

Autophagy inhibition enhances anthocyanin-induced apoptosis in hepatocellular carcinoma

Luigia Longo,¹ Francesca Platini,² Anna Scardino,¹ Oscar Alabiso,³ Giuseppe Vasapollo,¹ and Luciana Tessitore²

¹Department of Engineering of Innovation, University of Salento, Lecce, Italy, ²Department of Food, Chemical, Pharmaceutical, and Pharmacological Sciences (DISCAFF), University of East Piedmont "A. Avogadro", and ³Department of Clinical and Experimental Medicine and Ospedale Maggiore della Carità, Novara, Italy

Abstract

Anthocyanins extracted from the berries of *Phillyrea latifolia* L., *Pistacia lentiscus* L., and *Rubia peregrina* L., three evergreen shrubs widely distributed in the Mediterranean area, were examined for their antioxidant and anticancer activity. The *P. lentiscus* anthocyanins showed the highest H₂O₂ and 1,1-diphenyl-2-picrylhydrazil radical scavenging effects, indicating that these compounds can be considered as an alternative source of natural antioxidants for food and pharmaceutical products. Here, we also report a novel function of anthocyanins: the induction of autophagy, a process of subcellular turnover involved in carcinogenesis. Autophagy was characterized by the up-regulation of eIF2 α , an autophagy inducer, and down-regulation of mTOR and Bcl-2, two autophagy inhibitors. This led to the enhanced expression of LC3-II, an autophagosome marker in mammals, and monodansyl-cadaverine incorporation into autolysosomes. Anthocyanin-induced autophagy switched to apoptosis, as shown by the activation of Bax, cytochrome *c* and caspase 3, terminal deoxynucleotide transferase-mediated dUTP nick-end labeling-positive fragmented nuclei, and cells with sub-G₁ DNA content, which were prevented by z-VAD. Inhibition of autophagy by either 3-methyladenine or Atg5 small interfering RNA enhanced anthocyanin-triggered apoptosis. This provided evidence that autophagy functions as a survival mechanism in liver cancer cells against anthocyanin-induced apoptosis and a ratio-

nale for the use of autophagy inhibitors in combination with dietary chemopreventive agents. [Mol Cancer Ther 2008;7(8):2476–85]

Introduction

Reactive oxygen species, including free radicals, are a class of highly reactive molecules derived from the normal metabolism of oxygen or from exogenous sources such as tobacco smoking, certain pollutants, organic solvents, and pesticides. Reactive oxygen species induce oxidative damage to biomolecules, causing certain human diseases including cancer, cardiovascular diseases, neurodegenerative disorders, aging, etc. (1, 2). Furthermore, reactive oxygen species are known to induce both apoptosis and autophagic cell death (3, 4). Yet, reactive oxygen species are produced during rapamycin-mediated autophagy (5) and serve as novel signaling molecules in the survival pathway of starvation-induced autophagy (6). Consistently, antioxidant agents abolish autophagosome formation and proteolysis. Antioxidants can protect the human body from the effects of reactive oxygen species and retard the progress of many chronic diseases (7). Nowadays, the most commonly used antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene, propyl gallate, and *tert*-butylhydroquinone (8). However, these compounds have shown toxic and mutagenic effects and are suspected of being responsible for liver damage and carcinogenesis (9, 10). Therefore, the development and utilization of more effective and less toxic antioxidants is desired. In recent years, there has been increasing interest in finding antioxidant phytochemicals, the most effective being flavonoids and other phenolic compounds of many raw plant materials (7, 11, 12).

Among natural phenolic compounds, anthocyanins have shown scavenging activity against various artificially generated free radicals and the ability to prevent lipid oxidation (13, 14). We have found that the berries of *Phillyrea latifolia* L., *Pistacia lentiscus* L., and *Rubia peregrina* L., three evergreen shrubs widely distributed in the Mediterranean area, are rich in anthocyanins (15). The major anthocyanin of *P. lentiscus* berries has been identified as cyanidin 3-*O*-glucoside; delphinidin 3-*O*-glucoside and cyanidin 3-*O*-arabinoside have also been found in minor quantities. On the other hand, the major anthocyanin in the extracts of *P. latifolia* and *R. peregrina* berries was cyanidin 3-*O*-rutinoside; low quantities of cyanidin 3-*O*-glucoside were also found (15).

The aim of the present study is to investigate the antioxidant capacity of the anthocyanins extracted from those berries in order to consider them as an alternative source of natural antioxidants for food and pharmaceutical products. Here, we also determined the effect of the

Received 4/16/08; revised 6/3/08; accepted 6/4/08.

Grant support: University of East Piedmont and Lega Italiana per la Lotta contro i Tumori of Novara (L. Tessitore) and University of Salento (Lecce).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Luciana Tessitore, University of East Piedmont, Via Bovio, Novara 28100, Italy. Phone: 39-3213-75881; Fax: 39-3213-75821. E-mail: luciana.tessitore@pharm.unipmn.it

Copyright © 2008 American Association for Cancer Research.

doi:10.1158/1535-7163.MCT-08-0361

anthocyanins on the autophagic and cytotoxic response of hepatocellular carcinoma (HCC) cell lines. We focused our attention on HCC because it is a deadly disease with poor prognosis due to the ineffectiveness of therapy, except for liver transplantation (16). Therefore, the development of new agents for HCC is relevant to reduce its mortality. Our results indicated a novel function of anthocyanins as inducers of autophagy, a defense mechanism against apoptosis, and provided evidence for the combined use of autophagy inhibitors as potentiators of anticancer agents.

Materials and Methods

Extraction and Purification of Anthocyanins

The anthocyanins were extracted from wild-grown berries of *P. latifolia* L., *P. lentiscus* L., and *R. peregrina* L., hand-harvested from the woods of "Parco Regionale Bosco e Paludi di Rauccio", Lecce, Italy during September 2005, and purified as reported in previous studies (15, 17).

Determination of Anthocyanin Content

Anthocyanin content was determined by means of high-performance liquid chromatography-diode array detection analysis using an Agilent 1100 Series LC/DAD system as previously reported (15).

Assay of Hydrogen Peroxide Scavenging Activity

The H₂O₂ scavenging activity of the anthocyanins was determined according to the method of Ruch et al. (18), with slight modifications. The water solution of the anthocyanins (3.4 mL) at different concentrations (0.001–0.1 mg/mL) was added to 0.6 mL of 0.2 mol/L H₂O₂ (Sigma-Aldrich) in 0.1 mol/L phosphate buffer (pH 7.4). After 10 min, the absorbance of H₂O₂ was determined at 210 nm using a Varian Cary 100 Scan UV-visible spectrophotometer, against the blank solution containing the anthocyanin solution and the buffer phosphate without H₂O₂. The assay was also carried out with BHA and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) standards (Sigma-Aldrich) at the same conditions. The percentages of H₂O₂ scavenging activity of the anthocyanins and standards were calculated as $[(A_0 - A_1) / A_0] \times 100$, where A_0 is the absorbance of the control (containing 0.6 mL of 0.2 mol/L H₂O₂ in 0.1 mol/L phosphate buffer and 3.4 mL of water) and A_1 is the absorbance in the presence of the anthocyanins or standards. All measures were repeated thrice.

Assay of 1,1-Diphenyl-2-Picryl-Hydrazil Radical Scavenging Activity

Free radical scavenging activity of the anthocyanins was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH radical) using a slightly modified method from Gülçin et al. (19). One milliliter of a 0.1 mmol/L solution of DPPH radical (Sigma-Aldrich) in ethanol was added to 3 mL of the anthocyanin solution in methanol at different concentrations (0.001–0.01 mg/mL). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. The reduction of DPPH radical absorbance was measured at 517 nm using a Varian Cary 100 Scan UV-visible spectrophotometer against a blank solution prepared with 1 mL of

DPPH radical solution and 3 mL of methanol. The assay was also carried out with BHA and α -tocopherol (Sigma-Aldrich) standards at the same conditions. The percentage of inhibition activity was calculated as $[(A_0 - A_1) / A_0] \times 100$, where A_0 is the absorbance of the blank solution and A_1 is the absorbance in the presence of the anthocyanins or standards. All measures were repeated thrice.

Cell Lines and Treatment

Two human HCC cell lines PLC/PRF/5 (a kind gift from Prof. Roberto Mazzanti, 2nd Medical Oncology of Azienda Ospedaliero-Universitaria Careggi and Interuniversity Center of Liver Physiopathology, University of Florence, Florence, Italy) and HepG2, and a rat HCC cell line McArdle (ATCC-LGC Promochem) were cultured in DMEM containing 10% fetal bovine serum (Life Technologies) at 37°C in an atmosphere containing 5% CO₂. Cells were treated with 0.1, 0.2, 0.4, and 0.8 mg/mL of the aqueous anthocyanin solutions.

Colony Formation Efficiency Test

Cells were seeded in triplicate into 35-mm dishes containing agarose and RPMI 1640 and incubated at 37°C, in 5% CO₂, for 2 weeks.

Cell Viability

Trypan blue staining test was used to identify the dead cells. The reduced 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye (5 mg/mL), incubated at 37°C for 4 h, was solubilized in DMSO.

Cell Cycle Analysis by Flow Cytometry

Cells were fixed with 70% ethanol, incubated with RNaseA (20 μ g/mL) and propidium iodide (PI; 50 μ g/mL) and analyzed on a FACScan flow cytometer (FACSCalibur, BD Biosciences).

Immunocytochemistry and Fluorescent Staining

Cells were incubated with primary antibodies for Beclin1 and LC3 (Santa Cruz Biotechnology) and with secondary antibodies FITC or TRITC (Alexa Fluor, Molecular Probes). DNA fragmentation was detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique using the apoptosis detection kit (*In Situ* Cell Death Kit Pod; Roche). For fluorescent stainings, fixed cells were incubated with 4,6-diamidino-2-phenylindole (DAPI; 0.5 μ g/mL in PBS; for 25 min at room temperature) or PI (for 5 min at 37°C). For the incorporation of monodansylcadaverine: cells were incubated with 0.05 mmol/L of monodansylcadaverine in PBS at 37°C for 20 min. Cells were analyzed by Nikon fluorescence microscope.

Western Blot Analysis

Cells were lysed in a buffer [50 mmol/L Tris/HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L MgCl₂, 1% TX-100, 1 mmol/L EGTA, 1 mmol/L DTT], containing protease inhibitors and 0.1 mol/L of NaF. Protein concentrations were determined with the Bradford assay. The blots probed with Bcl-2, mTOR, P-mTOR, eIF2 α , P-eIF2 α (Calbiochem), and procaspase 3 and Bax antibodies (Santa Cruz Biotechnology, DBA) were detected by enhanced chemiluminescence according to the manufacturer's instructions (Pierce Biotechnology).

Small Interfering RNA Transfection

Cells were transfected with a total of 100 nmol/L of Atg5 small interfering RNA (siRNA; Dharmacon) using LipfectAMINE 2000 (Invitrogen Life Technologies, Inc.) in antibiotic-free medium, according to the manufacturer's instructions.

Statistical Analysis

The significance of the differences between groups was determined by ANOVA test. $P < 0.005$ was considered significant.

Results

P. latifolia and *R. peregrina* Berries Have the Highest Anthocyanin Contents

The total amount of anthocyanins in *P. latifolia* berries was 9.49 g kg^{-1} of berries; cyanidin 3-*O*-rutinoside was the most abundant pigment (8.26 g kg^{-1}) followed by cyanidin 3-*O*-glucoside (1.09 g kg^{-1}). *R. peregrina* berries showed the same anthocyanin composition of *P. latifolia* berries. Nevertheless, *R. peregrina* berries contained a lower amount of anthocyanins corresponding to 7.24 g kg^{-1} of berries. Also in this case, the major anthocyanin was cyanidin 3-*O*-rutinoside (6.06 g kg^{-1}) followed by cyanidin 3-*O*-glucoside (1.11 g kg^{-1}). The total anthocyanin content in *P. lentiscus* berries was 0.05 g kg^{-1} of berries; cyanidin 3-*O*-glucoside was the most abundant pigment (0.04 g kg^{-1})

followed by delphinidin 3-*O*-glucoside (0.01 g kg^{-1}) and cyanidin 3-*O*-araboside (traces). As can be seen, the berries of *P. latifolia* and *R. peregrina* had higher anthocyanin contents than *P. lentiscus* berries.

Anthocyanins from *P. lentiscus* Berries Exhibit the Highest Hydrogen Peroxide Scavenging Activity

The ability of the anthocyanins extracted from the berries of *P. latifolia*, *R. peregrina*, and *P. lentiscus* to scavenge H_2O_2 was compared with the scavenging effects of Trolox and BHA, used as reference standard antioxidants. The anthocyanins were capable of scavenging H_2O_2 in a concentration-dependent manner (Fig. 1A). At the 0.05 mg/mL concentration, the *P. lentiscus* anthocyanins exhibited 72% of H_2O_2 scavenging activity against 33% and 34% found for *P. latifolia* and *R. peregrina* anthocyanins, respectively (Fig. 1B). The H_2O_2 scavenging effect of BHA and Trolox was 38% and 78%, respectively, at the same concentration. These results showed that the analyzed anthocyanins had effective H_2O_2 scavenging activities that decreased in the order of Trolox > *P. lentiscus* anthocyanins > BHA > *R. peregrina* anthocyanins > *P. latifolia* anthocyanins, at 0.05 mg/mL concentration.

Anthocyanins from *P. lentiscus* Berries Exhibit the Highest DPPH Radical Scavenging Activity

The radical-scavenging activity of the anthocyanins extracted from the berries of *P. latifolia*, *R. peregrina*, and

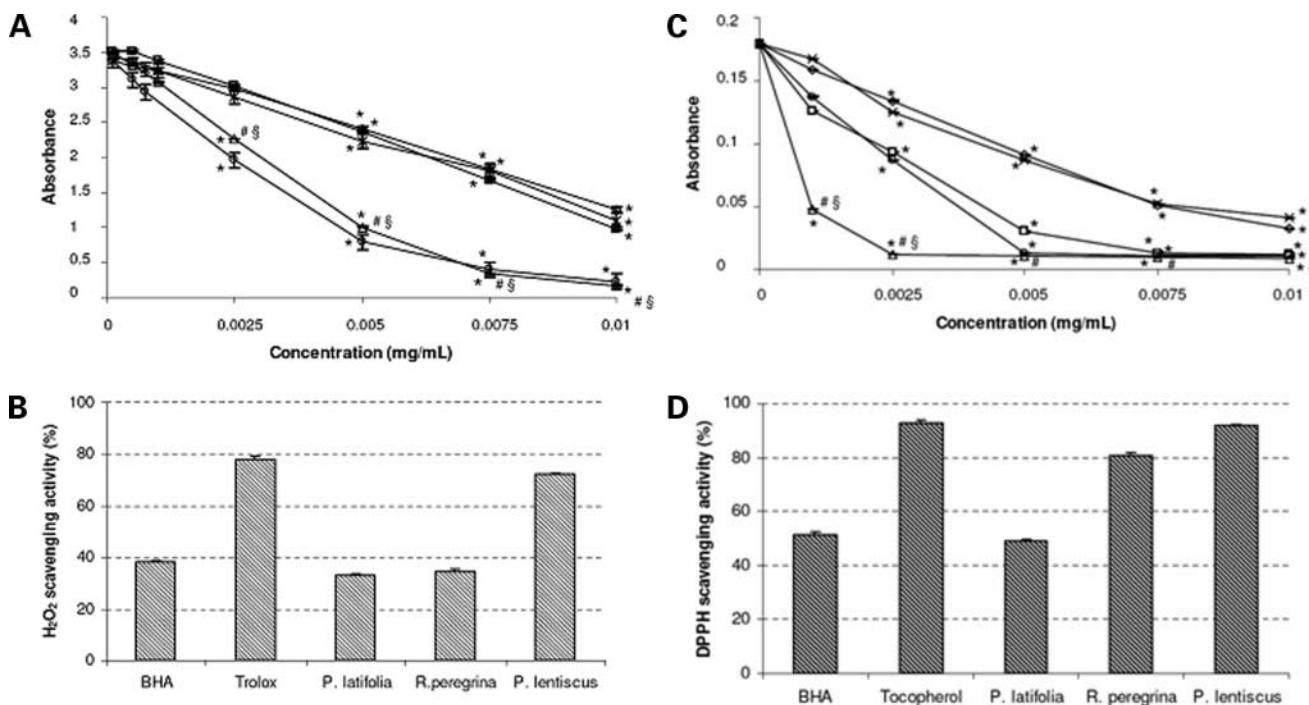


Figure 1. **A** and **B**, H_2O_2 scavenging activity of *P. latifolia* (\diamond), *R. peregrina* (\square), and *P. lentiscus* (\triangle) anthocyanins, BHA (\times), and Trolox (\square). **A**, H_2O_2 absorbance at 210 nm vs. concentration (mg/mL). Points, mean; bars, SD ($n = 3$); *, $P < 0.005$ vs. control; #, $P < 0.005$ *P. lentiscus* vs. *P. latifolia* anthocyanins; §, $P < 0.005$ *P. lentiscus* vs. *R. peregrina* anthocyanins. **B**, percentage (%) of H_2O_2 scavenging activity at the same concentration (0.05 mg/mL). **C** and **D**, DPPH scavenging activity of *P. latifolia*, *R. peregrina*, and *P. lentiscus* anthocyanins, BHA, and α -tocopherol. **C**, DPPH absorbance at 517 nm vs. concentration (mg/mL). Points, mean; bars, SD ($n = 3$); *, $P < 0.005$ vs. control; #, $P < 0.005$ *P. lentiscus* vs. *P. latifolia* anthocyanins; §, $P < 0.005$ *P. lentiscus* vs. *R. peregrina* anthocyanins. **D**, percentage (%) of DPPH scavenging activity at the same concentration (0.005 mg/mL).

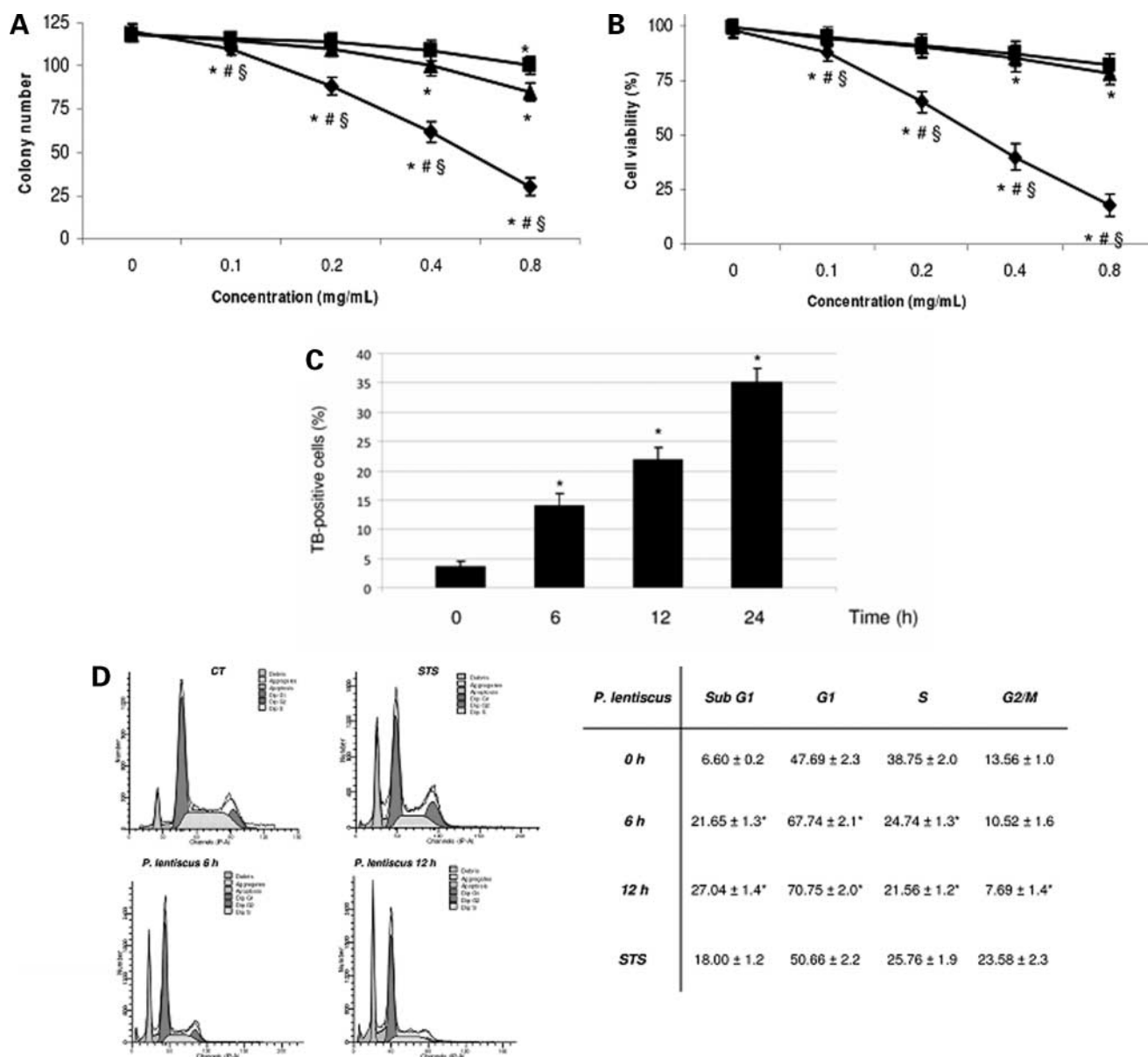


Figure 2. Loss of viability of PLC/PRF/5 cells exposed to the anthocyanins. PLC/PRF/5 cells were treated with *P. latifolia* (■), *R. peregrina* (▲), and *P. lentiscus* (◆) anthocyanins. Cell viability was measured by (A) colony formation efficiency test (14 d), (B) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (24 h), and (C) trypan blue-positive cells for the indicated times. Cell viability was evaluated by trypan blue exclusion assay and quantified as a percentage of trypan blue-positive cells. Columns, mean; bars, SD ($n = 3$); *, $P < 0.005$ vs. control; #, $P < 0.005$ *P. lentiscus* vs. *P. latifolia* anthocyanins; §, $P < 0.005$ *P. lentiscus* vs. *R. peregrina* anthocyanins. D, representative DNA distribution and cell cycle phase distribution of PLC/PRF/5 cells treated with 0.2 mg/mL of *P. lentiscus* anthocyanins at the indicated hours or not (CT, control); staurosporin (STS) was used as a positive control of apoptosis (200 nmol/L), cells were incubated for 12 h. Points, mean; bars, SD ($n = 3$); *, $P < 0.005$.

P. lentiscus was compared with the scavenging effects of α -tocopherol and BHA, which were used as reference standard antioxidants. The anthocyanins were capable of scavenging DPPH radicals in a concentration-dependent manner (Fig. 1C). At the 0.005 mg/mL concentration, the DPPH radical scavenging effects of *R. peregrina* and *P. lentiscus* anthocyanins were 81% and 92%, respectively, against the 49% found for *P. latifolia* anthocyanins. BHA and α -tocopherol exhibited 52% and 93% of DPPH scavenging

activity, respectively, at the same concentration. These results showed that the analyzed anthocyanins had effective DPPH radical scavenging activity that decreased in the order of α -tocopherol > *P. lentiscus* anthocyanins > *R. peregrina* anthocyanins > BHA > *P. latifolia* anthocyanins.

Anthocyanins from *P. lentiscus* Berries Exhibit the Highest Cytotoxicity

To examine the antitumor action of anthocyanin treatment, we assessed the growth-inhibitory effects of

various concentrations of the anthocyanins extracted from the berries. Treatment with the anthocyanins induced a concentration-dependent inhibition of anchorage-independent (Fig. 2A) and monolayer (Fig. 2B) cell growth, always more pronounced in PLC/PRF/5 cells exposed to *P. lentiscus* anthocyanins. The results of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide on *P. lentiscus* anthocyanins were consistent with those obtained using the trypan blue exclusion test, showing a time-dependent effect (Fig. 2C). To elucidate the molecular pathway underlying the antitumor effect, we investigated the progression of PLC/PRF/5 cells through the cell cycle after treatment with *P. lentiscus* anthocyanins because of its highest cytotoxic effect. The anthocyanins induced the appearance of a peak of cells with a DNA content lower than that of the G₀-G₁ peak, i.e., a population of sub-G₁ cells, suggesting a role for the anthocyanins in triggering cell death, accumulation of G₀-G₁ cells associated with a decrease in the percentage of S phase (Fig. 2D). These data showed that the suppressive effects of anthocyanins on HCC cell growth was due to a combination of events, resulting from the inhibition of the cell cycle at the G₁-S checkpoint and DNA synthesis, and induction of apoptosis.

Anthocyanin Treatment Induces Autophagy

The cytotoxicity of many anticancer agents is mediated via autophagy activation (20), which is frequently associated with G₁ arrest (21, 22). Autophagy is a multistep process for subcellular turnover by which intracellular membranes sequester organelles and molecules giving rise to the autophagosome that matures to amphisome, which fuses with a lysosome, generating an autolysosome

(23–25). In order to verify whether the anthocyanins induce the autophagic pathway, the generation of autolysosomes was evaluated by measuring the incorporation of monodansylcadaverine, a marker of the acidic compartment. The lysosomal vacuolar compartment was hardly detected as basal monodansylcadaverine staining in PLC/PRF/5 cells (Fig. 3A). The treatment with *P. lentiscus* anthocyanins induced the formation of monodansylcadaverine-labeled vacuoles (Fig. 3A).

To gain better a insight into the mechanism of anthocyanin-induced autophagy, we analyzed the effect of treatment with *P. lentiscus* anthocyanins on LC3-II, the processed form of the mammalian microtubule-associated protein 1 light chain, LC3-I. LC3-II is a structural component produced during autophagosome formation. Thus, being rapidly degraded, we measured LC3-II expression in the presence of protease inhibitors (Fig. 3B). Treatment of PLC/PRF/5 cells with *P. lentiscus* anthocyanins induced an increase in the immunofluorescence of LC3's punctate pattern. The accumulation of LC3-II was largely prevented by preincubation with 3-methyladenine, an inhibitor of the autophagic sequestration phase (26). Taken together, analysis of monodansylcadaverine incorporation and LC3 expression indicated that the anthocyanins induced autolysosome formation.

Next, using Western blotting analysis, we verified whether the anthocyanin-mediated activation of the autophagic process was related to an imbalance between mTOR and eIF2 α , which negatively and positively control the autophagic pathway, respectively (refs. 25, 27; Fig. 3C). We found that treatment with *P. lentiscus* anthocyanins

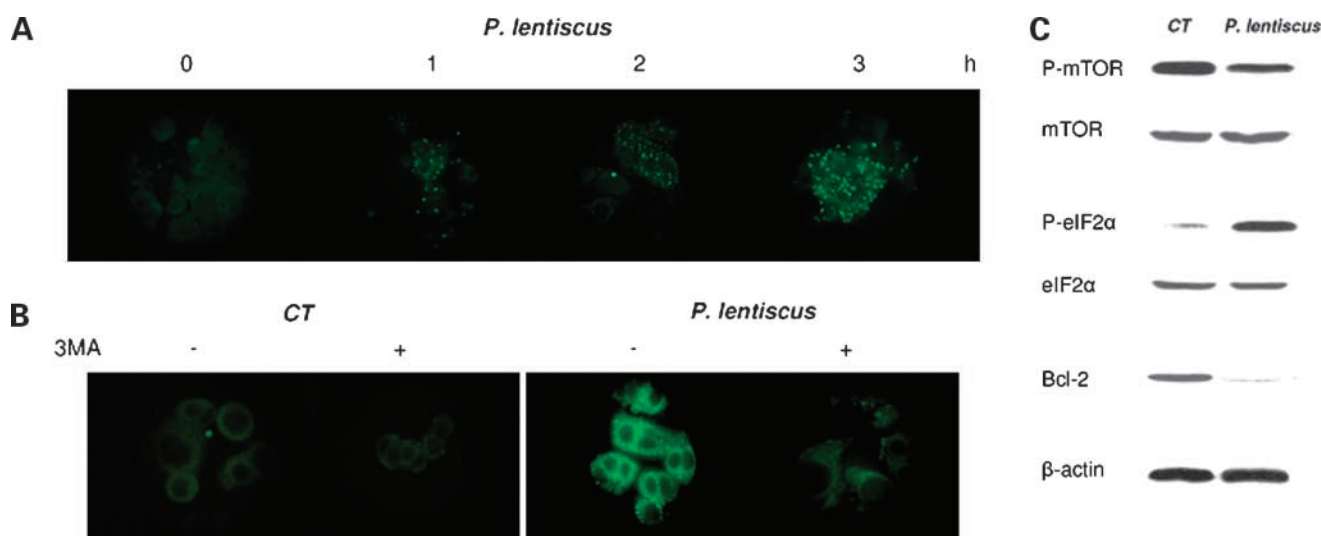


Figure 3. *P. lentiscus* anthocyanins induce autophagy. PLC/PRF/5 cells were exposed to the aqueous solution of *P. lentiscus* anthocyanins for the indicated times (A) or for 2 h (B and C). Confocal microscopy analysis. A, monodansylcadaverine (0.05 mmol/L) incorporation into autolysosomes. B, immunocytochemical analysis of LC3 in the absence or in the presence of 10 mmol/L of 3-MA. In the red channel, autofluorescent signals were minimal. Representative of three separate experiments. C, Western blotting analysis of mTOR and P-mTOR, eIF2 α and P-eIF2 α , and Bcl-2. β -Actin was used as a protein loading control (0.2 mg/mL of the aqueous *P. lentiscus* anthocyanin solution for 3 h). Data are representative of three separate experiments.

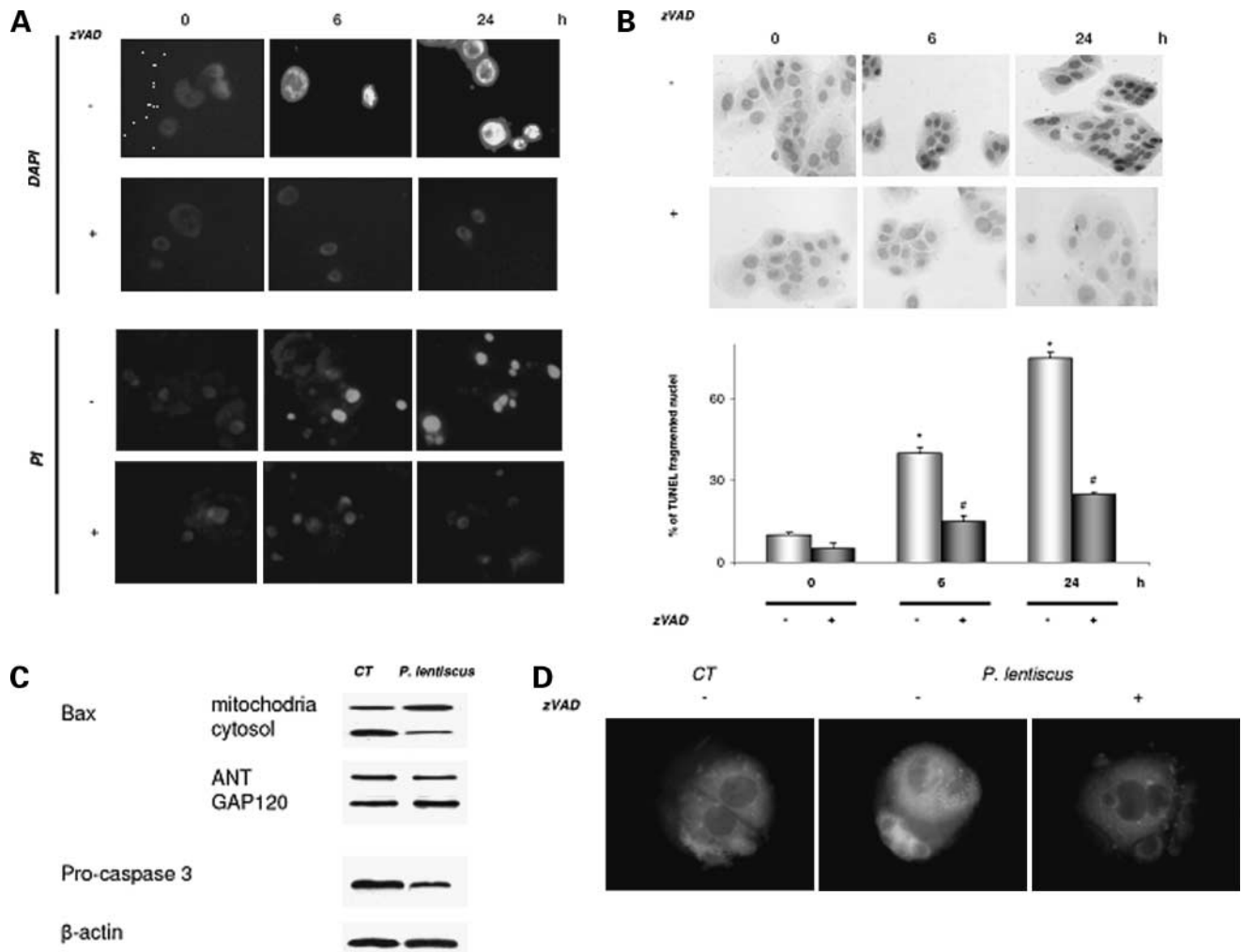


Figure 4. *P. lentiscus* anthocyanins induce apoptosis (0.2 mg/mL of the aqueous *P. lentiscus* anthocyanins solution). PLC/PRF/5 cells were exposed to the aqueous solution of *P. lentiscus* anthocyanins for the indicated times (**A** and **B**) or for 2 h (**C** and **D**) in the absence or in the presence of 20 $\mu\text{mol/L}$ of z-VAD (**A**, **B**, and **D**). Apoptotic analysis was done by nuclear DAPI and PI staining (**A**), nuclear fragmentation by TUNEL assay and percentage of cells showing TUNEL-positive fragmented nuclei (**B**) were calculated from the inspection of at least 10 randomly chosen fields (40–50 cells/field). **C**, Western blotting analysis of procaspase 3 and mitochondrial and cytosolic fractions for Bax expression; ANT, GAP120, and β -actin were used as mitochondrial, cytoplasmic, and total protein level internal controls, respectively. **D**, immunofluorescence analysis of cytochrome *c* release into the cytosol in PLC/PRF/5 cells; cells labeled with anti-cytochrome *c* were visualized by using a fluorescence microscope (original magnification, $\times 40$). Representative of at least three separate experiments. *Columns*, mean of four independent experiments; *bars*, SD; *, $P < 0.005$ vs. control; #, $P < 0.005$ comparison in the presence vs. absence of z-VAD.

down-regulated the phosphorylation of mTOR and up-regulated the phosphorylation of eIF2 α in PLC/PRF/5 cells. This data indicated that the anthocyanins interfere with the regulation of the initiation phase of autophagy through coordinated modulation of key molecules that are sensors of stress conditions.

The expression of Bcl-2, a well-known anti-autophagy inhibitor (25, 28), decreased in PLC/PRF/5 cells exposed to *P. lentiscus* anthocyanins (Fig. 3C). Suppression of Bcl-2 expression by anthocyanins could represent a key mechanism through which anthocyanins activate both autophagy and apoptosis, Bcl-2 being a potential molecular switch between the two pathways.

Autophagy Counteracts Anthocyanin-Induced Apoptosis

As expected by the anthocyanin-mediated increase of sub-G₁ phase PLC/PRF/5 cells, other apoptotic variables confirm the ability of the anthocyanins to trigger apoptosis (29, 30). Treatment of PLC/PRF/5 cells with *P. lentiscus* anthocyanins induced nuclei fragmentation as determined by DAPI and PI staining (Fig. 4A), TUNEL analysis and quantification (Fig. 4B), down-regulation of Bcl-2 (Fig. 3C), translocation of Bax from the cytoplasm to the mitochondria (Fig. 4C), where it activated the release of cytochrome *c* in the cytosol (Fig. 4D) which is essential for the cleavage of caspase 3 (Fig. 4C). Pretreatment with a

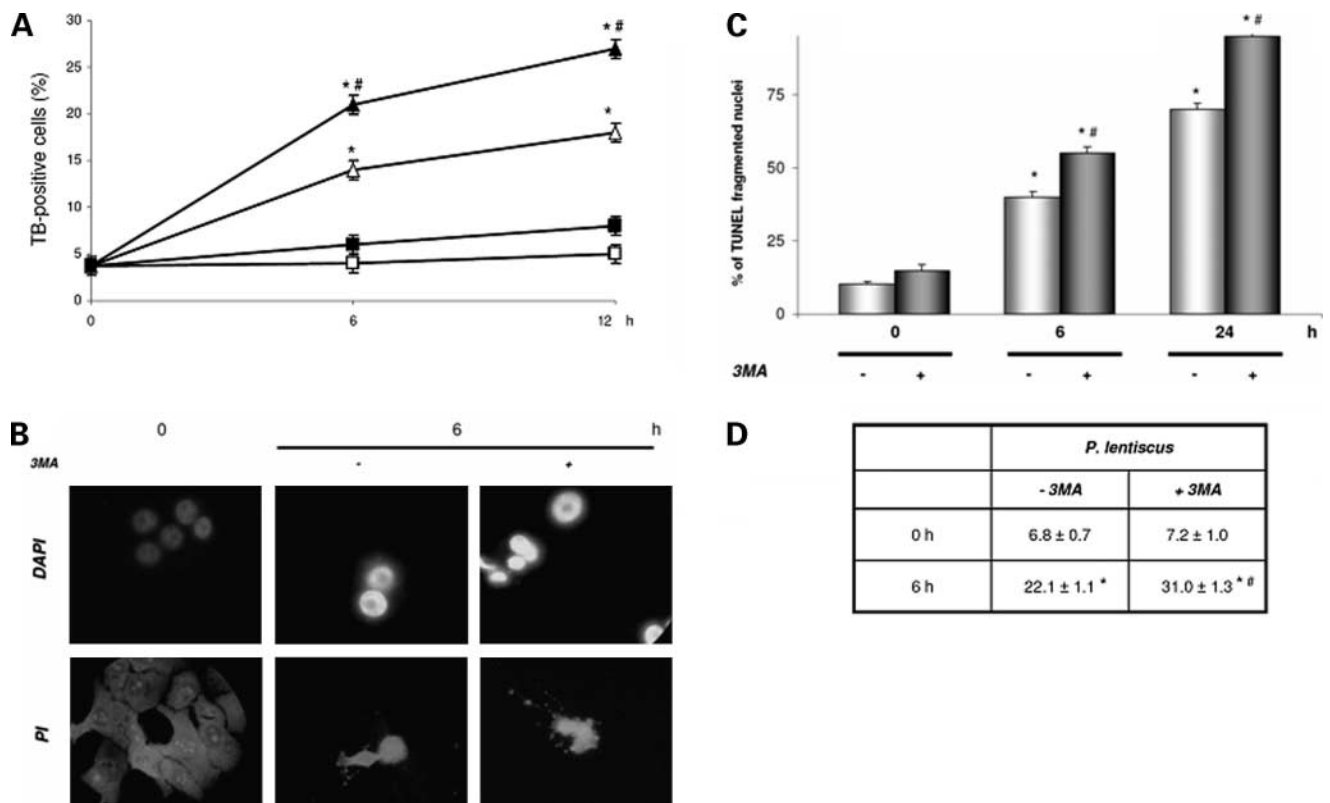


Figure 5. Autophagy inhibits apoptosis induced by *P. lentiscus* anthocyanins in PLC/PRF/5 cells. Cells (□, control; ■, control plus 3-MA) were exposed to the aqueous solution of *P. lentiscus* anthocyanins in the absence (Δ) or presence (▲) of 10 mmol/L of 3-MA. Cell viability was measured by trypan blue-positive cells (A), DAPI staining and PI staining (B), TUNEL assay and as a percentage of cells showing TUNEL-positive fragmented nuclei (C) and percentage of sub-G₁ cells (D). Columns, mean of at least three independent experiments; bars, SD; *, $P < 0.005$ vs. control; #, $P < 0.005$ present vs. absent 3-MA.

caspase inhibitor, z-VAD, significantly prevented cell death.

Cancer cells can respond to drugs by activating autophagy to promote cell survival, thus counteracting apoptosis (20). To elucidate the involvement of the autophagic process in anthocyanin-induced apoptosis, the effects of autophagy inhibitors were tested. Although the *P. lentiscus* anthocyanin-induced development of autophagic vacuoles was inhibited by 3-MA (Fig. 3B) and Wortammin (data not shown), cell viability was markedly worse. This was shown by a trypan blue assay (Fig. 5A), DAPI and PI staining (Fig. 5B), TUNEL assay (Fig. 5C), and sub-G₁ peak (Fig. 5D).

To further confirm the role of the autophagic machinery in anthocyanin-mediated apoptosis, we used siRNA to inhibit the *Atg5* gene, essential for autophagosome generation (23, 24). Silencing of *Atg5* (Fig. 6A) markedly inhibited *P. lentiscus* anthocyanin-induced generation of LC3-II-labeled vacuoles (Fig. 6B) and enhanced anthocyanin-induced cell demise (Fig. 6C and D). The data indicated that HCC cells respond to anthocyanins by activating autophagy to resist apoptosis.

Anthocyanin-Induced Autophagy Is Not Restricted to PLC/PRF/5 Cells

To test the hypothesis that anthocyanin-induced autophagy is not limited to PLC/PRF/5 cells, the activation of the autophagic machinery was also evaluated in a human (HepG2) and rat (McArdle) HCC cell line. Upon treatment with *P. lentiscus* anthocyanins, Bcl-2 expression was down-regulated (Supplementary Fig. S1A)⁴ and the number of LC3-labeled vacuoles/cell sections was increased (Supplementary Fig. S1B).⁴ Consistently, autophagy inhibition accelerated the execution of demise as shown by the increase in TUNEL-positive fragmented nuclei (Supplementary Fig. S1C)⁴ and in cells of sub-G₁ peak (Supplementary Fig. S1D).⁴ These data showed that anthocyanin-induced autophagy is a generalized cell response and was not limited to PLC/PRF/5 cells.

⁴ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

Discussion

The results obtained in this study show that the anthocyanins obtained from *P. lentiscus* berries have the strongest DPPH radical and H₂O₂ scavenging activities. Moreover, their scavenging effects are comparable to those of α -tocopherol, one of the strongest natural antioxidants, and Trolox, its water-soluble analogue. The higher scavenging activity of *P. lentiscus* anthocyanins is probably due to the presence of the monoglucoside of delphinidin, which possesses the highest radical scavenging activity among the anthocyanins present in the berries of this plant (14).

The present study provides evidence to show the induction of both autophagic and apoptotic machineries in HCC cells upon treatment with anthocyanins, which are highly promising cancer chemopreventive constituents of several vegetables (29, 30). This was not unexpected because many natural products are known to possess antioxidant and anticancer properties. Anthocyanins inhibit oxidative stress-induced apoptosis by elevating the cellular antioxidant capacity (31–33). On the other hand, they contribute to the antiproliferative activity of vegetables and fruits, including berries, by inducing the apoptosis of cancer cells (29, 30, 34–37). Despite their antioxidant action, paradoxically, they exhibit pro-oxidant activity resulting in selective cell death of leukemia cells (37).

The highest cytotoxicity against HCC cells is shown by *P. lentiscus* anthocyanins, which also displayed the stron-

gest antioxidant activity. The *in vitro* anticancer activity is due to the combined induction of the apoptotic program, G₁ arrest, and reduced DNA synthesis. The apoptotic response has been observed in response to various anticancer stimuli, including natural products (38); however, failure in HCC cure results from the development of drug resistance, the rates of HCC incidence and mortality being similar (16).

Here, we report for the first time a novel function of anthocyanins; the induction of autophagy, as shown by activation of the autophagosomal marker LC3 and incorporation of monodansylcadaverine in autolysosomes. This was possibly triggered by down-regulation of Bcl-2 and mTOR associated with up-regulation of eIF2 α signaling pathways. Autophagy displays a dual conflicting function in cancer cell biology. We have shown that HCC down-regulates autophagic activity (39, 40), and recently, autophagy has been proposed as a tumor suppression mechanism, underlying the possibility that activation of autophagy reverses the neoplastic phenotype (25, 41). On the contrary, in the present article, inhibition of autophagy enhanced apoptosis, indicating that autophagy contributes to tumor progression as a protective mechanism against stressful conditions (25, 42, 43). Autophagy optimizes nutrient utilization in rapidly growing cells when faced with hypoxic or metabolic stress, similar to therapeutic assault, and removes organelles that are sources or targets of lethal levels of free radical-induced damage (44). Thus, lack of autophagy for a long

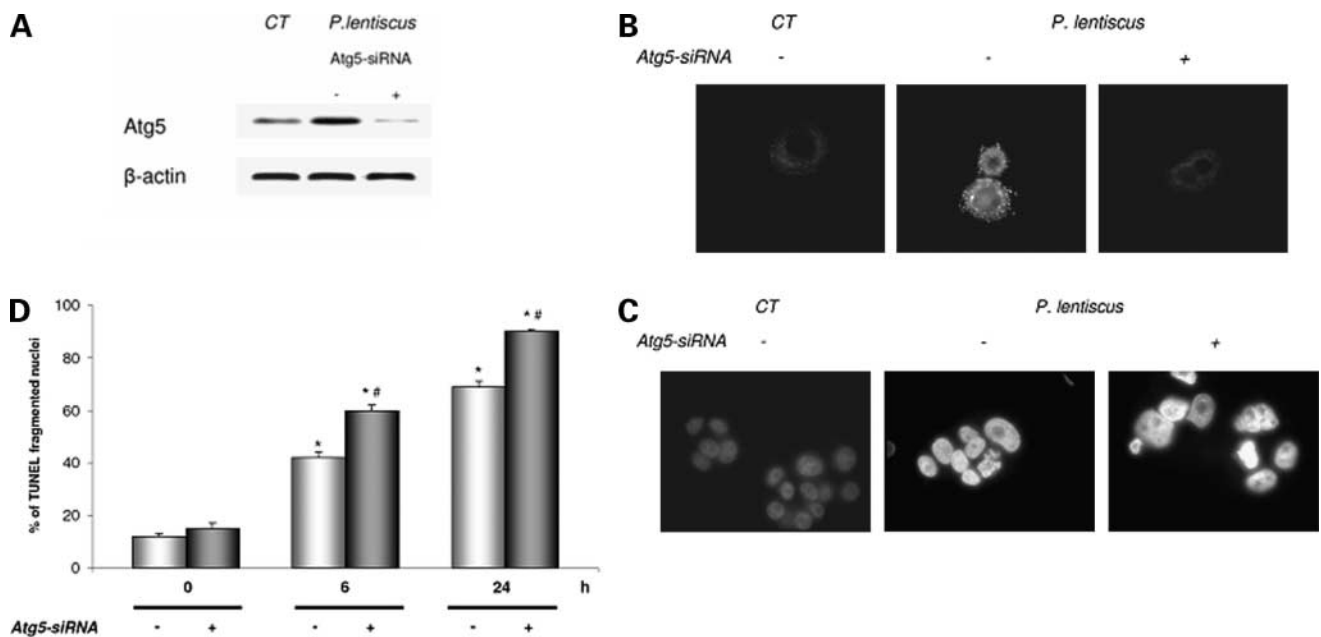


Figure 6. Enhancement of apoptosis induced by *P. lentiscus* anthocyanins of PLC/PRF/5 cells by silencing an autophagy gene. PLC/PRF/5 cells were treated with *Atg5* siRNA (100 nmol/L) for 24 h and then incubated with 0.2 mg/mL of the aqueous solution of *P. lentiscus* anthocyanins for 6 h. **A**, effect of the *Atg5* siRNA on *Atg5* expression evaluated by Western blot analysis at basal conditions and after 72 h of transfection with siRNA in PLC/PRF/5 cells. **B**, punctate LC3 fluorescence in PLC/PRF/5 cells transfected with *Atg5* siRNA and incubated without or with the aqueous solution of *P. lentiscus* anthocyanins and then examined by confocal fluorescent microscopy. The percentage of cells with punctate LC3 fluorescence was calculated relative to all cells. Cell viability was measured, in the absence and in the presence of z-VAD, by DAPI staining (**C**) and TUNEL assay, shown as a percentage of cells with TUNEL-positive fragmented nuclei (**D**), calculated from the inspection of at least 10 randomly chosen fields (40–50 cells/field). Columns, mean of at least three independent experiments; bars, SD; *, $P < 0.005$ vs. control; #, $P < 0.005$ present vs. absent transfection with *Atg5* siRNA.

period of time results in the accumulation of intracellular oxidants that enhance the probability of transformation. Yet, the same mechanism protects the cells of established cancers against various stressors.

The autophagic pathway is a novel therapeutic target for cancer treatment (20, 25, 42, 45). Anticancer treatments, including drugs, activate autophagy to kill cancer cells resistant to apoptosis. Yet, in HCC cells, a deficiency of autophagy enhances anthocyanin-induced cell demise. Paglin et al. (46) shows for the first time the possibility of treating cancer cells by autophagy inhibition because cancer cells respond to radiation by inducing autophagy. Autophagy inhibition enhances the anticancer effect of arsenic trioxide (20), hyperthermia (20), sulforaphane (47), and p53 or alkylating drugs (48). These and our observations indicate that autophagy protects cancer cells from therapy-induced apoptosis and that autophagy inhibitors strengthen the efficacy of proapoptotic chemotherapeutic strategies. Again, autophagy is commonly induced by hypoxia and it represents the ultimate nutritional source for tumor cells to survive low-nutrient conditions (20), thus suppression of autophagy in combination with other treatments accelerates tumor necrosis (27, 43).

In HCC cells, we eliminate the expression of Atg5 protein to inhibit autophagy because Atg5 is required for the formation of the autophagosome (23–25) and it is a novel proapoptotic factor (49). However, we rule out the possibility that Atg5 is involved in anthocyanin-induced apoptosis. Autophagy is regulated by class III phosphoinositide-3-kinase complex which is the putative target of 3-MA. Inhibition of anthocyanin-induced autophagy in the presence of 3-MA indicates the involvement of a class III phosphoinositide-3-kinase complex. This complex includes Beclin 1, a protein first identified as a novel Bcl-2-interacting protein, the binding of Bcl-2 to Beclin 1 disrupting its autophagic function (25, 28). Here, we have shown that anthocyanin treatment causes a decrease in the level of Bcl-2 protein in HCC cells. This suggests that anthocyanin disrupts the interaction between Bcl-2 and Beclin 1 in the trans-Golgi network to induce autophagy followed by the binding of Bcl-2 to Bax in the mitochondria to switch autophagy to apoptosis. Alternatively, because mTOR inhibition increases the levels of activated LC3 (25, 27), anthocyanins might directly target mTOR to induce autophagy.

In conclusion, our observations indicate that autophagy inhibition helps to enhance the chemopreventive/therapeutic activity of anthocyanin towards HCC. Future experiments are required to extend these preclinical *in vitro* results to evaluate the effect of anthocyanin treatment in mouse tumor models in support of potential clinical studies. In animal models, anthocyanins act as anticancer agents by protecting against genomic instability at various phases of the carcinogenic process, yet, the effect in human studies is weak (50). This study suggests that

the anthocyanins obtained from *P. latifolia*, *R. peregrina*, and *P. lentiscus* berries could be used as an easily accessible and abundant source of natural antioxidants as potential food additives and pharmaceutical agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

- Halliwell B, Gutteridge JMC, Cross CE. Free radicals, antioxidants and human disease: where are we now? *J Lab Clin Med* 1992;119:598–620.
- Auroma OI. Nutrition and health aspects of free radicals and antioxidants. *Food Chem Toxicol* 1994;32:671–83.
- Yu L, Wan F, Dutta S, et al. Autophagic programmed cell death by selective catalase degradation. *Proc Natl Acad Sci U S A* 2006;103:4952–7.
- Kiffin R, Bandyopadhyay U, Cuervo AM. Oxidative stress and autophagy. *Antioxid Redox Signal* 2006;8:152–62.
- Kissová I, Deffieu M, Samokhvalov V, et al. Lipid oxidation and autophagy in yeast. *Free Radic Biol Med* 2006;41:1655–61.
- Scherz-Shouval R, Elazar Z. ROS, mitochondria and the regulation of autophagy. *Trends Cell Biol* 2007;17:422–7.
- Kinsella JE, Frankel E, German B, Kanner J. Possible mechanisms for the protective role of antioxidants in wine and plant foods. *Food Technol* 1993;47:85–9.
- Sherwin FR. Antioxidants. In: R. Branen. *Food additives*. New York: Marcel Dekker; 1990. p. 139–93.
- Grice HC. Safety evaluation of butylated hydroxytoluene (BHT) in the liver, lung and gastrointestinal tract. *Food Chem Toxicol* 1986;24:1127–30.
- Wichi HP. Enhanced tumor development by butylated hydroxyanisole (BHA) from the prospective of effect on forestomach and oesophageal squamous epithelium. *Food Chem Toxicol* 1988;26:717–23.
- Lewis NG. Plant phenolics. In: Alscher RG and Hess JL. *Antioxidants in higher plants*. Boca Raton (FL): CRC Press; 1993. p. 135–69.
- Gülçin İ, Berashvili D, Gepdiremen A. Antiradical and antioxidant activity of total anthocyanins from *Perilla panchinensis* decne. *J Ethnopharmacol* 2005;101:287–93.
- Kähkönen MP, Heinonen M. Antioxidant activity of anthocyanins and their aglycons. *J Agric Food Chem* 2003;51:628–33.
- Espin JC, Soler-Rivas C, Wichers HJ, Garcia-Viguera C. Anthocyanin-based natural colorants: a new source of antiradical activity for foodstuff. *J Agric Food Chem* 2000;48:1588–92.
- Longo L, Scardino A, Vasapollo G. Identification and quantification of antocyanins in the berries of *Pistacia lentiscus* L., *Phillyrea latifolia* L., and *Rubia peregrina* L. *Innov Food Sci Emerg Technol* 2007;8:360–4.
- El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 2007;132:2557–76.
- Longo L, Vasapollo G. Anthocyanins from Bay (*Laurus nobilis* L.) berries. *J Agric Food Chem* 2005;53:8063–7.
- Ruch RJ, Cheng S, Klaunig JE. Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis* 1989;10:1003–8.
- Gülçin İ, Küfrevioğlu Ö, Oktay M, Büyükkokuroğlu ME. Antioxidant, antimicrobial, antiulcer and analgesic activities of nettle (*Urtica dioica* L.). *J Ethnopharmacol* 2004;90:205–15.
- Kondo Y, Kanzawa T, Sawaya R, Kondo S. The role of autophagy in cancer development and response to therapy. *Nat Rev Cancer* 2005;5:726–34.
- Botti J, Djavaheri-Mergny M, Pilatte Y, Codogno P. Autophagy signaling and the cogwheels of cancer. *Autophagy* 2006;2:67–73.
- Liang J, Shao SH, Xu ZX, et al. The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. *Nat Cell Biol* 2007;9:218–24.

23. Mizushima N. Autophagy: process and function. *Genes Dev* 2007;21:2861–73.
24. Klionsky DJ. Autophagy: from phenomenology to molecular understanding in less than a decade. *Nat Rev Mol Cell Biol* 2007;8:931–7.
25. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. *Cell* 2008;132:27–42.
26. Seglen PO, Gordon PB. 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. *Proc Natl Acad Sci U S A* 1982;79:1889–92.
27. Lum JJ, DeBerardinis RJ, Thompson CB. Autophagy in meta-zoans: cell survival in the land of plenty. *Nat Rev Mol Cell Biol* 2005;6:439–48.
28. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat Rev Mol Cell Biol* 2007;8:741–52.
29. Nichenametla SN, Taruscio TG, Barney DL, Exon JH. A review of the effects and mechanisms of polyphenolics in cancer. *Crit Rev Food Sci Nutr* 2006;46:161–83.
30. Neto CC. Cranberry and its phytochemicals: a review of *in vitro* anticancer studies. *J Nutr* 2007;137:186–93S.
31. Shih PH, Yeh CT, Yen GC. Anthocyanins induce the activation of phase II enzymes through the antioxidant response element pathway against oxidative stress-induced apoptosis. *J Agric Food Chem* 2007;55:9427–35.
32. Tarozzi A, Morroni F, Hrelia S, et al. Neuroprotective effects of anthocyanins and their *in vivo* metabolites in SHSY5Y cells. *Neurosci Lett* 2007;424:36–40.
33. Bouaziz A, Romera-Castillo C, Salido S, et al. Cinnamtannin B-1 from bay wood exhibits antiapoptotic effects in human platelets. *Apoptosis* 2007;12:489–98.
34. Srivastava A, Akoh CC, Fischer J, Krewer G. Effect of anthocyanin fractions from selected cultivars of Georgia-grown blueberries on apoptosis and phase II enzymes. *J Agric Food Chem* 2007;55:3180–5.
35. Reddivari L, Vanamala J, Chintharlapalli S, Safe SH, Miller JC, Jr. Anthocyanin fraction from potato extracts is cytotoxic to prostate cancer cells through activation of caspase-dependent and caspase-independent pathways. *Carcinogenesis* 2007;28:2227–35.
36. Agarwal C, Veluri R, Kaur M, Chou SC, Thompson JA, Agarwal R. Fractionation of high molecular weight tannins in grape seed extract and identification of procyanidin B2-3,3'-di-O-gallate as a major active constituent causing growth inhibition and apoptotic death of DU145 human prostate carcinoma cells. *Carcinogenesis* 2007;28:1478–84.
37. Feng R, Ni HM, Wang SY, et al. Cyanidin-3-rutinoside, a natural polyphenol antioxidant, selectively kills leukemic cells by induction of oxidative stress. *J Biol Chem* 2007;282:13468–76.
38. Fabregat I, Roncero C, Fernandez M. Survival and apoptosis: a dysregulated balance in liver cancer. *Liver Int* 2007;27:155–62.
39. Kisen GO, Tessitore L, Costelli P, et al. Reduced autophagic activity in primary rat hepatocellular carcinoma and ascites hepatoma cells. *Carcinogenesis* 1993;14:2501–5.
40. Canuto RA, Tessitore L, Muzio G, Autelli R, Baccino FM. Tissue protein turnover during liver carcinogenesis. *Carcinogenesis* 1993;14:2581–7.
41. Tessitore L, Platini F, Bottini C. Understanding autophagy in cell death control. In: *Curr Pharm Des*. In press 2008.
42. Levine B. Cell biology: autophagy and cancer. *Nature* 2007;446:745–7.
43. Mathew R, Karantza-Wadsworth V, White E. Role of autophagy in cancer. *Nat Rev Cancer* 2007;7:961–7.
44. Kim I, Rodriguez-Enriquez S, Lemasters JJ. Selective degradation of mitochondria by mitophagy. *Arch Biochem Biophys* 2007;462:245–53.
45. Rubinsztein DC, Gestwicki JE, Murphy LO, Klionsky DJ. Potential therapeutic applications of autophagy. *Nat Rev Drug Discov* 2007;6:304–12.
46. Paglin S, Hollister T, Delohery T, et al. A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. *Cancer Res* 2001;61:439–44.
47. Herman-Antosiewicz A, Johnson DE, Singh SV. Sulforaphane causes autophagy to inhibit release of cytochrome C and apoptosis in human prostate cancer cells. *Cancer Res* 2006;66:5828–35.
48. Amaravadi RK, Thompson CB. The roles of therapy-induced autophagy and necrosis in cancer treatment. *Clin Cancer Res* 2007;13:7271–9.
49. Yousefi S, Perozzo R, Schmid I, et al. Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. *Nat Cell Biol* 2006;8:1124–32.
50. Duthie SJ. Berry phytochemicals, genomic stability and cancer: evidence for chemoprotection at several stages in the carcinogenic process. *Mol Nutr Food Res* 2007;51:665–74.