Phycocyanin-mediated apoptosis in AK-5 tumor cells involves down-regulation of Bcl-2 and generation of ROS

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Abstract

C-phycocyanin, which is a major biliprotein of the blue-green algae, has been shown to possess cyclooxygenase-2 inhibitory activity. We have studied the effect of phycocyanin on a rat histiocytic tumor line. AK-5 cells are induced into apoptotic death program when treated with phycocyanin, which involves the activation of caspase-3. Phycocyanin-mediated apoptotic death is induced through the generation of reactive oxygen radicals. Free radical scavengers inhibited phycocyanin-induced apoptotic death in AK-5 cells. Bcl-2, an inhibitor of apoptosis, is shown to regulate ROS generation. Bcl-2 gene-transfected AK-5 cells are resistant to phycocyanin-induced death. Overexpression of Bcl-2 inhibited the production of ROS in phycocyanin-treated AK-5 cells. Thus, our observations demonstrate phycocyanin-induced apoptotic death in AK-5 cells, which is inhibited by Bcl-2 expression through the regulation of free radical generation. Phycocyanin, a natural product, could therefore be a possible chemotherapeutic agent through its apoptotic activity against tumor cells. (Mol Cancer Ther. 2003;2:1165–1170)

Introduction

C-phycocyanin is a major biliprotein of *Spirulina platensis*, blue-green algae possessing antioxidant (1), anti-arthritic, and anti-inflammatory properties (1, 2). Phycocyanin also acted as a hepatoprotective agent and as a hydroxyl radical scavenger (3). However, the mechanism of action of phycocyanin is not clearly understood.

Cyclooxygenase-2 (COX-2) catalyzed the conversion of arachidonic acid to prostaglandins and other eicosanoids (4). Overexpression of COX-2 is associated with high levels of prostaglandin E_2 (PGE_2) and has been demonstrated in several malignancies of colon, breast, lung, prostrate, skin, cervix, pancreas, bladder, and head and neck (5). Higher prostaglandin levels have been shown to stimulate proliferation of cells and mediate immune suppression (6, 7). Recently, COX-2 has been shown to be involved in the suppression of apoptosis, which is critical in tumor cell death. Several mechanisms have been proposed to account for suppression of apoptosis in response to COX-2 overexpression. One of the mechanisms being PGE_2-mediated up-regulation of Bcl-2, which in turn inhibited apoptosis (8). Alternatively, arachidonic acid-stimulated apoptosis and COX-2 expression inhibited apoptosis by increasing the conversion of arachidonic acid to PGE (8).

Several non-steroidal anti-inflammatory drugs and selective COX-2 inhibitors have been shown to induce apoptosis in colon cancer cell lines and transformed fibroblasts (8, 9). C-phycocyanin has also been shown to possess selective COX-2 inhibitory property (10). Thus, our interest was to study the induction of apoptosis by phycocyanin in tumor cells and the mechanisms involved in the apoptotic process. We have used a rat histiocytic tumor model for these studies (11) because AK-5 cells showed COX-2 expression. Our observations suggest involvement of caspase in the apoptotic death process by phycocyanin, which is inhibited by Bcl-2. Phycocyanin induced the generation of ROS by the tumor cells, which in turn induced apoptosis. Interestingly, phycocyanin also induced down-regulation of Bcl-2, which is known to play a crucial role in the apoptotic death processes.

Materials and Methods

Cell Culture

AK-5, a rat histiocytic tumor, is maintained as ascites in Wistar rats (11). However, to avoid ambiguity in results due to tumor heterogeneity, we have used in these studies a single cell clone of AK-5 called BC-8 which has been adapted to grow *in vitro* (12). BC-8 cells were grown in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 50 μg/ml streptomycin.

Phycocyanin Treatment

C-phycocyanin (isolated from *S. platensis*) was a kind gift from Green India Natural Products Ltd., Madurai. The particle size being 72 mesh with >95% purity and soluble in water with an absorption maxima at 618 nm. After a careful titration, we have used 30 μM phycocyanin concentration in all our experiments. For ROS generation, the cells were treated with phycocyanin for 6 h, whereas for RT-PCR analysis, the phycocyanin treatment was for 18 h. Significant apoptotic induction was observed after 24 h of phycocyanin treatment. Significance of the data obtained was analyzed using Student's *t* test.

Transfection of BC-8 Cells

BC-8 cells (2×10^6) were transfected with linearized pMEP4 vector with full-length murine Bcl-2 gene kindly provided by Dr. Y.A. Hannun by the electroporation method described earlier (13). The transfected clones were selected with hygromycin B (400 μg ml^{-1}) for 14 days.

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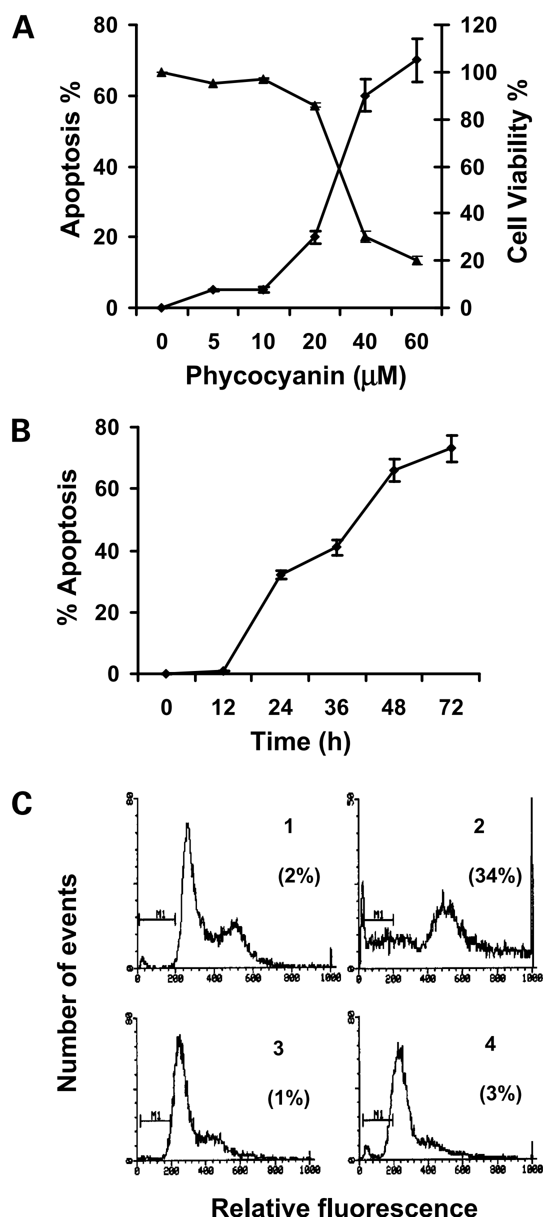

Figure 1. Phycocyanin-induced apoptosis in BC-8 cells. **A,** effect of different concentrations of phycocyanin on BC-8 viability; percentages of apoptotic cells are also shown. **B,** time-dependent apoptosis in BC-8 cells after phycocyanin treatment. **C,** flow cytometric analysis of apoptotic cells. BC-8 cells (*1*) were treated with 30 μM phycocyanin for 24 h and analyzed after PI staining (*2*). Similarly, Bcl-2 (*3*) transfectants were analyzed after phycocyanin treatment (*4*). Phycocyanin induced significant apoptotic death in BC-8 cells. These experiments were repeated 5–6 times.

Stably transfected single cell clones were obtained by limiting dilution procedure. Clones were screened by Northern hybridization and immunostaining and the positive clones were expanded and used in these studies.

Propidium Iodide Staining

Cells were washed with PBS, fixed in 80% ethanol, and stained with propidium iodide (PI) (Calbiochem, San Diego, CA) reagent (50 μg/ml in 0.1% sodium citrate buffer containing 0.1% Triton X-100) and the DNA content of the cells was evaluated by flow cytometry. Cell viability was also evaluated after PI staining of unfixed cells in the absence of Triton X-100.

DNA Extraction and Electrophoresis

Cells were fixed in ethanol, washed with PBS, and suspended in citrate-phosphate buffer. DNA was extracted, following the procedure described earlier (14) and electrophoresed on a 0.86% agarose gel at 2 V/cm for 16 h, stained with 5 μg/ml ethidium bromide, and visualized under UV light.

Detection of Apoptosis by Annexin-V Staining

BC-8 cells (2×10^6) induced to undergo apoptosis were stained with annexin V-FITC (Boehringer-Mannheim, Mannheim, Germany) in binding buffer [10 mM HEPES (pH 7.4), 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$ $CaCl_2$ (pH 7.4)] at 4°C for 15 min. The binding of annexin V-FITC to phosphatidylserine, exposed on the cell surface, was analyzed by laser scanning confocal microscopy.

Estimation of ROS

Superoxide-induced reduction of ferricytochrome *c* to ferrocytochrome *c* was monitored spectrophotometrically at 550 nm (15). Cells were suspended in complete phenol-red free DMEM and plated in 96-well plates at 5×10^4 cells/well. Superoxide generated was estimated in the presence of 80 μM cytochrome *c* with and without the addition of superoxide dismutase (300 units/ml). Intracellular superoxide was estimated fluorometrically using the oxidation-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCF; Molecular Probes, Eugene, OR) (16). Before exposure to phycocyanin, cells were washed with PBS and incubated with DCF (25 μM) for 30 min at 37°C.

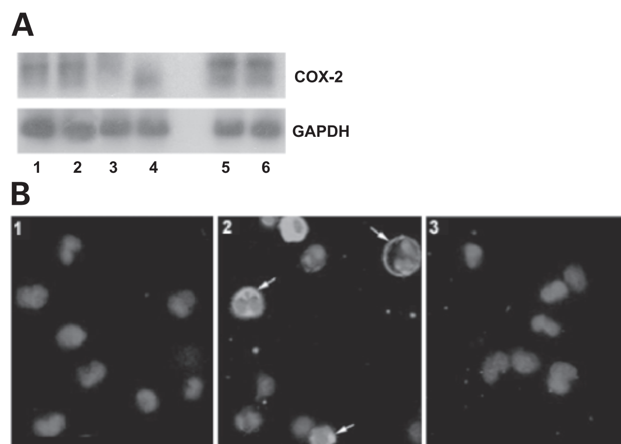

Figure 2. **A,** RT-PCR analysis of COX-2 expression with time. *Lane 1,* control; *lane 2,* 4 h; *lane 3,* 8 h; *lane 4,* 16 h treatment with phycocyanin. *Lane 5,* Bcl-2 cells control; *lane 6,* Bcl-2 cells treated with phycocyanin for 8 h. *Lower panel,* GAPDH control. **B,** Annexin-V staining of the cells analyzed by confocal microscopy. *1,* control BC-8 cells; *2,* BC-8 treated with phycocyanin for 24 h; *3,* Bcl-2 transfectants after treatment with phycocyanin.

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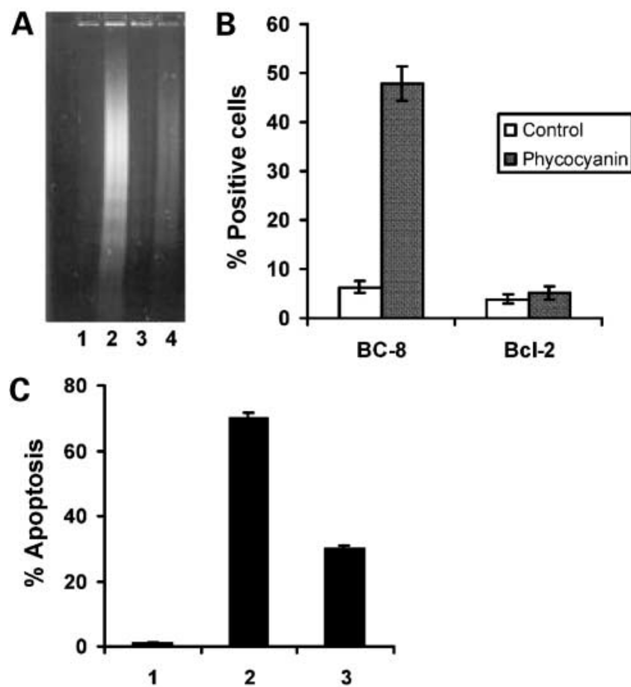

Figure 3. **A,** phycocyanin-induced DNA fragmentation. *Lane 1,* BC-8 control; *lane 2,* BC-8 + phycocyanin; *lane 3,* Bcl-2 control; *lane 4,* Bcl-2 + phycocyanin. Phycocyanin-treated BC-8 cells showed typical DNA ladder. The results shown are representative of three similar experiments. **B,** flow cytometric analysis of caspase-3-positive cells after treatment with phycocyanin for 24 h. The data shown are representative of three similar experiments. The difference between BC-8 control and phycocyanin-treated cells is highly significant ($P < 0.001$). **C,** inhibition of phycocyanin-induced apoptosis by Z-VAD. *1,* control BC-8 cells; *2,* BC-8 + phycocyanin; *3,* BC-8 + phycocyanin + Z-VAD (50 μM). The results shown are representative of three similar experiments. The differences between *1* and *2* and *2* and *3* are significant ($P < 0.001$).

Subsequently, the cells were washed, resuspended in culture medium and incubated with phycocyanin, and analyzed on a spectrofluorimeter at the excitation and emission wavelengths of 480 and 530 nm, respectively.

Caspase Activity

Caspase activity was detected by immunostaining with a specific antibody against active caspase (R&D Systems, Minneapolis, MN). Cells were incubated with phycocyanin for 18 h, washed, fixed in 70% ethanol, and stained with anti-caspase-3 antibody for 1 h. Cells were washed and treated with FITC-conjugated secondary antibody. Washed cells were analyzed by flow cytometry (17).

RT-PCR Analysis

Total cellular RNA was isolated with Trizol Reagent (Life Technologies, Inc.). Single-stranded cDNA was prepared in a 20-μl reaction volume using 500 ng of oligo(dT)$_{12-18}$ primer and 15 units of AMV reverse transcriptase (Promega, Madison, WI) and 2 μg of RNA. Fifty-microliter PCR reactions containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9.2), 200 mM of each deoxynucleotide triphosphate (dNTP), 0.1% Triton X-100, 200 nM of each primer, 1.25 units of Taq polymerase, and 2 μl of cDNA solution were set up. For each of the molecules studied, specific primers were used along with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. The PCR products were run on agarose gel, transferred to nylon membrane, and the blots were hybridized with specific cDNA probes. The relative density of the spots was normalized with corresponding GAPDH spots.

Western Analysis

Samples were electrophoresed on a 10% SDS-polyacrylamide gel for 3 h at 30 mA. Separated proteins were transferred to a nitrocellulose membrane (Hybond C; Amersham Corp., Buckinghamshire, UK) and probed with an appropriate dilution of the primary antibody for Bcl-2. The immune complex was detected using alkaline phosphatase-conjugated secondary antibody using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT).

Results

Phycocyanin-Induced Apoptosis in Tumor Cells

C-phycocyanin, which has been shown to be a selective COX-2 inhibitor, induced dose-dependent apoptosis in BC-8 cells with a concomitant decrease in cell viability as assessed by flow cytometry (Fig. 1A). However, BC-8 cells transfected with Bcl-2 gene were resistant to phycocyanin-mediated apoptosis (Fig. 1C4). These observations demonstrate phycocyanin-induced apoptotic death in BC-8 cells and its inhibition by Bcl-2. These observations were further confirmed by Annexin-V staining of these cells. Fig. 2B2 shows typical Annexin-V staining in BC-8 cells after treatment with phycocyanin, which was not observed in Bcl-2-transfected cells (Fig. 2B3). Apoptotic death in phycocyanin-treated BC-8 cells was observed in a time-dependent manner. Significant apoptotic cells were obtained after 24 h treatment (Fig. 1B). We have also studied the expression of COX-2 by BC-8 cells using RT-PCR. There was a time-dependent decrease in COX-2 transcripts on phycocyanin treatment; however, Bcl-2-transfected cells remained unaffected (Fig. 2A).

Phycocyanin-Induced DNA Fragmentation and Apoptosis

Phycocyanin-treated cells were also analyzed for DNA fragmentation, which is a typical hallmark of the apoptotic cell death. Phycocyanin induced fragmentation of BC-8 DNA which was not seen in Bcl-2 cells (Fig. 3A).

When caspase-3 activity was assayed after immunostaining, BC-8 cells showed a significant increase in caspase-3-positive cells after phycocyanin treatment, whereas Bcl-2 transfectants did not show a significant difference in caspase-3-positive cells in comparison to control (Fig. 3B). These observations were further confirmed by using pancaspase inhibitor Z-VAD, which inhibited phycocyanin-induced apoptosis in BC-8 cells (Fig. 3C). Thus, our studies demonstrate phycocyanin-induced apoptosis in BC-8 cells, which is mediated through the activation of caspase-3 and possibly other caspases.

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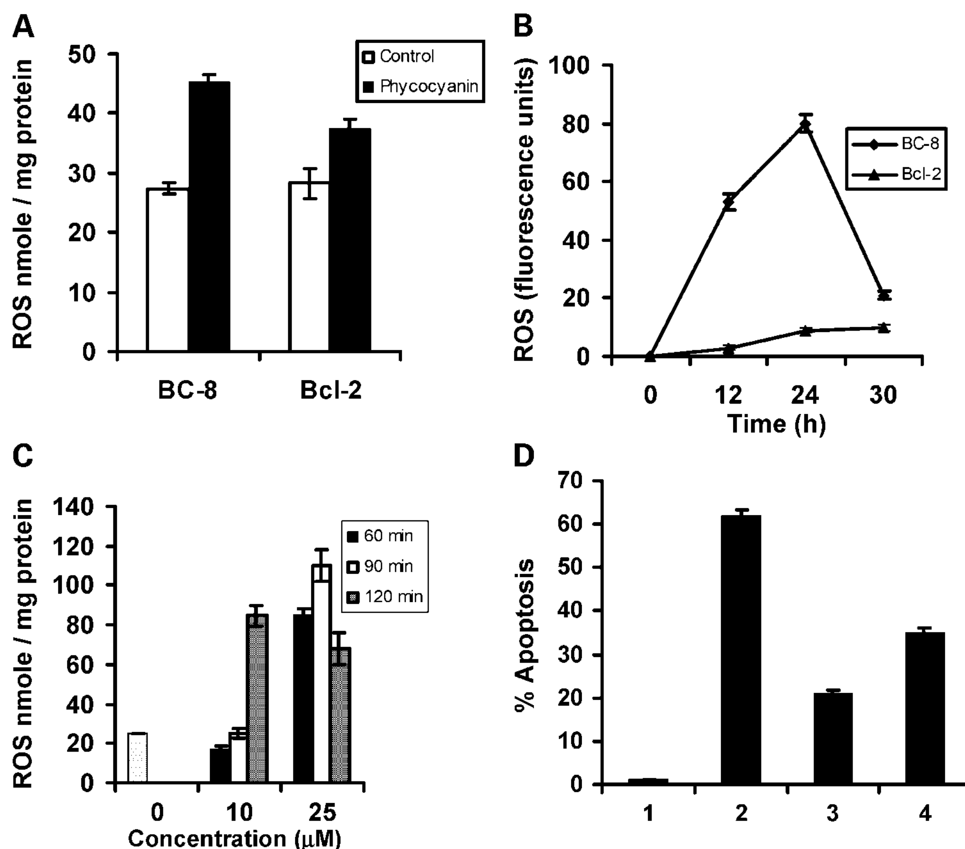

Figure 4. **A,** ROS released into the culture supernatant after treatment of BC-8 cells with phycocyanin. The data shown have been reproduced 3 times and the differences between BC-8 control and phycocyanin treated are significant ($P > 0.005$). **B,** intracellular ROS levels in BC-8 and Bcl-2 cells after treatment with phycocyanin for different time periods. The results shown are representative of three similar experiments. Time zero denotes untreated control cells. **C,** Celecoxib-induced generation of ROS by BC-8 cells at 10 and 25 μM concentrations. Cells were treated with Celecoxib for different time points. These experiments were repeated twice. **D,** effect of free radical scavengers on phycocyanin-induced apoptosis in BC-8 cells. *1,* control; *2,* BC-8 + phycocyanin; *3,* BC-8 + phycocyanin + NAc (1 mM); *4,* BC-8 + phycocyanin + GSH (2 mM). These experiments were repeated thrice and the differences with controls are significant ($P < 0.005$).

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Phycocyanin-Induced Apoptosis Is Mediated through ROS

To understand the mechanisms involved in phycocyanin-induced apoptosis, we studied the generation of ROS by these cells. Phycocyanin induced generation of significantly higher levels of ROS in BC-8 cells as compared to control (Fig. 4A). Extracellular ROS levels were not significantly different in Bcl-2-transfected cells after phycocyanin treatment (Fig. 4A). However, there was a significant difference in the intracellular levels of ROS after phycocyanin treatment between BC-8- and Bcl-2-transfected cells after 24 h (Fig. 4B). These observations were confirmed using Celecoxib, which is a well-known COX-2 inhibitor and is also known to induce apoptosis in tumor cells. Celecoxib when tested at two different concentrations induced significant levels of ROS at different time points (Fig. 4C) and also induced apoptosis in BC-8 cells (data not shown). These observations confirm ROS generation by cells treated with COX-2 inhibitors. To understand the role of ROS in the induction of apoptosis, we treated the cells with scavengers of free radicals. Treatment of BC-8 cells with either *N*-acetyl cysteine (NAc) or reduced glutathione (GSH) inhibited phycocyanin-induced apoptosis significantly (Fig. 4D).

Phycocyanin-Induced Down-Regulation of Bcl-2

Bcl-2 is a known inhibitor of apoptotic process. On treatment of BC-8 cells with phycocyanin, we observed down-regulation of Bcl-2 expression (Fig. 5A). When the expression of Bcl-2 protein was evaluated by Western analysis, there was total absence of Bcl-2 protein after treatment of BC-8 cells with phycocyanin for 24 h (Fig. 5B), thereby confirming time-dependent down-regulation of Bcl-2 expression by phycocyanin. These observations also

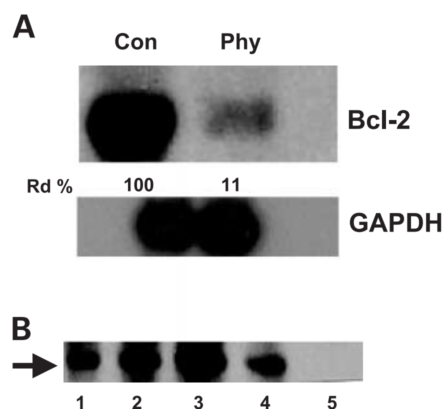

Figure 5. **A,** RT-PCR analysis for the expression of Bcl-2 by BC-8 cells after treatment with phycocyanin for 18 h. Blots were hybridized with specific cDNA probes. *Rd* denotes percent relative density of the spots with respect to control. GAPDH control is shown as loading control. The results shown are representative of three similar experiments. **B,** Western analysis of the Bcl-2 protein expression. BC-8 cells were treated with phycocyanin for different time points and 10^5 cells were loaded in each lane after boiling in the sample buffer. *Lane 1,* BC-8 control; *lanes 2–5,* treatment with phycocyanin for 4, 8, 16, and 24 h, respectively.

correlate with our apoptosis data, where phycocyanin induced apoptosis in BC-8 cells only after 24 h of treatment. Bcl-2 protein is seen up to 16 h of phycocyanin treatment (Fig. 5B).

Discussion

Enough evidence has accumulated suggesting that the inducible form of COX-2, which is a central enzyme in prostaglandin biosynthesis, plays a key role in tumor development. COX-2 has been shown to play a significant role in promoting tumor growth in various organ systems and is overexpressed in human colorectal (18), breast (19), lung (20), and prostrate tumors (21), as well as in several rodent tumor models (19–21). COX-2 expression inhibited DR-5 expression and conferred resistance to TRAIL-induced apoptosis in human colon cancer cells (22). On the other hand, use of COX-2 inhibitors imparted protection against breast and colon cancers (22, 23). These inhibitors also controlled metastatic disease in a murine breast cancer model (24). Celecoxib which is a specific COX-2 inhibitor possessed antiproliferative activity as well as induced apoptosis in tumor cells (23).

C-phycocyanin, which is a major biliprotein of *S. platensis*, has also been shown to be a specific COX-2 inhibitor (10). Our interest was to study the effect of phycocyanin on rat histiocytic tumor cells. Our studies demonstrate that phycocyanin induced apoptosis in BC-8 cells with typical apoptotic features like DNA fragmentation and Annexin-V staining. It was interesting to study the mechanism of phycocyanin-induced apoptosis in BC-8 cells. Treatment of tumor cells with phycocyanin induced the generation of free radicals, which are known to induce macromolecular damage leading to the death of the cells by apoptosis (25, 26). Many inducers of apoptosis caused intracellular oxidation which could be checked by the overexpression of Bcl-2. Bcl-2 has been shown to reduce ROS generation, thereby inhibiting apoptotic induction by different stimuli (27). Bcl-2 has also been shown to promote sequestration of glutathione into the nucleus, leading to the alteration of nuclear redox and blockade of caspase activity (28).

In our studies, we also observed inability of phycocyanin to induce apoptosis in Bcl-2 transfectants which correlated with a significant decrease in ROS production in these cells (Fig. 4B). Bcl-2 family of proteins appears to control cell death by regulating mitochondrial physiology (29). The precise mechanism by which Bcl-2 modulates cell death is not clearly understood but its antiapoptotic functions are attributed to the reduction of ROS (30), effect on the mitochondrial protein flux, and modulation of mitochondrial calcium homeostasis (31). Similarly, inhibition of phycocyanin-mediated apoptosis in the presence of Z-VAD indicates the role of caspases in apoptotic death of BC-8 cells.

Phycocyanin down-regulated the expression of Bcl-2 in BC-8, thereby making them vulnerable to apoptotic death. Since many of the tumors have been shown to be resistant to apoptosis, which is attributed to the expression of Bcl-2, therefore, treatment with phycocyanin, which leads to down-regulation of Bcl-2, may make them sensitive to other antineoplastic drugs that kill tumor cells through apoptosis.

In conclusion, our studies show that a naturally occurring biliprotein phycocyanin is able to induce apoptosis in tumor cells through the production of ROS, which is suppressed by Bcl-2. Phycocyanin also down-regulated the expression of Bcl-2, a well-known antiapoptotic molecule. Like other COX-2 inhibitors, phycocyanin being a natural product, should be more acceptable as an anticancer compound.

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