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Molecular mechanisms in C-Phycocyanin induced apoptosis in human chronic myeloid leukemia cell line-K562

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Abstract

C-Phycocyanin (C-PC), the major light harvesting biliprotein from *Spirulina platensis* is of greater importance because of its various biological and pharmacological properties. It is a water soluble, non-toxic fluorescent protein pigment with potent anti-oxidant, anti-inflammatory and anti-cancer properties. In the present study the effect of highly purified C-PC was tested on growth and multiplication of human chronic myeloid leukemia cell line (K562). The results indicate significant decrease (49%) in the proliferation of K562 cells treated with 50 μM C-PC up to 48 h. Further studies involving fluorescence and electron microscope revealed characteristic apoptotic features like cell shrinkage, membrane blebbing and nuclear condensation. Agarose electrophoresis of genomic DNA of cells treated with C-PC showed fragmentation pattern typical for apoptotic cells. Flow cytometric analysis of cells treated with 25 and 50 μM C-PC for 48 h showed 14.11 and 20.93% cells in sub-G0/G1 phase, respectively. C-PC treatment of K562 cells also resulted in release of cytochrome *c* into the cytosol and poly(ADP) ribose polymerase (PARP) cleavage. These studies also showed down regulation of anti-apoptotic Bcl-2 but without any changes in pro-apoptotic Bax and thereby tilting the Bcl-2/Bax ratio towards apoptosis. These effects of C-PC appear to be mediated through entry of C-PC into the cytosol by an unknown mechanism. The present study thus demonstrates that C-PC induces apoptosis in K562 cells by cytochrome *c* release from mitochondria into the cytosol, PARP cleavage and down regulation of Bcl-2.

Keywords: C-PC; K562 human chronic myeloid leukemia cell line; Apoptosis; PARP; Cytochrome *c*; Bcl-2

1. Introduction

Chemoprevention is an effective way to reduce cancer risk. Natural products have been the mainstay of cancer chemotherapy for the past 30 years. Blue-green algae are the most primitive life forms on earth with nutrient-dense, edible forms like Nostoc, Spirulina, Aphanizomenon species, etc. Spirulina is non-nitrogen fixing blue-green algae with over 30 years long history of safe human consumption. Spirulina is gaining attention as a nutraceutical and source of potential pharmaceuticals. Recent studies have demonstrated antioxidant [1], antimutagenic [2], antiviral [3], anticancer [4,5], anti-allergic [6], immune enhancing [7], hepato-protective [8], blood vessel relaxing [9] and blood lipid-lowering effects [10] of Spirulina extracts. The biological and pharmacological properties of Spirulina were attributed mainly to calcium-spirulan and C-Phycocyanin (C-PC).

C-PC, a water-soluble non-toxic biliprotein pigment isolated from *Spirulina platensis* has significant antioxidant and radical scavenging properties [11]. Phycocyanin was shown to inhibit inflammation in mouse ears [12] and prevent acetic acid induced colitis in rats [13]. C-PC is used for the treatment of diseases such as Alzheimer's and Parkinson's [14] and prevents experimental oral and skin cancers [15]. Of major interest to ongoing research in inflammation as well as cancer is the finding that C-PC selectively inhibits cyclooxygenase-2 [16]. Recently we have reported that C-PC induces apoptosis in mouse macrophage cell line RAW 264.7 stimulated with LPS [17] and rat histiocytoma cell line AK5 [18]. C-PC caused a dose-dependent decrease in the levels of PGE_2 in LPS stimulated macrophage cell line [17]. This decrease in

Abbreviations: C-PC, C-Phycocyanin; CML, chronic myeloid leukemia; COX, cyclooxygenase; FACS, fluorescence activated cell sorter

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PGE_2 with no change in COX-1 and COX-2 protein levels could be due to inhibition of COX-2 activity by C-PC [16].

There is an expanding body of information on the potential applications of nonsteroidal anti-inflammatory drugs (NSAIDs) in cancer chemoprevention [19,20]. Epidemiological studies indicate that the use of aspirin and other NSAIDs reduces the risk of cancer by 40–50% [21]. Selective inhibitors of COX-2 have been demonstrated to induce apoptosis in a variety of cancer cells, including those of colon [22], stomach [23], prostate and breast [24]. These observations are consistent with the cancer chemopreventive effects of NSAIDs. However, the biochemical mechanism underlying COX-2 inhibitor induced apoptosis remains elusive. Tumor inhibition by NSAIDs may be mediated by distinct cellular processes. These processes involve the ability of NSAIDs to restore apoptosis, induce cell cycle arrest, and inhibit angiogenesis [25,26]. One of the main ways by which NSAIDs exert their effects is through modulation of apoptosis, although there is considerable debate about how these effects are mediated.

Since C-PC has many therapeutic roles including anticancer properties, the present study is undertaken to test the effect of highly purified (>95% pure) C-PC on growth and multiplication of human chronic myeloid leukemia K562 cell line and molecular mechanisms involved in C-PC induced cellular effects.

2. Materials and methods

2.1. Chemicals

Phosphate buffered saline (PBS), RPMI medium, fetal bovine serum (FBS) were purchased from Gibco Ltd. DEAE-cellulose, poly-L-lysine, glutaraldehyde, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), DAPI (4,6-diamidino-2-phenylindole), proteinase K, RNase A, propidium iodide were from Sigma Chemical Co. Nitrocellulose membranes and the enhanced chemiluminescence (ECL) kit were from Amersham Life Science. Mouse monoclonal antibodies against cytochrome *c* and Bax were from Santa Cruz Biotechnology. Polyclonal antibodies of Bcl-2 and PARP were from R&D systems. C-PC was a generous gift from Green India Natural Products Ltd.

2.2. Cell culture and treatment

The human chronic myeloid leukemia K562 cells were grown in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. The cultured cells were passed twice each week, seeding at a density of about 2×10^5 cells/ml. Cell viability was determined by the trypan blue dye exclusion method. A stock solution of 10^{-3} M C-PC was prepared in PBS freshly for each experiment.

2.3. Cell proliferation assay

Cell proliferation was determined using the MTT assay [27]. K562 cells (5×10^3 cells/well) were incubated in 96-well plates in the presence or absence of C-PC (10, 25, 50, 100 μM) for 24, 48, 72 and 96 h in a final volume of 100 μl. After treatment 20 μl of MTT (5 mg/ml in PBS) was added to each well and incubated for an additional 4 h at 37 °C. The purple-blue MTT formazan precipitate was dissolved in 100 μl of DMSO and the absorbance values at 570 nm were determined on a multi-well plate reader (μ Quant Biotek Instruments).

2.4. Nuclear staining assay

Cells (1×10^5) exposed to C-PC (25 and 50 μM) for 48 h, were harvested, washed with ice-cold PBS and fixed in a solution of methanol: acetic acid (3:1) for 30 min. Fixed cells were placed on slides and stained with 1 μg/ml DAPI for 15 min. Nuclear morphology of the cells was observed by fluorescence microscopy.

2.5. Scanning electron microscopy (SEM)

After treatment, cells were collected, washed with PBS and concentrated to 1×10^5 cells/ml, one drop of such suspension was placed onto a plastic cover slip previously coated with 1% poly-L-lysine. Cells were fixed with glutaraldehyde for 1 h and post fixed with 1% osmium tetroxide for 1 h. Cells were dehydrated by passing through graded alcohols and dried by the critical-point technique. After trimming, mounting, and coating with gold–platinum the specimens were observed on SEM (JSM-5600, JEOL Co.).

2.6. Analysis of DNA fragmentation

DNA laddering was detected by isolating fragmented DNA using the SDS/proteinase K/RNase A extraction method, which allows the isolation of only fragmented DNA without contaminating genomic DNA [28]. Briefly, cells were washed in cold PBS and lysed in a buffer containing 50 mM Tris–HCl (pH 8.0), 1 mM EDTA, 0.2% Triton X-100 for 20 min at 4 °C. After centrifugation at 14,000 × *g* for 15 min, the supernatant was treated with proteinase K (0.5 mg/ml) and 1% SDS for 1 h at 50 °C. DNA was extracted twice with buffered phenol and precipitated with 140 mM NaCl and 2 vol. of ethanol at −20 °C overnight. DNA precipitates were washed twice in 70% ethanol, dissolved in TE buffer, and treated for 1 h at 37 °C with RNase A. Finally, DNA preparations were electrophoresed in 1% agarose gels, stained with ethidium bromide and visualized under UV light.

2.7. Analysis of cellular DNA content by FACS

Cells were seeded at 3.6×10^4 cells in 6 well culture plates, cultured in 10% FBS with or with out C-PC (25 and 50 μM) for 48 h. After treatment cells were harvested, washed with PBS, and the viability was determined by the trypan blue dye exclusion method. For DNA content analysis, 10^6 cells were fixed in 70% ethanol, washed with PBS, incubated with 0.1 mg/ml RNase A and stained with propidium iodide (final concentration: 50 μg/ml). Flow cytometric analyses were performed using a Becton–Dickinson FACS flow cytometer as previously described [17].

2.8. Detection of cytochrome c release

Release of cytochrome *c* from mitochondria to cytosol was measured by Western blot as previously described [29] with some modifications. Briefly, cells were washed once with ice-cold PBS and gently lysed for 30 s in 80 μl ice-cold lysis buffer (250 mM sucrose, 1 mM EDTA, 0.05% digitonin, 25 mM Tris, pH 6.8, 1 mM dithiothrietol, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM PMSF, 1 mM benzamidine). Lysates were centrifuged at 12,000 × *g* at 4 °C for 5 min to obtain the extracts (cytosolic extracts free of mitochondria). Supernatants were electrophoresed on a 15% polyacrylamide gel and then analyzed by Western blot using anti-cytochrome *c* antibody.

2.9. Preparation of whole cell extracts and immunoblot analysis

To prepare the whole cell extract, cells were washed with PBS and suspended in a lysis buffer (20 mM Tris, 1 mM EDTA, 150 mM NaCl, 1% NP 40, 0.5% deoxycholic acid, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml leupeptin, 20 μg/ml aprotinin). After 30 min of shaking at 4 °C, the mixtures were centrifuged (10,000 × *g*) for 10 min, and the supernatants were collected as the whole-cell extracts. The protein content was determined according to the Bradford method [30]. An equal amount of total cell lysate was resolved on 8–12% SDS–PAGE gels along with protein molecular weight standards, and then transferred onto nitrocellulose membranes. The membranes were blocked with 5% (w/v) nonfat dry milk and then incubated with the primary antibodies (PARP, Bcl-2, Bax) in 10 ml of antibody-diluted buffer (1× Tris-buffered saline and 0.05% Tween with 5% milk) with gentle shaking at 4 °C for 8–12 h and then incubated with peroxidase conjugated secondary antibodies. Signals were detected using an ECL Western blotting kit.

2.10. Immunofluorescent confocal microscopy

The K562 cells, treated with C-PC for 48 h were washed with PBS and fixed in 4% paraformaldehyde, pH 7.4 for 20 min at 4 °C. After fixation cells were washed twice with PBS. The cells were permeabilized for 5 min at room temperature in 0.2% saponin, PBS, 0.03 M sucrose and 1% BSA. After permeabilization cells were washed with PBS and blocked for 60 min in blocking solution containing 0.5% NP-40, 5% normal goat antiserum in PBS. The cells were washed with PBS and incubated with polyclonal C-PC antibody at a dilution of 1:200 (in blocking solution) for 30 min at room temperature. The cells were then washed twice with PBS and once with distilled water, and were observed on Meridain ULPIMA confocal microscope with the use of a mounting medium for fluorescence.

2.11. Statistical analysis

The results were expressed as mean ± S.D. of data obtained from three independent experiments. Statistical analysis of differences was carried out by analysis of variance (ANOVA). The level of significance was set at $P < 0.05$.

3. Results

3.1. Effect of C-PC on K562 cell growth

Cells were cultured in 10% FBS containing medium with or without C-PC (10–100 μM) for 4 days and cell proliferation was evaluated by the MTT assay. Under those experimental conditions a dose dependent decrease in K562 cell proliferation was observed until 48 h after C-PC (10, 25, 50 and 100 μM) treatment (Fig. 1), with maximum decrease in cell proliferation being at 50 μM C-PC where the percent inhibition was 49%. Since the maximum inhibition was observed with 50 μM C-PC, further experiments were carried on cells exposed to 50 μM C-PC. Similar results were obtained when cell growth was monitored by trypan blue dye exclusion method also (data not shown).

3.2. Morphological changes

A distinguishing feature of apoptosis is the condensation and fragmentation of nuclear chromatin, which can be monitored by fluorescence microscope. K562 cells were exposed to various concentrations of C-PC (25 and 50 μM) for 48 h, and then assessed for morphological signs of apoptosis by staining with DAPI. Nuclear condensation and apoptotic bodies, a hallmark of apoptosis, were observed in cells treated with C-PC (Fig. 2B and C). Also the number of apoptotic cells increased with the increasing concentration of C-PC. Chromatin of apoptotic cells was segregated and compacted into sharply delineated masses, very close to the nuclear envelope, as indicated by the arrows. Phase contrast microscopic studies also revealed

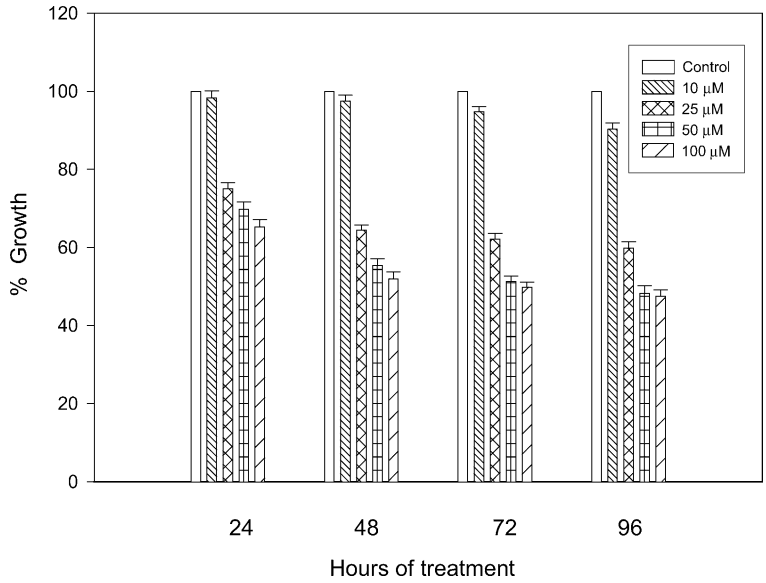

Fig. 1. Effect of C-PC on K562 cell growth. K562 cells were cultured in 10% FBS medium and treated with 10, 25, 50, 100 μM C-PC for 24, 48, 72, 96 h by MTT assay. The percent viable cells were calculated in comparison to untreated cells. The number of cells in the control were taken as 100%.Values were expressed as mean ± S.D. of three independent experiments, each performed in triplicate ($P < 0.05$).

the presence of cells with web like activated membrane structure and decrease in cell number (data not shown).

3.3. Electron microscopic studies

In the light of changes observed under fluorescence and phase contrast microscopes, further studies were undertaken for detailed analysis of morphological changes on SEM. To determine whether the antiproliferative effects of C-PC were associated with apoptosis, we examined the ultrastructural changes of K562 cells treated with 50 μM C-PC for 48 h. Apoptotic cell death was confirmed by scanning electron microscopy, which revealed characteristic ultrastructural features of apoptosis. SEM studies of C-PC treated cells revealed the presence of membrane blebbing, which might be due to a deep cytoskeleton rearrangement, causing progressive changes in cell shape, organelle distribution and cell shrinkage and severing junctions with its neighbors and loss of microvilli (Fig. 3B).

3.4. Internucleosomal DNA fragmentation

In addition to morphological evaluation, apoptosis induction by C-PC was ascertained by using an assay developed to measure DNA fragmentation, a biochemical

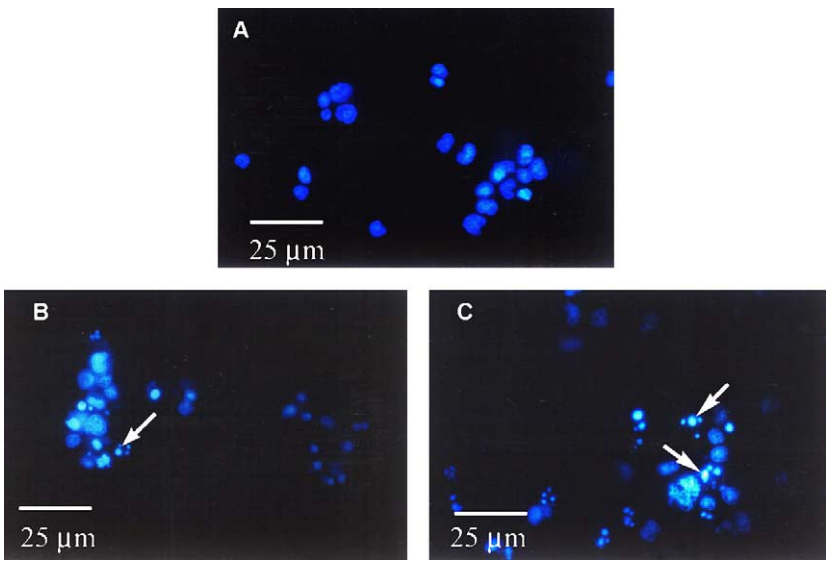

Fig. 2. Nuclear morphology of K562 cells treated with C-PC. Morphological changes of K562 cells treated with 25 and 50 μM C-PC for 48 h were stained with DAPI and observed under Olympus BH2RFC fluorescence microscope (400×). Arrows indicate nuclear condensation and apoptotic bodies. Each experiment was repeated at least three times. (A) Control K562 cells, (B) K562 cells treated with 25 μM C-PC, and (C) K562 cells treated with 50 μM C-PC.

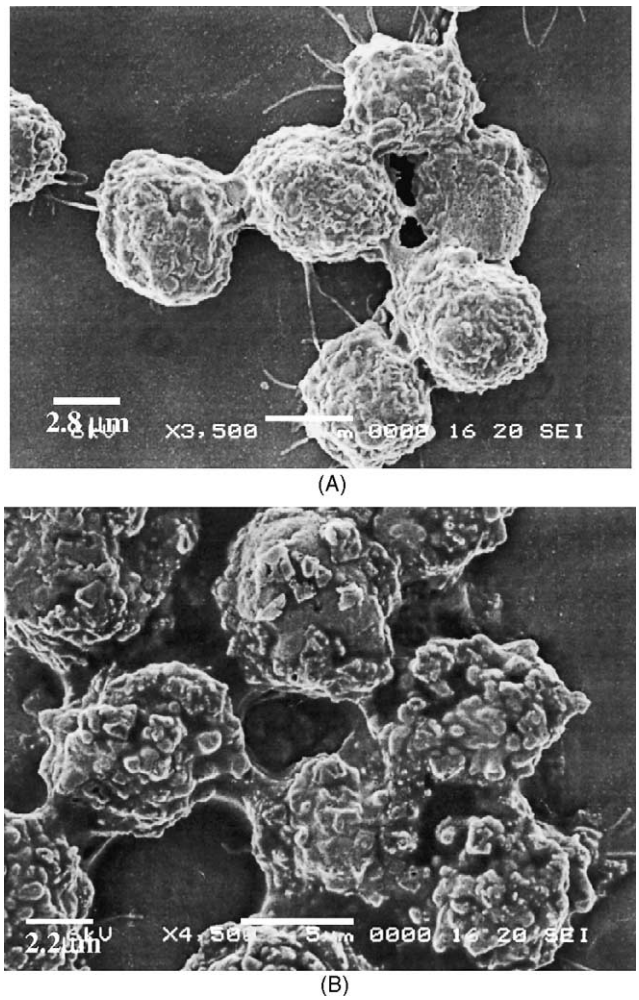

Fig. 3. Ultrastructural morphology in K562 cells treated with C-PC 50 μM for 48 h. (A) Control cells with occasional microvilli (3500×), (B) C-PC treated cells (48 h, 50 μM) showing clumping and shortening of microvilli, cell shrinkage and membrane blebbing, holes and cytoplasmic extrusions (4500×).

hallmark of apoptosis. During later stages of apoptosis internucleosomal cleavage of cellular DNA by endonucleases to 180 bp or oligomers of 180 bp fragments could be detected by extraction of nuclear DNA and agarose gel electrophoresis. As illustrated in Fig. 4, agarose gel electrophoresis of DNA extracted from K562 cells treated with C-PC at concentrations of 10, 25, 50 μM for 48 h revealed a progressive increase in the non-random fragmentation into a ladder of 180–200 bp (lanes 2–4). The degree of nuclear DNA fragmentation was directly proportional to the concentration of C-PC. Such a pattern corresponds to internucleosomal cleavage, reflecting the endonuclease activity characteristic of apoptosis. Control cells did not show any DNA fragmentation (lane 1).

3.5. DNA content assay by fluorescence activated cell sorter (FACS)

The induction of apoptosis in C-PC treated cells was further verified by flow cytometric analysis of DNA content. Loss of DNA is a typical feature of apoptotic cells. In the present study K562 cells treated with C-PC (25 and 50 μM) for 48 h were taken for FACS analysis. Fig. 5 illustartes the DNA content histograms obtained after PI staining of permeabilized cells. In agreement with DNA fragmentation results, a typical sub-diploid apoptotic peaks were observed in K562 cells treated with 25 and 50 μM C-PC for 48 h. The FACS analysis of control cells, on the other hand, showed prominent G1, followed by S and G2/M phases (Fig. 5A). Only 2.97% of these cells showed hypodiploid DNA (sub-G0/G1 peaks). This value of 2.97% hypodiploid DNA in control cells increased to 14.11 and 20.93% in C-PC (25 and 50 μM) treated cells. Flow cytometric analysis of C-PC treated cells showed the increase of hypodiploid apoptotic cells in a concentration-dependent manner and the decrease of the cells at S and G2 phase of cell cycle. This result suggested a possibility that C-PC induced apoptosis occurs at S and G2 phase of the cell cycle.

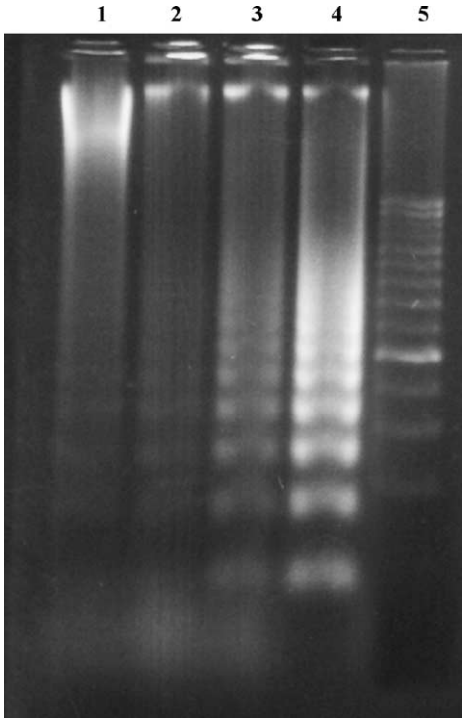

Fig. 4. Agarose gel electrophoresis of DNA extracted from K562 cells treated with C-PC. After treatment cells were lysed and total cellular DNA was extracted and electrophoresed on a 1% agarose gel containing 0.05 mg/ml ethidium bromide at 5 V/cm. The gels were then photographed under UV illumination. Lane 1: K562 control cells; lane 2: K562 cells treated with 10 μM C-PC; lane 3: K562 cells treated with 25 μM C-PC; lane 4: K562 cells treated with 50 μM C-PC; lane 5: 100 bp DNA ladder.

3.6. C-PC treatment evokes cytochrome c release

One of the major apoptotic pathways is activated by the release of apoptogenic protein, cytochrome *c*, from mitochondria into the cytosol. The release of cytochrome *c*, one of the most important respiratory-chain proteins, from the

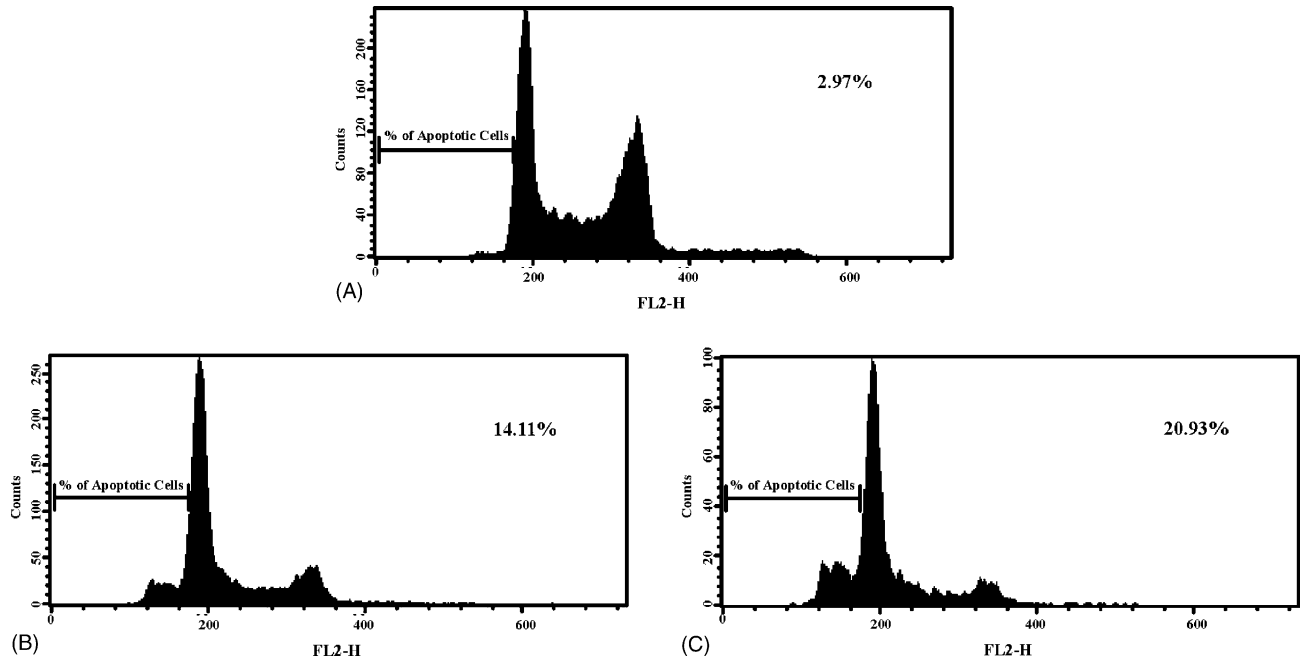

Fig. 5. Flow cytometric analysis of the control and C-PC treated K562 cells. Cells exposed to different concentrations of C-PC for 48 h were fixed, and stained with propidium iodide and the DNA content was quantified by FACS. The number of hypodiploid (sub-G0/G1 phase) cells is expressed as a percentage of the total number of cells. (A) Control cells, (B) C-PC 25 μM, and (C) C-PC 50 μM.

mitochondria into the cytosol is the hallmark of cells undergoing apoptosis [31,32]. To specify the molecular basis of apoptosis the release of cytochrome *c* into the cytosol was measured in K562 cells treated with C-PC, by Western blot analysis employing mouse monoclonal cytochrome *c* antibodies. As shown in Fig. 6 in untreated cells cytochrome *c* (lane 1) was not detectable in the cytoplasm, whereas the levels of cytosolic cytochrome *c* significantly increased after C-PC treatment. As depicted in the figure, the levels of cytochrome *c* in the cytosol were elevated within 6 h after treatment with C-PC (lanes 2 and 3) and the levels were further increased by 12 h (lanes 4 and 5) with later stabilization at 24 h (lanes 6 and 7).

3.7. PARP cleavage in response to C-PC treatment

PARP, poly(ADP-ribose) polymerase, has been implicated in many cellular processes including apoptosis and DNA repair. PARP is primarily found in the nucleus and is activated by DNA strand breaks. PARP is a 116-kDa protein, which converts nicotinamide adenine dinucleotide (NAD) to nicotinamide and protein-linked ADP-ribose polymers. The DNA repair enzyme, PARP has been recognized as a representative death substrate that is cleaved and inactivated by down-stream caspases. In response to growth factor withdrawal or on exposure to a variety of chemotherapeutic compounds, PARP is cleaved to generate 85 and 23 kDa fragments [33]. To determine whether PARP is cleaved in C-PC induced cell death, we treated K562 cells with 25 and 50 μM C-PC for 48 h and PARP cleavage was monitored with PARP antibodies, which specifically recognizes the 23-kDa fragment of the cleaved PARP and uncleaved 116 kDa PARP. Fig. 7 illustrates the gradual increase in the proportion of the $M_r = 23{,}000$ cleavage product and decrease in the proportion of 116 kDa uncleaved PARP with increasing concentrations of C-PC (lanes 2 and 3). In the control cells, however, no fragment of PARP was observed, except the uncleaved 116 kDa protein (lane 1).

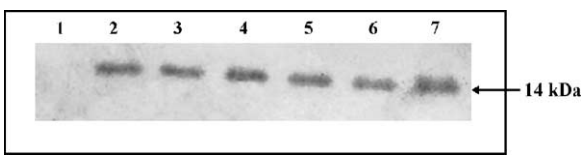

Fig. 6. Effect of C-PC on cytochrome *c* release. Equal amounts of protein (50 μg) from the K562 cells treated with 25 and 50 μM C-PC for the indicated times (0, 6, 12, 24 h) were analyzed by 15% SDS–PAGE, and after electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with mouse monoclonal anti-cytochrome *c* antibodies. Lane 1: 0 h; lane 2: C-PC 25 μM, 6 h; lane 3: C-PC 50 μM, 6 h; lane 4: C-PC 25 μM, 12 h; lane 5: C-PC 50 μM, 12 h; lane 6: C-PC 25 μM, 24 h; lane 7: C-PC 50 μM, 24 h.

3.8. Bcl-2/Bax ratio modulation

Different proteins of the Bcl-2 family have been implicated in triggering or preventing apoptosis. Bax and Bcl-2 are the proteins associated with the mitochondrial membrane and their ratio is crucial for cell survival. In light of the recent reports that attributed COX-2 inhibitor-induced apoptosis to Bcl-2 downregulation [25,34], studies were undertaken to test whether Bcl-2 expression is affected

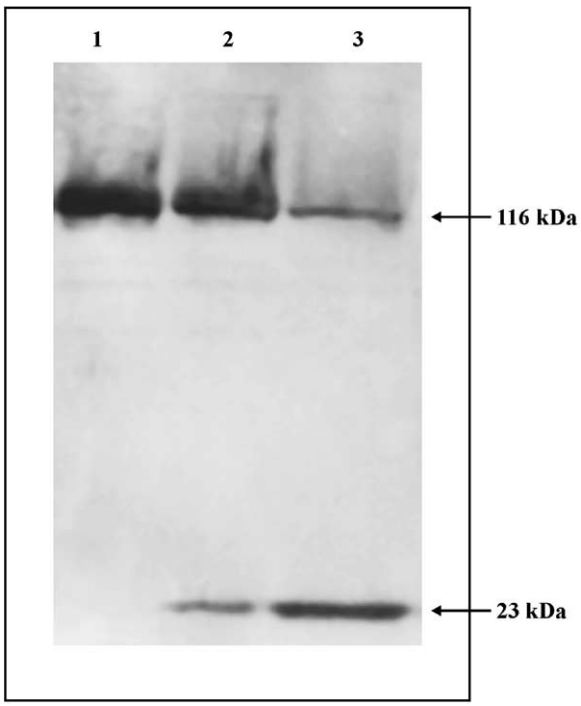

Fig. 7. Western blot analysis showing PARP cleavage in K562 cells treated with C-PC. Whole cell extracts from K562 cells treated with or without C-PC for 48 h, were fractionated on 12% SDS–PAGE, transferred to nitrocellulose membrane and subsequently probed with anti-PARP antibodies. This antibody recognizes both uncleaved PARP (116 kDa) and the 23 kDa cleaved PARP. Lane 1: control cells; lane 2: cells treated with 25 μM C-PC; lane 3: cells treated with 50 μM C-PC.

after C-PC treatment in K562 cells. Changes in the expression of cellular anti-apoptotic proteins, Bcl-2 and of the pro-apoptotic protein Bax, following C-PC treatment (25 and 50 μM) for 48 h were examined by Western blotting. As shown in Fig. 8A untreated cells (lane 1) expressed high levels of Bcl-2 protein, which in C-PC treated cells is down regulated (lanes 2 and 3). Bax protein levels (Fig. 8B), however, were not altered on C-PC treatment. As a result of decreased Bcl-2 with no change in Bax, the ratio of Bcl-2/Bax reduced significantly during C-PC treatment.

3.9. Immunolocalization

The subcellular distribution of C-PC was determined in K562 cells using laser scanning confocal microscopy. Polyclonal antibodies of C-PC were used to determine its in situ localization in C-PC treated cells. This was done by using fluorescence labeled anti-rabbit antisera and monitoring the cells exposed to anti-Phycocyanin antiserum on confocal microscopy. These studies showed a strong immunofluorescence signal in the cytosol with no signal in the nuclear compartment (Fig. 9B) of C-PC treated cells.

4. Discussion

Chemoprevention, the use of drugs or natural substances to inhibit carcinogenesis, is an important and rapidly

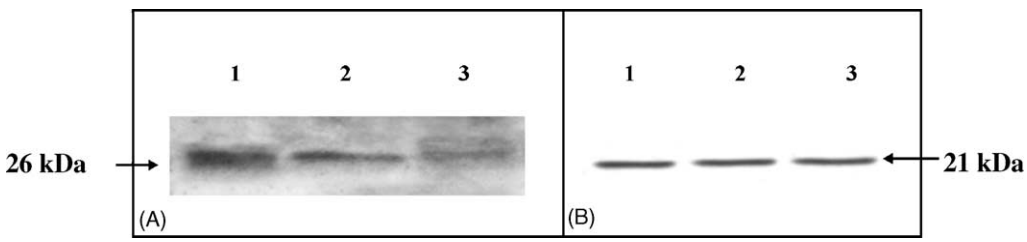

Fig. 8. Western blot analysis of Bcl-2 and Bax of K562 cells treated with or without C-PC. Protein (50 μg) from cell lysates were electrophoresed in SDS–PAGE gels, transferred to nitrocellulose membrane, and probed with anti-Bcl-2/anti-Bax antibodies as described in materials and methods. Lane 1: control; lane 2: C-PC 25 μM; lane 3: C-PC 50 μM.

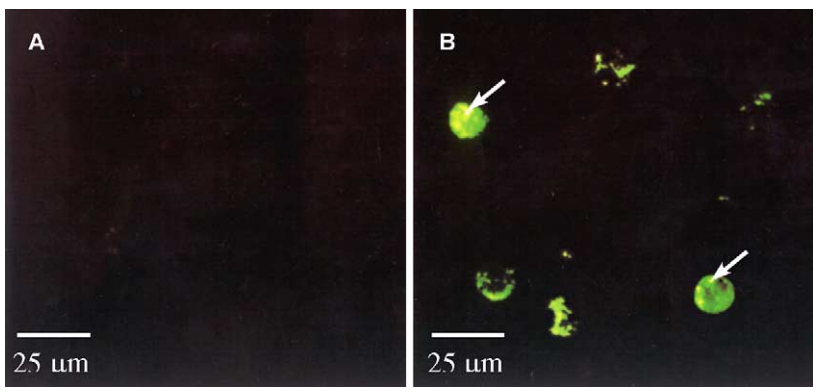

Fig. 9. Confocal image showing the localization of C-PC in K562 cells fixed in paraformaldehyde using C-PC polyclonal antibodies. After treatment cells were permeabilized, washed with PBS, incubated with rabbit polyclonal anti-C-PC antibodies and then incubated with anti-rabbit FITC conjugated secondary antibody. Cells were washed and were observed under Meridian ULPIMA laser scanning confocal microscope with the use of mounting medium for fluorescence. (A) Control K562 cells and (B) K562 cells treated with C-PC 50 μM.

evolving subject of cancer research. There has recently been a surge of interest in marine bioresources, particularly seaweeds, as sources of bioactive substances. Several preparations of seaweeds such as polysaccharride, peptide and phycobiliproteins were shown to affect the multiplication of tumor cells [5,35,36]. Aqueous extracts of green, brown and red algae were shown to possess bioactivity against murine immunocytes [37]. C-PC from *S. platensis* was shown to reduce the viability of mouse myeloma cells after irradiation by 300 J/cm at 514 nm for 3 days [15].

C-PC is one of the major water-soluble biliprotein present in *S. platensis*. This water-soluble protein pigment is gaining a lot of importance these days because of its various biological and pharmacological properties. C-PC is extensively used as a food colorant and in cosmetics because of its blue color and its strong fluorescence in the visible region. It is also non-carcinogenic. However, most of its pharmacological properties are not known except a few. Morcos et al. [15,38] have shown its photodynamic properties and its use in cancer treatment. They have shown that, C-PC specifically binds to cancer cells, and thus can be used for anatomical imaging of tumors in vivo [38]. Recently, its hepatoprotective [39], anti-oxidant [40], radical scavenging [11] and anti-inflammatory properties have been demonstrated [41]. Earlier studies from our laboratory revealed that C-PC is a selective inhibitor of COX-2 [16] and induces apoptosis in mouse macrophage cell line, RAW 264.7 stimulated with LPS [17] and in rat histiocytoma cell line, AK5 [18].

In an effort to gain insight into effects of C-PC and to understand the biochemical mechanism underlying C-PC induced apoptosis, in the present study we have evaluated the effects of C-PC on the proliferation and apoptosis of chronic myeloid leukemia cells (K562 cells). The effects of C-PC on the viability of K562 cells were evaluated after 24, 48, 72, 96 h in culture. These studies have clearly shown the inhibition in the growth of K562 cells in a dose and time dependent manner. A dose dependent decrease in K562 cell proliferation was observed until 48 h after C-PC treatment (Fig. 1) with maximum decrease in cell proliferation being at 50 μM where the percent inhibition was 49%. This reduction in the growth of K562 cells in the presence of C-PC could be due to either apoptosis or necrosis. In order to test the factors responsible for reduced growth of K562 cells, further studies were undertaken on the characteristic markers of apoptosis.

Recent accumulating evidence suggests that defects in the process of apoptosis may be closely associated with carcinogenesis and that many cancer cells have defective machinery for self-destruction [42]. It is suggested that the susceptibility to apoptosis-inducing effects of chemotherapeutic drugs may depend on the intrinsic ability of tumor cells to respond to apoptosis [42,43]. It has been reported that sulindac sulfide can induce apoptosis in promyelocytic leukemia cell line HL-60, which suggests that nonsteroidal anti-inflammatory drugs (NSAIDs) have anti-leukemic effect [44]. Recently, it has been shown that aspirin and salicylate induce apoptosis of B-CLL cells [45]. The present study demonstrates the induction of apoptosis in K562 cells treated with C-PC. Apoptosis is a specific mode of cell death recognized by a characteristic pattern of morphological, biochemical, and molecular changes. C-PC treated cells showed pronounced morphological changes like cell shrinkage, formation of membrane blebs, and micronuclei characteristic of apoptosis as evidenced by fluorescence and electron microscopic studies. A ladder-like DNA fragmentation pattern, a biochemical marker of apoptosis (cleavage of DNA into nucleosomal size fragments of 180–200 bp) was observed in K562 cells treated with C-PC. Flow cytometric analysis of treated cells showed the increase of hypodiploid apoptotic cells in a concentration-dependent manner and the decrease of the cells at S and G2 phase of cell cycle. This result suggested a possibility that C-PC induced apoptosis occurs at S and G2 phase of the cell cycle. This result is similar to that found by Hanif et al. [46] in the study of colon cancer cells for NSAIDs.

In mitochondria, cytochrome *c* is required as an electron carrier in oxidative phosphorylation, a process which generates the majority of intracellular ATP [47]. Several apoptosis inducing agents are known to trigger mitochondrial uncoupling leading to the rupture of outer membrane. This in turn causes the release of pro-apoptotic factors such as apoptosis inducing factor (AIF), cytochrome *c* and the apoptosis protease-activating factor (Apaf-1) into the cytosol. In cytoplasm, cytochrome *c* is known to become associated with caspase-9, Apaf-1 and dATP to form the apoptosome complex [48], which in turn activates caspase-9, -3 and -7. Caspase activation results in the cleavage of cellular substrates and eventually leading to apoptosis. In the present study, the release of cytochrome *c* into the cytosol in response to C-PC treatment is examined by employing Western blot analysis. These studies have shown the release of cytochrome *c* in response to the treatment of C-PC, as early as 6 h, with later increase up to 24 h. In this report, we show that the release of cytochrome *c* from mitochondria to cytosol is an early event in the apoptotic process, preceding morphological signs of apoptosis. Similar release of cytochrome *c* into cytosol is reported in RAW 264.7 cells treated with C-PC [17]. NS-398, the selective inhibitor of COX-2, was also shown to induce apoptosis in colon cancer cells by cytochrome *c* dependent pathway [49].

The present study provides biochemical evidence for cellular damage in the form of activation of potential substrates for an ICE/CED-3-like protease during apoptosis called, PARP. Activation of caspases leads to cell demise [50] via cleavage of cellular substrates, such as actin [51], fodrin [52], PARP [53] and gelsolin [54]. By processes that are not altogether clear, poly(ADP) ribosylation of variety of proteins facilitates DNA repair. Activation of PARP by DNA damage depletes energy stores and

thus may prevent apoptosis. PARP cleavage, on the other hand, seems to be important to preserve the energetic substrates for apoptotic events. In the present study, C-PC accelerated the cleavage of PARP leading to the formation of a 23 kDa product. This cleavage of PARP might then preclude the catalytic domains of PARP being recruited to the sites of DNA damage, and presumably disable PARP from coordinating subsequent repair and maintain genome integrity. Also PARP is known to negatively regulate the Ca^{2+} and Mg^{2+} dependent endonucleases [55]. Since C-PC is promoting the PARP cleavage in K562 cells, it may result in activation of Ca^{2+} and Mg^{2+} dependent endonucleases, which would eventually cleave DNA into oligonucleosomal fragments. Caspase-3 cleavage product was not detected in the present study (data not shown), although PARP cleavage was observed. It is possible that other caspases may be participating in C-PC induced apoptosis.

Bcl-2 belongs to a growing family of proteins, which can either inhibit (Bcl-2, Bcl-X_L, Bcl-2w, Mcl-1, Bfl-1, A1, etc.) or favor (Bax, Bcl-X_S, Bad, Bak, Bik, etc.) apoptosis, which in cells reside predominantly in the outer mitochondrial membrane, endoplasmic reticulum, and the outer nuclear envelope [56,57]. The capacity of Bax and Bcl-2 to compete with one another via heterodimers suggests a reciprocal relationship in which Bcl-2 monomers or homodimers favor survival and Bax homodimers favor cell death. In this study, we found that both Bcl-2 and Bax were expressed in K562 cells. At 50 μM concentration, C-PC potently down regulated Bcl-2 protein levels without any change in the protein levels of Bax. The resulting net effect could thus lead to a lowered ratio of Bcl-2/Bax, which might be responsible for C-PC induced apoptosis in K562 cells. Immunological studies employing C-PC polyclonal antibodies and fluorescent in situ hybridization on confocal microscope suggest that C-PC enters into the K562 cells and is concentrated in the cytosol. However, it is not clear whether this is mediated by any cell surface receptors or by other mechanisms. It was demonstrated that C-PC is specifically taken up by actively proliferating cancer cells [38], suggesting such a possibility in these K562 cells. Further studies, however, should be undertaken to unravel the mode of entry of C-PC into the cell.

In summary, this work presents evidence that C-PC induces apoptosis in human chronic myeloid leukemia cells. C-PC induced ultrastructural changes such as cell shrinkage, formation of membrane blebs, and micronuclei are some of the characteristics of cells undergoing apoptosis. This induction of apoptosis in K562 cells by C-PC appears to be mediated by cytochrome *c* release, PARP cleavage, Bcl-2 down regulation. Since C-PC being a natural pigment and a component of edible Spirulina extracts, it forms a good alternative to highly toxic chemotherapeutic products in the market or might be used in combination therapy. Also it can form a good candidate for enhancing the sensitivity of cancer cells to conventional anticancer drugs and thus ultimately reducing their toxic side effects.

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