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Journal of Cellular Biochemistry 112:2471–2479 (2011)

Journal of Cellular Biochemistry

Induction of Autophagy by Dimethyl Cardamonin Is Associated With Proliferative Arrest in Human Colorectal Carcinoma HCT116 and LOVO Cells

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ABSTRACT

Dimethyl cardamonin (2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone; DMC) is a naturally occurring chalcone, and it is the major compound isolated from the leaves of *Syzygium samarangense* (Blume) Merr. & L.M. Perry (Myrtaceae). Experiments were conducted to determine the effects of DMC on cell proliferation, cell-cycle distribution, and programmed cell death in cultures of human colorectal carcinoma HCT116 and LOVO cells. Results showed that DMC inhibited HCT116 and LOVO cell proliferation and induced G_2/M cell cycle arrest, which was associated with the conversion of microtubule associated protein light chain 3 (LC3)-I–LC3-II, an autophagosome marker, and the incorporation of monodansylcadaverine (MDC), a marker for the acidic compartment of autolysosomes or acidic vesicular organelles. The treatment of HCT116 and LOVO cells using a combination of DMC with an autophagy inhibitor, such as 3-methyladenine (3-MA), beclin 1 siRNA, or atg5 siRNA, suppressed the effect of DMC-mediated anti-proliferation. These results imply that DMC can suppress colorectal carcinoma HCT116 and LOVO cell proliferation through a G_2/M phase cell-cycle delay, and can induce autophagy, the hallmark of Type II programmed cell death (PCD). Taken together, our results suggest that DMC may be an effective chemotherapeutic agent for HCT116 and LOVO colorectal carcinoma cells. J. Cell. Biochem. 112: 2471–2479, 2011.

KEY WORDS: DIMETHYL CARDAMONIN; AUTOPHAGY; G_2/M CELL CYCLE ARREST; HCT116; LOVO

Macroautophagy (hereafter called autophagy), which is known as type-II programmed cell death (PCD), is induced in response to nutrition limitations or cellular stress. It is an evolutionarily conserved process that occurs in all eukaryotic cells. Autophagic cells generally enlarge, develop vacuoles, or have enlarged or swollen nuclei [Elliott and Reiners, 2008]. At the beginning of autophagy, a portion of the cytoplasm and other intracellular organelles are sequestered in double-membraned structures that are called as autophagosomes. This process requires the formation of a multiprotein complex consisting of beclin-1, UVRAG (UV irradiation resistance-associated tumor suppressor gene), and myristylated kinase, and the conjugation of phosphatidylethanolamine (PE) to LC3/Atg8 (LC3 is a mammalian homolog of Atg8). Lipid conjugation leads to the conversion of soluble LC3 (LC3-I) to autophagic vesicle-associated LC3 (LC3-II). These autophagosomes then fuse with lysosomes to form autolysosomes. Finally, the sequestered contents are then degraded by lysosomal hydrolases and are recycled [Maiuri et al., 2007].

Autophagy is used for the elimination of pathogens and the engulfment of apoptotic cells. Importantly, the effects of these events on cancer are not well known. Most evidence indicates a role for autophagy in sustaining cell survival. For example, ginsenoside Rk1, which was obtained from heat-processed Sun Ginseng (SG), has a novel function as an inducer of autophagy in the early stages of Rk1-induced apoptosis in HepG2 cells. In contrast, cell death resulting from progressive cellular consumption has been attributed to unrestrained autophagy. Recent studies suggested that autophagy can also contribute to another cell death pathway under certain circumstances, the so-called Type II PCD (“autophagic cell death”), that is morphologically and biochemically distinct from Type I PCD apoptosis [Qu et al., 2007]. A number of studies have reported that in response to various anticancer therapies, autophagic cell death is

Grant sponsor: Korea Institute of Science and Technology, Korea; Grant number: 2Z03401.
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Received 10 September 2010; Accepted 25 April 2011 • DOI 10.1002/jcb.23171 • © 2011 Wiley-Liss, Inc.
Published online 2 May 2011 in Wiley Online Library (wileyonlinelibrary.com).

activated in cancer cells derived from tissues such as breast, colon, prostate, and brain [Bursch et al., 1996; Komata et al., 2004; Scarlatti et al., 2004; Kanzawa et al., 2005]. For example, tamoxifen induces autophagic cell death in breast cancer cells through the down-regulation of protein kinase B/AKT [Scarlatti et al., 2004].

DMC (2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone) is a naturally occurring chalcone, and it is the major compound isolated from the leaves of *Syzygium samarangense* (Blume) Merr. & L.M. Perry (Myrtaceae) [Ghayur et al., 2006]. The plant has been reported to have immunostimulating [Srivastava et al., 1995] and anti-bacterial [Chattopadhayay et al., 1998] activities. In Taiwanese folk medicine, DMC has been used as an antipyretic and a diuretic agent [Kuo et al., 2004], where it has been reported to have anti-bacterial [Gafner et al., 1996], anti-diabetic [Resurreccion-Magno et al., 2005], and anti-tumoral [Dan-Dan et al., 2009] properties. However, the mechanisms of action of its anti-tumoral effects have not been studied despite a similar structure to cardamonin, a chalcone that has been shown to possess anti-tumoral activity [Na et al., 2008].

In this study, we investigated the mechanism by which DMC induced anti-proliferative effect in colorectal carcinoma HCT116 and LOVO cells. We found that DMC possessed autophagic anti-proliferative effect in HCT116 and LOVO cells. Here, we focused on human colorectal carcinoma because it is one of the most frequent malignant neoplasms worldwide [Pisani et al., 1999]. Furthermore, medicinal plants or active components of plant origin have been found to exert beneficial effects in the prevention and treatment of human colorectal carcinoma [Na et al., 2008]. To the best of our knowledge, this study provides the first evidence that DMC may act as a chemopreventive agent by inducing an autophagy-mediated anti-proliferative effect in human HCT116 and LOVO colorectal carcinoma cells.

MATERIALS AND METHODS

CELL CULTURE AND CHEMICALS

The human colorectal carcinoma cell line HCT116 and LOVO were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in Dulbecco's modified Eagle medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Hyclone), 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a humidified atmosphere with 5% CO_2. DMC was isolated from the leaves of *S. samarangense*. The structure of DMC (Fig. 1A) was established by comparing ^{1}H and ^{13}C-NMR optical rotation and ESIMS data with previously obtained values [Kim et al., 2008]. The compound was dissolved in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO) at a concentration of 20 mM to generate a stock solution. For treatment of cells, the compound was diluted in culture medium to the appropriate concentration. Bafilomycin A1 and 3-MA were obtained from Sigma.

ANALYSIS OF CELL VIABILITY

Proliferation of cells was measured with Cell Counting Kit-8 (CCK8) (Dojindo Laboratories, Kumamoto, Japan) [Hyun et al., 2010]. Briefly, exponentially growing cells were seeded in a 96-well plate at a density of 1.0×10^4 cells/well. The next day, the cells were treated in triplicate with various concentrations of DMC. After incubation for 24 h, 10 μl of the kit reagent was added to each well, and the cells were incubated for an additional hour. Cell viability was assessed by scanning with a microplate reader at 450 nm. Control cells were exposed to culture media containing 0.5% v/v DMSO.

WESTERN BLOT ANALYSIS

Whole-cell lysates were prepared by incubating cells in RIPA buffer (Cell Signaling, Beverly, MA) supplemented with 1× protease inhibitor cocktail (Roche, Mannheim, Germany) and 1 mM phenylmethylsulfonyl fluoride (PMSF) according to the manufacturer's instructions. Proteins (40 μg/lane) were separated by electrophoresis with NuPAGE 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA), transferred onto PVDF membranes, and analyzed with the indicated antibodies. The bound antibodies were visualized using ECL Advance Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK) and LAS-4000 film (Fujifilm, Tokyo, Japan) [Shin et al., 2005; Ko et al., 2007]. Monoclonal antibodies to cyclin B1 and polyclonal antibodies to LC3B, cdc2, phospho-Cyclin B1 (Ser 147), phospho-Chk2 (Thr 68), Chk2, cleaved Caspase-3, cleaved Caspase-9, ATG5, Beclin-1, and GAPDH were purchased from Cell Signaling Technology. Antibody to the cleaved form of PARP was obtained from BD Biosciences Pharmingen (BD Biosciences, San Jose, CA).

CELL CYCLE ANALYSIS

To detect changes in the cell cycle, cells were collected, washed with cold phosphate buffered saline (PBS), and fixed in 70% ethanol at 4°C for 30 min. They were then washed twice with PBS, resuspended in 500 μl of propidium iodide (PI) staining solution containing 40 μg/ml PI and 20 μg/ml RNase A in PBS, incubated at room temperature (RT) for 30 min in the dark, and then analyzed with a FACSCalibur flow cytometer (Becton–Dickinson, San Jose, CA) and a ModFit LT V2.0 computer program. At least 20,000 events were assessed in this experiment.

CONFOCAL MICROSCOPY ANALYSIS

To detect the expression of LC3, cells were seeded onto sterile coverslips that were placed in 12-well plates. The next day, the cells were treated with DMC. At 24 h post-treatment, the cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 30 min, blocked with 2% BSA in PBS for 1 h, and incubated with the LC3B primary antibody at RT for 1 h and with the Alexa fluor 488 secondary antibody (Invitrogen) at RT for 1 h in the dark. This was followed by incubation with 1 μg/ml of 4′,6′-diamidino-2-phenylindole (DAPI) at RT for 20 min in the dark. Slides were then prepared with one drop of ProLong Gold Antifade Reagent (Invitrogen), and the coverslips were sealed onto the slides with clear nail lacquer. The images were obtained using a Leica TCS SP5 confocal microscope (Leica, Mannheim, Germany). To detect autophagic vacuoles, samples were seeded onto sterile coverslips and treated with 200 nM Bafilomycin A1 at RT for 40 min, followed by incubation with 50 μM monodansylcadaverine (MDC) (Sigma) in PBS at 37°C for 10 min. The coverslips were then washed 4 times with PBS and immediately sealed onto the slides. These images were

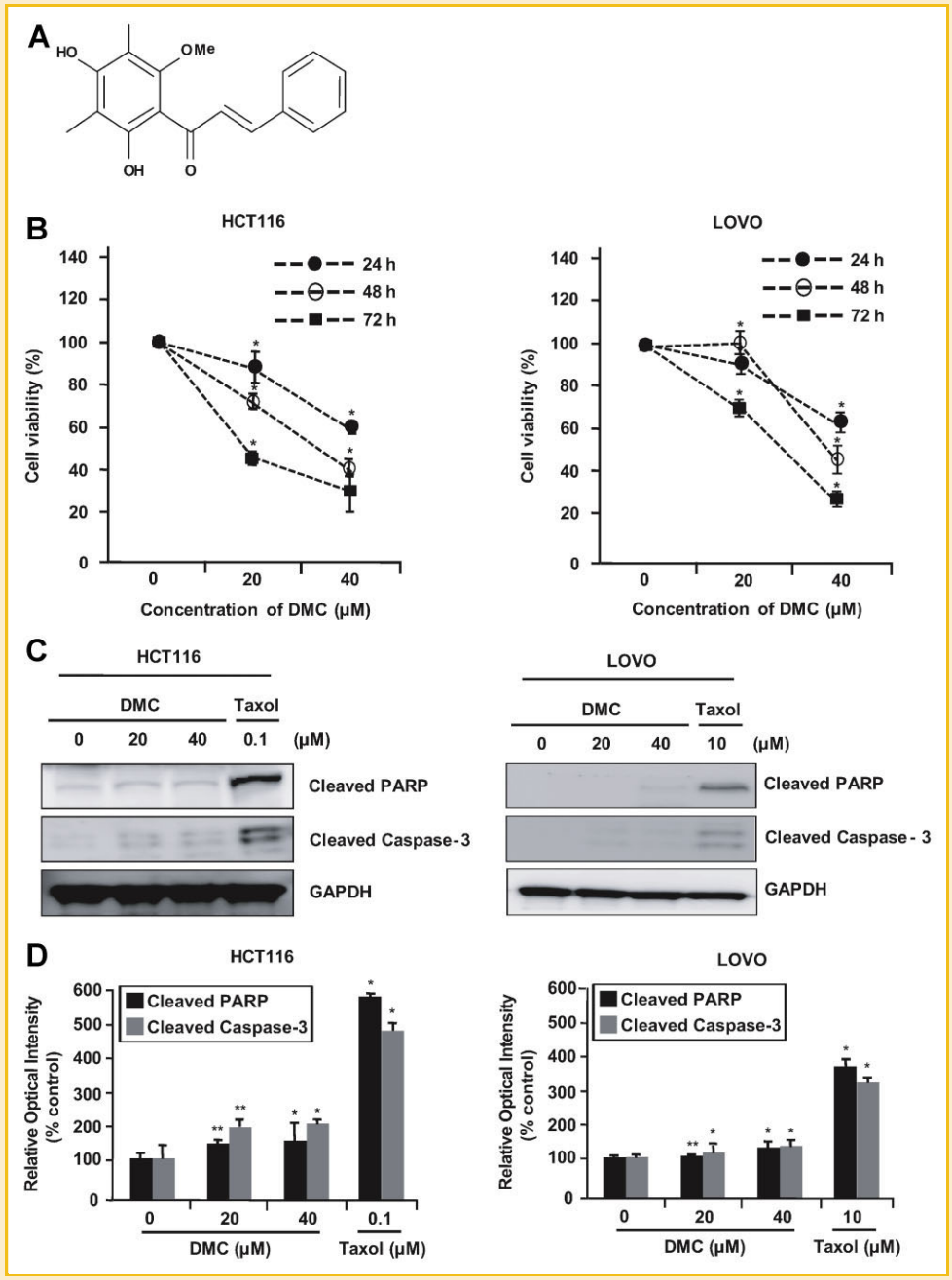

Fig. 1. DMC showed an anti-proliferative effect in HCT116 and LOVO cells after a 24 h incubation. The inhibitory effect of DMC on cell proliferation. A: The structure of DMC. B: CCK8 assay for viability of DMC over time. Data represent mean ± SD of three independent experiments. $^{*}P < 0.05$. C, D: DMC did not induce apoptosis. After the cells were treated with DMC for 24 h, total cell extracts were probed with antibodies against cleaved PARP, cleaved caspase-3. This is representative of three independent experiments (Taxol, positive control, 0.1 μM and 10 μM).

also obtained by using a confocal microscope (Leica), as described above.

SMALL INTERFERING RNA (SIRNA) TRANSFECTION—BECLIN 1 AND ATG5 KNOCKDOWN

siRNAs were purchased from Dharmacon (Lafayette, CO). The target sequence for beclin 1 and atg5 were gAUACCgACUUgUUCCUUA and ACAAAGAUGUGCUUCGAGA, respectively. The cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. A non-specific siRNA was used as a negative control.

STATISTICAL ANALYSIS

Statistical data were expressed as mean ± SD; student's *t*-test and analysis of variance (ANOVA) were used to determine statistical significance. Values of $P < 0.05$ and $P < 0.01$ were considered to be statistically significant.

RESULTS

DMC EXHIBITED ANTIPROLIFERATIVE ACTIVITY IN COLORECTAL CARCINOMA HCT116 AND LOVO CELLS

To evaluate the potential cell growth inhibition of DMC, we first examined the effect of DMC on cell proliferation in HCT116 and LOVO cells. As shown in Figure 1B, HCT116 and LOVO cells were treated with 0, 20, and 40 μM of DMC for 24, 48, and 72 h. Cell viability was subsequently measured by using the CCK8 assay. CCK8 is more sensitive than 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay. WST-8[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] is reduced by dehydrogenases in cells to give a yellow colored product (formazan), which is soluble in the tissue culture medium. The amount of the formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells. DMC inhibited cell growth in both a concentration and time-dependent manner. When HCT116 and LOVO cells were treated with DMC for 24 h, at a dose of 40 μM, DMC inhibited cell proliferation by about 40% and 37%, respectively, as compared with the vehicle control (0.01% v/v DMSO). Additional experiments were performed to determine whether DMC induced apoptosis in HCT116 and LOVO cells. Western blot analysis was used to detect cleaved forms of PARP, and the activated forms of caspase-3, which are involved in the apoptosis response. As shown in Figure 1C and D when HCT116 and LOVO cells were treated with DMC for 24 h, DMC did not induce cleavage of PARP or the activation of caspase-3. This result correlated very well with the western blot results. Taxol, which was used as a positive control, induced apoptosis in HCT116 and LOVO cells (Figure 1C and D). These findings suggest that DMC inhibits cell proliferation in both a concentration- and time-dependent manner. Especially, in 24 h treatment, DMC inhibits cell proliferation, although it does not induce apoptosis.

DMC MODEST INDUCES G_2/M PHASE ARREST AND REGULATES THE EXPRESSION OF G_2/M PHASE RELATED PROTEINS

To examine the mechanism responsible for DMC-mediated cell growth inhibition, the cell cycle distribution was analyzed by using PI staining and flow cytometric analysis. When HCT116 and LOVO cells were treated for 24 h with 40 μM DMC, the population of cells in the G_2/M phase increased from $23.0 \pm 5.3\%$ to $30.5 \pm 5.2\%$ and from $14.0 \pm 4.1\%$ to $35.8 \pm 1.9\%$, as compared with the vehicle control (0.01% v/v DMSO), respectively (Fig. 2A).

Next, we assessed the effects of DMC on cell cycle-related regulatory factors. Western blot analysis of molecular markers related to G_2/M arrest showed significant changes, including suppressed levels of Cdc2 and cyclin B1, and increased levels of phospho-Chk2 (Fig. 2B). The western blot data are consistent with flow cytometry results. Together, these findings suggest that DMC induces modest G_2/M arrest.

DMC INDUCED AUTOPHAGY

When HCT116 and LOVO cells were treated with 20 and 40 μM of DMC for 24 h, we found that DMC inhibited cell proliferation, but not apoptosis. Growing evidence indicates that non-apoptotic PCD is principally attributed to autophagy (type II PCD) [Kondo et al., 2005]. Therefore, we measured the incorporation of MDC, a marker for the acidic compartment of autolysosomes [Longo et al., 2008] to determine whether DMC induced autophagy in HCT116 and LOVO cells. As shown in Figure 3A, DMC treatment increased the production of autophagic vacuoles.

Bafilomycin A1, an inhibitor of vacuolar H+ ATPase, prevents the transition of autophagosomes to autophagolysosomes by disrupting the fusion of autophagosomes to lysosomes [Kanzawa et al., 2003]. Treatment of HCT116 cells with Bafilomycin A1 markedly reduced the accumulation of the vacuoles induced by DMC treatment. Therefore, we found that autophagosomes produced by DMC treatment undergo the same maturation process lysosomal fusion.

To gain a better insight into the autophagic pathway of DMC-induced autophagy, we experimented with the effects of DMC treatment on the LC3-II protein, the lipidated form of the mammalian microtubule-associated protein 1 light chain, LC3-I. LC3-II is produced during autophagosome formation. The amount of LC3-II protein has been found to correlate with the extent of autophagy [Tanida et al., 2004]. As shown in Figure 3B, western blot analysis showed that increased amounts of LC3 proteins, particularly LC3-II, were detected with increased concentrations of DMC in HCT116 and LOVO cells. In addition, confocal imaging analysis showed that LC3 expression was markedly upregulated in a dose-dependent manner (Fig. 3C). Specifically, in HCT116 and LOVO cells, the number of LC3+ dots or vacuoles increased from 0.6/cell to 2.7/cell and from 0.4/cell to 2.4/cell after DMC treatment at a concentration of 40 μM, as compared with the vehicle control (0.01% v/v DMSO), respectively (Fig. 3D). In contrast, the expression of beclin 1 and atg5, another protein involved in autophagy [Mizushima et al., 2008], was not altered by DMC treatment in HCT116 and LOVO cells (Fig. 3B).

INHIBITION OF AUTOPHAGY PROTECTS HCT116 FROM DMC-INDUCED ANTI-PROLIFERATIVE EFFECT

As mentioned above, we found that DMC induced autophagy in HCT116 and LOVO cells. Recent research has demonstrated that autophagy can play either a pro-survival or a pro-death role following treatment with anticancer drugs [Mathew et al., 2007]. In agreement with the above data, DMC-treated cells arrested at G_2/M phase, but did not undergo apoptosis at a dose of 20 and 40 μM. To assess whether autophagy contributed to DMC-associated anti-proliferative effect death, we performed experiments employing knockdown strategies that impaired autophagy. RNA interference-mediated knockdown of atg5 and beclin 1, which are involved in the autophagy cascade (Fig. 4A) [Mizushima et al., 2008], in DMC treated HCT116 cells markedly reduced conversional ratio of LC3-I to LC3-II and cell proliferation inhibitory rate by about 12.5 and 42.5%, compared with cells treated with 40 μM DMC alone or 40 μM DMC plus non-specific siRNA (Fig. 4B and C). Also, DMC treated LOVO cells markedly reduced conversional ratio of LC3-I to LC3-II and cell proliferation inhibitory rate by about 48 and 21%, compared with cells treated with 40 μM DMC alone or 40 μM DMC plus non-specific siRNA (Fig. 4B and C). Therefore, this result suggested that the inhibition of DMC-induced autophagy induced a protective effect in DMC-treated cells.

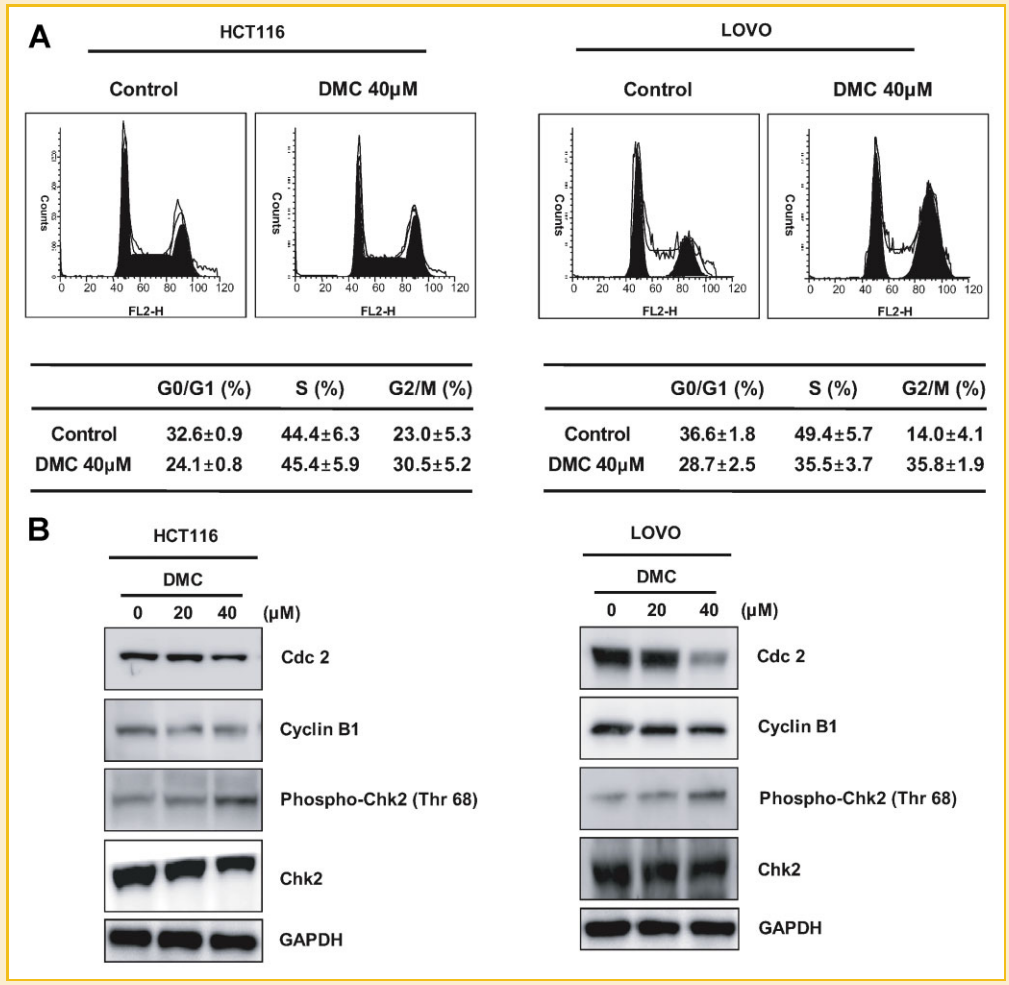

Fig. 2. DMC induced G_2/M arrest in HCT116 and LOVO cells treated for 24 h. A: cell cycle distribution was assessed by flow cytometry. This is representative of three independent experiments. B: immunoblot analysis of cell cycle markers associated with the G_2/M phase. Total cell extracts were probed with antibodies against Cdc 2, Cyclin B1, Chk2, phospho-Chk2, and GAPDH. This is representative of three independent experiments.

To confirm whether the inhibition of autophagy protects cells from DMC-induced anti-proliferative effects, we also employed 3-MA, a Class III PI3K-mediated inhibitor of autophagy [Mei et al., 2008]. As shown in Fig. 4D, 3-MA treatment alleviated the DMC-induced anti-proliferative effect as compared to cells that were treated with 40 μM DMC alone (Fig. 4D). Next, we verified whether DMC-mediated activation of the autophagic process was related to Class III PI 3-kinase. We found that cells treated with both 3-MA and DMC had much lower levels of the conversional ratio of LC3-I to LC3-II when compared with cells treated with DMC alone. Therefore, these data indicated that DMC-mediated autophagy resulted from Class III PI 3-kinase regulation (Fig. 4E).

In summary, the survival benefits achieved with either 3-MA treatment, atg5 siRNA, or beclin 1 siRNA in the DMC-treated HCT116 and LOVO cells provide convincing evidence that DMC not only induces autophagy but that the accompanying autophagy-dependent anti-proliferation contributes significantly to the efficacy of DMC in inducing anti-proliferative effect.

DISCUSSION

In the present study, we found that DMC inhibited proliferation by inducing G_2/M arrest and autophagy in colorectal carcinoma HCT116 and LOVO cells. Furthermore, we found that inhibition of DMC-induced autophagy enhanced protection to the DMC-induced anticancer effect.

DMC is used as an antipyretic and a diuretic agent in Taiwanese folk medicine. Furthermore, it has been reported to have anti-bacterial, anti-diabetic, and anti-tumoral properties. Recently, it was shown that in breast cancer cells, DMC inhibits erbB-2 tyrosine kinase phosphorylation, blocking its downstream pathway and triggering apoptosis via the induction of Bim [Dan-Dan et al., 2009]. Despite its structural similarity to cardamonin, which has been reported to possess anti-tumoral activity, the anti-tumor effects of DMC have not been previously studied in HCT116 colorectal carcinoma cells [Na et al., 2008].

Our results demonstrated that DMC inhibited proliferation in a dose-dependent manner, and that this anti-proliferative effect was

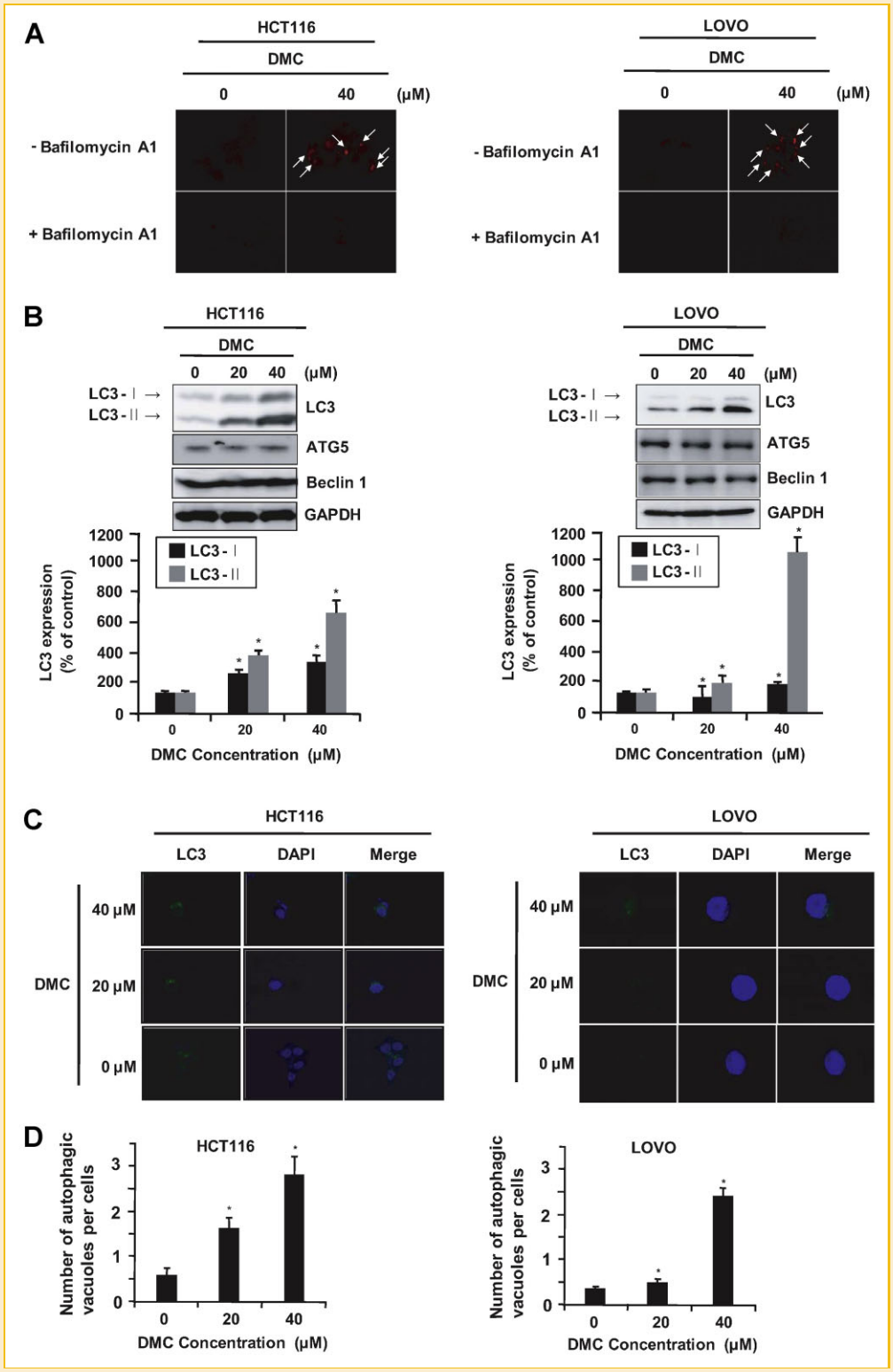

Fig. 3. DMC induced autophagy in HCT116 and LOVO cells. Analysis of autophagic vacuoles (A), LC3 aggregation (C), and enumeration of LC3+ dots (D) by confocal microscopy. The expression level of the autophagy marker, LC3, by immunoblot analysis and by densitometric analysis (B). A: HCT116 and LOVO cells were treated with DMC (40 μM) for 24 h and stained with MDC in the presence or absence of Bafilomycin A1. Under MDC staining, mature autophagic vacuoles, such as autophagolysosomes, were observed (white arrow). This is representative of three independent experiments. B: (top) HCT116 and LOVO cells were treated with various concentrations of DMC for 24 h, and total cell extracts were assayed by immunoblot analysis for expression of LC3-I, LC3-II, Beclin1, and ATG5. This is representative of three independent experiments. (bottom) The expression levels of LC3-I and LC3-II were quantified by densitometric analysis. Data represent the mean ± SD for three independent experiments. $^{*}P < 0.05$. C: HCT116 and LOVO cells were treated with DMC (40 μM) for 24 h, and labeled with 4′,6-diamidino-2-phenylindole (DAPI, blue), and Alexa fluor 488 secondary antibody against LC3B (green). This is representative of three independent experiments. D: LC3+ dots per cell were counted; the data represent the mean ± SD of three independent experiments. $^{*}P < 0.05$.

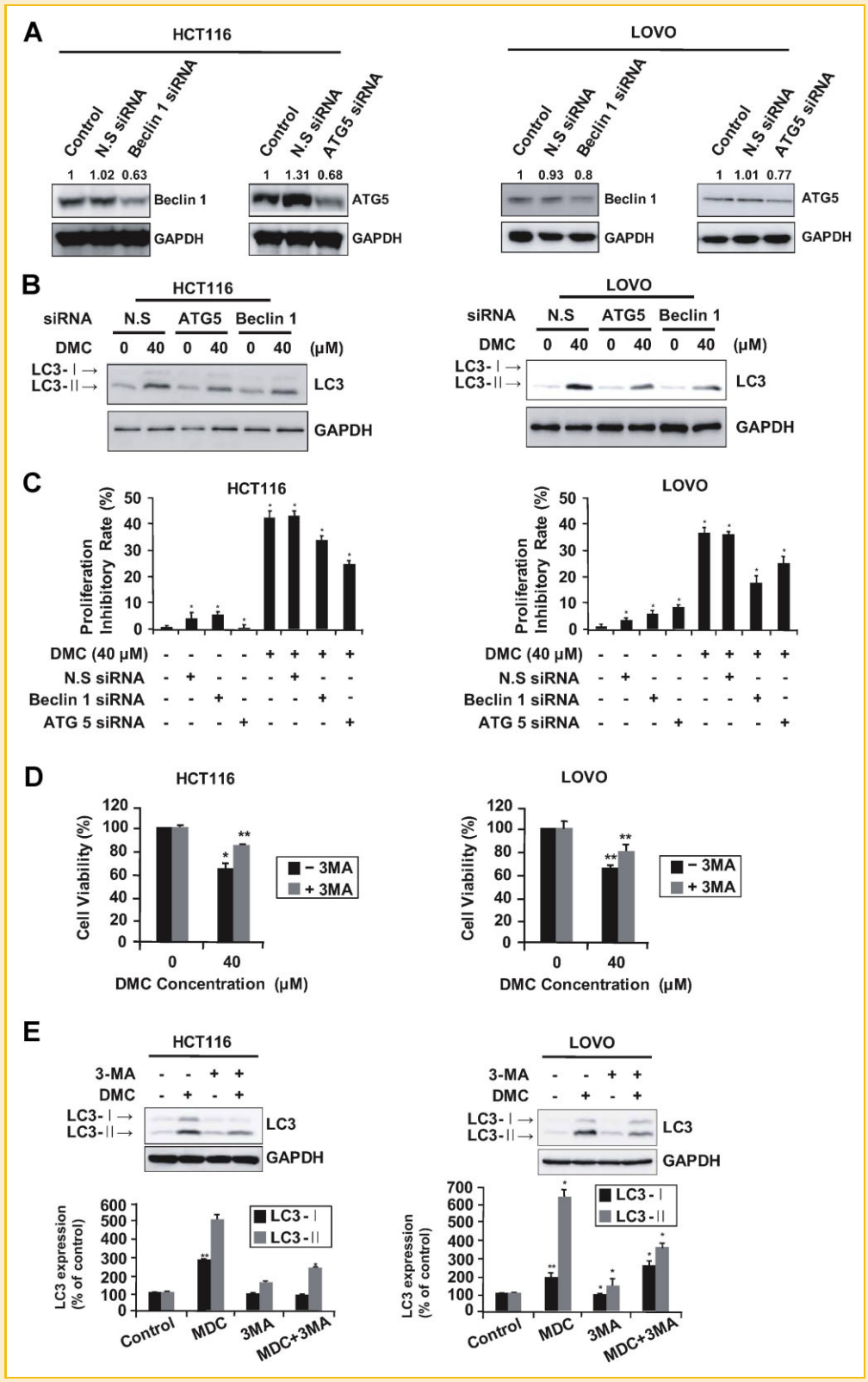

Fig. 4. Autophagy induced DMC-mediated anti-proliferative effect in HCT116 and LOVO cells. A, B, C: The combination treatment of DMC with beclin 1 and atg5 silencing protected the cells from DMC-induced anti-proliferation. A: atg5 or beclin 1 siRNA-induced downregulation of atg5 or beclin 1, as determined by immunoblotting 48 h after treatment with non-specific (N.C.) siRNA, atg5, or beclin 1-targeted siRNA. B: HCT116 and LOVO cells transfected with either non-specific siRNA, atg5 siRNA, or beclin 1 siRNA were exposed to DMC (40 μM) and then immunoblot analysis for LC3 was performed. This is representative of three independent experiments. C: HCT116 and LOVO cells transfected with either non-specific siRNA, atg5 siRNA, or beclin 1 siRNA were exposed to DMC (40 μM) and then cultured for 24 h prior to CCK8 analysis. Data represent the mean ± SD for three independent experiments. $^{*}P < 0.05$. D: The combination treatment of DMC with 3-MA (5 mM) protected the cells from the anti-proliferative effect of DMC. D: The viability of HCT116 and LOVO cells was measured by CCK8 analysis following 24 h of culture with DMC with or without the autophagy inhibitor 3-MA. Data represent the mean ± SD for three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$. E: (top) 3-MA blocked the conversion of LC3-I to LC3-II that is induced by DMC. After exposure of the cells to 40 μM of DMC with and without 3-MA for 24 h, immunoblot analysis for LC3 was performed. This is representative of three independent experiments. (bottom) The expression levels of LC3-I and LC3-II were quantified by densitometric analysis. Data represent the mean ± SD for three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

associated with a G_2/M phase arrest. These data are consistent with previous studies demonstrating the ability of temozolomide, heat shock, or the activation of neurotrophin receptor TrkA to induce G_2/M cell cycle arrest and autophagy in malignant glioma cells [Kanzawa et al., 2004; Komata et al., 2004; Hansen et al., 2007]. Moreover, the anti-proliferative effect of DMC was associated with the induction of autophagy as evidenced by the increased formation of LC3-II autophagic vacuoles, accumulation of acidic compartments, and the up-regulation of the LC3-II protein. The effects of DMC on autophagy induction in HCT116 and LOVO cells are marked at 40 μM.

In the present study, we speculated that the inhibition of proliferation and activation of autophagy by DMC depends on Class III PI3K, which is known to be required for the induction of autophagy during nutrient deprivation in both yeast and mammalian cells [Backer, 2008], because our results indicated that the inhibition of Class III PI3K by 3-MA inhibits the conversion of LC3-II from LC3-I and the DMC-mediated anti-proliferative effect. Also, we reported a novel function of DMC, namely the induction of autophagy, as demonstrated by increased formation of the autophagosomal marker LC3-II and the incorporation of MDC in the autolysosomes. We aimed to determine the function of DMC-induced autophagy in HCT116 and LOVO cells. Autophagy has conflicting roles under anti-cancer conditions in various cancer cells [Mathew et al., 2007]. Some have reported that the inhibition of autophagy enhanced apoptosis, suggesting that autophagy contributes to tumor progression by protecting cells against anticancer agents [Levine, 2007; Levine and Kroemer, 2008]. In contrast, other studies reported that autophagy can have tumor repression activities. For example, anticancer treatments, including drugs, can activate autophagy to kill cancer cells that are resistant to apoptosis [Levine and Kroemer, 2008; Longo et al., 2008]. In our study, we found that the inhibition of autophagy by 3-MA, beclin 1 siRNA, or atg5 siRNA treatment protected cells against the DMC-induced anti-proliferative effect (Fig. 4). In previous studies, it was shown that beclin 1 haploinsufficiency may have an impact on cells with an apoptotic defect, preventing an apoptotic response to starvation, thus allowing survival by autophagy [Degenhardt et al., 2006]. In contrast, other research showed that beclin 1 is a tumor suppressor protein that mediates autophagy repressing the growth of tumor cells [Mizushima et al., 2008]. In addition, multiple proteins, including atg5, regulate the process of autophagosome formation. In previous studies, it was shown that forced expression of Atg5 not only promotes autophagy, but also enhances susceptibility toward apoptotic stimuli irrespective of cell type [Yousefı et al., 2006]. Here, we found that silencing beclin 1 or atg5 inhibits DMC-mediated anti-proliferation and DMC-induced autophagy (Fig. 4C and D). When HCT116 and LOVO cells were treated with DMC (40 μM) and an autophagy inhibitor for 24 h, the viability of the cells significantly increased relative to the cells treated with DMC alone. Hence, we speculated that DMC-induced autophagy, which occurs after exposure to DMC for 24 h, might have a function in inducing anti-proliferative effect. However, after exposure for 24 h to DMC and autophagy inhibitors, autophagy was inhibited and protection against DMC-induced anti-proliferation occurred. Briefly, this result suggests that DMC treatment associated autophagy is a mechanism of anti-proliferative effect.

In conclusion, we found that DMC induces in vitro growth inhibition, G_2/M cell cycle arrest, and autophagy in colorectal carcinoma HCT116 and LOVO cells. We also found that autophagy is responsible for the DMC-induced proliferation inhibition, as evidenced by the combinatorial treatment of cells with autophagy inhibitors and DMC.

REFERENCES

Backer JM. 2008. The regulation and function of Class III PI3Ks: Novel roles for Vps34. Biochem J 410:1–17.

Bursch W, Ellinger A, Kienzl H, Török L, Pandey S, Sikorska M, Walker R, Hermann RS. 1996. Active cell death induced by the anti-estrogens tamoxifen and ICI 164 384 in human mammary carcinoma cells (MCF-7) in culture: The role of autophagy. Carcinogenesis 17:1595–1607.

Chattopadhayay ED, Sinha BK, Vaid. LK. 1998. Antibacterial activity of syzygium species. Fitoterapia 119:365–367.

Dan-Dan Li, Xiao-Qi Wu, Jun Tang, Xiao-Yi Wei, Xiao-Feng Zhu. 2009. ON-III inhibits erbB-2 tyrosine kinase receptor signal pathway and triggers apoptosis through induction of Bim in breast cancer cells. Cancer Biol Ther 8:739–743.

Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, Mukherjee C, Shi Y, Gélinas C, Fan Y, Nelson DA, Jin S, White E. 2006. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell 10:51–64.

Elliott A, Reiners JJ. 2008. Suppression of autophagy enhances the cytotoxicity of the DNA-damaging aromatic amine p-anilinoaniline. Toxicol Appl Pharmacol 232:169–179.

Gafner S, Wolfender JL, Mavi S, Hostettmann K. 1996. Antifungal and antibacterial chalcones from Myrica serrata. Planta Med 62:67–69.

Ghayur MN, Gilani AH, Khan A, Amor EC, Villaseñor IM, Choudhary MI. 2006. Presence of calcium antagonist activity explains the use of Syzygium samarangense in diarrhea. Phytother Res 20:49–52.

Hansen K, Wagner B, Hamel W, Schweizer M, Haag F, Westphal M, Lamszus K. 2007. Autophagic cell death induced by TrkA receptor activation in human glioblastoma cells. J Neurochem 103:259–275.

Lee HM, Surh BY, Chun Y-J. 2010. Roles of neutral sphingomyelinase 1 on CD95-mediated apoptosis in human jurkat T lymphocytes. Biomol Therapeut 18:262–270.

Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S. 2004. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. Cell Death Differ 11:448–457.

Kanzawa T, Kondo Y, Ito H, Kondo S, Germano I. 2003. Induction of autophagic cell death in malignant glioma cells by arsenic trioxide. Cancer Res 63:2103–2108.

Kanzawa T, Zhang L, Xiao L, Germano IM, Kondo Y, Kondo S. 2005. Arsenic trioxide induces autophagic cell death in malignant glioma cells by up-regulation of mitochondrial cell death protein BNIP3. Oncogene 24:980–991.

Kim YJ, Kwon HC, Ko H, Park JH, Kim HY, Yoo JH, Yang. HO. 2008. Anti-tumor activity of the ginsenoside Rk1 in human hepatocellular carcinoma cells through inhibition of telomerase activity and induction of apoptosis. Biol Pharm Bull 31:826–830.

Ko H, Kim HS, Kim NH, Lee SH, Kim KH, Hong SH, Yook JI. 2007. Nuclear localization signals of the E-cadherin transcriptional repressor Snail. Cell Tissue Organ 185:66–72.

Komata T, Kanzawa T, Nashimoto T, Aoki H, Endo S, Nameta M, Takahashi H, Yamamoto T, Kondo S, Tanaka R. 2004. Mild heat shock induces autophagic

growth arrest, but not apoptosis in U251-MG and U87-MG human malignant glioma cells. J Neurooncol 68:101–111.

Kondo Y, Kanzawa T, Sawaya R, Kondo S. 2005. The role of autophagy in cancer development and response to therapy. Nat Rev Cancer 5:726–734.

Kuo YC, Yang LM, Lin. LC. 2004. Isolation and immunomodulatory effect of flavonoids from Syzygium samarangense. Planta Med 70:1237–1239.

Levine B. 2007. Cell biology: Autophagy and cancer. Nature 446:745–747.

Levine B, Kroemer G. 2008. Autophagy in the pathogenesis of disease. Cell 132:27–42.

Longo L, Platini F, Scardino A, Alabiso O, Vasapollo G, Tessitore L. 2008. Autophagy inhibition enhances anthocyanin-induced apoptosis in hepatocellular carcinoma. Mol Cancer Ther 7:2476–2485.

Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. 2007. Self-eating and self-killing: Crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol 8:741–752.

Mathew R, Karantza-Wadsworth V, White E. 2007. Role of autophagy in cancer. Nat Rev Cancer 7:961–967.

Wang M, Tan W, Zhou J, Leow Leow, Go M, Lee HS, Casey PJ. 2008. A small molecule inhibitor of isoprenylcysteine carboxymethyl-transferase induces autophagic cell death in PC3 prostate cancer cells. J Biol Chem 283:18678–18684.

Mizushima N, Levine B, Cuervo AM, Klionsky DJ. 2008. Autophagy fights disease through cellular self-digestion. Nature 451:1069–1075.

Li N, Liu J-H, Zhang J, Yu B-Y. 2008. Comparative evaluation of cytotoxicity and antioxidative activity of 20 flavonoids. J Agric Food Chem 56:3876–3883.

Pisani P, Parkin DM, Bray F, Ferlay J. 1999. Estimates of the worldwide mortality from 25 cancers in 1990. Int J Cancer 83:18–29.

Qu X, Zou Z, Sun Q, Luby-Phelps K, Cheng P, Hogan RN, Gilpin C, Levine B. 2007. Autophagy gene-dependent clearance of apoptotic cells during embryonic development. Cell 128:931–946.

Resurreccion-Magno MHC, Villasenor IM, Harada N, Monde K. 2005. Anti-hyperglycaemic flavonoids from Syzygium samarangense (Blume) Merr. and Perry. Phytother Res 19:246–253.

Scarlatti F, Bauvy C, Ventruti A, Sala G, Cluzeaud F, Vandewalle A, Ghidoni R, Codogno P. 2004. Ceramide-mediated macroautophagy involves inhibition of protein kinase B and up-regulation of beclin 1. J Biol Chem 279: 18384–18391.

Shin S, Lee Y, Kim W, Ko H, Choi H, Kim K. 2005. Caspase-2 primes cancer cells for TRAIL-mediated apoptosis by processing procaspase-8. EMBO J 24:3532–3542.

Srivastava R, Shaw AK, Kulshreshtha K. 1995. Triterpe noids and chalcone from Syzygium samarangense. Phytochemistry 38:687–689.

Tanida I, Ueno T, Kominami E. 2004. LC3 conjugation system in mammalian autophagy. Int J Biochem Cell Biol 36:2503–2518.

Yousefı S, Perozzo R, Schmid I, Perozzo R, Schmid I, Ziemiecki A, Schaffner T, Scapozza L, Brunner T, Simon HU. 2006. Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. Nat Cell Biol 8:1124–1132.