Anti-proliferative effects of C-phycocyanin on a human leukemic cell line and induction of apoptosis via the PI3K/AKT pathway

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ABSTRACT

C-phycocyanin (c-PC) is a biliprotein found in edible blue-green algae. Its anti-cancer activity has been evidenced by many in vitro and in vivo studies. Here, the effects of c-PC on cytotoxicity and cell signaling through the apoptotic pathway were studied in a human erythromyeloid leukemia cell line (K562). Cell viability after incubation with various concentrations of c-PC was determined, and the signal transduction of the BCR-ABL fusion protein via the downstream targeting pathway, PI3K/AKT, was observed. Western blot analysis was performed to examine the levels of BCR-ABL, PI3K, AKT, and their phosphorylated derivatives in K562 cells during treatment with 100 µM c-PC. The results showed that c-PC inhibits K562 cell growth in a dose-dependent manner with the half maximal inhibitory concentration (IC₅₀) = 128.6 µM. We found that c-PC exhibits inhibitory effects on protein phosphorylation in K562 cells. Phosphorylated BCR-ABL and phosphorylated PI3K were decreased at 20 and 16 h of treatment, respectively, whereas neither the BCR-ABL nor the PI3K level was significantly changed. AKT and its phosphorylated form were decreased at 1 hr. These results indicated that anti-proliferative effects of c-PC are mediated by inactivation of BCR-ABL signaling and the downstream pathway PI3K/AKT.

Key words: apoptosis, BCR-ABL fusion protein, leukemia, phycocyanin, PI3K kinase

INTRODUCTION

Chronic myelocytic leukemia (CML) is classified as a myeloproliferative neoplasm (MPN) characterized by the accumulation of myeloid lineage cells in peripheral blood and bone marrow. It is caused by proliferation of aberrant pluripotent hematopoietic stem cells (HSC) and inhibition of programmed cell death or apoptosis. In over 90 percent of CML patients, there is a translocation between chromosome 9 and 22 resulting in the fusion of the cellular oncogene, ABL, and the breakpoint cluster region, BCR [1]. The product of the BCR-ABL fusion gene has effects on important signal transduction pathways including PI3K/AKT, Ras/Raf/Mek/ERK, and JAK/STAT[2]. The protein kinase B/AKT signaling pathway has emerged as critical signaling node within all cells and as one of the most important protein kinases at the core of human physiology and diseases[3]. Sequential activation of many downstream targets of the PI3K/AKT pathway has been found to involve leukemogenesis. In CML patients, the PI3K/AKT pathway is initiated by BCR-ABL kinase activation through GAB2, leading to unregulated proliferation and inactivation of anti-apoptotic proteins such as Bcl-2 [2,4,5]. Therefore, inhibition of the signaling pathway associated with the BCR-ABL fusion protein in leukemic cells should restrain cell growth and proliferation, which would be helpful as a therapeutic strategy for CML[6].

Many natural compounds have been introduced as anti-cancer agents. Approximately two-thirds of human cancers could be prevented by dietary changes [7]. Several in vivo and in vitro studies have demonstrated that some natural dietary compounds such as isoflavones, indole-3-carbinol (I3C) and curcumin have inhibitory effects on human
These compounds exerted their effects by altering cell signaling pathways such as NF-κB, PI3K/AKT, MAPK, p53, Ras/Raf/Mek/ERK and JAK/STAT. C-Phycocyanin (c-PC), a biliprotein isolated from cyanobacteria and red algae, is a natural compound that is endowed with various biological and pharmacological properties [10]. C-PC possesses significant antioxidant activities [11,12] and can enhance immunity and inflammatory responses [13]. Previous studies have shown that c-PC can induce apoptosis in cancer cells such as a mouse macrophage cell line (RAW 264.7), prostate cell line (LNCaP), breast cell line (MCF-7), and erythremyeloid leukemia cell line (K562) [10, 14-16]. Subashini et al. demonstrated that c-PC-induced apoptosis in K562 cells is mediated by cytochrome c release, PARP cleavage, and Bcl-2 down-regulation[16].

To further elucidate the pro-apoptotic mechanism of c-PC, we determined its cytotoxic and anti-proliferative activities on the human leukemic cell line K562. The effect of c-PC on expression and phosphorylation of proteins in the apoptotic pathway that play a key role in carcinogenesis was explored.

**EXPERIMENTAL SECTION**

**Reagents and chemicals**

RPMI 1640 medium, fetal bovine serum (FBS), and the Western blot detection kit (Supersignal West Femto) were purchased from Thermo Fisher Scientific Inc. (MA, USA). XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt) was purchased from Sigma Aldrich (MO, USA). DEAE Sepharose Fast Flow, Hybond, ECL nitrocellulose membrane and Hyper film ECL was purchased from GE Healthcare Life Sciences (NJ, USA). Anti-rabbit IgG, HRP-linked antibody #7074, PI3 Kinase p110α (C73F8) rabbit monoclonal antibody #4249, phospho-PI3 Kinase p85 (Tyr458)/p55 (Tyr199) antibody #4228, AKT antibody #9272, phospho-AKT (Thr308) antibody #9278, phospho-c-Abl (Tyr412) (247C7) rabbit monoclonal antibody #2865, c-Abl antibody #2862, LY294002 #9901, and β-actin (13E5) rabbit monoclonal antibody #4970 were from Cell Signaling Technology (MA, USA).

**C-PC extraction and purification**

C-PC was purified from *Spirulina platensis* by ammonium sulfate precipitation and ion-exchange chromatography. *S. platensis* cells (a gift from W. Suppamaethakorn, Taweewattana Farm, Bangkok, Thailand) were lysed with 100 mg/ml lysozyme in 1 M sodium phosphate buffer (pH 7.4) and 100 mM EDTA by stirring for 15 h at 37 °C. Cell debris was removed by centrifugation at 3000 x g for 20 minutes. Crude extracts were subjected to protein precipitation using 35% and 50% of ammonium sulfate at 4 °C. After stirring in ammonium sulfate for 30 min, the precipitate was pelleted by centrifugation at 12000 x g for 20 min at 4 °C. The precipitate from 50% ammonium sulfate containing c-PC was re-dissolved in 0.025 M Tris-HCl buffer (pH 8.0). Anion-exchange chromatography was performed on a DEAE Sepharose Fast Flow column. The DEAE column was pre-equilibrated with 0.025 M Tris-HCl buffer (pH 8.0). The c-PC-containing extract was loaded onto the column, and c-PC was eluted with a buffer containing a gradually increasing concentration of NaCl from 0 to 0.8 M. The absorbance of the eluted fractions was measured using a UV–vis spectrophotometer. Fractions with A620/A280 ≥ 4.0 were combined. A652 and A620 were used to calculate c-PC concentration using the following equation:

\[
c \cdot \text{PC (mg} \cdot \text{ml}^{-1}) = \frac{[A_{620} - 0.474(A_{652})]}{5.34}
\]

The purified c-PC was lyophilized, and a stock solution of 1 mM c-PC in PBS was freshly prepared for each experiment.

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**

Protein electrophoresis was performed to assess the molecular weight and purity of c-PC using a 12% separating and 5% stacking polyacrylamide gel containing 0.1% SDS. Samples were mixed with loading dye (2% SDS, 10% glycerol, 4.5% β-mercaptoethanol, 0.025% bromophenol blue in 60 mM Tris, pH 6.8) and boiled for 5 min prior to electrophoresis. Proteins were electrophoresed at 72 and 150 volts for the stacking and resolving gel, respectively, and were visualized by staining with Coomassie brilliant blue R250.

**Cell culture**

The human chronic myeloid leukemia K562 cell line was used in this study. It was kindly provided by Professor Apiwat Mutirangura (Faculty of Medicine, Chulalongkorn University, Thailand). Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin and maintained in a humidified atmosphere with 5% CO₂ at 37 °C. The cultured cells were passaged...
every 3 days and seeded at a density of approximately 2-3 x10^5 cells/ml. Cell viability was determined by Trypan blue staining and counted using a hemocytometer.

**Cell cytotoxicity assay**

Cell cytotoxicity was evaluated by the 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay. K562 cells (5x10^5 cells/well) were incubated in 96-well plate with c-PC at concentrations varying from 5 to 150 µM in a final volume of 100 µl. Each concentration was tested in triplicate. After 24 h, 20 µl of XTT solution was added to each well and further incubated for 4 h in a humidified atmosphere with 5% CO₂ at 37 °C. Cells grown without c-PC were used as blanks. The absorbance was measured at 450 nm using a microplate reader (EnSpireTM 2300, Perkin Elmer, MA, USA). The half-maximal inhibitory concentration (IC₅₀) of c-PC to K562 cells was determined by a dose-response curve.

**Western blot analysis**

To study the expression and phosphorylation of proteins in the PI3K/AKT pathway, K562 cells were treated with c-PC at a concentration less than the IC₅₀ (100 µM) at various times (1, 4, 8, 12, 16, 20, 24 h). The whole cell extract was then prepared as follows. Cells were washed with PBS, pH 7.4 (137 mMNaCl, 2.7 mMkCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄) and suspended in Cell Culture Lysis Reagent (Promega, WI, USA). The mixture was vortexed for 15 seconds and then centrifuged at 12,000 x g for 2 minutes at 4 °C. The supernatant was collected. The protein concentration was determined using a Bradford assay (Bio-Rad, CA, USA). The levels of proteins associated with BCR-ABL and the PI3K/AKT signaling pathway were determined by Western blotting. The whole cell extract was electrophoresed on an SDS–polyacrylamide gel as described above. The separated proteins were transferred to a nitrocellulose membrane and then incubated with specific primary antibodies overnight at 4 °C and washed 3 times. After that, they were incubated with peroxidase-conjugated secondary antibodies for 1 hour at room temperature. The chemiluminescent signal was detected using Western blot detection reagents (Supersignal West Femto). The protein levels were determined with the ImageJ program.

**Statistical analysis**

The results are expressed as the mean ± S.D. Data were obtained from three independent experiments. ANOVA was used for the comparison of means between groups. The difference was considered statistically significant when p value < 0.05 or < 0.01. SPSS software (Ver. 17.0) was used.

**RESULTS AND DISCUSSION**

**C-PC purification**

In this study, the purification of c-PC was carried out by ammonium sulfate precipitation and anion-exchange chromatography. SDS-PAGE analysis of the purified fraction clearly showed 2 protein bands corresponding to the α and β subunits of c-PC, which migrated as approx. 18.4 kDa and 21.3 kDa bands, respectively (Fig. 1). More than 98% purity was achieved as judged by SDS-PAGE. A purity ratio (A620 / A280) of 4.36 was obtained.
Lane 1: molecular weight markers, lane 2: crude extract of *S. platensis*, lane 3: soluble fraction of 35% ammonium sulfate precipitation, lane 4: precipitate from 50% ammonium sulfate precipitation, lane 5: purified fraction from ion-exchange chromatography.

**Cell cytotoxicity**

Cell cytotoxicity was performed to determine the toxicity of c-PC to K562 cells. The decrease in cell viability indicated cell death or inhibition of cell growth. The effects of c-PC on the viability of K562 cells after 24 h in culture were evaluated. Cells were treated with various concentrations of c-PC (5, 10, 20, 40, 60, 80, 100, 150 µM) as described above. C-PC reduced the viability of K562 cells in a dose-dependent manner (Fig. 2). The calculated IC\textsubscript{50} of c-PC was 128.60 µM.

![Figure 2 - Effect of c-PC on K562 cell viability](image)

K562 cells were treated with 5 to 150 µM c-PC for 24 hours. The XTT assay was performed in triplicate to determine the percent viability of the cells. The dose-response curve was plotted as the mean ± S.D. of percent viability. Statistical analysis was carried out with one-way ANOVA followed by Tukey’s multiple comparison test compared to non-treated cells (* p < 0.01).

**Western blot analysis**

Figure 3 shows the levels of BCR-ABL, PI3K, AKT and their phosphorylated derivatives in the whole cell extract of K562 cells treated with c-PC after normalization with β-actin. The results showed that the level of BCR-ABL of the treated cells was not significantly altered, but p-BCR-ABL declined until the level of p-BCR-ABL reached approximately 10% of the original level within 24 h (Fig. 3A). The same effects were observed for PI3K and phosphorylated PI3K (p-PI3K), as shown in Figure 3B. The PI3K level did not change, whereas the level of p-PI3K significantly decreased to less than 30% within 24 h. The p-BCR-ABL/BCR-ABL ratio and p-PI3K/PI3K ratio also decreased (Fig. 3A and 3B). Figure 3C shows the levels of AKT and phosphorylated AKT (p-AKT) in the whole cell extract of K562 cells treated with c-PC after normalization with β-actin. The AKT and p-AKT levels significantly decreased from the first hour of treatment and reached approximately 30% within 24 h. Thus, the ratio of p-AKT to AKT did not change.

After cells were incubated with 100 µM c-PC for 24 hours, expression of proteins in the BCR-ABL signaling pathway was then detected. The upper panel shows Western blot results of BCR-ABL and pBCR-ABL (A), PI3K and pPI3K (B), and AKT and pAKT (C). The protein level was determined and normalized with β-actin using the ImageJ program. The middle panel demonstrates the level of each protein during the treatment period of 24 h. The lower panel demonstrates the ratio of the phosphorylated- and non-phosphorylated form at the given time. The control (c) was non-treated cells. Statistical analysis was carried out with a one-way ANOVA followed by Tukey’s multiple comparison test compared to controls (*p<0.05, **p < 0.01).
Cell signal transduction is a communication network that controls basic biological activities such as adhesion, cytokine release, mortality and cell proliferation [2]. Signal transduction in cancer cells deviated from normal, generating an immortal cell that does not undergo programmed cell death [17]. Moreover, those altered cells have a high tendency to proliferate. In chronic leukemias, including chronic myeloid leukemia (CML), Philadelphia-negative myeloproliferative neoplasms (MPNs), and chronic lymphocytic leukemia (CLL), evidence of defects in the regulation of cellular signaling pathways has been reported [2]. In CML, the product of the Philadelphia chromosome, the BCR-ABL fusion protein, causes a defect in cell proliferation by inactivating signal transduction related to anti-apoptosis and hyperproliferative pathways in leukemic cells [18]. Currently, treatment of a CML patient is usually based on a potent protein-tyrosine kinase inhibitor such as imatinib [19]. Imatinib inhibits the kinase activity of the BCR-ABL oncoprotein, thus hindering cell proliferation. Although imatinib is an effective medication, drug resistance is a problem [20]. Therefore, an alternative medicine is needed. A novel natural compound with a kinase-inhibiting effect might be a safe and effective treatment for CML patients.

C-PC is a natural compound found in edible algae. It has many biological and pharmacological properties including free radical scavenging, anti-inflammatory, and anti-cancer effects [21]. According to previous studies, the anti-cancer effects of c-PC appear to be mediated by various mechanisms such as an increase in the proapoptotic Fas protein, down-regulation of Bcl-2 [13], and selective inhibition of COX2 [10]. These studies emphasized that the pathway through which c-PC exerts its activities will enable its application as a therapeutic agent for MPNs.

Many methods have been used for the separation and purification of c-PC. The purity of c-PC, as judged by an A620/A280 purity ratio greater than 4.0, is sufficient for further testing [22]. In this study, c-PC was purified using an optimized 2-step purification composed of ammonium sulfate precipitation and ion-exchange chromatography. Very high purity c-PC (A620/A280 = 4.36) was obtained and used for further testing on cells.

We found that c-PC at a micromolar level exhibited a cytotoxic effect on K562 cells. Our result is in good agreement with that obtained in a previous study. Subhashini et al. demonstrated a decrease to approximately 65%
of the control growth of K562 cells treated with 100 μM C-PC at 24 h. They reported that the decrease in cell growth is a result of apoptosis via downregulation of anti-apoptotic Bcl-2, release of cytochrome c into the cytosol, and cleavage of poly(ADP) ribose polymerase (PARP)[16]. There are also other studies suggesting that c-PC may induce apoptosis and inhibit proliferation of cancer cells [10,15,21,23]. It is possible that these events are mediated by altering signal transduction related to both apoptosis enhancement and terminated proliferation in cancer cells, possibly through the Ras/Raf/Mek/ERK, JAK/STAT, MAPK and PI3K/AKT pathways in K652 cells [2].

PI3K/AKT is the major downstream target of the BCR-ABL activation cascade and is targeted by tyrosine kinase inhibitors such as imatinib[24,25] in leukemia treatment, resulting in apoptosis and inhibition of leukemic cell proliferation. The BCR-ABL kinase activates the PI3K/AKT pathway through GAB2, and subsequently, many downstream targets of the PI3K/AKT pathway have been shown to participate in BCR-ABL-induced leukemogenesis. PI3K/AKT activation induces phosphorylation of BAD, which in turn blocks anti-apoptotic protein Bcl-2. Moreover, the FOXO transcription factors involved in cell cycle arrest are phosphorylated by AKT, inhibiting FOXO3a activity [2]. PI3K/AKT also plays the key role in the inhibition of the caspase cascade pathway, NF-κB production and the mTOR pathway [26-27]. Here, the effect of c-PC on the signal transduction from BCR-ABL through the PI3K/AKT pathway in K562 cell lines was explored. The results indicated that c-PC inactivates the phosphorylation of both BCR-ABL and PI3K. The decrease in AKT and p-AKT levels was influenced by the decrease in upstream signaling from PI3K. In CML, it has been reported that kinase phosphorylation of BCR-ABL, PI3K, and AKT is necessary to stimulate cellular signaling and leukemogenesis[18]. Therefore, the effects of c-PC on signal transduction in apoptotic pathways of K562 cell lines can be summarized as shown in Figure 4.

![Figure 4 - Effect of c-PC on downstream targeting associated with the BCR-ABL fusion protein](image)

**CONCLUSION**

According to the results of the present study, c-PC exhibited anti-proliferative and apoptotic effects on chronic myelocytic leukemia cells (K562) in a dose-dependent manner. This effect is caused by inhibition of BCR-ABL and the downstream PI3K pathway, which is involved in the proliferation and apoptosis of K562 cells. However, ongoing research on other related signaling pathways should be established given the decrease in AKT activity after the first hour of treatment, which is not correlated with p-PI3K. These findings might be helpful for the development of novel nontoxic therapeutic compounds for chronic myelocytic leukemia.

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