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Ramalin-Mediated Apoptosis Is Enhanced by Autophagy Inhibition in Human Breast Cancer Cells

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Breast cancer, the most commonly diagnosed cancer in women worldwide, is treated in various ways. Ramalin is a chemical compound derived from the Antarctic lichen *Ramalina terebrata* and is known to exhibit antioxidant and antiinflammatory activities. However, its effect on breast cancer cells remains unknown. We examined the ability of ramalin to induce apoptosis and its mechanisms in MCF-7 and MDA-MB-231 human breast cancer cell lines. Ramalin inhibited cell growth and induced apoptosis in both cell lines in a concentration-dependent manner. By upregulating Bax and downregulating Bcl-2, ramalin caused cytochrome c and apoptosis-inducing factor to be released from the mitochondria into the cytosol, thus activating the mitochondrial apoptotic pathway. In addition, activated caspase-8 and caspase-9 were detected in both types of cells exposed to ramalin, whereas ramalin activated caspase-3 only in the MDA-MB-231 cells. Ramalin treatment also increased the levels of LC3-II and p62. Moreover, the inhibition of autophagy by 3-methyladenine or Atg5 siRNA significantly enhanced ramalin-induced apoptosis, which was accompanied by a decrease in Bcl-2 levels and an increase in Bax levels. Therefore, autophagy appears to be activated as a protective mechanism against apoptosis in cancer cells exposed to ramalin. These findings suggest that ramalin is a potential anticancer agent for the treatment of patients with non-invasive or invasive breast cancer. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: ramalin; apoptosis; autophagy; breast cancer cells.

INTRODUCTION

Breast cancer is the most common cancer among women in both developed and developing countries (Jemal *et al*., 2011). Although the risk factors that can lead to breast cancer include certain dietary patterns, physical inactivity, family history, gene mutations, and alcohol consumption, most of the established risk factors are related to exposure to the hormone oestrogen, such as the use of contraceptives, hormone replacement therapy, age at menarche, age at menopause, and the experience of pregnancy and lactation (Hulka and Moorman, 2001). Breast cancer is divided into many subtypes depending on the characteristics of the cells (Rouzier *et al*., 2005). However, the presence of oestrogen receptors (ERs) is one of the most important general classifications, because ERs play a significant role in several signal transduction pathways of breast cancer cells and affect gene expression and cell characteristics upon exposure to oestrogen. ER-positive breast cancer is responsive to anti-oestrogen therapy and leads to a better outcome in general, whereas ER-negative breast cancer is more aggressive and is usually associated with a poor clinical prognosis (Badve and Nakshatri, 2009; Shen *et al*., 2012). Despite many clinical trials involving numerous agents, the search continues for more effective therapeutic agents targeted to both kinds of breast cancer.

Homeostasis is maintained when cell survival and cell death are in balance. Among the several kinds of cell death, apoptosis is programmed cell death that controls homeostasis, mutation, ageing, tissue development, and immune responses that involve the removal of infected cells (Elmore, 2007). In cancer, homeostasis is off-balance, and abnormal proliferation occurs consistently, so evading apoptosis is a key to tumour progression (Hanahan and Weinberg, 2000). Therefore, inducing apoptosis in cancer cells can be an effective approach to treating cancer (Nicholson, 2000).

Autophagy is an evolutionarily conserved process involved in disassembling proteins or cell organelles and reconstructing cells in response to the lack of nutrition to maintain the homeostasis of cells (Yoshimori, 2004). Usually, autophagy maintains homeostasis at the basal level, but it can be upregulated by several stimuli, including cell starvation, hypoxia, growth factor deprivation, and oxidant-mediated injury, to perform an adaptive function for cell survival (Kaushal, 2012). In addition, when autophagy is impaired or overly activated, it can function as a pathway to cell death (Herzog *et al*., 2012). However, the role of autophagy in regulating cancer cell survival or death in response to anticancer drugs remains to be defined. Autophagy can play a role either in causing cell death in tumour cell (Jin and White, 2007) or in helping cancer cells to survive (Jain *et al*., 2013). Although autophagy is known to be an essential step to trigger apoptosis and play a negative role depending on various conditions (Gozuacik and

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Kimchi, 2004; Eisenberg-Lerner *et al*., 2009), further research is needed to investigate the role of autophagy in certain cancer cells for treatment.

Ramalin is a compound isolated from the methanol/water extract of the Antarctic lichen, *Ramalina terebrata*. Lichens have a symbiotic relationship with a fungus and an alga, which synthesize diverse secondary metabolites (Huneck, 1999). Various lichen metabolites have a wide range of biological functions, such as anti-inflammatory, fungicidal, cytotoxic, and antiproliferative activity (Müller, 2001). Recently, Paudel and colleagues showed that ramalin has more significant antioxidant activity than other well-known antioxidants (Paudel *et al*., 2011). However, the effect of ramalin on cancer cells has not been addressed.

In this study, we investigated the cytotoxic effects of ramalin and the mechanisms by which ramalin exerts its effect in two human breast cancer cell lines, MDA-MB-231 and MCF-7. Our results revealed that ramalin-induced apoptosis was enhanced by the inhibition of autophagy.

MATERIALS AND METHODS

Reagents. Unless otherwise indicated, all the chemicals used in this study were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Foetal bovine serum, penicillin G and streptomycin, Trypsin-EDTA, RPMI 1640, and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from GE Healthcare Bio-Sciences Corp. (Piscataway Township, NJ, USA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was obtained from Merck KGaA (Darmstadt, Germany). LC-3 antibody was obtained from Novus Biologicals (Littleton, CO, USA), and Atg5 antibody was obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against apoptosis-inducing factor (AIF), α-tubulin, Bax, Bcl-2, beclin-1, cytochrome c, COX IV, p53, p62, caspase-3, caspase-8, and caspase-9 antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Extraction and isolation of ramalin. Ramalin was extracted and purified from the freeze-dried and ground lichen sample (*Ramalina terebrata*), as previously described (Paudel *et al*., 2011). The molecular formula of ramalin [γ-glutamyl-*N*′-(2-hydroxyphenyl) hydrazide] was determined as $C_{11}H_{15}N_3O_4$ by analysis of its High-Resolution Electrospray Ionization Mass Spectrometry data, indicating six degrees of unsaturation, which were accounted for by the presence of two carbonyl groups on an aromatic ring system (Fig. 1). This formula was supported by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy data. The purity of the isolated compound was confirmed to exceed 99%. Ramalin (MW 254.11141) was dissolved in distilled water to a concentration of 100 mg/mL and diluted to the appropriate concentrations within the culture medium.

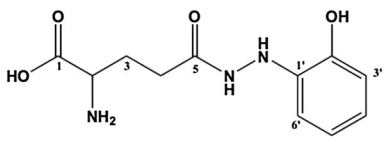

Figure 1. Chemical structure of ramalin.

Cell culture. The two human breast cancer cell lines (MCF-7 and MDA-MB-231) were obtained from the American Type Culture Collection (Manassas, VA, USA). MCF-7 cells were propagated in RPMI-1640, and MDA-MB-231 cells were cultured in DMEM. Both media were supplemented with 10% (v/v) foetal bovine serum and antibiotics (100 IU/mL of penicillin and 100 μg/mL of streptomycin). Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

Assessment of cell proliferation and viability. Breast cancer cells were seeded in a 96-well plate (1×10^5 cells/well), and different concentrations of ramalin (10, 50, and 100 μg/mL) were added. Cell proliferation was measured by MTT assay as described elsewhere (Nguyen *et al*., 2015). After incubation for 24 h, the treated cells were washed once with phosphate buffered saline (PBS), and the media were replaced with 200 μL of MTT solution (25 mg/mL in media) and incubated at 37 °C for 4 h. The supernatant was discarded, and the formazan blue crystals formed by the reduction of MTT were dissolved in 150 mL of DMSO. Optical density was measured by absorbance at 560 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The trypan blue dye exclusion test was also used to determine the number of viable cells. Cells were washed with PBS and then collected by centrifuge at 200 × g for 5 min. The pellet was re-suspended in 1 mL of PBS, and one part of 0.4% trypan blue was mixed with one part cell suspension. The unstained (viable) and stained (nonviable) cells were counted separately.

Determination of apoptosis by Annexin V/propidium iodide staining analysis. Cells undergoing apoptosis were identified using the FITC Annexin V/Propidium Iodide (PI) Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Cells (8×10^5 cells/dish) were seeded in 60-mm dishes and treated with various concentrations (10, 50, and 100 μg/mL) of ramalin. In some experiments, a pan-caspase inhibitor, Z-VAD-fmk, was included along with ramalin. After incubation for 24 h, the cells were harvested by centrifugation (5 min at 448 × g) and washed in duplicate with ice-cold PBS. Cells were then re-suspended in 100 μL of binding buffer containing 5 μL of Annexin V-FITC conjugate and 10 μL of PI and were incubated in the dark at room temperature. After 15 min, 400 μL of the binding buffer was added, and the cells were analysed by means of flow cytometry (BD FACSCanto II, BD Biosciences). At least 10 000 cells were subjected to analysis to identify viable, apoptotic, and necrotic populations, and the fraction of the cell population was measured by FCS Express 4 Flow Cytometry software (De Novo Software, Glendale, CA, USA).

Transient transfection with Atg5 small interfering RNA. The small interfering RNA (siRNA) targeting Atg5 and a negative control siRNA were purchased from Dharmacon (Dharmacon RNA Technologies, Lafayette, CO, USA). Cells were transfected with siRNAs (10 nM) using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendations. Cells were treated with ramalin for a further 24 h and then analysed for the expression of proteins.

Western blot analysis. Western blotting was performed as previously described but with some modifications (Lee *et al.*, 2013). Briefly, the cells were treated with different concentrations of ramalin (10, 50, and 100 μg/mL) for the indicated times in figure legends. After treatment, the cells were harvested and washed twice in PBS followed by lysis in a lysis buffer (50 mM of Tris-Cl, pH 6.8, 10% glycerol, 2% Sodium dodecyl sulfate (SDS), and 1 mM of Phenylmethylsulfonyl fluoride (PMSF)) and were then incubated on ice for 15 min. Nonidet P-40 (0.5%) was added to lyse the cells that were vortexed for 10 s and centrifuged twice at 500 × g at 4 °C for 10 min. After centrifugation, the nuclear pellet was obtained and the supernatants were further centrifuged 10 000 × g at 4 °C for 30 min. The resulting mitochondrial pellets were suspended in a lysis buffer, sonicated, and stored at −70 °C until used. The supernatant from the previous step was further centrifuged at 100,000 × g at 4 °C for 1 h to obtain the cytoplasmic fraction, which was stored at −70 °C until used. Protein concentrations were quantified with the DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) and bovine serum albumin as standard. Protein samples (20 μg) from cytoplasmic, mitochondrial, or whole-cell extract preparations were resolved with SDS-PAGE, after which they were transferred onto NC/PVDF membranes (Millipore Corp., Billerica, MA, USA). After blocking in 5% skim milk at room temperature for 1 h, membranes were washed with Tris-buffered saline containing 0.05% Tween-20 (TBST) and probed with the appropriate antibodies. A horseradish peroxidase-conjugated secondary antibody was added, and secondary antibodies were detected by an enhanced chemiluminescence kit (AbClon, Seoul, Korea) and a Davinch-Chemi Chemiluminescence Imaging System (Davinch-K, CellTAGen, Seoul, Korea). In all immunoblotting experiments, blots were re-probed with anti-β-actin, anti-COX IV, or anti-α-tubulin antibody as a control for equal loading.

Autophagy assays by fluorescence microscopy. GFP-LC3 transfected cells were observed by means of fluorescence microscopy (Olympus-America, Melvile, NY). To determine the activation of autophagy, GFP-LC3-expressing cells were treated with ramalin in culture medium for 24 h. Cells were fixed with 4% paraformaldehyde in PBS, and the punctate cytoplasmic patterns of LC3 were visualized by fluorescence microscopy. The number of GFP-LC3 dots per cell was counted and presented in a graphic format. The quantitative results obtained were then statistically analysed and compared.

Statistical analysis. Results are represented as means ± SEM. One-way analysis of variance was used to determine significance among the groups, after which a

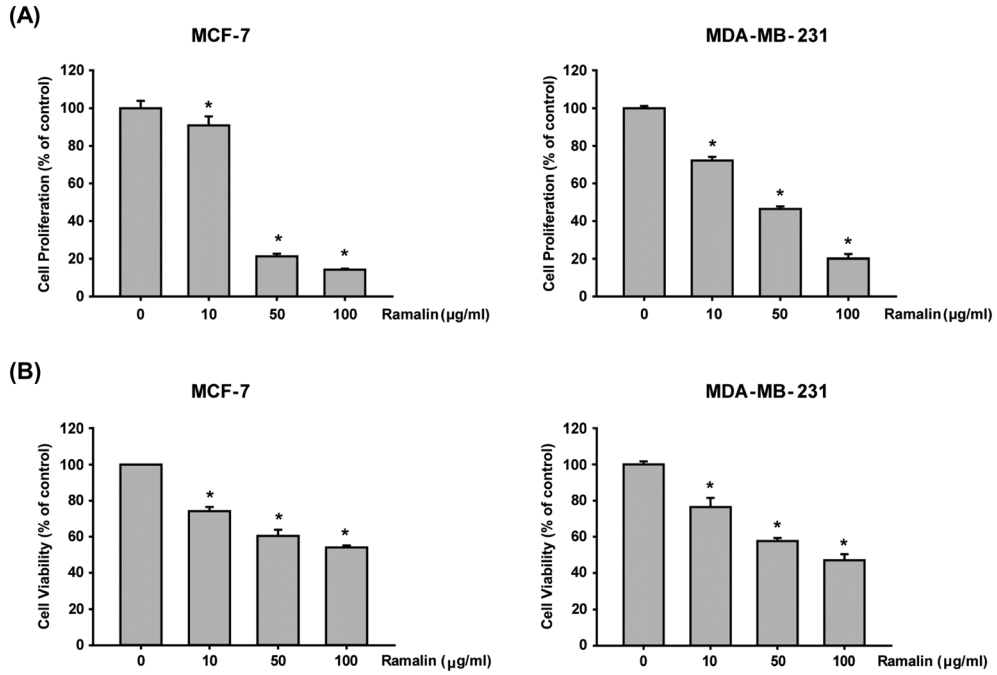

Figure 2. The effect of ramalin on proliferation and viability in MCF-7 and MDA-MB-231 cells. Human breast cancer cells were treated with different concentrations of ramalin, as indicated, for 24 h. (A) Cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The level of cell viability is presented as the percentage of untreated control cells. (B) Cell viability was also assessed by the trypan blue exclusion test. Data shown are the means ± SEM of at least three independent experiments. * $p < 0.05$ versus untreated control.

modified *t*-test with the Bonferroni correction was used for comparisons between individual groups. Significant values are represented by an asterisk (* $p < 0.05$).

RESULTS

Effect of ramalin on proliferation and viability of human breast cancer cells

To examine the effect of ramalin on the proliferation and viability of human breast cancer cell lines MCF-7 and MDA-MB-231, cells were treated with ramalin. The proliferation rates of both cell lines were determined by MTT assay. When the cells were exposed to ramalin (10, 50, or 100 μg/mL), the inhibitory effect of ramalin on cell proliferation occurred within 24 h or earlier (Fig. 2A). However, both types of cells were almost completely dead at 50 and 100 μg/mL after 36 h (data not shown). After 24 h of incubation, ramalin treatment resulted in concentration-dependent decreases in cell proliferation to 90.9%, 21.3%, and 14.3% in MCF-7 cells and 72.2%, 46.4%, and 20.1% in MDA-MB-231 cells at ramalin concentrations of 10, 50, and 100 μg/mL, respectively. Next, we analysed the effect of ramalin on the viability of breast cancer cells. Trypan blue assay showed that ramalin decreased the cell viability rate in both breast cancer cell lines in a concentration-dependent manner. Accordingly, in all subsequent experiments, the cells were treated with 10, 50, and 100 μg/mL of ramalin for 24 h.

Induction of apoptosis by ramalin in MCF-7 and MDA-MB-231 cells

To determine whether ramalin triggers apoptotic cell death, MCF-7 and MDA-MB-231 cells were treated with three different concentrations of ramalin (10, 50, and 100 μg/mL). After incubation for 24 h, the cells were stained with binding buffer containing FITC Annexin V/PI and then analysed by means of flow cytometry. Treatment with ramalin led to a concentration-dependent increase in early and late apoptosis/necrosis rates from 0.54% to 39.47% in the MCF-7 cells and from 2.04% to 34.94% in the MDA-MB-231 cells (Fig. 3). Although MCF-7 cells were more sensitive to the apoptotic effect of ramalin at the highest concentration (100 μg/mL), the proportion of cells undergoing apoptosis was increased in a concentration-dependent manner in both human breast cancer cell lines.

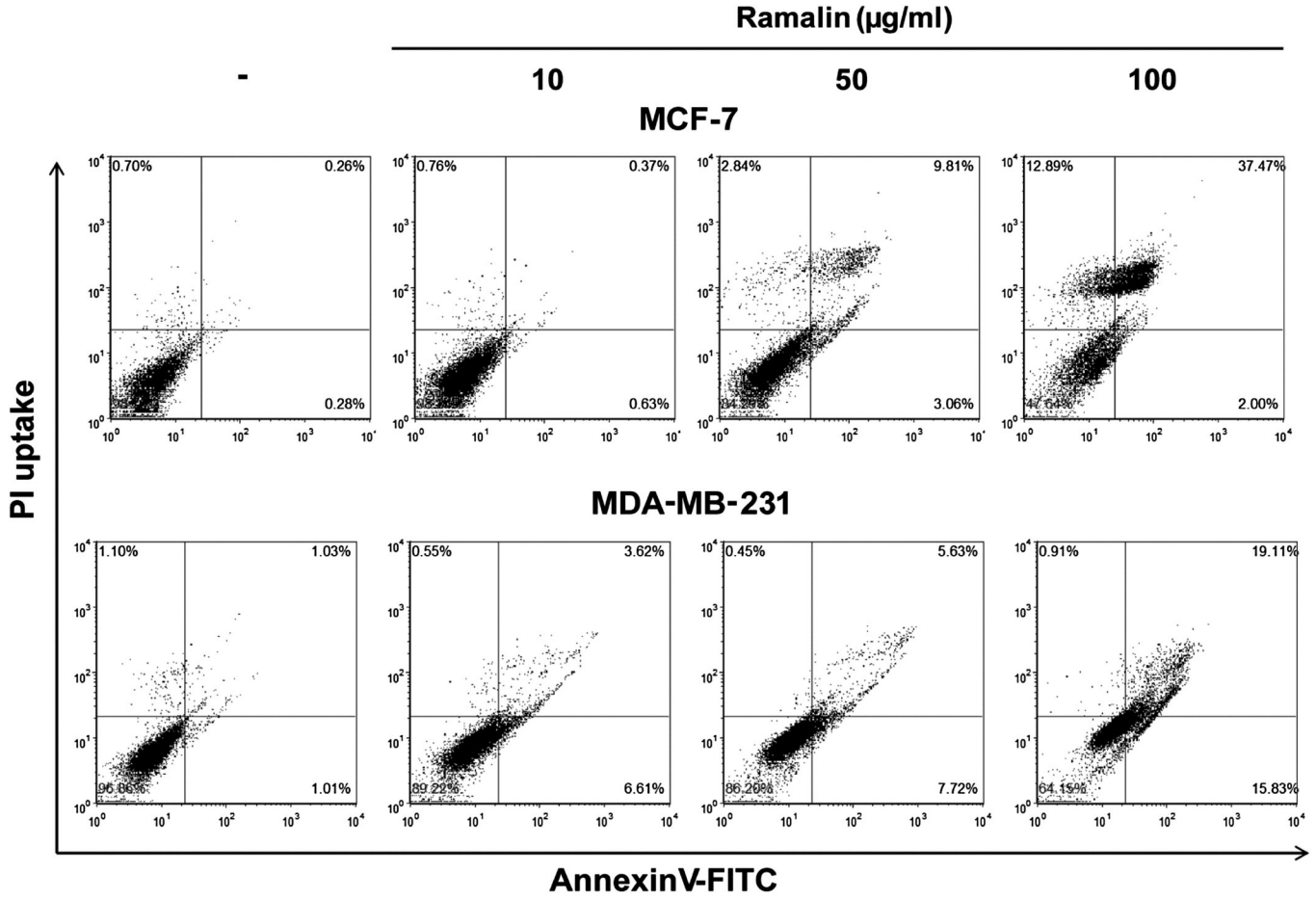

Figure 3. Ramalin induces apoptosis in MCF-7 and MDA-MB-231 cells. After MCF-7 cells and MDA-MB-231 cells were treated with the indicated concentrations of ramalin for 24 h, the cells were harvested and stained for Annexin V/propidium iodide. Apoptotic cells were analysed by flow cytometry. Cell populations of FITC−/PI−, FITC+/PI−, and FITC+/PI+ were regarded, respectively, as living (lower left quadrant), early-stage apoptotic (lower right quadrant), and late-stage apoptotic/necrotic cells (upper right quadrant). Data are representative of three independent experiments.

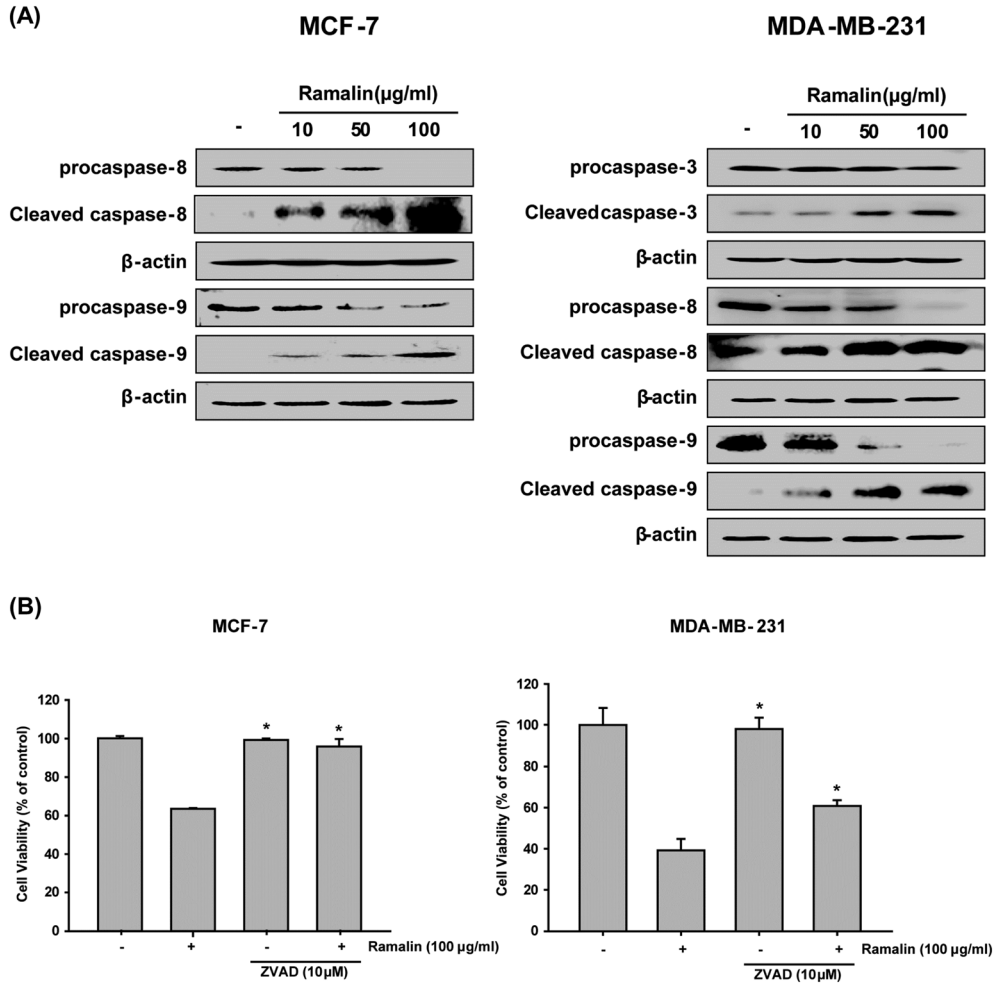

Figure 4. Effect of ramalin on caspase activation. MCF-7 and MDA-MB-231 cells were treated with ramalin (10, 50, and 100 μg/mL) for 24 h. (A) Whole-cell protein lysates were prepared, and protein expression was measured by performing western blot analysis with specific antibodies. β-actin was used as an internal control. (B) Cell viability was assessed by the trypan blue exclusion test. (C) The cells were pretreated with 10 μM of Z-VAD-fmk for 2 h, followed by treatment with ramalin for 24 h. The apoptotic cells were analysed by means of flow cytometry. Cell populations FITC-/PI-, FITC + /PI-, and FITC + /PI + were regarded, respectively, as living (lower left quadrant), early-stage apoptotic (lower right quadrant), and late-stage apoptotic/necrotic cells (upper right quadrant). Data are representative of three independent experiments.

Effect of ramalin on activation of caspases

Caspases, which represent both initiators and executors of the apoptotic process, have been well known to play an important role in external and internal apoptosis pathways (Lavrik *et al.*, 2005). To determine whether caspases are activated in ramalin-induced apoptosis in breast cancer cells, we exposed the two cell lines to ramalin. After incubation for 24 h, the expression of caspases was determined by western blotting (Fig. 4A). Treatment with ramalin activated both caspase-8 and caspase-9 in both breast cancer cell lines in a concentration-dependent manner. However, cleaved caspase-3 was expressed in MDA-MB-231 cells but not in caspase-3-deficient MCF-7 cells in a concentration-dependent manner. To confirm the relevance of caspase in ramalin-induced apoptosis, cells were treated with the pan-caspase inhibitor, Z-VAD-fmk. By trypan blue assay, pretreatment of both types of cells with Z-VAD-fmk for 2 h before ramalin exposure significantly attenuated ramalin-induced cell death in both cell lines (Fig. 4B). Moreover, flow cytometric analysis also showed that Z-VAD-fmk pretreatment resulted in a decrease in early- and late-stage apoptosis in the MCF-7 cells (from 11.6% to 2.94%) and the MDA-MB-231 cells (from 6% to 5.17%), respectively (Fig. 4C). These results suggest that ramalin triggers both extrinsic and intrinsic pathways of apoptotic cell death and causes caspase-3-dependent and caspase-3-independent apoptosis in breast cancer cells.

Effect of ramalin on the expression of Bcl-2 family proteins and p53

It has been known that p53 directly or indirectly regulates the activity of the Bcl-2 family proteins (Hemann and Lowe, 2006). To understand the relation between p53 and the expression of Bax and Bcl-2 in ramalin-treated breast cancer cells, we examined the effect of ramalin on the expression of Bcl-2 family members and on p53 status. MCF-7 (wild-type p53) cells and MDA-MB-231 (p53 mutant 280, Arg-Lys) cells were exposed to 10, 50, and 100 μg/mL of ramalin for 24 h. In the MCF-7 cells, p53 protein levels were upregulated by ramalin, whereas the levels of endogenous p53

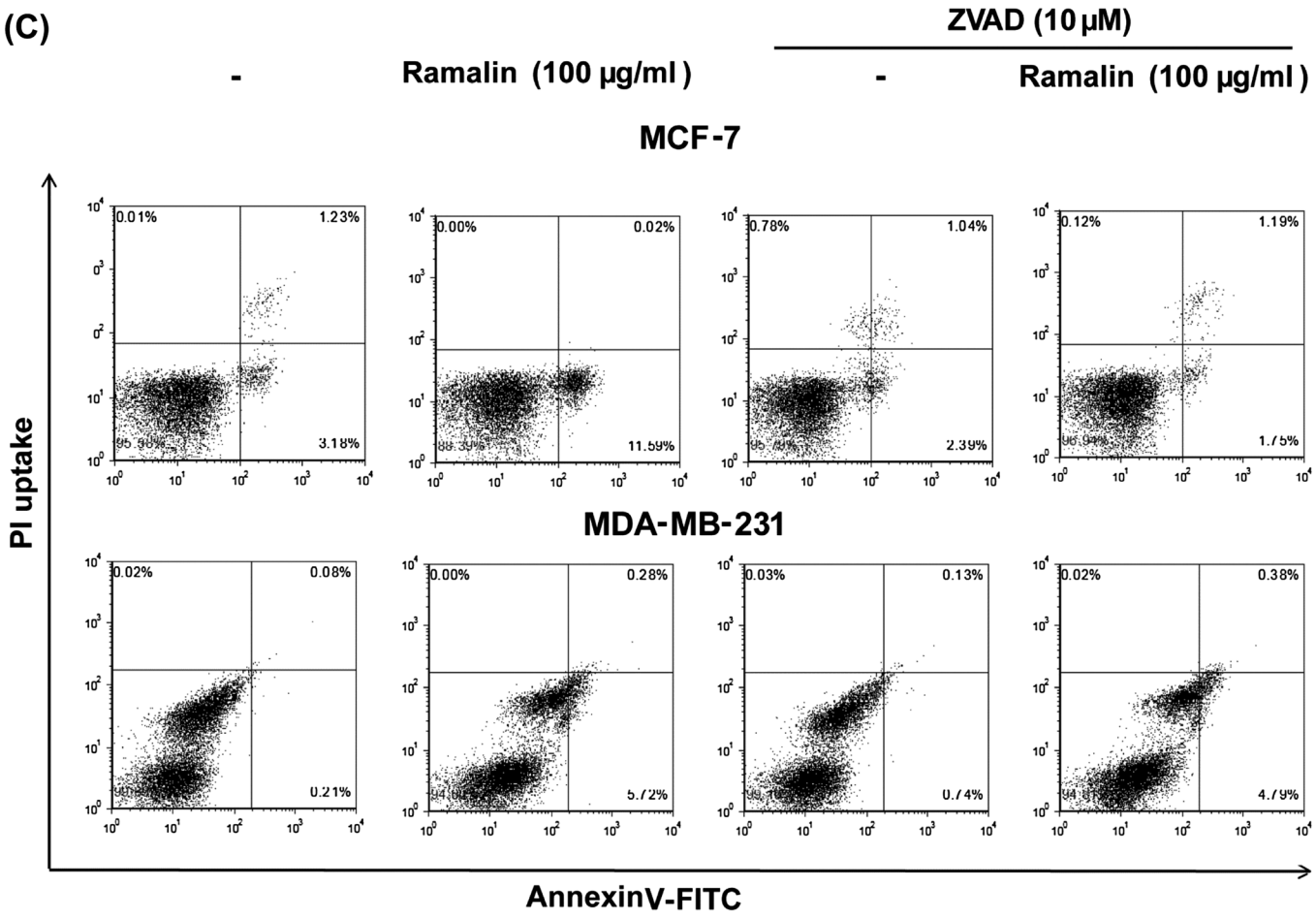

Figure 4. (Continued)

expression were decreased in the ramalin-treated MDA-MB-231 cells (Fig. 5). Regardless of p53 status, the expression of Bax, the pro-apoptotic Bcl-2 family protein, was increased and the expression of Bcl-2, the anti-apoptotic protein, was decreased in the ramalin-treated human breast cancer cells in a concentration-dependent manner (Fig. 5). These data demonstrate that the apoptotic pathway of ramalin-treated human breast cancer cells is regulated by the Bcl-2 family proteins and is independent of p53 expression.

Effect of ramalin on translocation of cytochrome c and apoptosis-inducing factor

Mitochondria play a crucial role in the commitment to apoptosis through the release of cytochrome c and AIF translocation (Burlacu, 2003; Tait and Green, 2010; Hemann and Lowe, 2006; Du *et al.*, 2000). To determine whether the mitochondrial apoptotic pathway is involved in ramalin-induced apoptosis, we examined the release of cytochrome c and AIF from the mitochondria to the

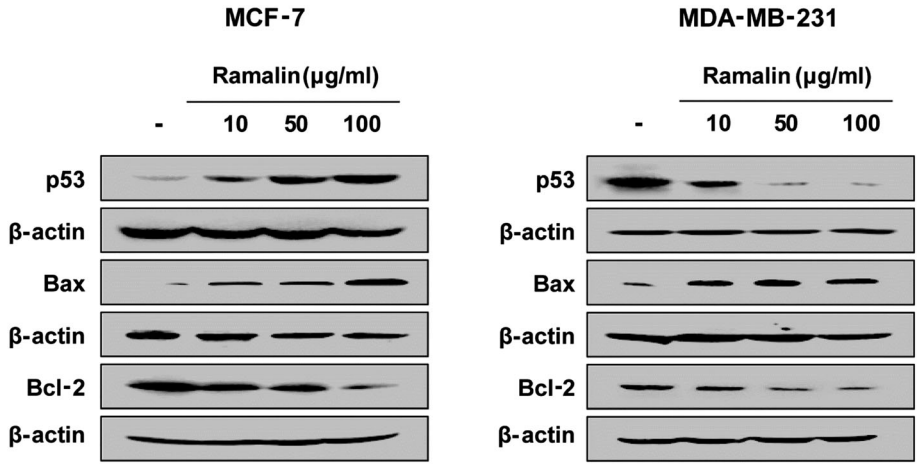

Figure 5. Effects of ramalin on p53, Bcl-2, and Bax protein levels. MCF-7 cells and MDA-MB-231 cells were exposed to different concentrations (0, 10, 50, and 100 μg/mL) of ramalin for 24 h. The cells were collected, and total proteins were extracted. Levels of proteins were determined by western blot analysis using the antibodies indicated. β-actin was used as an internal control. Data are representative of three independent experiments.

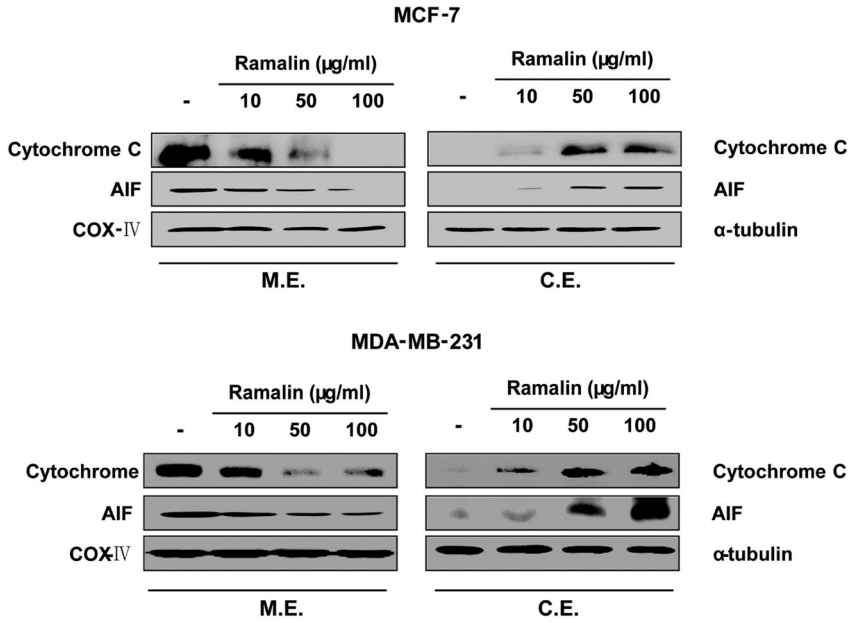

Figure 6. Ramalin triggers release of cytochrome c and apoptosis-inducing factor (AIF) from mitochondria into the cytosol. Cytosolic and mitochondrial fractions were isolated from breast cancer cells treated with the indicated concentrations of ramalin for 24 h (see Materials and Methods section). Protein levels of cytochrome c and AIF were analysed by western blot analysis. COX IV and α-tubulin were used as internal controls for the mitochondrial and cytosolic fractions, respectively. Data are representative of three independent experiments. M.E., mitochondria extracts; C.E., cytosol extracts.

cytosol. MCF-7 and MDA-MB-231 cells were treated with three different concentrations of ramalin (10, 50, and 100 μg/mL). After incubation for 24 h, the accumulation of cytochrome c and AIF in the mitochondrial and cytosolic extracts was examined by western blotting analysis with specific antibodies. As shown in Fig. 6, treatment with ramalin resulted in an increase in cytosolic levels of cytochrome c and AIF and a decrease in the mitochondrial levels of cytochrome c and AIF in a concentration-dependent manner in both breast cancer cell lines, indicating that ramalin did in fact trigger the release of cytochrome c and AIF.

Ramalin treatment activates autophagy in breast cancer cells

Because it has been widely accepted that autophagy interacts in a complex way with apoptosis, many anticancer agents induce autophagy-mediated cell death. Therefore, in order to investigate whether ramalin activates the autophagy pathway, MCF-7 and MDA-MB-231 cells were treated with different concentrations of ramalin (10, 50, and 100 μg/mL) for 24 h. After ramalin exposure, the accumulation of GFP-LC3 puncta in the cytosol was increased in the two cell lines, as compared with the control cells, in a concentration-dependent manner (Fig. 7A). In addition, expression of the autophagic markers LC3 and p62 (SQSTM1) was determined by western blotting. Consistent with the GFP-LC3 puncta results, the accumulation of LC3-II was found to be increased in both breast cancer cell lines exposed to ramalin in a concentration-dependent manner (Fig. 7B). Interestingly, ramalin caused increased p62 expression in both types of cells (Fig. 7B), which is consistent with other reports in which increased p62 expression was required to induce autophagy (Wong *et al*., 2010; Robert *et al*., 2009; Puissant *et al*., 2010; Puissant *et al*., 2012). These results indicated that ramalin activated the autophagy process in breast cancer cells.

Phosphatidylinositol 3-kinase (PI3K) class III (PI3KcIII), also known as Vps34, is a member of the PI3K protein family of enzymes that are involved in the initiation of canonical autophagy by recruiting autophagy-related gene complexes to induce membrane phagophore formation. To determine whether PI3KcIII is involved in ramalin-stimulated apoptosis/autophagy, MCF-7 and MDA-MB-231 cells were treated with 100 μg/mL of ramalin in the presence or absence of 1 mM of 3-methyladenine (3MA), a Vps34 inhibitor, for 24 h. As shown in Fig. 8A, exposure to ramalin with 3MA increased the levels of expression of p62 and LC3-II even higher when compared with ramalin alone, whereas the combined treatment further reduced the expression of both Atg5 and beclin-1 in both cell lines when compared with cells treated with ramalin alone, suggesting that ramalin could activate the early stage of the autophagy process in breast cancer cells.

Further support for this conclusion comes from the observation that pretreatment with actinomycin D resulted in decreased expression of Atg5, beclin-1, LC-3, and p62 in both breast cancer cells exposed to ramalin, whereas the expression of these autophagy-related genes was significantly higher with bafilomycin A1 treatment (Fig. 8B). Similarly, the increased number of GFP-LC3 puncta upon ramalin treatment was reduced by actinomycin D in the MCF-7 and MDA-MB-231 cells (Fig. 8C). However, pretreatment with bafilomycin A1 resulted in a significantly higher number of GFP-LC3 puncta as compared with that in the ramalin-treated cells.

Next, we investigated the contribution of autophagy to ramalin-induced apoptosis. Both cell types were treated with ramalin in the presence or absence of 3MA. The

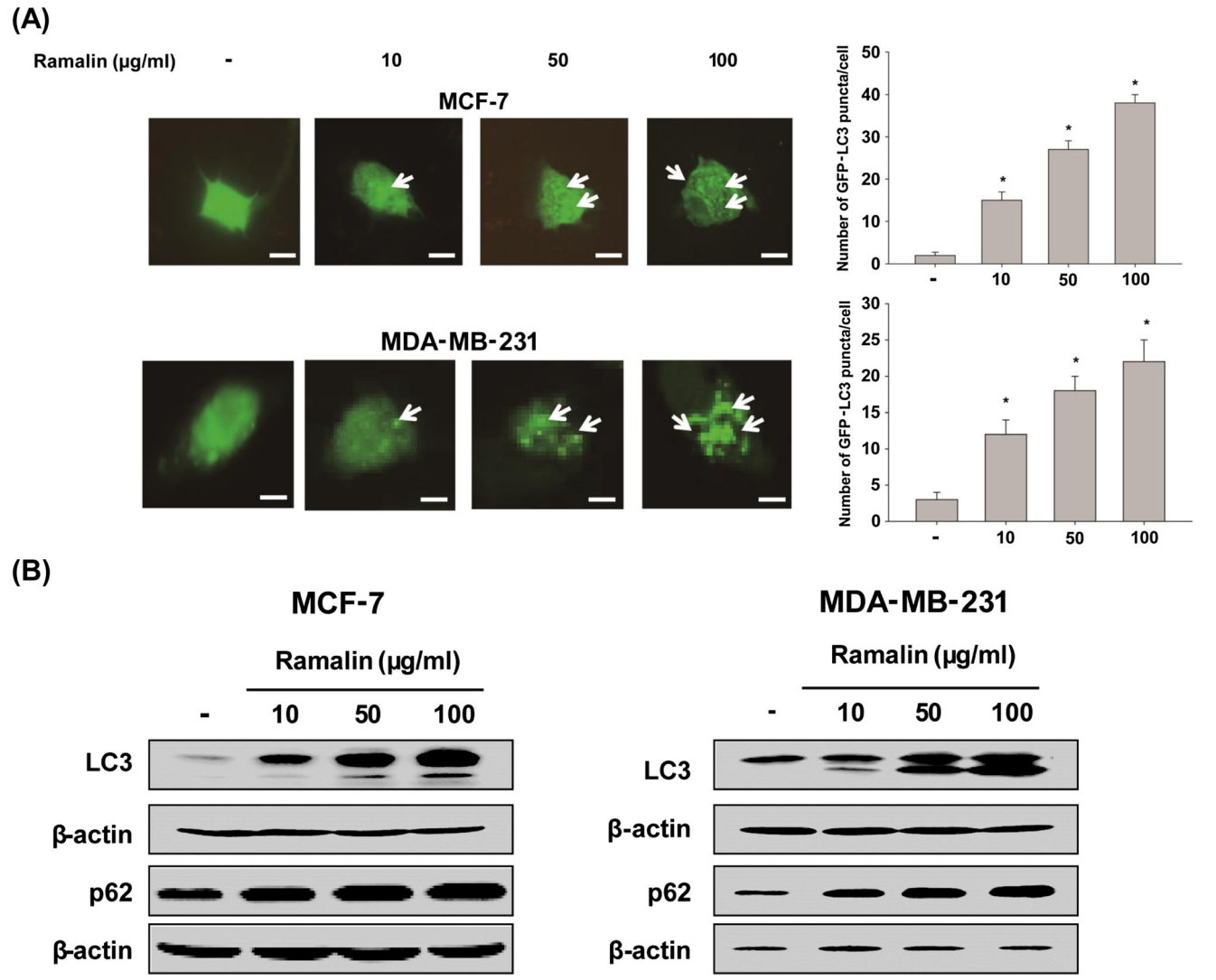

Figure 7. Ramalin induces autophagy in breast cancer cells. (A) Immunofluorescence microscopy was used to determine the punctate pattern of LC3 (arrows) in breast cancer cells transiently transfected with the GFP-LC3 vector and then treated with either vehicle (control) or ramalin for 24 h. Representative fluorescent images are shown (scale bars = 20 μm). Quantification shown in the graph on the right represents the mean ± SEM number of GFP-LC3 puncta per cell from three independent experiments. * $p < 0.05$ versus untreated control. (B) MCF-7 cells and MDA-MB-231 cells were treated with the indicated concentrations of ramalin for 24 h. Total cell lysates were prepared and subjected to immunoblotting of LC3, p62, and β-actin. β-actin was used as an internal control. Data are representative of three independent experiments. This figure is available in color online at wileyonlinelibrary.com/journal/ptr.

expression of the pro-apoptotic Bax protein was increased, and the expression of anti-apoptotic Bcl-2 protein was decreased to a greater extent by pretreatment with ramalin plus 3MA in the MCF-7 and MDA-MB-231, as compared with the cells treated with ramalin alone (Fig. 9A). In addition, the ramalin-treated MCF-7 cells and MDA-MB-231 cells showed reduced viability when autophagy was inhibited by 3MA (Fig. 9B).

To provide more direct evidence that autophagy influences ramalin-induced apoptosis, we used Atg5-targeting siRNA to decrease endogenous Atg5 expression. Atg5 is essential for autophagosome formation. Knockdown of Atg5 increased the expression of Bax and decreased the expression of Bcl-2 protein in both ramalin-treated breast cancer cells, as compared with the cells transfected with scrambled siRNA (Fig. 9C). These results suggest that the autophagic process is closely related to ramalin-induced breast cancer cell apoptosis.

DISCUSSION

In the present study, we found that ramalin induced both autophagy and apoptosis in breast cancer cells and that autophagy inhibition enhanced ramalin-induced apoptosis. Breast cancer has become the most important cancer in women (Jemal *et al.*, 2011). In general, ER-positive cells are present in 60% of patients with breast cancer, and these patients can be treated with anti-oestrogen therapy; ER-negative breast cancer is harder to treat (Badve and Nakshatri, 2009; Shen *et al.*, 2012). Although there have been many attempts to develop treatments, more effective anticancer agents are still needed for both ER-positive and ER-negative breast cancer.

In this study, we investigated the apoptotic effects of ramalin, a novel compound, on two kinds of human breast cancer cell lines. Our results showed that treatment

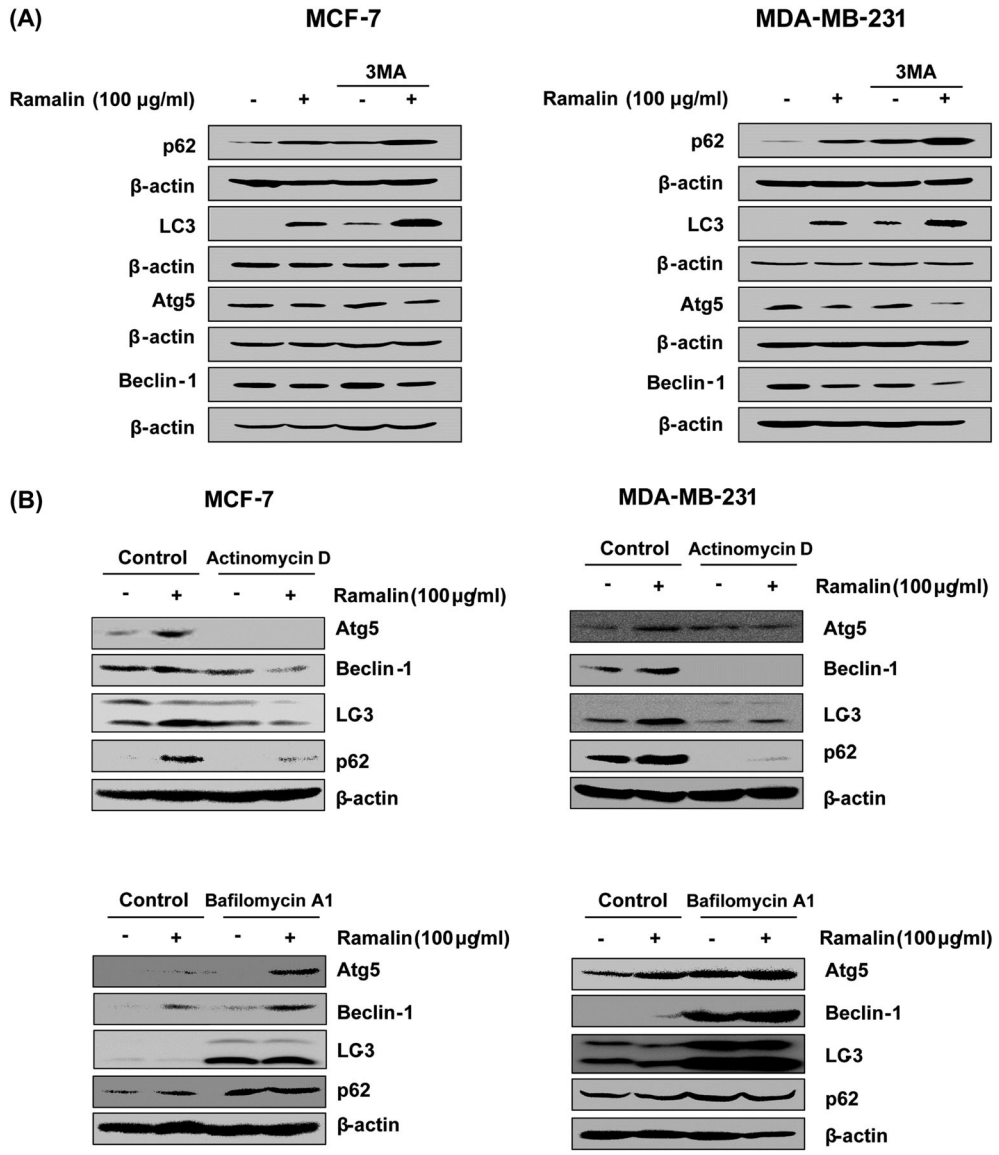

Figure 8. Inhibition of autophagy in ramalin-treated cells. (A and B) MCF-7 and MDA-MB-231 cells were treated with 100 μg/mL of ramalin in the presence or absence of 3-methyladenine (3MA; 1 mM), actinomycin D (1 μM), or bafilomycin A (1 ng/mL) for 2 h. Levels of proteins were determined by western blot analysis using the antibodies indicated. β-actin was used as the control for protein loading. (C) Immunofluorescence microscopy was used to analyse the punctate pattern of LC3 (arrows) in inhibitor-pretreated cells that were transiently transfected with the GFP-LC3 vector and then treated with either vehicle (control) or ramalin for 24 h. Representative fluorescent images are shown (scale bars = 20 μm). Quantification shown in the bottom graph represents the mean ± SEM number of GFP-LC3 puncta per cell from three independent experiments. * $p < 0.05$ versus ramalin-only treatment. $^{\#}$ $p < 0.05$ versus ramalin-only treatment.

with ramalin resulted in a dose-dependent decrease in cell proliferation in both MCF-7 and MDA-MB-231 cells. In addition, both types of ramalin-treated breast cancer cells showed dose-dependent increases in early-stage or late-stage apoptosis. These findings were confirmed by experiments using the pan-caspase inhibitor Z-VAD-fmk, which enhanced cell viability in the ramalin-treated cells. Collectively, these results suggest that ramalin can inhibit the growth of human breast cancer cells and induce apoptosis. It is noteworthy that ramalin functions independently of ER status to induce growth inhibition and cytotoxicity in breast cancer cells. In this investigation, MCF7 and MDA-MB-231 human breast cancer cell lines were used, which are the most widely used breast cancer cell lines. One of the main differences between MCF-7 and MDA-MB-231 cell lines is the presence of ER (Morelli *et al.*, 2003). It has been argued that ER status might predict the response of breast cancer cells to cytotoxic compounds (Duffy, 2006; Jensen and Jordan, 2003; Jordan and O'Malley, 2007). Some papers have reported that there was complete correlation between the presence of ER and the sensitivity of the breast cancer cells to cytotoxic compounds. In contrast, others have demonstrated that the presence of ER did not correlate with response to cytotoxic compounds. In the present study, our data showed that both breast cancer cells are sensitive to ramalin and there is a similar cytotoxicity between the two breast cancer cell lines after treatment with ramalin. In addition, because the MDA-MB-231 cell line does not contain ERs, the presence of ER has no significant effect on ramalin-induced cell growth inhibitory activity, suggesting that the effect of ramalin may be via ER-independent, non-cell-type-specific cytotoxic mechanism.

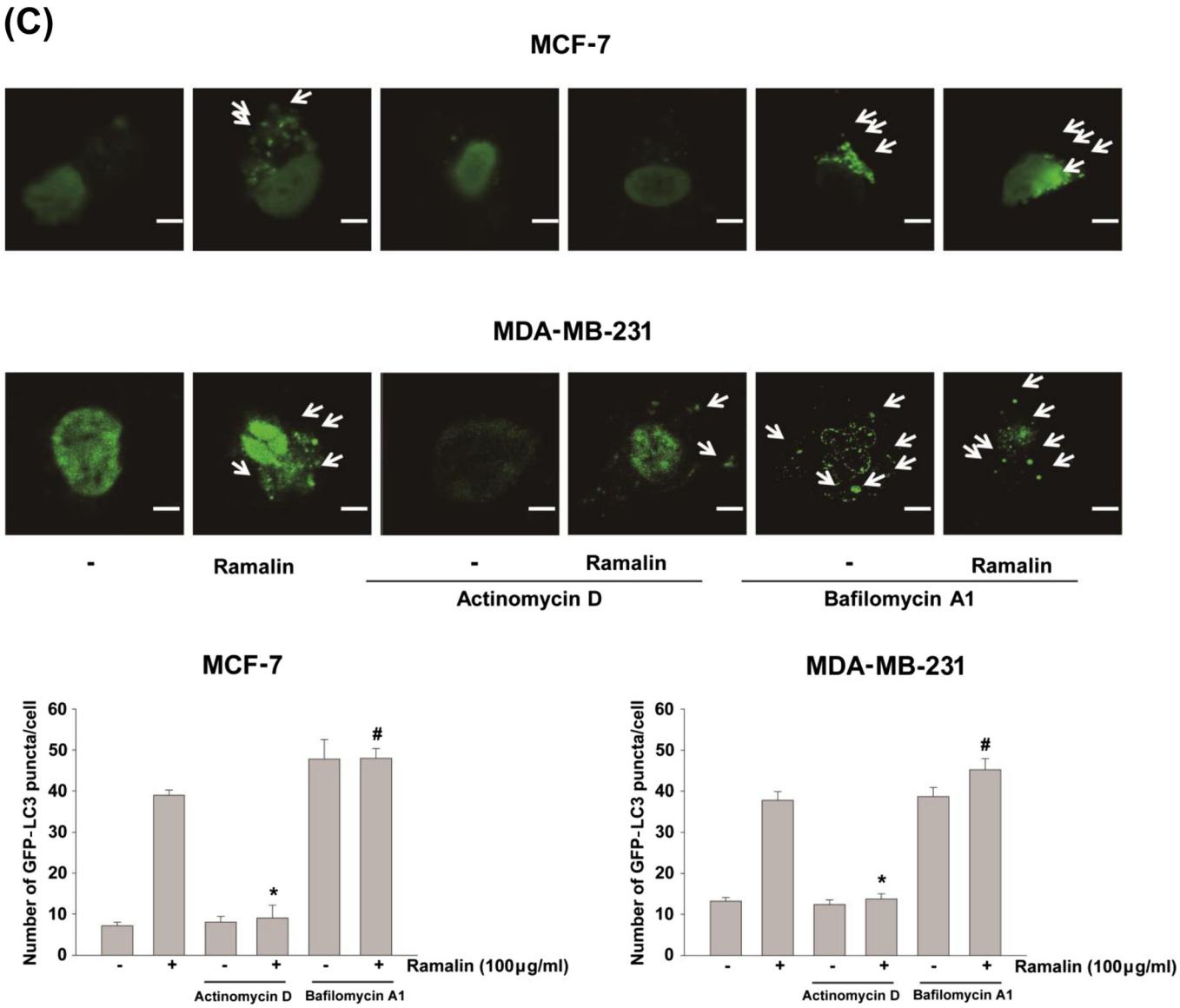

Figure 8. (Continued). This figure is available in color online at wileyonlinelibrary.com/journal/ptr.

The early signal transduction of the apoptotic pathway is delicately controlled by several proteins, such as p53 or members of the Bcl-2 family (Chipuk and Green, 2008). The tumour suppressor p53 plays a role in regulating the response to several stimuli and triggering apoptosis by causing pro-apoptosis to maintain cellular integrity (Haupt *et al.*, 2003). In evading apoptosis, about half of human tumours inactivate p53 by mutation, and these tumour cells are more resistant to chemotherapy than are cancer cells that express wild-type p53 (Scata and El-Deiry, 2007). In addition, the expressions of wild-type p53 and mutated p53 are regulated by the same promoter, with the wild-type p53 being expressed in MCF-7 cells and the mutant p53 (p53 mutant 280, Arg-Lys) being expressed in MDA-MB-231 cells (Scata and El-Deiry, 2007). Functional p53 is important in the p53-dependent pathway leading to apoptosis. In our study, treatment with ramalin led to an increase in p53 expression in the MCF-7 cells; however, in the MDA-MB-231 cells, treatment with ramalin resulted in a decrease in the expression of dysfunctional p53, suggesting that the apoptosis induced by ramalin occurs via a p53-independent pathway. Nevertheless, our results suggest that ramalin affects the survival of breast cancer cells that retain the wild-type p53 or the mutant p53, and these two breast cancer cell lines with different p53 status show differential molecular responses to ramalin. However, further studies are needed to establish the role of p53 in ramalin-induced apoptosis in this system.

Mitochondria play a crucial role in a cell's commitment to apoptosis through increased permeability of the outer mitochondrial membrane, decreased transmembrane potential, and release of cytochrome c and AIF (Burlacu, 2003; Tait and Green, 2010). These mitochondrial events can be inhibited by anti-apoptotic Bcl-2 family members (Bcl-2 and Bcl-XL), whereas pro-apoptotic Bcl-2 family members (Bax, Bak, and Bad) can trigger these events. We observed mitochondrial changes in both types of breast cancer cells. Treatment with ramalin enhanced the release of both AIF and cytochrome c from the mitochondria to the cytosol. Because the translocation of Bax to the mitochondrial membrane can alter the conformational changes and

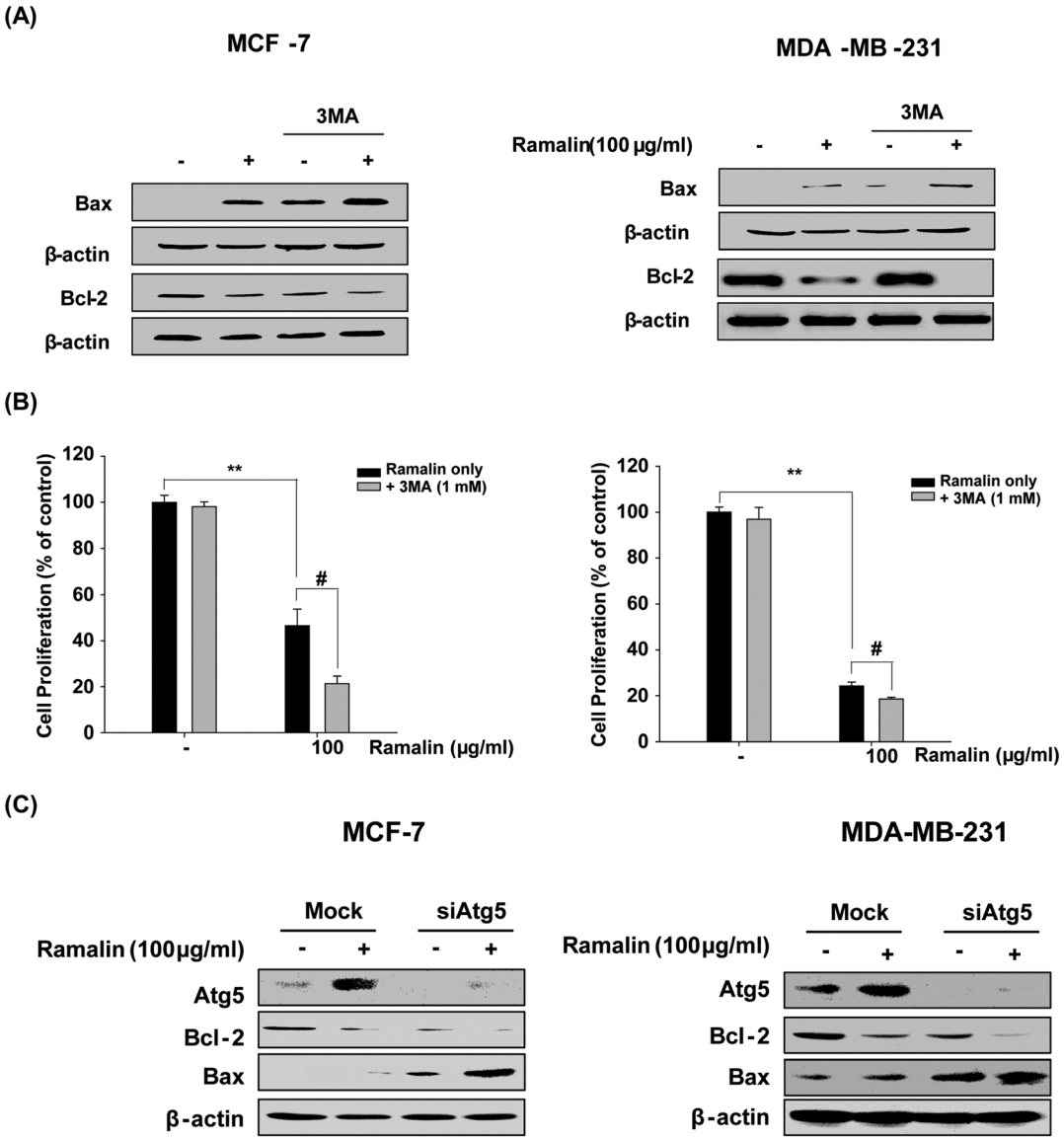

Figure 9. Inhibition of autophagy enhances ramalin-induced apoptosis. MCF-7 and MDA-MB-231 cells were treated with 100 μg/mL of ramalin in the presence or the absence of 3-methyladenine (3MA; 1 mM) for 24 h. (A) Protein levels were determined by western blot analysis using the antibodies indicated. β-actin was used as the control for protein loading, and band intensities were quantified by densitometry using ImageJ software. (B) Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data shown are the means ± SEM of at least three independent experiments. * $p < 0.05$ versus ramalin-only treatment. (C) Cells were transfected with control siRNA and Atg5 siRNA (see Materials and Methods section). Total cell lysates were prepared and subjected to immunoblotting of Bcl-2, Bax, and β-actin. β-actin was used as an internal control. Data are representative of three independent experiments.

cause the release of cytochrome c into the cytosol, activating the caspase cascade that leads to apoptotic cell death (Finucane *et al*., 1999), it is plausible that cytochrome c release as induced by ramalin is regulated by Bax. In our study, the expression of Bax was increased in the ramalin-treated MCF-7 and MDA-MB-231 cells, whereas Bcl-2 expression decreased following treatment with ramalin.

Thus, our results suggest that ramalin induces apoptosis in both breast cancer cell lines by the release of cytochrome c and AIF from the mitochondria by regulating Bcl-2 family proteins. The release of cytochrome c triggers activation of the caspase cascade from apical caspases to effector caspases (Finucane *et al*., 1999). One of the main consequences of mitochondrial cytochrome c release is the activation of caspase-3, which is the most frequently activated caspase protease in apoptotic cells. In this study, caspase-8 and caspase-9 were activated in both ramalin-treated human breast cancer cells, and the expression of caspase-3 was also increased in the ramalin-treated MDA-MB-231 cells; however, ramalin treatment did not induce activation of caspase-3 in the MCF-7 cells in accordance with the previous study in which MCF-7 cells did not express caspase-3 (Janicke *et al*., 1998). Based on these findings, it is conceivable that ramalin-induced apoptosis occurs through a caspase-3-dependent pathway in MDA-MB-231 cells and a caspase-3-independent pathway in MCF-7 cells. Interestingly, our data demonstrate that in MDA-MB-231 cells, ramalin induced the expression of AIF, which acts in a caspase-independent manner. Therefore, this finding suggests that the two pathways might exist in ramalin-treated MDA-MB-231 cells during apoptosis. In addition, it has been reported that the activation of other caspases, such as caspase-6 and caspase-7, is involved in apoptosis induced by a

variety of different apoptotic stimuli in caspase-3-deficient MCF-7 cells (Suyama *et al*., 2007; Chou *et al*., 2010; Tsao *et al*., 2008; Jin *et al*., 2010; Jang *et al*., 2007). Thus, the results presented here suggest that activation of caspase-6 and caspase-7 may be associated with ramalin-induced apoptosis in MCF-7 cells.

The interplay between apoptosis and autophagy is rather complex, but it is a critical factor influencing outcomes in death-related diseases such as cancer (Eisenberg-Lerner *et al*., 2009). However, the relationship between autophagy and cancer remains controversial. In some studies, the autophagic process can help cancer cells survive despite extremely stressful conditions (Jain *et al*., 2013), but in other studies, autophagy may inhibit tumourigenesis (Jin and White, 2007). In our study, we looked at whether autophagy contributed to the survival of breast cancer cells during ramalin-induced apoptosis. Our data showed that the expression of LC3-II and the accumulation of GFP-LC3 puncta in the cytosol were increased by treatment with ramalin in a concentration-dependent manner in the MCF-7 cells and MDA-MB-231 cells. The expression of p62 was also increased in both types of ramalin-treated cells. Moreover, 3MA or actinomycin D treatment inhibited the increased expression of autophagy-related genes in both types of ramalin-treated breast cancer cells, whereas pretreatment with bafilomycin A1 resulted in a significantly higher expression of autophagy-related genes. Thus, these data indicate that ramalin activated the autophagic process in breast cancer cells. Although it is widely accepted that p62 gets degraded during the process of autophagy, our data are in keeping with the previous notion that an increase in p62 expression is required for autophagy induction (Wong *et al*., 2010; Robert *et al*., 2009; Puissant *et al*., 2010; Puissant *et al*., 2012). This difference could be due to differences in the stimuli and the cell types used. Nevertheless, results of the current study suggest that ramalin could activate the autophagic process.

In order to determine whether autophagy promoted either cell survival or cell death in response to treatment with ramalin, we used pharmacologic and genetic methods to inhibit autophagy. Cell viability in both breast cancer cell lines was decreased to a greater extent by treatment with ramalin plus 3MA than by treatment with ramalin alone. Furthermore, ramalin combined with 3MA caused upregulation of Bax expression and downregulation of Bcl-2 expression in the MCF-7 and MDA-MB-231 cells. These data indicate that ramalin-triggered autophagy was protective. Further support for this conclusion comes from the observation that inhibition of Atg5 caused the increase in Bax levels and the decrease in Bcl-2 levels in the breast cancer cells treated with ramalin. These findings suggest that inhibition of autophagy can enhance ramalin-induced apoptosis

Recently, many natural products from plants have been found to induce cell death and autophagy. Treatment with sulforaphane or fisetin resulted in the induction of apoptosis and the inhibition of autophagy in breast cancer cells (Kanematsu *et al*., 2010; Yang *et al*., 2012). Based on these findings, it is plausible that ramalin could act through mechanisms similar to those of sulforaphane and fisetin. Taken together, these data suggest that there is crosstalk between ramalin-induced apoptosis and autophagy in human breast cancer cells.

In summary, this study demonstrates that ramalin induces apoptosis in MCF-7 and MDA-MB-231 cells via the mitochondrial pathway and involves the upregulation of Bax and the downregulation of Bcl-2, leading to the release of cytochrome c and AIF from mitochondria into the cytosol and to the activation of caspase. Moreover, inhibition of autophagy can enhance ramalin-induced apoptosis in different human breast cancer cells. Taken together, our data suggest that ramalin has the potential to be an effective anticancer agent for human breast cancer cells irrespective of their p53 or oestrogen-receptor status. However, further study is needed to examine the efficacy, concentration, and mechanism of ramalin as an anticancer agent *in vivo*.

Acknowledgements

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Conflict of Interest

The authors have no conflicts of interest to declare.

REFERENCES

Badve S, Nakshatri H. 2009. Oestrogen-receptor-positive breast cancer: towards bridging histopathological and molecular classifications. *J Clin Pathol* **62**: 6–12.

Burlacu A. 2003. Regulation of apoptosis by Bcl-2 family proteins. *J Cell Mol Med* **7**: 249–257.

Chipuk JE, Green DR. 2008. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? *Trends Cell Biol* **18**: 157–164.

Chou C, Yang J, Lu H, *et al.* 2010. Quercetin-mediated cell cycle arrest and apoptosis involving activation of a caspase cascade through the mitochondrial pathway in human breast cancer MCF-7 cells. *Arch Pharm Res* **33**: 1181–1191.

Du C, Fang M, Li Y, Li L, Wang X. 2000. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* **102**: 33–42.

Duffy MJ. 2006. Estrogen receptors: role in breast cancer. *Crit Rev Clin Lab Sci* **43**: 325–347.

Eisenberg-Lerner A, Bialik S, Simon H, Kimchi A. 2009. Life and death partners: apoptosis, autophagy and the cross-talk between them. *Cell Death Differ* **16**: 966–975.

Elmore S. 2007. Apoptosis: a review of programmed cell death. *Toxicol Pathol* **35**: 495–516.

Finucane DM, Bossy-Wetzel E, Waterhouse NJ, Cotter TG, Green DR. 1999. Bax-induced caspase activation and apoptosis via cytochrome c release from mitochondria is inhibitable by Bcl-xL. *J Biol Chem* **274**: 2225–2233.

Gozuacik D, Kimchi A. 2004. Autophagy as a cell death and tumour suppressor mechanism. *Oncogene* **23**: 2891–2906.

Hanahan D, Weinberg RA. 2000. The hallmarks of cancer. *Cell* **100**: 57–70.

Haupt S, Berger M, Goldberg Z, Haupt Y. 2003. Apoptosis-the p53 network. *J Cell Sci* **116**: 4077–4085.

Hemann M, Lowe S. 2006. The p53–Bcl-2 connection. *Cell Death Differ* **13**: 1256–1259.

Herzog C, Yang C, Holmes A, Kaushal GP. 2012. zVAD-fmk prevents cisplatin-induced cleavage of autophagy proteins but impairs autophagic flux and worsens renal function. *Am J Physiol Renal Physiol* **303**: F1239–F1250.

Hulka BS, Moorman PG. 2001. Breast cancer: hormones and other risk factors. *Maturitas* **38**: 103–113.

Huneck S. 1999. The significance of lichens and their metabolites. *Naturwissenschaften* **86**: 559–570.
Jain K, Paranandi KS, Sridharan S, Basu A. 2013. Autophagy in breast cancer and its implications for therapy. *Am J Cancer Res* **3**: 251–256.
Jang M, Park BC, Lee AY, *et al.* 2007. Caspase-7 mediated cleavage of proteasome subunits during apoptosis. *Biochem Biophys Res Commun* **363**: 388–394.
Janicke RU, Ng P, Sprengart ML, Porter AG. 1998. Caspase-3 is required for alpha-fodrin cleavage but dispensable for cleavage of other death substrates in apoptosis. *J Biol Chem* **273**: 15540–15545.
Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. 2011. Global cancer statistics. *CA Cancer J Clin* **61**: 69–90.
Jensen EV, Jordan VC. 2003. The oestrogen receptor: a model for molecular medicine. *Clin Cancer Res* **9**: 1980–1989.
Jin S, White E. 2007. Role of autophagy in cancer: management of metabolic stress. *Autophagy* **3**: 28–31.
Jin S, Zhang QY, Kang XM, Wang JX, Zhao WH. 2010. Daidzein induces MCF-7 breast cancer cell apoptosis via the mitochondrial pathway. *Ann Oncol* **21**: 263–268.
Jordan VC, O'Malley BW. 2007. Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. *J Clin Oncol* **25**: 5815–5824.
Kanematsu S, Uehara N, Miki H, *et al*. 2010. Autophagy inhibition enhances sulforaphane-induced apoptosis in human breast cancer cells. *Anticancer Res* **30**: 3381–3390.
Kaushal GP. 2012. Autophagy protects proximal tubular cells from injury and apoptosis. *Kidney Int* **82**: 1250–1253.
Lavrik I, Golks A, Krammer PH. 2005. Death receptor signaling. *J Cell Sci* **118**: 265–267.
Lee J, Kim J, Rhee D, Pyo S. 2013. *Streptococcus pneumoniae* ClpP protease induces apoptosis via caspase-independent pathway in human neuroblastoma cells: cytoplasmic relocalization of p53. *Toxicon* **70**: 142–152.
Morelli C, Garofalo C, Bartucci M, Surmacz E. 2003. Estrogen receptor-α regulates the degradation of insulin receptor substrates 1 and 2 in breast cancer cells. *Oncogene* **22**: 4007–4016.
Müller K. 2001. Pharmaceutically relevant metabolites from lichens. *Appl Microbiol Biotechnol* **56**: 9–16.
Nguyen CT, Luong TT, Kim GL, Pyo S, Rhee DK. 2015. Korean Red Ginseng inhibits apoptosis in neuroblastoma cells via estrogen receptor beta-mediated phosphatidylinositol-3 kinase/Akt signaling. *J Ginseng Res* **39**: 69–75.
Nicholson DW. 2000. From bench to clinic with apoptosis-based therapeutic agents. *Nature* **407**: 810–816.
Paudel B, Bhattarai HD, Koh HY, *et al.* 2011. Ramalin, a novel non-toxic antioxidant compound from the Antarctic lichen *Ramalina terebrata*. *Phytomedicine* **18**: 1285–1290.
Puissant A, Robert G, Fenouille N, *et al.* 2010. Resveratrol promotes autophagic cell death in chronic myelogenous leukemia cells via JNK-mediated p62/SQSTM1 expression and AMPK activation. *Cancer Res* **70**: 1042–1052.
Puissant A, Fenouille N, Auberger P. 2012. When autophagy meets cancer through p62/SQSTM1. *Cancer Res* **2**: 397–413.
Robert G, Sahra IB, Puissant A, *et al.* 2009. Acadesine kills chronic myelogenous leukemia (CML) cells through PKC-dependent induction of autophagic cell death. *PLoS One* **4**e7889.
Rouzier R, Perou CM, Symmans WF, *et al.* 2005. Breast cancer molecular subtypes respond differently to preoperative chemotherapy. *Clin Cancer Res* **11**: 5678–5685.
Scata KA, El-Deiry WS. 2007. p53, BRCA1 and breast cancer chemoresistance. *Adv Exp Med Biol* **608**: 70–86.
Shen K, Rice SD, Gingrich DA, *et al.* 2012. Distinct genes related to drug response identified in ER positive and ER negative breast cancer cell lines. *PLoS One* **7**e40900: .
Suyama K, Noguchi Y, Tanaka T, *et al.* 2007. Isoprenoid-independent pathway is involved in apoptosis induced by risedronate, a bisphosphonate, in which Bim plays a critical role in breast cancer cell line MCF-7. *Oncol Rep* **18**: 1291–1298.
Tait SW, Green DR. 2010. Mitochondria and cell death: outer membrane permeabilization and beyond. *Nat Rev Mol Cell Biol* **11**: 621–632.
Tsao CH, Su HL, Lin YL, *et al.* 2008. Japanese encephalitis virus infection activates caspase-8 and -9 in a FADD-independent and mitochondrion-dependent manner. *J Gen Virol* **89**: 1930–1941.
Wong CH, Iskandar KB, Yadav SK, Hirpara JL, Loh T, Pervaiz S. 2010. Simultaneous induction of non-canonical autophagy and apoptosis in cancer cells by ROS-dependent ERK and JNK activation. *PLoS One* **5**e9996: .
Yang PM, Tseng HH, Peng CW, Chen WS, Chiu SJ. 2012. Dietary flavonoid fisetin targets caspase-3-deficient human breast cancer MCF-7 cells by induction of caspase-7-associated apoptosis and inhibition of autophagy. *Int J Oncol* **40**: 469–478.
Yoshimori T. 2004. Autophagy: a regulated bulk degradation process inside cells. *Biochem Biophys Res Commun* **313**: 453–458.

