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Delphinidin Induces Necrosis in Hepatocellular Carcinoma Cells in the Presence of 3-Methyladenine, an Autophagy Inhibitor†

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The present study was performed to determine whether anthocyanins could trigger different modes of cell death in different cancers. It was found that whereas cyanidin-3-rutinoside and delphinidin could induce apoptosis in leukemia cells, they caused growth retardation in hepatocellular carcinoma cells (HCC), which was accompanied with a significant cellular vacuolization. The latter was likely caused by macroautophagy and was completely suppressed by 3-methyladenine, an inhibitor of class III phosphoinositide 3-kinase that is important for autophagy activation, and by bafilomycin A1, which blocks lysosomal degradation. Delphinidin induced significant lipidation of LC3, an indication of macroautophagy, which was also suppressed by 3-methyladenine. Macroautophagy was required for the survival of delphinidin-treated HCC cells as inhibition with 3-methyladenine led to massive necrosis without caspase activation. Thus, anthocyanins could induce different modes of cell death for different cancers. Furthermore, anthocyanins could be used in combination with a macroautophagy inhibitor for treating cancers such as HCC.

KEYWORDS: Delphinidin; bilberry; autophagy; necrosis; hepatocellular carcinoma

INTRODUCTION

Substantial amounts of work have demonstrated that naturally occurring flavonoid compounds exhibit remarkably high scavenging activities toward chemically generated radicals and benefiting effects to human health with no or low toxicity (*1*−*5*). In recent years, considerable efforts have also been directed to establishing the usefulness of these compounds for cancer chemoprevention and chemotherapy (*6*−*8*).

Flavonoid compounds are widely available in human dietary agents, including berries. Bilberry (*Vaccinium myrtillus*) is a member of the *Vaccinium* genus, which is a minor crop in the United States. Bilberry has been used for a wide range of common ailments such as scurvy, night blindness, infections, and diabetes in alternative medicine (*2*, *9*). However, recent studies suggest that bilberry extracts have anti-inflammation and cancer chemoprevention activities (*10*−*12*). In addition to common nutritional compounds such as vitamins, bilberry extracts are rich in anthocyanidins and other flavonoids. Among the anthocyanidins, delphinidin is the dominant fraction (*10*, *13*). Delphinidin (**Figure 1A**) is a diphenylpropane-based polyphenolic ring structure that carries a positive charge in its central ring. Delphinidin is gaining considerable attention as it appears to possess a strong antioxidant property and other potentially beneficial traits, such as anti-inflammation, antimutagenesis, and antiangiogenesis activities (*14*−*21*).

Our previous studies indicated that fractions with antioxidant activities from various berry species have anticancer potentials (*22*, *23*). In particular, we found that cyanidin-3-rutinoside (C-3-R) isolated from black raspberry could selectively kill leukemic cells but not normal peripheral blood mononuclear cells (*24*). In the work presented here, we examined whether C-3-R and delphinidin could exhibit a similar cytotoxicity toward solid tumor cells, such as human hepatocellular carcinoma (HCC) cells. We found that neither C-3-R nor delphinidin could induce apoptosis or caspase activation in HCC cells. Further studies using delphinidin indicated that it instead induced growth retardation and macroautophagy. Combined use of 3-methyladenine (3-MA), a macroautophagy inhibitor, unleashed the cytotoxicity of delphinidin, leading to a strong necrotic cell death. This study thus indicated that whereas anthocyanins could be used singularly for treating certain types of cancer, such as leukemia, they could by also used in combination with autophagy inhibitors for treating other types of cancers, such as HCC. It is thus important to delineate individual cases so that anthocyanins could be used most effectively for cancer therapy.

MATERIALS AND METHODS

Extraction and Purification of Delphinidin from Bilberries. Bilberry fruits (*V. myrtillus*) used in this study were grown in Idaho and

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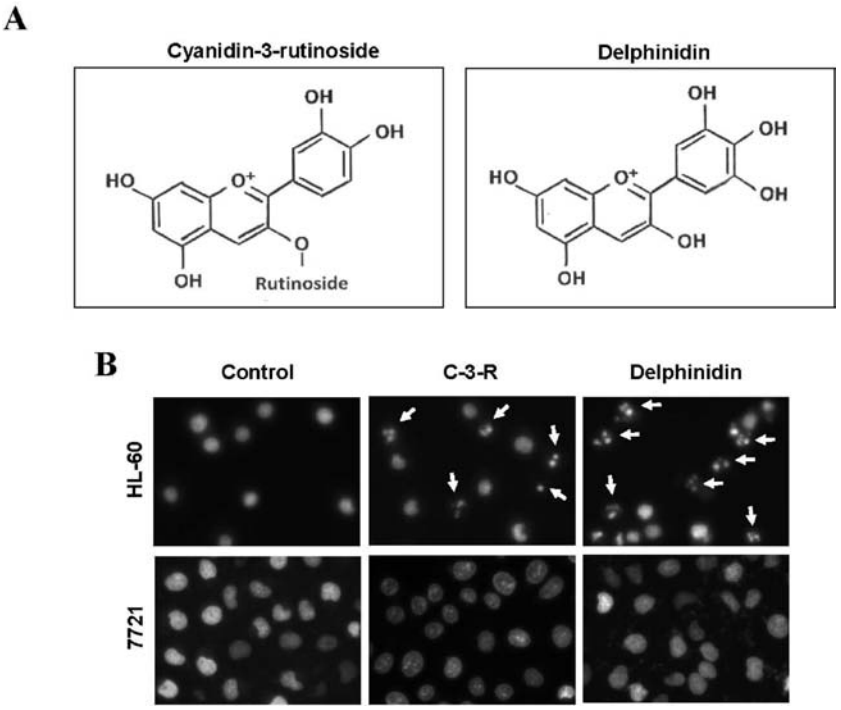

Figure 1. Cyanidin-3-rutinoside and delphinidin induce apoptosis in HL-60 but not in SMMC7721 cells: (**A**) structures of cyanidin-3-rutinoside and delphinidin; (**B**) leukemia cell line, HL-60 (upper panels) and hepatocellular carcinoma cell line, SMMC7721 (lower panels), were treated with medium control, or cyanidin-3-rutinoside (60 μM for HL-60 and 100 μM for SMMC7721), or delphinidin (20 μM for HL-60 and 100 μM for SMMC7721) for 16−24 h. Cells were then stained with Hoechst 33342. Arrows indicate apoptotic cells with condensed or fragmented nuclei.

were kindly provided by Dr. Danny L. Barney of the University of Idaho, Moscow, ID. Berries were hand-harvested at a commercially mature stage. The fruits were sorted to eliminate damaged, shriveled, and unripe fruit and selected for uniform size and color. Undamaged berries were randomized and frozen in liquid nitrogen and then transported in a cooler to Beltsville, MD, on the same day. Berries were then stored at −80 °C until they were used for assays. The flesh part of berries was homogenized with 1× phosphate-buffered saline (PBS) (1:1, w/v) in a clean glass tank. The homogenate was centrifuged at 1000*g* to recover the supernatant as the crude extract. The material was immediately frozen at −80 °C and diluted with culture medium before use.

Delphinidin was extracted from bilberries and purified by high-performance liquid chromatography (HPLC) from bilberries as previously described (*25*). Briefly, the bilberries were extracted twice with 80% methanol and 0.01% HCl in a Polytron homogenizer for 1 min. The concentrated extract was dissolved in acidified water (0.01% HCl) and passed through a C_{18} Sep-Pak cartridge. Delphinidin and other anthocyanins that were adsorbed onto the cartridge were recovered with acidified (0.01% HCl) methanol. The methanol extract was passed through a 0.45 μm membrane filter and further separated by a HPLC system (Waters Corp.) coupled with a photodiode array detector. The mobile phase consisted of 5% aqueous formic acid (A) and HPLC grade acetonitrile (B). The delphinidin-containing fraction was collected and identified by comparing the HPLC elution pattern of authentic standard at 530 nm and validated with LC-MS spectra as previously reported (*10*). The lyophilized delphinidin was 99% pure and stored at −80 °C until use.

Chemical Reagents and Antibodies. The following chemicals were used: 3-methyladenine (Sigma), LY294002 (Calbiochem), and z-VAD-fmk (Biomol). The following antibodies were used: anti-LC3B (*26*), anti-Atg5 (*27*), anti-Atg6/Beclin 1 (BD Biosciences), anti-Atg7 (Rockland Immunochemicals), anti-β-actin (Sigma), anti-ATF-4 (Santa Cruz Biotechnology), anti-CHOP (Santa Cruz Biotechonology), and anti-Bip (Sigma).

Cell Culture, Transfection, and Cell Death Assay. Human hepatocellular carcinoma cell lines SMMC7721, HCCLM3, and MHCC97L (*28*) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and standard supplements. Cellular vacuolization was determined by phase contrast microscopy. Nuclear morphology was determined by Hoechst 33342 staining (5 μg/mL). Membrane integration was assessed by propidium iodide (PI) staining (1 μg/mL). Viability was determined by the percentage of PI-positive cells. Micrographs were obtained using a Nikon TE200 inverted microscope equipped with a digital camera (SPOT, Diagnostic Instrument, Inc.) and the accompany software.

Caspase activities were measured using 20 μg of proteins and 20 μM fluorescent substrates (Ac-VDVAD-AFC, Ac-DEVD-AFC, Ac-IETD-AFC, and Ac-LEHD-AFC for caspase-2, -3, -8, and -9, respectively) (Biomol). The fluorescence signals were detected by a fluorometer (Tecan GENios) at 400/510 nm (excitation/emission), as described previously (*24*).

A siRNA against human LC3B (5′-GAAGGCGCUUACAGCU-CAA-3′) and a negative control from Invitrogen were transfected at 0.24 μM per 1×10^6 cells using Oligofectamine (Invitrogen) for 48 h before analysis as described before (*29*).

Immunoblot Assay. This was essentially performed as described previously (*24*). Cells were washed in PBS and lysed in RIPA buffer. Thirty to forty micrograms of protein was separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were detected with the indicated primary and secondary antibodies and developed with SuperSignal West Pico chemiluminescent substrate (Pierce).

RESULTS

Differential Effects of Cyanidin-3-rutinoside and Delphinidin in Leukemia Cells and Hepatocellular Carcinoma Cells. Our previous studies indicated that C-3-R could cause apoptosis in human leukemia cells, but not in normal human peripheral blood mononuclear cells, through the induction of intercellular redox stress (*24*). We confirmed that another structurally closely related anthocyanidin, delphinidin, could also induce apoptosis in the leukemia HL-60 cells at low doses in 24 h (**Figure 1**). However, neither C-3-R nor delphinidin could induce apoptosis in several human HCC cell line lines, including SMMC7721 (**Figure 1B**), HCCLM3, and MHCC97L (data not shown) in the same time frame at an even higher dose. In fact, these HCC cells could maintain their viability well in the presence of C-3-R or

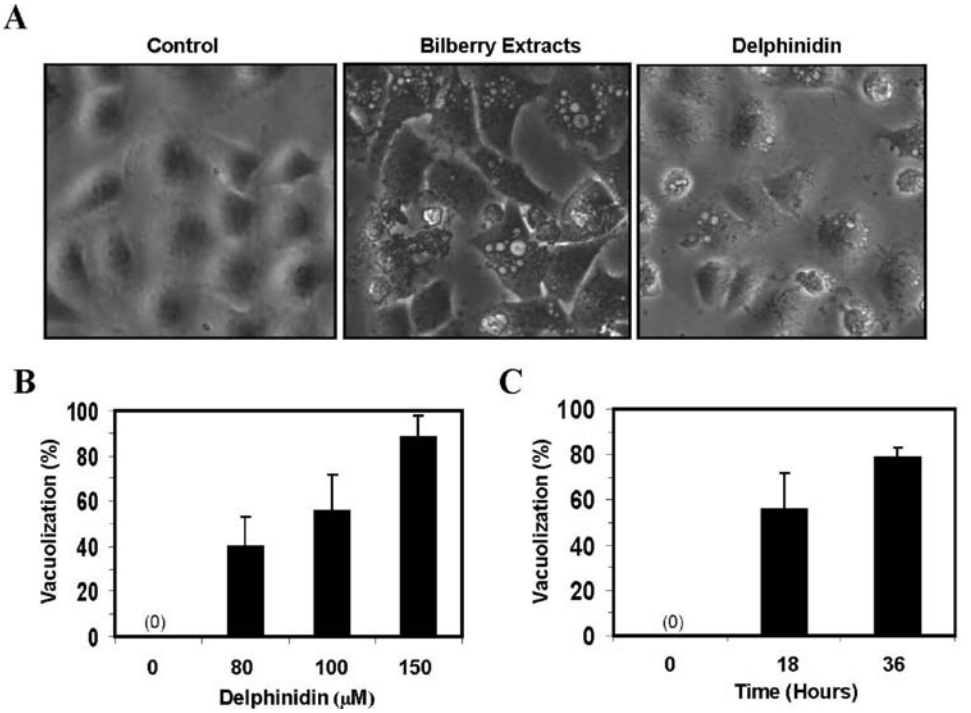

Figure 2. Induction of vacuolization by bilberry extract and delphinidin. (**A**) SMMC7721 cells were treated for 18 h with the vehicle control (0.05% DMSO), bilberry extract (1:20 dilution of original extract), or purified delphinidin (100 μM). The phase contrast images were then taken, demonstrating the vacuoles induced by the treatment. (**B**, **C**) The percentage of cells with three or more vacuoles after exposure to delphinidin for 18 h at the indicated concentrations (**B**) or at 100 μM for different times (**C**) was determined (mean $\pm$ SD).

delphinidin for a sustained period of time (see below). Failure to induce apoptosis by these compounds was not because these cells did not have competent apoptosis machinery because they could mount an effective apoptosis response when simulated by other agents, such as tumor necrosis factor-α (TNFα) or etopside (*28*). Thus, these results indicated that anthocyanidin could have different effects in different types of tumors.

Induction of Cellular Vacuolization in HCC Cells by Anthocyanidins. We noted that significant cellular vacuolization was induced by a crude bilberry extract that was enriched with delphinidin as well as the purified delphinidin in SMMC7721 (**Figure 2**) and in other HCC cells (data not shown). Similarly, C-3-R could induce significant cellular vacuolization in these HCC cells (data not shown). The appearance of these small intracellular vacuoles was both dose-dependent and time-dependent (**Figure 2B**,**C**).

Because anthocyanidins possess strong free radical-scavenging activity, we wondered whether the induction of cellular vacuolization by delphinidin or C-3-R was related to their antioxidant activities. However, neither resveratrol (a natural non-anthocyanidin polyphenolic antioxidant), nor butylated hydroxyanisole (BHA, a chemical antioxidant commonly used for food preservation and chemoprevention) could induce similar vacuolization in the HCC cells (data not shown). Although these studies were not exhaustive and other types of antioxidants had not been examined, they suggested that the antioxidant property of delphinidin or C-3-R might not be singularly responsible for the intracellular vacuolization in the HCC cells.

We previously observed that tumor cells under endoplasmic reticulum (ER) stress could exhibit massive cellular vacuolization as a manifestation of the dilated ER lumens (*29*). ER stress could be determined on the basis of the up-regulation of a battery of proteins, including ATF-4, CHOP, and Bip, as a part of the unfolded protein response (UPR). UPR is activated to alleviate ER stress (*30*). However, we had not

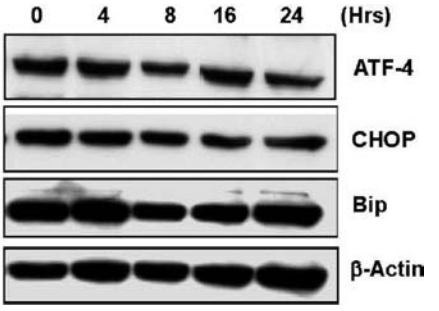

Figure 3. Delphinidin does not induce unfolded protein response. SMMC7721 cells were treated with delphinidin (150 μM) for the indicated time. Total lysates were made and subjected to immunoblot assay using the indicated antibodies. No changes were observed in the expression levels of the specific molecules analyzed.

observed changes in the expression levels of any of these proteins in SMMC7721 cells following delphinidin treatment (**Figure 3**), suggesting that there was no ER stress induced by this anthocyanidin.

We then suspected that the vacuolization was related to macroautophagy (hereafter referred to as autophagy), an intracellular degradation process characterized by the formation of double-membraned autophagosomes, which are then degraded together with their contents in the lysosome (*31*). A key molecular complex involved in autophagy induction is the beclin 1 complex, which is composed of beclin 1, Atg14, VPS34, and VPS15 (*32*). VPS34 and VPS15 form the class III phosphoinositide 3-kinase (PI3-K), the activity of which is regulated by beclin 1 and Atg14. We thus determined whether 3-methyladenine (3-MA), preferentially targeting the class III PI3-K (*33*), could inhibit delphinidin-induced cellular vacuolization. Indeed, 3-MA effectively suppressed the formation of the vesicles in a dose-dependent manner (**Figure 4A**,**B**). Similarly, a generic PI3-kinase

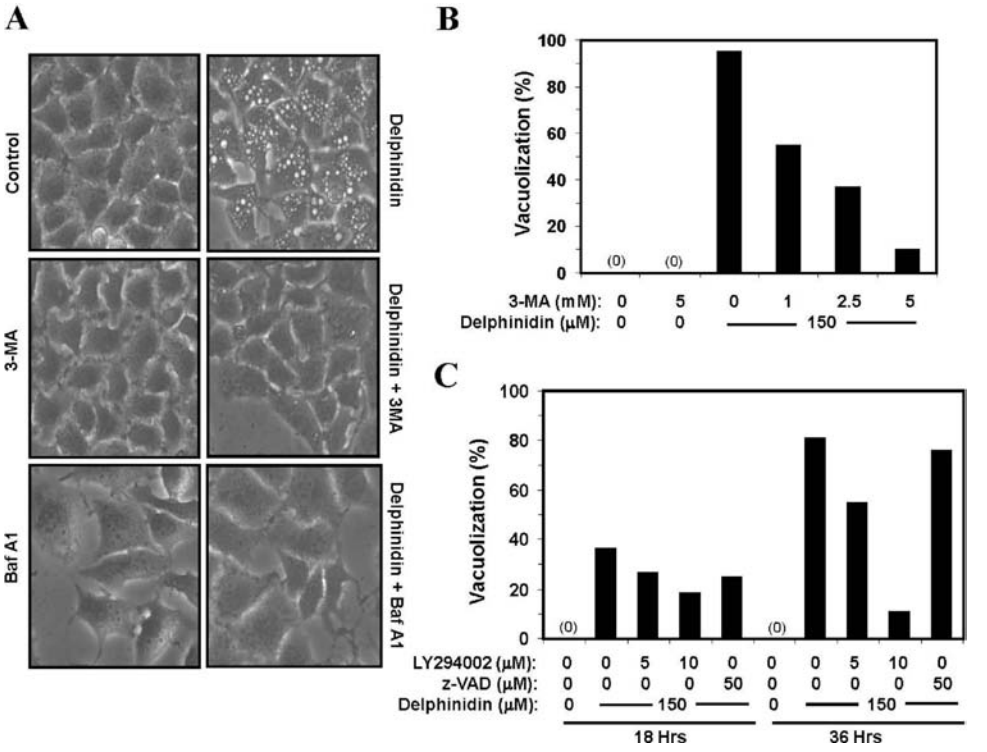

Figure 4. Inhibition of delphinidin-induced vacuolization by autophagy inhibitors, but not by a pan-caspase inhibitor. (**A**) SMMC7721 cells were pretreated with medium, bafilomycin A1 (Baf A1, 100 nM), or 3-MA (5 mM) for 1 h and then exposed to medium or delphinidin (150 μM) as indicated for another 18 h. The phase-contrast images were then taken. (**B**, **C**) SMMC7721 cells were pretreated with medium, 3-MA (1−5 mM), LY294002 (5−10 μM), or a pan-caspase inhibitor, z-VAD-fmk (50 μM), for 1 h and then exposed to delphinidin (150 μM) for 18 h (**B**, **C**) or 36 h (**C**). The percentages of cells with three or more vacuoles after treatment were determined. Results represent one of at least two repeat assays performed.

inhibitor, LY294002, had similar inhibitory effects, whereas z-VAD-fmk, a pan-caspase inhibitor, did not (**Figure 4C**).

Interestingly, the vacuolization could be also suppressed by bafilomycin A1 (Baf A1) (**Figure 4A**). Baf A1 is a vacuolar type H^+-ATPase important for the acidic environment of lysosome (*34*). Baf A1 is frequently used in suppressing lysosome function that is required for autophagic degradation. Thus, the inhibitory effects of Baf A1 supported the conjecture that delphinidin-induced cellular vacuolization could result from autophagic degradation.

Induction of LC3 Lipidation by Delphinidin in SMMC7721 Cells. More than 30 genes have been defined that participate in autophagy or autophagy-related processes in the yeast, many of which have mammalian homologues (*31*,*32*). The core machinery seems to be built around two ubiquitin-like conjugation systems. In one system, a ubiquitin-like protein, Atg8, or one of its mammalian homologues, the microtubule-associated protein 1 light chain 3 (LC3), is first cleaved by Atg4 to expose the conserved Gly120 at its C-terminus. Atg8/LC3 is then conjugated to phosphatidylethanolamine, also via Atg7, a ubiquitin-activating enzyme (E1)-like protein, and Atg3, a ubiquitin carrier protein (E2)-like protein (*26*, *32*). The unconjugated form of Atg8/LC3 (i.e., LC3-I, about 18K_d) is in the cytosol, whereas the conjugated form (i.e., LC3-II, about 16 K_d) targets the autophagosomal membrane (*26*). This process is also affected by another conjugation system, Atg5−Atg12−Atg16, and the beclin 1−class III PI3-K complex (*32*). This association of Atg8/LC3 to the autophagosomes is important for autophagosome formation.

To determine whether bilberry extracts or delphinidin could induce autophagy, we examined the induction of LC3-II, the lipidated form of LC3, in SMMC7721 cells by immunoblot (**Figure 5**). Indeed, both agents could induce strong time-dependent formation of LC3-II, which could be suppressed by the class III PI3-K inhibitor, 3-MA (**Figure 5**). The specificity of this assay was confirmed with a specific siRNA against LC3, which completely knocked down the appearance of LC3-II (**Figure 5C**). On the other hand, the expression levels of Atg5, beclin 1, and Atg7 were not significantly changed as in other cases (**Figure 5B**). The considerable formation of LC3-II thus strongly supported the notion that delphinidin could induce autophagy in SMMC7721cells.

Cotreatment of SMMC7721 Cells with Delphinidin and 3-MA Led to Necrotic Cell Death. Whereas delphinidin alone did not cause immediate apoptotic cell death, it significantly inhibited the growth of SMMC7721 cells (**Figure 6**). At the end of a 5 day culture, although the number of control cells was increased >6-fold, that of the delphinidin-treated cells was only half the control level. This growth retardation was reversible, and cells resumed normal growth once delphinidin was removed from the culture (data not shown). Interestingly, cotreatment with 3-MA did not improve the retarded growth, which might be in part due to an increased cell death at the same time. Although short-term treatment (for 24 h) with delphinidin alone did not induce cell death in SMMC7721 cells (**Figure 6**), prolonged treatment (120 h) led to a decrease in viability. However, cotreatment of 3-MA caused a significant increase in cell death in terms of a faster kinetics and a lower viability (**Figure 7A**). More than 50% of cells died in 48 h, and by 120 h virtually all cells died. The death was characterized by a positive PI staining without apparent nuclear fragmentation or condensation (**Figure 7B**). Delphinidin alone did not induce any caspase activation in either extrinsic or intrinsic pathways. Thus, no caspase-8, -9, -2, or -3 was found to be activated (**Figure 7C**). Similarly, C-3-R could not induce caspase activity in SMMC7721, HCCLM3, and MHCC97L cells

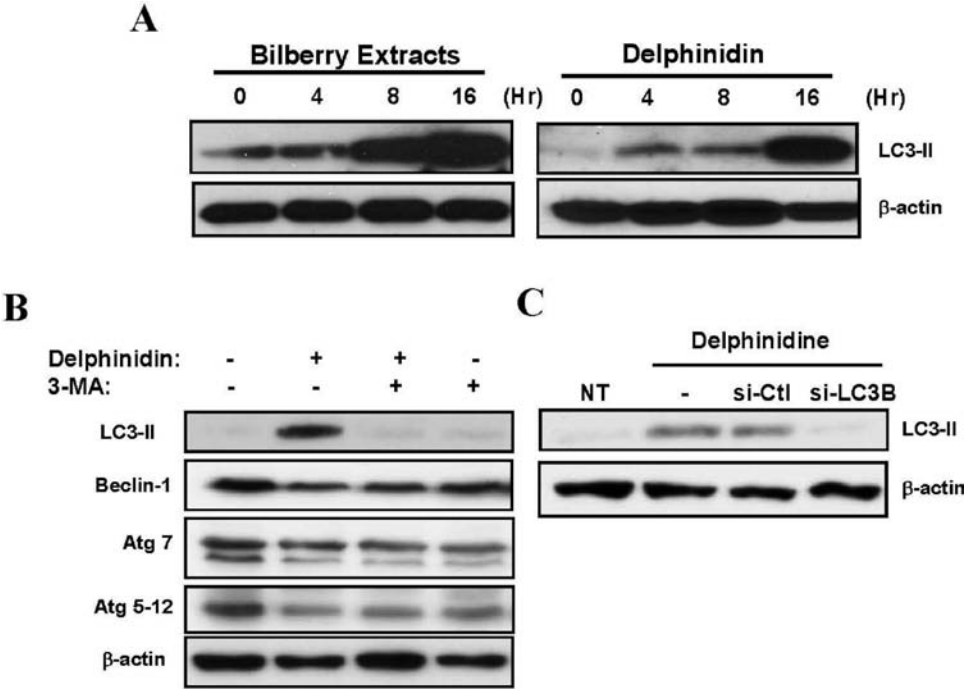

Figure 5. Delphinidin induces LC3 lipidation in SMMC7721 cells. (**A**) SMMC7721 cells were treated with either bilberry extract or purified delphinidin (100 μM) for different times. Whole cell lysates were made and subjected to immunoblot assay using an anti-LC3 antibody. An increased level of LC3-II was observed. (**B**) SMMC7721 cells were pretreated with 3-MA (5 mM) for 1 h and then exposed to delphinidin (100 μM) for another 16 h. The levels of LC3-II and other Atg proteins in the whole cell lysates were probed by the specific antibodies. (**C**) SMMC7721 cells were transfected with a negative control siRNA or a specific siRNA against LC3B for 36 h. Cells were then treated with vehicle control or delphinidin (100 μM) as indicated for 16 h. The total lysates were subjected to immunoblot assay with an anti-LC3 antibody. LC3-II expression could be specifically inhibited by the siRNA.

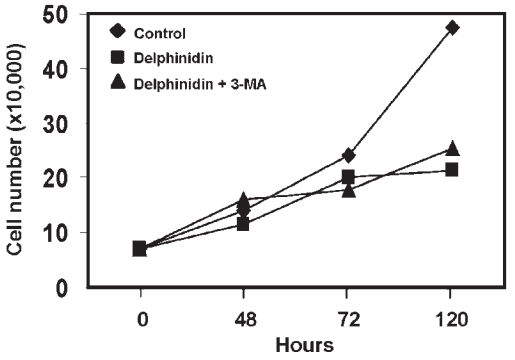

Figure 6. Delphinidin causes growth retardation. SMMC7721 cells (7×10^4/well) were seeded in complete medium overnight and then treated with delphinidin (100 μM) in the absence or presence of 3-MA (5 mM) for 48, 72, or 120 h. The total number of cells was counted and plotted against time. Results represent one of at least two repeat assays performed.

(data not shown). Notably, cotreatment with 3-MA did not result in any increase in caspase activity, despite the enhanced cell death (**Figure 7D**). These results suggested that 3-MA promoted necrotic, but not apoptotic, cell death in delphinidin-treated HCC cells.

DISCUSSION

Natural products such as plant polyphenolic compounds have been widely studied for their chemopreventive and chemotherapeutic potentials (*6*−*8*). A variety of compounds have been examined in different circumstances. We demonstrated in this study that anthocyanins could have different effects in different tumors. C-3-R and delphinidin, two structurally very closely related compounds, could potently induce rapid apoptosis in leukemia cells, but not in HCC cells (**Figure 1**) (*24*). Instead of entering a rapid apoptosis, HCC cells exhibited two major phenotypes that were not observed in the leukemia cells.

First, there was an apparent cellular vacuolization, although the extent varied in different HCC cell lines (**Figure 2**). Our studies suggested that this phenomenon might not be related to the general antioxidant activity of C-3-R or delphinidin as other natural or chemical antioxidants did not cause similar effects. We also did not find any evidence of ER stress (**Figure 3**), which could cause ER dilation that could be manifested as cellular vacuolization (*29*). Our findings would strongly support the notion that the vacuolization was caused by the autophagic activity induced by the anthocyanins. This was because the vacuolization could be completely inhibited by 3-MA and LY-294002, two PI3-kinase inhibitors (**Figure 4**). In particular, 3-MA preferentially inhibits the class III PI3-kinase (*33*), which is required for the activation of autophagy (*35*). Notably, Baf A1, a vacuolar type H^+-ATPase inhibitor, which would suppresses autophagic degradation (*34*), could also completely inhibit the vacuolization (**Figure 4A**). It is not entirely clear how autophagic degradation could lead to the formation of cytosolic vacuoles, which themselves did not likely represent autophagosomes. The autophagosomes are usually only visible under an electron microscope. A possible explanation could be that the vacuoles represent the "empty space" resulting from autophagic digestion, as seen in nutrient-deprived cells that were dependent on autophagic digestion for survival (*36*). Alternatively, the vacuolization could have a completely different functional role, downstream of autophagic degradation, related to the cell's adaptation to the anthocyanidin treatment.

That delphinidin could induce autophagy in SMMC7721 cells was mainly based on the finding that it could induce the lipidation of LC3, a mammalian Atg8 homologue important for autophagosome formation (*26*). This induction was suppressed by 3-MA and siRNA-mediated knockdown of LC3 (**Figure 5**). Another

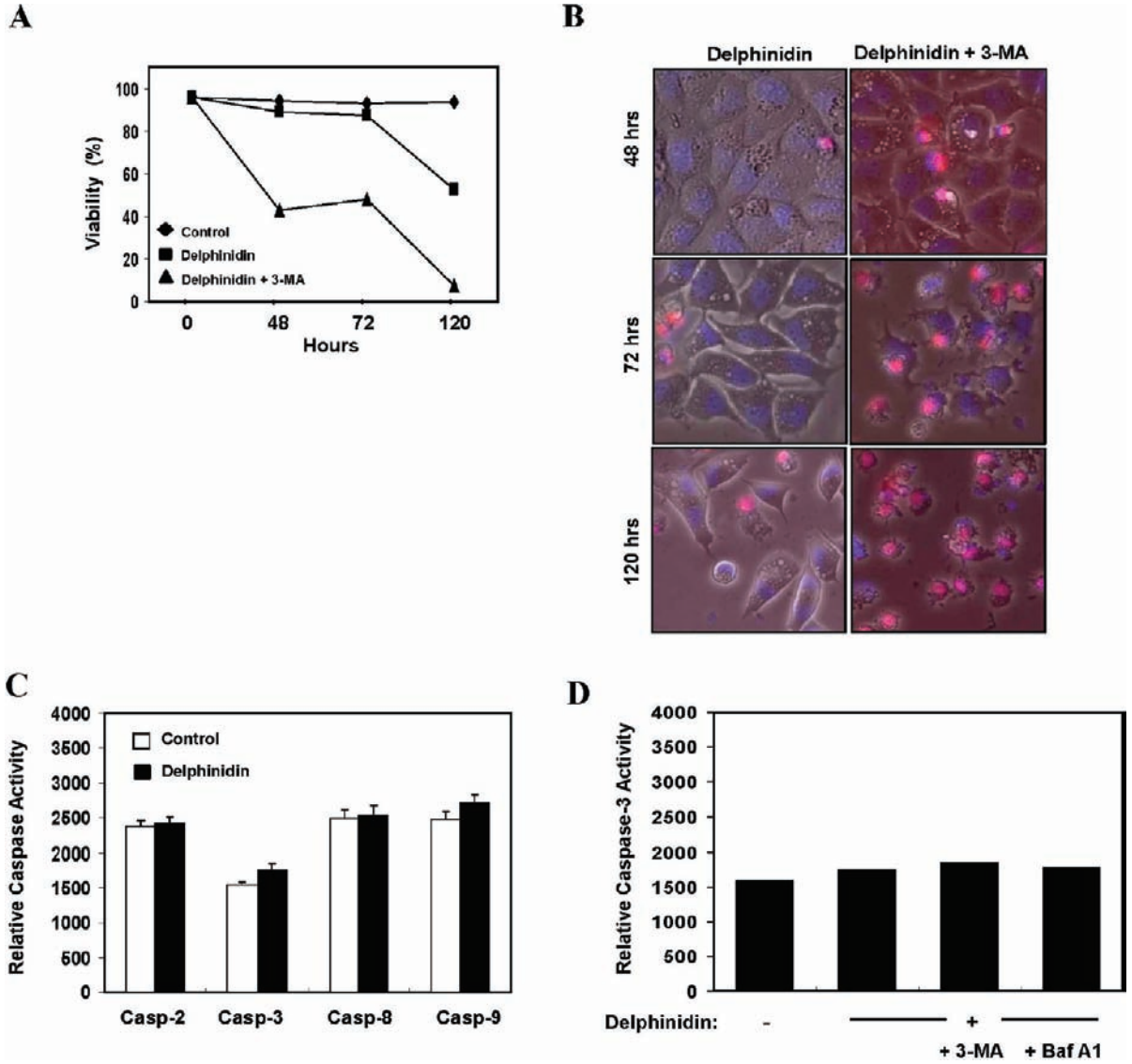

Figure 7. Cotreatment of delphinidin and 3-MA promotes necrotic cell death. (**A**, **B**) SMMC7721 cells were treated with delphinidin (100 μM) in the absence or presence of 3-MA (2.5 mM) for 48, 72, or 120 h. Delphinidin was replenished every 48 h to maintain the potency in the medium. Cells were then stained by Hoechst 33342 (purple) and propidium iodide (red) before digital photographs were taken. Viability was determined on the basis of PI-positive cells (**A**). Fluorescence images were merged with the phase contrast images (**B**). (**C**) SMMC7721 cells were treated with delphinidin (150 μM) for 24 h. Cell lysates were then prepared and incubated with the specific fluorescence substrates of various caspases. The results indicated there was no caspase activation that could be detected in SMMC7721 cells treated with delphinidin. (**D**) SMMC7721 cells were treated with delphinidin (100 μM) in the absence or presence of 3-MA (5 mM) or Baf A1 (100 nM) for 24 h. Total lysates were made, and the caspase-3 activity was measured using Ac-DEVD as the substrate. Results represent one of at least two repeat assays performed.

commonly used tool for the detection of autophagy is the fusion molecule, GFP-LC3 (*26*). GFP-LC3, when introduced into cells by transfection, would be diffusively distributed in the cytosol in cells with low autophagic activities, but would be in punctated autophagosome in cells with elevated autophagic activity. However, in the SMCC7721 cells, transfection of GFP-LC3 alone resulted in a high background level of puncta formation without delphinidin treatment (data not shown). This somewhat precluded an accurate assessment of the GFP-LC3 distribution for autophagic induction. Future studies should apply more sophisticated assays, such as electron microscopy and autophagy flux analysis (*37*), to ascertain the ability of delphinidin to induce autophagy in HCC cells. However, the autophagy-inducing ability of delphinidin is likely not unique among the plant polyphenolic compounds because it has been reported that autophagy could be induced in tumor cells by mixed anthocyanins from *P. lentiscus* berries (*38*) or by resveratrol (*39*). In the case of *P. lentiscus* anthocyanin-induced autophagy in HCC cells, it was considered that the down-regulation of Bcl-2 and mTOR might be responsible for the induction of autophagy (*38*). Whether the same mechanism of autophagy induction is employed in the delphinidin-treated SMMC7721 cells has yet to be determined in future studies.

The second notable feature of delphinidin treatment of the HCC cells was growth retardation but not immediate cell death (**Figure 6**). Cell death would generally not occur until 5 days later even with freshly replenished delphinidin. However, when 3-MA (**Figure 7**), LY294002, or Baf A1 (data not shown) was coadministrated, rapid necrotic cell death occurred with no caspase activation. These findings would be best interpreted by the following scenario. The detrimental effect of delphinidin may be related to nutrient or energy stress, which, however, was

inhibited by the simultaneously activated autophagy. Autophagy sustained the viability of the delphinidin-treated cells by providing the necessary nutrients and/or energy via autophagic degradation, an evolutionarily conserved function of autophagy (*31*). These cells survived, but could proliferate only modestly, and eventually succumbed to death when no more cellular materials could be consumed (*36*). Inhibition of autophagy led to an immediate blockage of nutrient and/or energy supply, resulting in rapid necrosis. The lack of apoptosis activation was not due to any deficiency in the apoptosis pathways as these HCC cells were known to be responsive to apoptotic stimulations (*28*). The lack of caspase activation by delphinidin (**Figure 7C**) or C-3-R (data not shown), even in the presence of autophagy inhibitor 3-MA or Baf A1 (**Figure 7D**), was likely due to the deficiency of ATP, which is required for caspase activation.

In summary, the mode of cell death induced by delphinidin in HCC cells is quite different from the rapid apoptosis in leukemia cells, suggesting that a different chemotherapeutic strategy should be adopted, that is, a combinatory use of delphinidin and an autophagy inhibitor. Similar strategies of suppressing autophagy together with chemotherapeutic agents have been reported in several other cases, including the use of mixed anthocyanins preparation (*38*), indicating that this could be a valuable way to apply natural products in cancer therapy.

ABBREVIATIONS USED

3-MA, 3-methyladenine; Baf A1, bafilomycin A1; C-3-R, cyanidin-3-rutinoside; ER, endoplasmic reticulum; HCC, hepatocellular carcinoma; PI3-kinase, phosphoinositide 3-kinase; UPR, unfolded protein response.

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