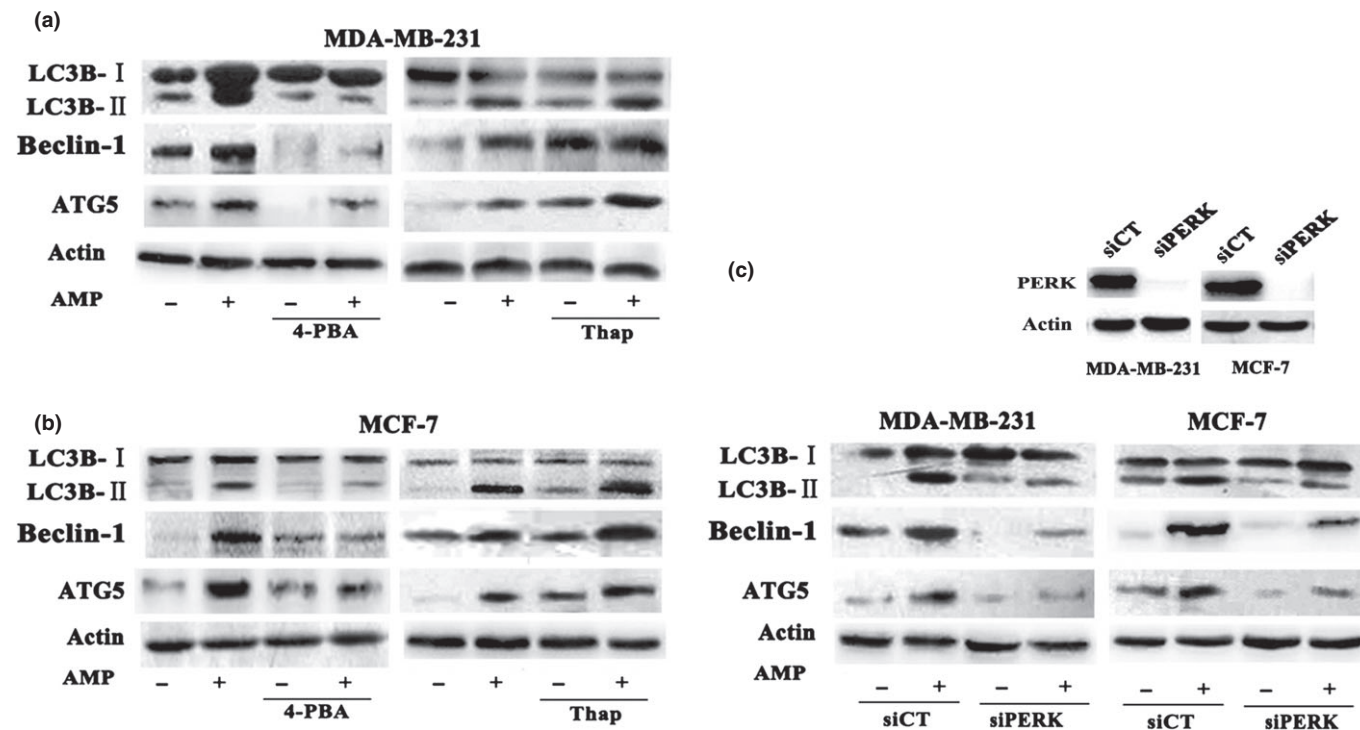

Fig. 4. Activation of endoplasmic reticulum (ER) stress is involved in Ampelopsin (AMP)-induced autophagy. Effects of ER stress inhibitor 4-PBA or ER stress activator thapsigargin on AMP-induced autophagy in MDA-MB-231 (a) and MCF-7 (b). (c) Effects of silencing of PERK on AMP-induced autophagy. All blots representative of three independent experiments with similar results are shown.
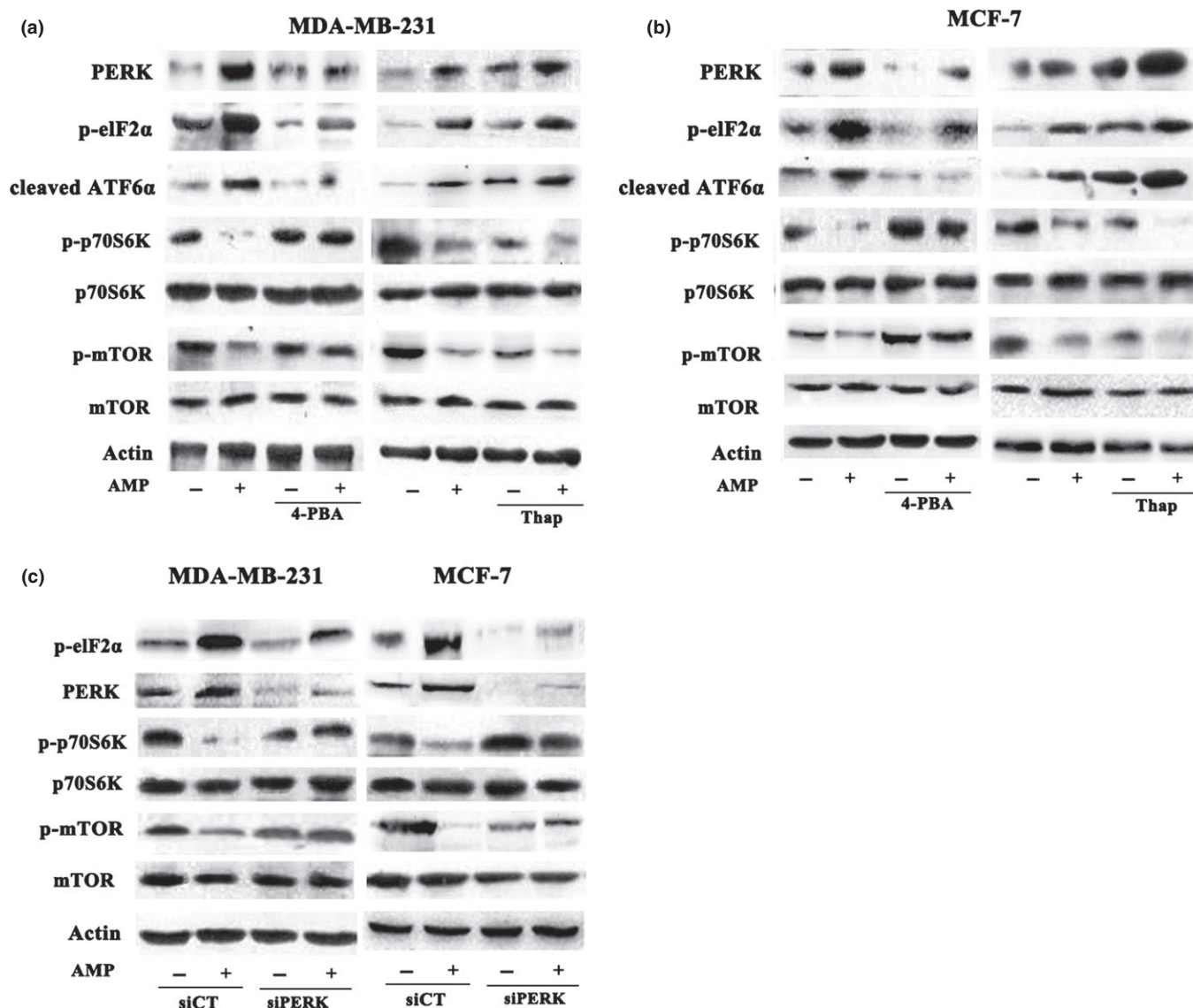
Fig. 5. Ampelopsin (AMP)-induced inhibition of Akt-mTOR pathway is attributed to endoplasmic reticulum (ER) stress. Effects of ER stress inhibitor 4-PBA or ER stress activator thapsigargin on Akt-mTOR pathway in MDA-MB-231 (a) and MCF-7 (b). (c) Effects of silencing of PERK on Akt-mTOR pathway. All blots representative of three independent experiments with similar results are shown.

been widely used as a tea product in South China.[39,40] AMP has been confirmed to exert a number of biological and pharmacological actions including anticancer property.[12,14] In our previous study, we found that AMP could dose-dependently induce cell death in human breast cancer cells.[15] Here, we found that AMP could promote autophagy, demonstrated by the formation of autophagosomes and autophagic vacuoles stained with LTG, upregulation of the autophagy marker protein LC3B-II, promotion of the conversion of LC3B-I to autophagosome-associated LC3B-II, and downregulation of p62 /SQSTM1. In addition, blocking autophagy by Beclin-1 or ATG5 siRNA decreased AMP-induced LC3B-II expression, while Baf A1 significantly increased LC3B-II and p62 /SQSTM1 levels. These results demonstrate that AMP increases autophagic flux in breast cancer cells. To determine the precise role of altered autophagy in AMP-induced cell death, we assessed the effect of the chemical autophagy inhibitors (Baf A1 and 3-MA), or RNA interference against Beclin-1 and ATG5, respectively, on AMP-induced cell death. Interest-

ingly, inhibiting autophagy not only decreased cell viability, but also increased the ratio of apoptotic cells. Taken together, these findings provided strong evidence suggest that the activation of autophagy may be an adaptive response to AMP in breast cancer cells.

Accumulating data confirmed that Akt-mTOR pathway directly controls stimulation of autophagy and anticancer agents can suppress tumor growth and survival via inhibition of Akt-mTOR pathway in accompany with induction of autophagy.[41,42] In our study, AMP time- and dose-dependently downregulate the levels of the phosphorylation of p70S6K, mTOR and Akt, while upregulating the expressions of autophagy marker proteins in MDA-MB-231 and MCF-7 cells. Conversely, Akt activator IGF-1 pretreatment not only partially restored Akt-mTOR pathway inhibited by AMP, but also obviously suppressed autophagy induction by AMP as evidenced by increase of the levels of the phosphorylation of p70S6K, mTOR and Akt, and decrease of the accumulation of LC3B-II and expression levels of Beclin-1 and ATG5 induced by AMP

in both breast cancer cells. These results imply that inhibition of Akt-mTOR pathway is involved in induction of autophagy by AMP. This finding is in agreement with the strong inhibition of mTOR in cells leading to induction of autophagy.

Unfolded protein response (UPR), the major ER stress pathway, is identified essentially to restore ER homeostasis to promote cell survival. But, if ER damage is uncontrollable and excessive, UPR can also induce cell to death.[23,24] Autophagy has often been observed as a consequence of ER stress and regarded as an alternate pathway to relieve ER stress or take part in cell death induced by ER stress.[24,43] Activation of ER stress has recently been found in a variety of chemotherapeutic candidates' treatment in cancer cells along with induction of autophagy, although the mechanistic link between autophagy and ER stress remains not exactly clarified.[19,23,44] Our previous study has shown that AMP could activate ER stress in both MCF-7 and MDA-MB-231 cells, and PERK-CHOP pathway plays a vital role in AMP-induced cell apoptosis.[15] Here, we found blockage of ER stress pathway by 4-BPA and silencing of PERK, respectively, could sharply decrease expressions of LC3B-II, Beclin-1 and ATG5 induced by AMP in both breast cancer cells. These results indicate that induction of autophagy is mediated by activation of ER stress induced by AMP in both breast cancer cells. Recent study has shown that Akt-mTOR pathway is involved not only in ER stress-induced

apoptosis, but also in the induction of autophagy.[19,45] In addition, ER stress has been reported to negatively regulate mTOR and thereby promote autophagy in cancer cells in response to chemotherapeutic agents.[23,46,47] In the present study, declines of the levels of the phosphorylation of p70S6K and mTOR induced by AMP were also partially restored in response to blockage of ER stress pathway by 4-BPA and silencing of PERK, suggesting that activation of ER stress induced by AMP was closely linked to inhibition of Akt-mTOR pathway.

In summary, we demonstrated that protective autophagy could occur simultaneously in breast cancer cells exposed to AMP, and that these changes were partially mediated by ER stress and Akt-mTOR pathway. These findings may be helpful in the development of AMP as a chemotherapeutic drug for breast cancer, and to the rationale for enhancing its anti-breast cancer efficacy by inhibiting protective autophagy.

Acknowledgments

Disclosure statement

The authors have no conflict of interest.

References

1 Sui X, Chen R, Wang Z et al. Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. Cell Death Dis 2013; 4: e838.
2 Maes H, Rubio N, Garg AD, Agostinis P. Autophagy: shaping the tumor microenvironment and therapeutic response. Trends Mol Med 2013; 19: e51076.
3 Shi Y, Tang B, Yu PW et al. Autophagy protects against oxaliplatin-induced cell death via ER stress and ROS in Caco-2 cells. PLoS One 2012; 7: e51076.
4 White E, Karp C, Strohecker AM, Guo Y, Mathew R. Role of autophagy in suppression of inflammation and cancer. Curr Opin Cell Biol 2010; 22: 212–7.
5 Chen N, Karantza V. Autophagy as a therapeutic target in cancer. Cancer Biol Ther 2011; 11: 157–68.
6 Debnath J. The multifaceted roles of autophagy in tumors-implications for breast cancer. J Mammary Gland Biol Neoplasia 2011; 16: 173–87.
7 Amaravadi RK, Lippincott-Schwartz J, Yin X-M et al. Principles and current strategies for targeting autophagy for cancer treatment. Clin Cancer Res 2011; 17: 654–66.
8 Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin 2013; 63: 11–30.
9 Jiang D, Sui M, Zhong W, Huang Y, Fan W. Different administration strategies with paclitaxel induce distinct phenotypes of multidrug resistance in breast cancer cells. Cancer Lett 2013; 335: 404–11.
10 Kim TH, Shin YJ, Won AJ et al. Resveratrol enhances chemosensitivity of doxorubicin in multidrug-resistant human breast cancer cells via increased cellular influx of doxorubicin. Biochim Biophys Acta 2014; 1840: 615–25.
11 Vu P, Walters J, Brownlow B, Elbayoumi T. Enhanced cytotoxicity of optimized liposomal genistein via specific induction of apoptosis in breast, ovarian and prostate carcinomas. J Drug Target 2013; 21: 1001–11.
12 Zhang B, Dong S, Cen X et al. Ampelopsin sodium exhibits antitumor effects against bladder carcinoma in orthotopic xenograft models. Anticancer Drugs 2012; 23: 590–6.
13 Qi S, Xin Y, Guo Y et al. Ampelopsin reduces endotoxic inflammation via repressing ROS-mediated activation of PI3K/Akt/NF-kappa B signaling pathways. Int Immunopharmacol 2012; 12: 278–87.
14 Ni F, Gong Y, Li L, Abdolmaleky HM, Zhou J-R. Flavonoid Ampelopsin inhibits the growth and metastasis of prostate cancer in vitro and in mice. PLoS One 2012; 7: e38802.
15 Zhou Y, Shu F, Liang X et al. Ampelopsin induces cell growth inhibition and apoptosis in breast cancer cells through ROS generation and endoplasmic reticulum stress pathway. PLoS One 2014; 9: e89021.
16 Sivendran S, Agarwal N, Gartrell B et al. Metabolic complications with the use of mTOR inhibitors for cancer therapy. Cancer Treat Rev 2014; 40: 190–6.
17 Peddi PF, Shatsky RA, Hurvitz SA. Noninfectious pneumonitis with the use of mTOR inhibitors in breast cancer. Cancer Treat Rev 2014; 40: 320–6.
18 Li Z-Y, Yang Y, Ming M, Liu B. Mitochondrial ROS generation for regulation of autophagic pathways in cancer. Biochem Biophys Res Commun 2011; 414: 5–8.
19 Jung CH, Kim H, Ahn J et al. Anthricin isolated from Anthriscus sylvestris (L.) Hoffm. inhibits the growth of breast cancer cells by inhibiting Akt/mTOR signaling, and its apoptotic effects are enhanced by autophagy inhibition. Evid Based Complement Alternat Med 2013; 2013: e385219.
20 Cai Y, Wang Z, Sun T et al. Diarylquinoline compounds induce autophagy-associated cell death by inhibiting the Akt pathway and increasing reactive oxygen species in human nasopharyngeal carcinoma cells. Oncol Rep 2013; 29: 983–92.
21 Zhu G-Y, Li Y-W, Tse AK-W et al. 20(S)-Protopanaxadiol, a metabolite of ginsenosides, induced cell apoptosis through endoplasmic reticulum stress in human hepatocarcinoma HepG2 cells. Eur J Pharmacol 2011; 668: 88–98.
22 Jain K, Paranandi KS, Sridharan S, Basu A. Autophagy in breast cancer and its implications for therapy. Am J Cancer Res 2013; 3: 251–65.
23 Verfaillie T, Salazar M, Velasco G, Agostinis P. Linking ER stress to autophagy: potential implications for cancer therapy. Int J Cell Biol 2010; 2010: 930509.
24 Zhao XC, Omikorede O, Smith D, Herbert TP. Endoplasmic reticulum stress can upregulate activating transcription factor 4 (ATF4) and C/EBP homologous protein (CHOP) expression via a PKR-like ER kinase (PERK) independent pathway in pancreatic beta cells. Diabet Med 2013; 30: 33–3.
25 Lan Y-C, Chang C-L, Sung M-T et al. Zoledronic acid-induced cytotoxicity through endoplasmic reticulum stress triggered REDD1-mTOR pathway in breast cancer cells. Anticancer Res 2013; 33: 3807–14.
26 Shi JM, Bai LL, Zhang DM et al. Saxifragifolin D induces the interplay between apoptosis and autophagy in breast cancer cells through ROS-dependent endoplasmic reticulum stress. Biochem Pharmacol 2013; 85: 913–26.
27 Klionsky DJ, Abeliovich H, Agostinis P et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy 2008; 4: 151–75.
28 Ryter SW, Cloonan SM, Choi AMK. Autophagy: a critical regulator of cellular metabolism and homeostasis. Mol Cells 2013; 36: 7–16.
29 Williams JA, Hou Y, Ni H-M, Ding W-X. Role of intracellular calcium in proteasome inhibitor-induced endoplasmic reticulum stress, autophagy, and cell death. Pharm Res 2013; 30: 2279–89.
30 Bruening A, Rahmeh M, Friese K. Nelfinavir and bortezomib inhibit mTOR activity via ATF4-mediated sestrin-2 regulation. Mol Oncol 2013; 7: 1012–8.

31 O Farrell F, Rusten TE, Stenmark H. Phosphoinositide 3-kinases as accelerators and brakes of autophagy. *FEBS J* 2013; **280**: 6322–37.

32 Selimovic D, Porzig B, El-Khattouti A *et al.* Bortezomib/proteasome inhibitor triggers both apoptosis and autophagy-dependent pathways in melanoma cells. *Cell Signal* 2013; **25**: 308–18.

33 Mandl J, Meszaros T, Banhegyi G, Csala M. Minireview: endoplasmic reticulum stress: control in protein, lipid, and signal homeostasis. *Mol Endocrinol* 2013; **27**: 384–93.

34 Yorimitsu T, Nair U, Yang Z, Klionsky DJ. Endoplasmic reticulum stress triggers autophagy. *J Biol Chem* 2006; **281**: 30299–304.

35 Bachar-Wikstrom E, Wikstrom JD, Kaiser N, Cerasi E, Leibowitz G. Improvement of ER stress-induced diabetes by stimulating autophagy. *Autophagy* 2013; **9**: 626–8.

36 Mazure NM, Pouyssegur J. Hypoxia-induced autophagy: cell death or cell survival? *Curr Opin Cell Biol* 2010; **22**: 177–80.

37 Ciechomska IA, Gabrusiewicz K, Szczepankiewicz AA, Kaminska B. Endoplasmic reticulum stress triggers autophagy in malignant glioma cells undergoing cyclosporine A-induced cell death. *Oncogene* 2013; **32**: 1518–29.

38 Rudolf E. Selenite-mediated cellular stress, apoptosis, and autophagy in colon cancer cells. Autophagy: Cancer, Other Pathologies, Inflammation, Immunity, Infection, and Aging. Volume 1 - Molecular Mechanisms. 2014; 221–33.

39 Liu B, Du J, Zeng J, Chen C, Niu S. Characterization and antioxidant activity of dihydromyricetin-lecithin complex. *Eur Food Res Technol* 2009; **230**: 325–31.

40 Zhao L, Wang A, Liu B, Li G, Zhang Z, Chen S. Antioxidant and cytotoxic activity of dihydromyricetin from Ampelopsis Grossedentata leaves. *Agro Food Ind Hi Tech* 2009; **20**: 14–7.

41 Poornima P, Weng CF, Padma VV. Neferine from Nelumbo nucifera induces autophagy through the inhibition of PI3K/Akt/mTOR pathway and ROS hyper generation in A549 cells. *Food Chem* 2013; **141**: 3598–605.

42 Chen Z, Yang L, Liu Y *et al.* LY294002 and Rapamycin promote coxsackievirus-induced cytopathic effect and apoptosis via inhibition of PI3K/AKT/mTOR signaling pathway. *Mol Cell Biochem* 2014; **385**: 169–77.

43 Chua CEL, Tang BL. Linking membrane dynamics and trafficking to autophagy and the unfolded protein response. *J Cell Physiol* 2013; **228**: 1638–40.

44 Sanchez-Alvarez R, Martinez-Outschoorn UE, Lin Z *et al.* Ethanol exposure induces the cancer-associated fibroblast phenotype and lethal tumor metabolism implications for breast cancer prevention. *Cell Cycle* 2013; **12**: 289–301.

45 Kapuy O, Banhegyi G. Depletion of luminal pyridine nucleotides in the endoplasmic reticulum activates autophagy with the involvement of mTOR pathway. *Biomed Res Int* 2013; **2013**: 942431.

46 Dewaele M, Martinet W, Rubio N *et al.* Autophagy pathways activated in response to cell resistance against ROS damage. *J Cell Mol Med* 2011; **15**: 1402–14.

47 King FW, Fong S, Griffin C *et al.* Timosaponin AIII is preferentially cytotoxic to tumor cells through inhibition of mTOR and induction of ER stress. *PLoS One* 2009; **4**: e7283.