



Molecular and Cellular Pharmacology

C-Phycocyanin inhibits MDR1 through reactive oxygen species and cyclooxygenase-2 mediated pathways in human hepatocellular carcinoma cell line

Reddy P. Nishanth^{a,b}, B.S. Ramakrishna^{a,b}, Radhika G. Jyotsna^a, Karnati R. Roy^a, Gorla V. Reddy^a, Pratap K. Reddy^b, Pallu Reddanna^{a,*}^a School of Life Sciences, University of Hyderabad, Hyderabad-500046, India^b Department of Zoology, Osmania University, Hyderabad-500007, India

ARTICLE INFO

Article history:

Received 20 November 2009

Received in revised form 30 August 2010

Accepted 7 September 2010

Available online 19 September 2010

Keywords:

C-Phycocyanin

Cyclooxygenase-2

HepG2 cell

MDR1 [multidrug resistance 1]

Reactive oxygen species

ABSTRACT

The effects of C-Phycocyanin (C-PC), a biliprotein from *Spirulina platensis* on the regulation of multidrug resistance-1 (MDR1), a poly glycoprotein in human hepatocarcinoma cell line, HepG2 were reported. The results revealed that a significant down regulation of MDR1 expression in C-PC treated HepG2 cells was through reactive oxygen species and cyclooxygenase-2 (COX-2) mediated pathways. C-PC in a concentration dependent manner increased the accumulation of doxorubicin in HepG2 cells and enhanced sensitivity of the cells to doxorubicin by 5 folds. The induction of MDR1 expression by PGE₂ and its down regulation by C-PC and DPI (Diphenylene iodonium, NADPH oxidase inhibitor) or by COX-2 knockdown suggest that the enhanced sensitivity of HepG2 cells to doxorubicin by C-PC is mediated by the down regulation of MDR1 expression. Further studies reveal the involvement of NF-κB and AP-1 in the C-PC induced down regulation of MDR1. Also the inactivation of the signal transduction pathways involving Akt, ERK, JNK and p38 by C-PC was observed. The present study thus demonstrates the efficacy of C-PC in overcoming the MDR1 mediated drug resistance in HepG2 cells by the down regulation of reactive oxygen species and COX-2 pathways via the involvement of NF-κB and AP-1.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Hepatocellular carcinoma is the sixth most common cancer and the third leading cause of cancer related deaths worldwide. It accounts for approximately 6% of all human cancers and up to 1 million deaths per year (Parkin et al., 2005; Bosch et al., 2005). Chemotherapy of many cancers is frequently abortive due to the development of resistance. Cancer cells possess intrinsic drug resistance to several compounds or they can rapidly acquire it. Although extensive efforts have been made to increase our knowledge in this field over the past few years the molecular mechanisms involved in the phenomenon are only partially known. Among the several types of drug resistance, a membrane associated 170 kDa protein, multidrug resistance 1 (MDR1), also called P-glycoprotein (P-gp), is involved in development of resistance to various anti-cancer drugs (Juliano and Ling, 1976).

Expression of MDR1 was shown in the apical membranes of liver, kidney, gut and at the blood-brain barrier (Thiebaut et al., 1987; Cordon-Cardo et al., 1989). Overexpression of MDR1 was observed in isolated hepatocytes of endotoxin treated rats (Vos et al., 1998), adenocarcinomas derived from adrenal, kidney, liver and bowel (Fojo et al., 1987), breast cancer (Filipits et al., 1996) and in prostate cancer (Bhargal et al., 2000). Intrinsic up regulation of MDR1 expression has been associated

with primary resistance in untreated hepatocellular carcinoma of human and rodent origin (Bradley et al., 1992; Huang et al., 1992). It was shown that regulation of MDR1 expression was dependent on reactive oxygen species and cyclooxygenase-2 (COX-2) activity (Roy et al., 2007a, 2010; Wartenberg et al., 2001; Patel et al., 2002). Studies have shown that multidrug transporters are inducible by oxidative stress (Kuo, 2009). Cancer cells are known to generate reactive oxygen species endogenously to significant amounts which may be one or the only cause of their excessive growth, elucidating the relation between the risk of carcinogenesis and reactive oxygen species (Szatrowski and Nathan, 1991). Thus, it is not astonishing that elevated expression of these drug resistance genes is found in tumors even before chemotherapy starts.

COX-2 has been reported to play a major role in the regulation of MDR. Simultaneous overexpression of the COX-2 and MDR1, reported in the regenerative nodules of cirrhotic livers as well as in well-differentiated hepatocellular carcinoma (Koga et al., 1999; Nagasue et al., 1995; Roy et al., 2010) suggests a possible role for COX-2 in MDR. In the light of the above scenario, the present study is undertaken to test whether redox signaling and COX-2 are the viable targets to overcome drug resistance in cancer cells.

In view of serious side effects of synthetic drugs, there is a shift towards identifying natural compounds without undesired effects. One such compound is C-Phycocyanin (C-PC), a soluble biliprotein from *Spirulina platensis*. We have reported earlier that C-PC is a selective COX-2 inhibitor (Reddy et al., 2000), hepatoprotective (Sathyaikumar et al.,

* Corresponding author. Tel./fax: +91 40 23010745.

E-mail address: prsl@uohyd.ernet.in (P. Reddanna).

2007), induces apoptosis in doxorubicin resistant HepG2 cell line (Roy et al., 2007b), K562 cell line (Subhashini et al., 2004) and also in lipopolysaccharide stimulated, RAW 264.7 macrophages (Reddy et al., 2003). Recently, we have also shown that C-PC ameliorates 2-AAF induced oxidative stress and MDR1 expression in RAW 264.7 cells (Roy et al., 2007a) and in albino mice (Roy et al., 2008). The present study was undertaken in hepatocarcinoma cell line (HepG2) to evaluate the effects of C-PC on MDR1 expression and to elucidate the mechanisms underlying these effects.

2. Materials and methods

2.1. Chemicals

PBS, RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco BRL (California, USA). Nitrocellulose membrane was from Millipore (Bangalore, India). Phosphatase inhibitor cocktail 1 and 2, β -actin antibodies and DPI were purchased from Sigma-Aldrich (Bangalore, India). Primary antibodies to Akt, p-Akt, JNK, p-JNK, ERK, p-ERK, P38, p-P38 and MDR1 were procured from Santa Cruz Biotechnology Inc. (California, USA). Monoclonal COX-2 antibody, PGE₂ estimation kit and PGE₂ were from Cayman Chemical Co. USA. siRNA for COX-2 (sc-44256) and MDR1 (sc-29395) were purchased from Santa Cruz Biotechnologies Inc., USA. C-PC was provided by Paris Agro Ltd, Chennai, India. All the other chemicals and reagents were purchased from local companies and are of molecular biology grade.

2.2. Cell culture and treatment

HepG2 cells expressing MDR1 and COX-2 constitutively were grown in RPMI 1640 medium supplemented with 10% heat inactivated FBS, 100 IU/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine and maintained in a humidified atmosphere with 5% CO₂ at 37 °C. The cultured cells were sub-cultured twice each week and the exponentially growing cells were used for all treatments. C-PC and doxorubicin dissolved in PBS was used for the treatments. At the time of treatment working solutions were prepared accordingly in RPMI 1640 medium. The drugs were added to the cells, 6 h after the subculture. Stocks of C-PC and doxorubicin were freshly prepared before every treatment. The primary human hepatocytes were obtained from Centre for Liver Research and Diagnostics (CLRD), Deccan College of Medical Sciences, Hyderabad, India.

2.3. Effect of C-PC or doxorubicin on proliferation of HepG2 cells

Cell proliferation was determined by MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Campling et al., 1988). HepG2 cells (5×10^3 cells/well) were seeded to 96 well culture plates in the presence or absence of C-PC (1 μ M, 10 μ M, 25 μ M, 50 μ M and 100 μ M) or doxorubicin (100 nM, 500 nM, 1 μ M, 10 μ M, 25 μ M and 50 μ M) for 12, 24 and 48 h in a final volume of 100 μ l. After treatment, the medium was removed and 20 μ l of MTT (5 mg/ml of PBS) was added to the fresh medium. After 2 h incubation at 37 °C, 100 μ l of DMSO was added to each well and plates were agitated for 1 min. Absorbance was read at 570 nm on a multiwell plate reader. Percent inhibition of proliferation was calculated as a fraction of control (control was without C-PC or doxorubicin).

2.4. Synergistic effect of C-PC and doxorubicin on proliferation of HepG2 cells

HepG2 cells (5×10^3 cells/well) were seeded on 96 well culture plates. Doxorubicin (100 nM, 500 nM, 1 μ M, 10 μ M, 25 μ M and 50 μ M) was added in the presence or absence of C-PC (25 μ M) for 24 h in a final volume of 100 μ l. After treatment, the MTT assay was performed as mentioned above. Percent inhibition of proliferation was calculated

as a fraction of control (Cells treated with 25 μ M C-PC was taken as control for determining the synergistic effects of doxorubicin and C-PC).

2.5. Confocal analysis to check the localization/uptake of C-PC

To perform localization studies, HepG2 cells (1×10^5) were exposed to C-PC (50 μ M) for 24 h. After treatment cells were observed for C-PC under confocal microscope at an excitation wave length of 620 nm.

2.6. Intracellular drug accumulation assays

HepG2 cells (1×10^6 cells/well) were seeded on 6 well culture plates. Cells were incubated with C-PC at concentrations of 5, 25 and 50 μ M, DPI (10 μ M) and PGE₂ (6 μ g/ml) for 24 h. To determine intracellular drug accumulation, 50 μ M of doxorubicin (standard anti-cancer drug) was added to HepG2 cells and incubated for 3 h. Then the cells were harvested and the final doxorubicin accumulated in HepG2 cells was visualized and analyzed using laser scanning confocal microscope (Max Excitation λ 480 nm) and flow cytometer (FL2 filter), respectively.

2.7. RT-PCR analysis

HepG2 cells were seeded at a density of 5×10^6 in 90 mm culture dishes. Cells were treated with C-PC (5, 25 and 50 μ M), DPI (10 μ M) and PGE₂ (6 μ g/ml) for 24 h. Cells were harvested and the total RNA was extracted using TRI reagent. cDNA was synthesized using oligo (dT), dNTP mixture, RevertAid H Minus M-MuLV Reverse Transcriptase. A 2 μ l aliquot of the 20 μ l total cDNA was used for standard PCR reaction of 28 cycles using the COX-2 FP: 5'-TTC AAA TGA GAT TGT GGG AAA ATT GGT-3', RP: 5'-AGA TCA TCT TTG TCT GAG TAT TTT-3' and MDR1 FP: 5'-TGA CTA CCA GGC TCG CCA A-3', RP: 5'-TAG CGA TCT TCC CAG CAC CTT-3' primer sets with a annealing temperature of 54 °C. The PCR products were visualized on 1% agarose gels with ethidium bromide, under UV light. The GAPDH primers served as control.

2.8. COX-2 and MDR1 knockdown analysis

HepG2 cells (1×10^6 cells/well) were seeded into 6 well culture plates. After overnight incubation cells were transfected with siRNA for COX-2 and MDR1 at concentration of 100 nM for 48 h. Total RNA was isolated and RT-PCR analysis was performed to estimate the expression of MDR1 in control, COX-2 siRNA transfected, MDR1 siRNA transfected, DPI (10 μ M) and PGE₂ (6 μ g/ml) treated cells.

2.9. Preparation of whole cell extracts and immunoblot analysis

HepG2 cells at a density of 5×10^6 were seeded on 90 mm culture dishes. They were incubated with C-PC (5 μ M, 25 μ M and 50 μ M), DPI (10 μ M) and PGE₂ (6 μ g/ml). Cells harvested were used for preparation of whole cell extract. The harvested, control and treated, HepG2 cells were washed with PBS and suspended in lysis buffer (20 mM Tris, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μ g/ml leupeptin, 20 μ g/ml aprotinin and phosphatase inhibitor cocktail 1 and 2 with 100 fold dilution). After 30 min of shaking at 4 °C, the mixtures were centrifuged (10,000 g) for 10 min, and the supernatants were used as the whole cell extracts. The protein content was determined according to the Bradford method (Bradford, 1976). 100 μ g of protein from each treatment was resolved on 7–10% SDS-PAGE gels (MDR1, COX-2, Akt, p-Akt, ERK, p-ERK, JNK, p-JNK, P38 and p-P38) along with protein molecular weight standards, and then transferred onto nitrocellulose membranes. Membranes were stained

with 0.5% Ponceau S in 1% acetic acid to check the transfer. The membranes were blocked with 5% w/v nonfat dry milk and then incubated with the primary antibodies in 10 ml of antibody-diluted buffer (1X Tris-buffered saline and 0.05% Tween-20 with 1% milk) with gentle shaking at 4 °C for 8–12 h and then incubated with peroxidase conjugated secondary antibodies. Signals were detected by using western blot detection reagents. Equal protein loading was confirmed by reprobing the nitrocellulose membranes with β -actin antibodies.

2.10. Measurement of intracellular reactive oxygen species

Reactive oxygen species production in control and treated HepG2 cells was measured using the dye 2, 7 dichloro dihydro fluorescein diacetate (DCFH-DA). DCFH-DA, a non-fluorescent cell permeable compound becomes the fluorescent compound, 2, 7-dichlorofluorescein (DCF), upon oxidation by reactive oxygen species. Cells seeded at a density of 2×10^6 in 60 mm culture dishes were treated with C-PC (5, 25 and 50 μ M) for 6 h, DPI (10 μ M) and PGE₂ (6 μ g/ml) for 1 h. Cells were harvested after 10 min incubation with DCFH-DA (10 μ M) and washed with PBS. Reactive oxygen species measurement was carried out on FACS Calibur flow cytometer. Data were collected using the data acquisition program CELL Quest (Becton Dickinson, San Jose, CA). DCF was measured with the following excitation and emission wavelengths: $\lambda_{exc} = 488$ nm, $\lambda_{em} = 525$ nm. 20,000 cells were analyzed per sample.

2.11. Electrophoretic mobility shift assay (EMSA)

HepG2 cells at a density of 5×10^6 were seeded in 90 mm culture dishes. Cells were incubated with C-PC (5 μ M, 25 μ M and 50 μ M), for 12 h, DPI (10 μ M) and PGE₂ (6 μ g/ml) for 6 h. Cells were harvested and then used for nuclear protein extraction. The cells were washed with PBS and 200 μ l of ice cold lysis buffer (20 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 1% NP-40, 1 mM PMSF) was added, and incubated for 5 min on ice with 3–4 vortexings of 10 s each. The nuclei were then harvested by centrifugation at 16,000 rpm for 1 min. The nuclear pellet was resuspended in 40 μ l of nuclear protein extraction buffer (420 mM NaCl, 10 mM HEPES, 10 mM MgCl₂, 1 mM EDTA, 0.1 mM DTT and 25% glycerol) and incubated on ice for 30 min with intermittent vortexing of 10 s each. The sample was then centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant collected was used for the mobility shift assay after protein estimation using Bradford assay. Nuclear extracts (8 μ g) were incubated with γ -³²P labeled double stranded oligonucleotide with specific NF- κ B (5'-CTG AAT CAA CTG CTT CAA-3') and AP-1 (5'-CTG AAT CAA CTG CTT CAA-3') binding sequence for 30 min at 37 °C. DNA-protein complex formed was separated from free oligonucleotides on 6.6% native acrylamide gel. The dried gel was exposed to X-ray film. The specificity of binding was examined by competition with unlabeled oligonucleotide (cold competition).

2.12. PGE₂ estimation

HepG2 cells at a density of 5×10^6 were seeded in 90 mm culture dishes. They were incubated with C-PC (5 μ M, 25 μ M & 50 μ M), DPI (10 μ M) and celecoxib (10 μ M) for 24 h. At the end of the treatment period, culture medium was collected to determine the amount of PGE₂ secreted by these cells and stored at -80 °C. The quantitative analysis of PGE₂ released into the medium was assessed by using PGE₂ immunoassay kit as per manufacturer's instructions (Cayman chemicals, USA).

2.13. Statistical analysis

Data reported as the mean \pm S.E.M. of three independent experiments. Statistical analysis of differences was carried out by one-way analysis of variance (ANOVA). A P-value less than 0.05 was considered to indicate significant.

3. Results

3.1. C-PC and doxorubicin showed synergistic effects on the proliferation of HepG2 cells

HepG2 cells were treated with C-PC (1–100 μ M) and doxorubicin (0.1–50 μ M) separately for 12, 24 and 48 h and cell proliferation was determined by MTT assay. Under these experimental conditions, a dose dependent decrease in proliferation of HepG2 cells was observed with an IC₅₀ of 50 μ M for C-PC (Fig. 1A) and 5 μ M for doxorubicin at 24 h exposure (Fig. 1B).

In order to test the combination effects, HepG2 cells were treated with varying concentrations of doxorubicin (100 nM, 500 nM, 1 μ M, 10 μ M, 25 μ M and 50 μ M) and fixed concentration of C-PC (25 μ M) for 24 h and cell proliferation was determined by MTT assay. In the presence of 25 μ M C-PC, the percent inhibition in the growth of HepG2 cells was much higher at all the concentrations of doxorubicin studied. As a result the IC₅₀ of doxorubicin for HepG2 cells was reduced from 5 μ M to 1 μ M in the presence of 25 μ M C-PC (Fig. 1B) i.e. a fivefold reduction.

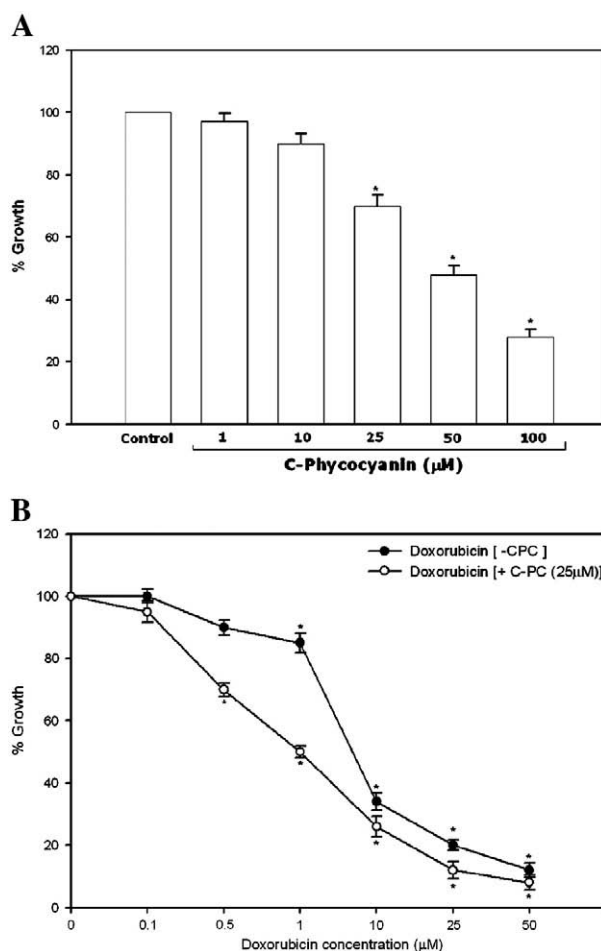


Fig. 1. IC₅₀ values of C-PC and doxorubicin alone and in combination in HepG2 cells. (A) Cells were treated with 1, 10, 25, 50 and 100 μ M C-PC for 24 h. The percentage of viable cells was calculated in comparison with untreated cells. The number of cells in the control was taken as 100%. (B) HepG2 cells (5×10^3 cells/well) were treated with doxorubicin (0.1 μ M, 0.5 μ M, 1 μ M, 10 μ M, 25 μ M and 50 μ M) in the presence or absence of C-PC (25 μ M) for 24 h. The percent viable cells were calculated in comparison to control cells. (Control was without doxorubicin or C-PC for the determination of IC₅₀ for C-PC or doxorubicin. Cells treated with 25 μ M C-PC were taken as control for determining its synergistic effects with doxorubicin). *Denotes statistical significance over control (P < 0.05).

3.2. Localization of C-PC

The entry of C-PC into HepG2 cells was determined using laser scanning confocal microscopy. These studies showed strong fluorescence signal in the cells treated with C-PC (Fig. 2B) for 24 h, which was absent in untreated control cells (Fig. 2A). These results indicate the entry of C-PC into the HepG2 cells.

3.3. C-PC treatment increased the accumulation of doxorubicin in HepG2 cells

Increased accumulation of doxorubicin in C-PC treated HepG2 cells was confirmed by confocal analysis (Fig. 3A) and FACS (Fig. 3B). HepG2 cells treated with C-PC (5, 25 and 50 μ M), DPI (10 μ M) and PGE₂ (6 μ g/ml) for 24 h followed by incubation with 50 μ M doxorubicin for 3 h, showed concentration dependent increase in doxorubicin accumulation in the presence of C-PC. Cells treated with DPI, a known reactive oxygen species inhibitor, also showed comparative increase in doxorubicin accumulation. Whereas, PGE₂ treated cells showed lesser accumulation of doxorubicin, compared to control.

In addition, to check the role of MDR1/P-gp in efflux of doxorubicin, HepG2 cells were treated with doxorubicin for 3 h at 37 °C (P-gp active state) and at 4 °C (P-gp inactive state), followed by FACS analysis. Cells incubated with the drug at 4 °C (MDR1 inactive state) showed more accumulation of the drug compared to cells incubated at 37 °C (MDR1 active state), confirming the role of MDR1 pumps in efflux of doxorubicin in HepG2 cells (Fig. 3C).

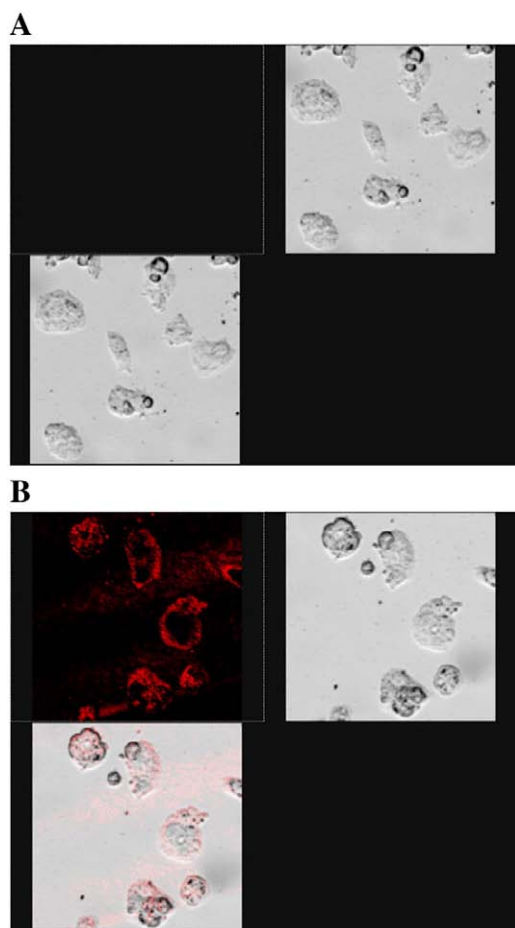


Fig. 2. C-PC localization in HepG2 cells. Confocal images of the HepG2 cells incubated with (B) and without (A) C-PC (50 μ M) for 24 h. The fluorescence in the C-PC treated cells is due to the presence of C-PC inside the cells or on the cell surface.

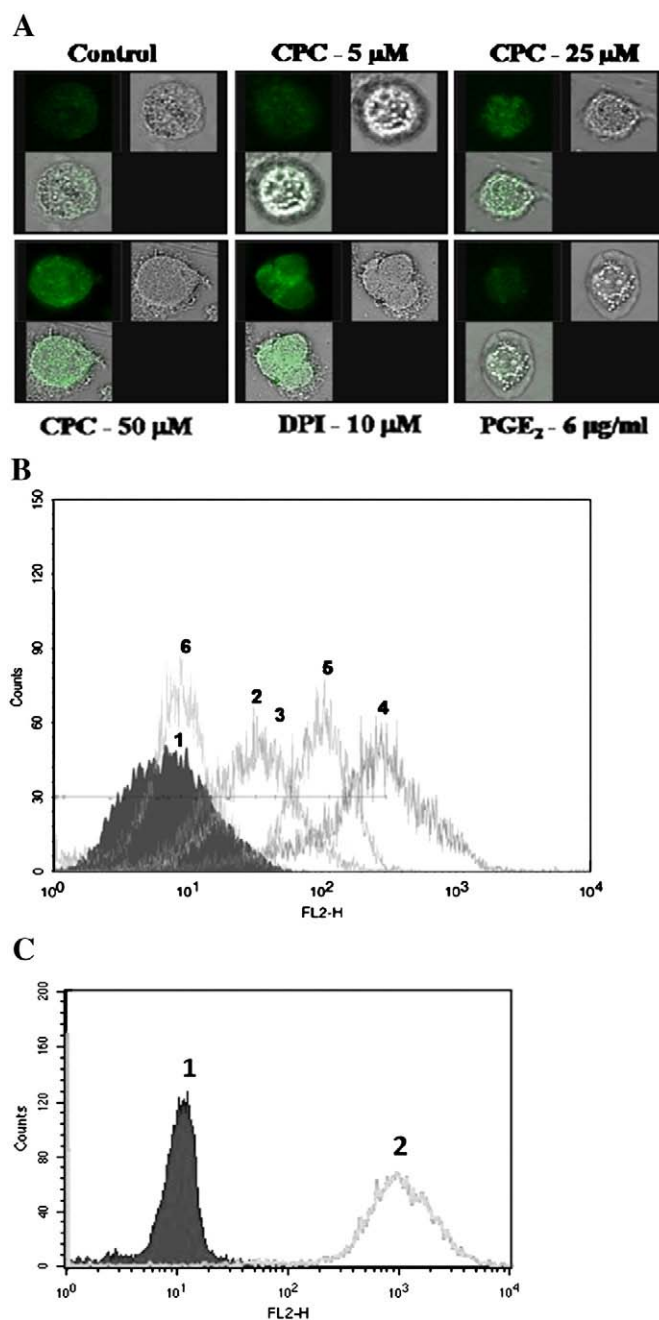


Fig. 3. Analysis of intracellular doxorubicin accumulation by confocal microscopy and flow cytometer. HepG2 cells were treated with 5 μ M, 25 μ M and 50 μ M of C-PC, 10 μ M of DPI and 6 μ g/ml PGE₂ for 24 h and then incubated with 50 μ M doxorubicin for 3 h. The fluorescence of doxorubicin retained in HepG2 cells was visualized and measured by confocal microscopy and flow cytometry. A) Confocal analysis shows the accumulation of doxorubicin with the treatments of C-PC, DPI and PGE₂, at excitation wavelength of 480 nm. B) FACS analysis with FL2 filter shows histogram 1 (doxorubicin retained in control cells exposed to 50 μ M doxorubicin) overlaid with histogram 2, 3, 4, 5 and 6 (doxorubicin retained in cells treated with 5, 25, 50 μ M C-PC, DPI 10 μ M and PGE₂ 6 μ g/ml, respectively, and exposed to 50 μ M doxorubicin). C) FACS analysis shows histogram 1 (doxorubicin retained in HepG2 cells exposed to 50 μ M doxorubicin at 37 °C) overlaid with histogram 2, (doxorubicin retained in HepG2 cells exposed to 50 μ M doxorubicin at 4 °C). Greater the FL2-height, greater is the drug retention.

3.4. C-PC inhibits MDR1 expression at transcription and translation levels

Treatment of HepG2 cells with C-PC (5 μ M, 25 μ M and 50 μ M) for 24 h resulted in a concentration dependent reduction of MDR1 at both mRNA (Fig. 4A) and protein (Fig. 4B) levels. Treatment of cells with DPI

(10 μM) also diminished the expression of MDR1. Addition of PGE₂ (6 $\mu\text{g}/\text{ml}$) to the medium, on the other hand, induced the expression of MDR1, compared to untreated controls. Unaltered levels of GAPDH and β -actin in all treatments confirm specific down regulation of MDR1 expression by C-PC.

3.5. C-PC induced down regulation of cellular reactive oxygen species generation

In view of the antioxidant characteristics of C-PC, FACS analysis was performed to analyze the potential of C-PC as a reactive oxygen species scavenger in HepG2 cells. As shown in Fig. 5, cells treated with C-PC for 6 h showed a concentration dependent decrease in the levels of reactive oxygen species, conferring its antioxidant potential. Cells treated with DPI (10 μM) for 1 h showed a significant decrease in the reactive oxygen species generation. Addition of PGE₂ (6 $\mu\text{g}/\text{ml}$) to the medium, on the other hand, induced the reactive oxygen species generation, compared to control.

3.6. C-PC down regulates COX-2 expression and PGE₂ levels in HepG2 cells

In the context of COX-2 role in MDR1 regulation, studies were taken up to check the effect of C-PC on COX-2 expression. Treatment of HepG2 cells with C-PC (5 μM , 25 μM and 50 μM) for 24 h showed a concentration dependent down regulation of COX-2 at both mRNA (Fig. 6A) and protein levels (Fig. 6B). C-PC treatment decreased the levels of PGE₂ in HepG2 cells in a dose dependent manner (Fig. 6C). Treatment of cells with DPI (10 μM) also showed a prominent down regulation of COX-2 (Fig. 6A and B) expression along with decreased levels of PGE₂ (Fig. 6C). PGE₂ treatment, on the other hand, induced the expression of COX-2 at mRNA (Fig. 6A) and protein levels

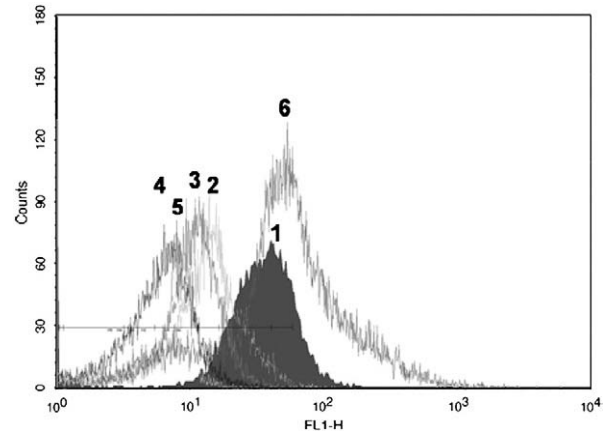


Fig. 5. Effect of C-PC on intra cellular reactive oxygen species generation. FACS analysis of generation of reactive oxygen species using DCFH-DA in HepG2 cells treated with or without C-PC, DPI and PGE₂. Histogram 1, Control; 2, C-PC (5 μM); 3, C-PC (25 μM); 4, C-PC (50 μM); 5, DPI (10 μM) and 6, PGE₂ (6 $\mu\text{g}/\text{ml}$).

(Fig. 6B). Celecoxib, a selective COX-2 inhibitor, decreased the levels of PGE₂ in HepG2 cells significantly (Fig. 6C).

Additionally, the effect of COX-2 depletion on MDR1 expression was tested by siRNA knockdown studies. Knockdown of COX-2 by siRNA reduced the expression of MDR1 and knockdown of MDR1 by siRNA reduced the expression of COX-2 (Fig. 6D). Cells treated with DPI showed a decrease in both MDR1 and COX-2 expression. PGE₂ treatment, on the other hand, increased the expression of MDR1 and COX-2 compared to the untreated control cells (Fig. 6D).

To further confirm that the expression of MDR1 in HepG2 cells is reactive oxygen species and COX-2 dependent, the levels of reactive oxygen species, expression of COX-2 and MDR1 were checked in human

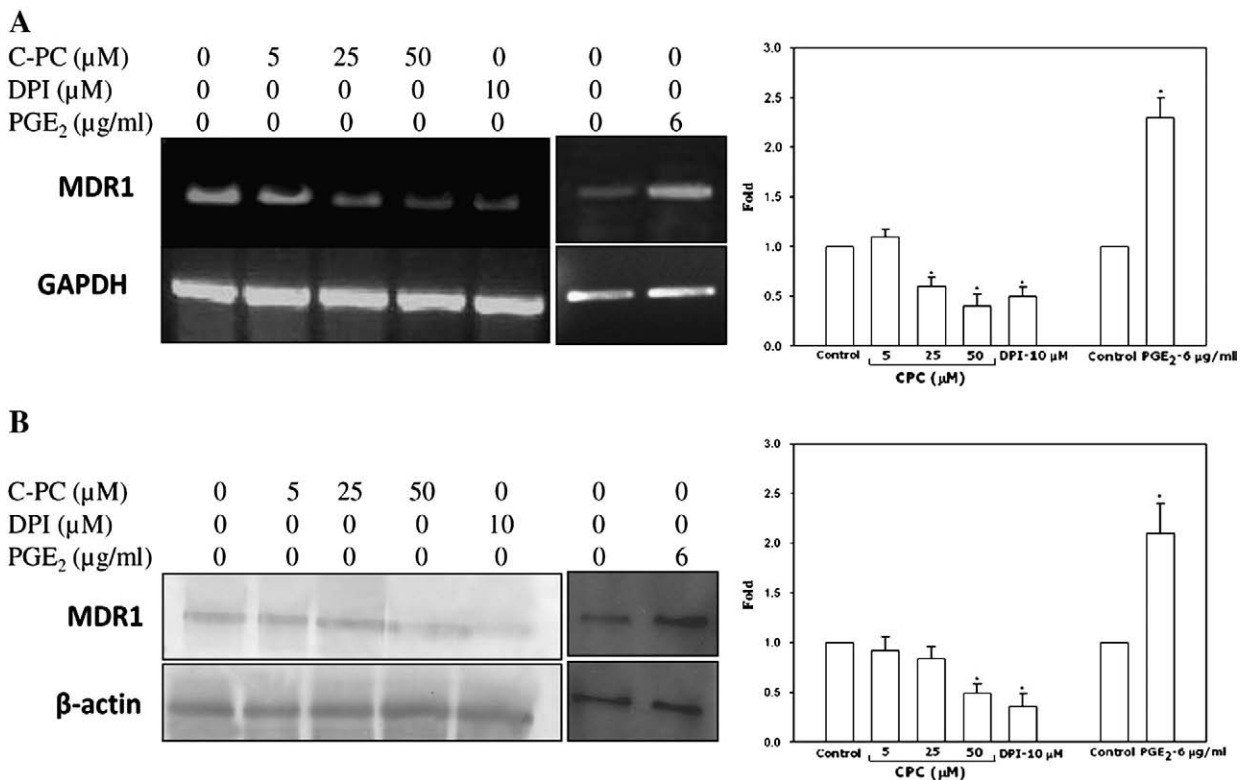


Fig. 4. Effect of C-Phycocyanin on MDR1 expression. (A) RT-PCR analysis of C-PC, DPI and PGE₂ mediated effect on MDR1 mRNA expression in HepG2 cells. GAPDH was used as an internal control. (B) Western blot analysis of C-PC, DPI and PGE₂ mediated effect on MDR1 protein expression in HepG2 cells. β -actin was used as an internal control to monitor equal loading. Bar graph represents densitometric values of MDR1 levels. *Denotes statistical significance over control ($P < 0.05$).

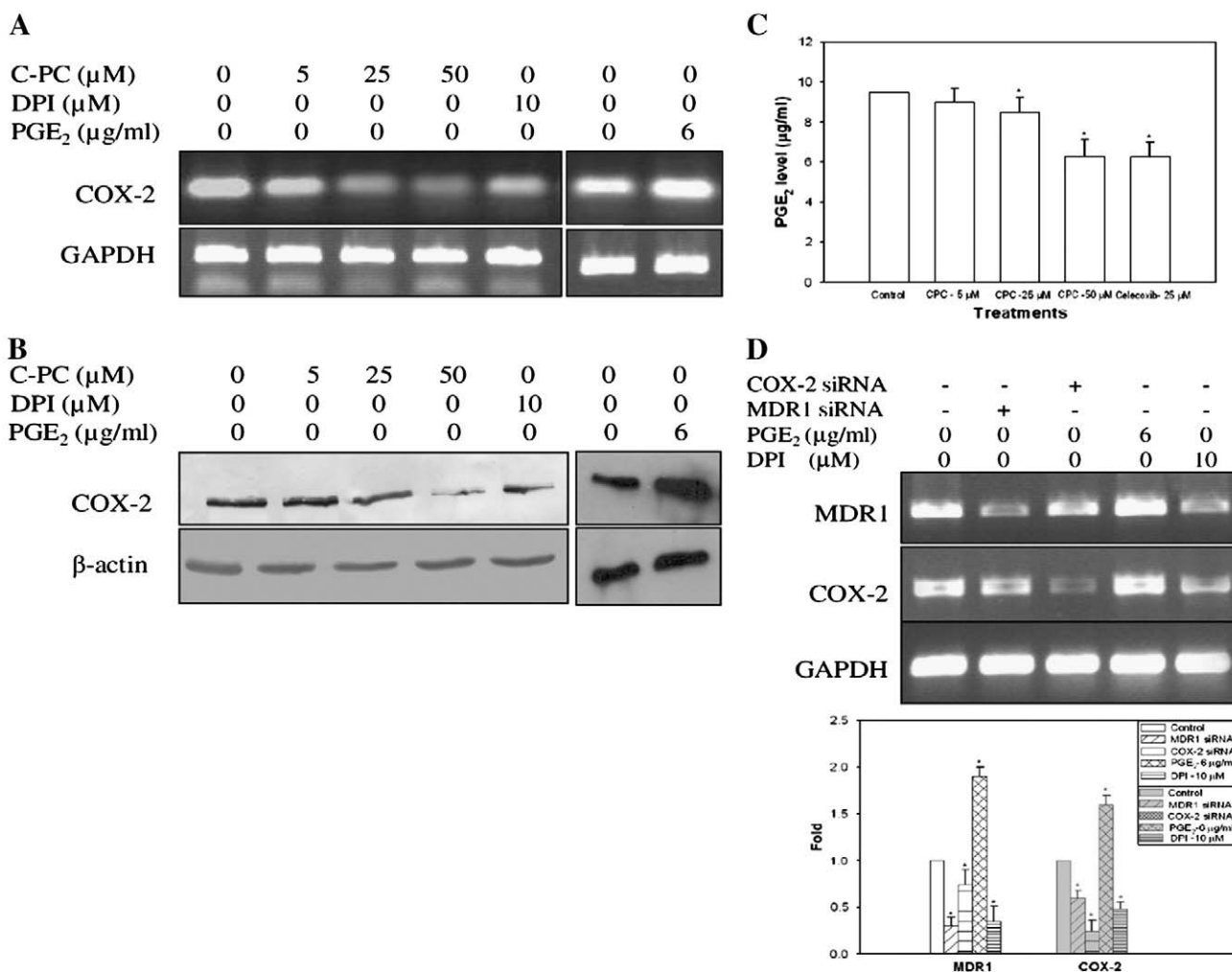


Fig. 6. Effect of C-PC on COX-2 expression and PGE₂ release, and effect of COX-2 knockdown on MDR1 expression in HepG2 cells. (A) RT-PCR analysis of C-PC, DPI and PGE₂ mediated effect on COX-2 mRNA expression in HepG2 cells. GAPDH was used as an internal control. (B) Western blot analysis of C-PC, DPI and PGE₂ mediated effect on COX-2 protein expression in HepG2 cells. β -actin was used as an internal control to monitor equal loading. (C) Bar graphs showing the difference in the release of PGE₂ in HepG2 cells upon treatment with C-PC (5 μM , 25 μM and 50 μM), DPI (10 μM) and celecoxib (10 μM). * Denotes statistical significance over control ($P < 0.05$). (D) RT-PCR analysis of COX-2 and MDR1 knockdown and DPI-PGE₂ mediated effect on MDR1 and COX-2 mRNA expression in HepG2 cells.

primary hepatocytes (shown in supplementary data-S1). The levels of reactive oxygen species (Fig. S1A) and expression of COX-2 and MDR1 (Fig. S1B) were minimal or at basal level in primary hepatocytes, compared to those in HepG2 cells.

3.7. C-PC induced down regulation of MDR1 expression is mediated by signal transduction pathways involving Akt/NF- κ B and MAPK/AP-1

As NF- κ B and AP-1 are positive regulators of MDR1 expression and Akt and MAP Kinase pathways are downstream targets, we examined the effect of C-PC, DPI and PGE₂. Nuclear levels of NF- κ B and AP-1 were reduced in a dose dependent manner in HepG2 cells treated with C-PC (5, 25 and 50 μM) for 12 h. Treatment with DPI (10 μM) and PGE₂ (6 $\mu\text{g/ml}$) for 6 h, on the other hand, showed opposing effects with a decrease in the levels of NF- κ B (Fig. 7A) and AP-1 (Fig. 7B) with DPI treatment and an increase with PGE₂ treatment in HepG2 cells. Further, in support of above data, the decreased activity and expression of p65, active subunit of NF- κ B (Fig. S2A) and phosphorylated active subunit (p-c-Jun) of AP-1 (Fig. S2B), were recorded upon treatment of HepG2 cells with C-PC, DPI. PGE₂ treatment, on the other hand, increased the same (shown in supplementary data-S2).

Similarly, a concentration dependent decrease in phosphorylated levels of Akt, JNK, ERK and p38 was observed with C-PC treatment. In the same way, HepG2 cells treated with DPI decreased the levels of the

above. PGE₂ treatment, on the other hand, increased the phosphorylated levels of these proteins (Fig. 8).

4. Discussion

The multidrug transporter P-glycoprotein, encoded by the MDR1, has long been recognized as one of the major causes for the acquisition of the multidrug resistant phenotype of cancer cells (Gillet et al., 2007). There are many studies to overcome MDR by inhibiting MDR transporters, to suppress or circumvent MDR mechanisms (Sharom et al., 1999; Sikić, 1999; Tan et al., 2000). In the last two decades, search for effective and clinically applicable MDR therapies took place to clarify the mechanisms underlying MDR and to develop agents to overcome drug resistance. Recent studies indicate that the activation of the reactive oxygen species and cyclooxygenase system might be critical event in the development of MDR1 mediated drug resistance (Roy et al., 2007a, 2010; Patel et al., 2002). Many studies suggest that the expression of vital multidrug transporters is inducible by oxidative stress (Kuo, 2009) and a number of synthetic drugs used in cancer chemotherapy were known to induce oxidative stress by generation of reactive oxygen species. The intrinsic drug resistance has been found in various solid tumors, particularly in liver cancers, where MDR1 up regulation is frequently observed, suggesting that oxidative stress plays an important role in hepatocarcinogenesis. Conversely, COX-2 also has

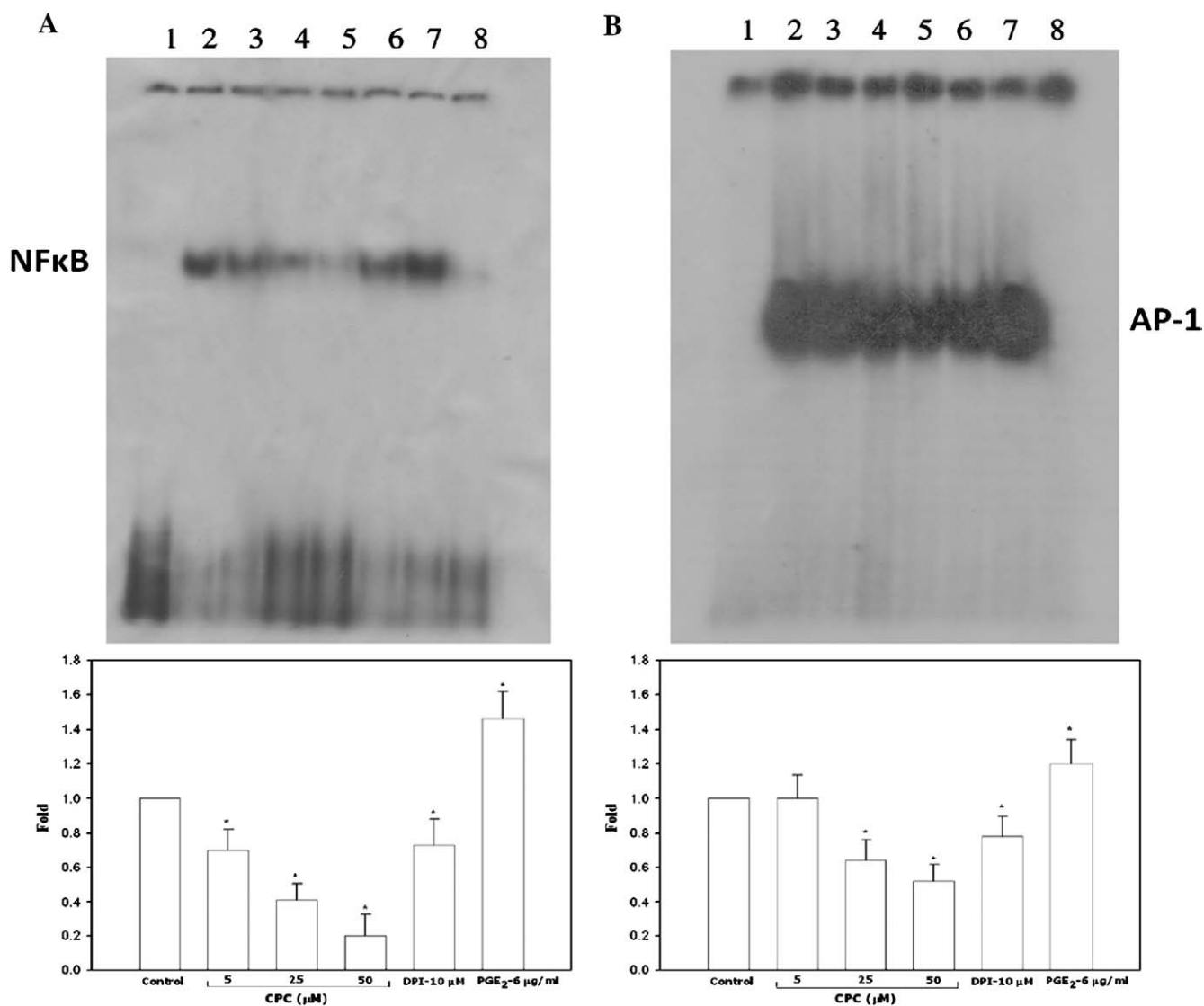


Fig. 7. Effect of C-PC on nuclear translocation of NF- κ B and AP-1 in HepG2 cells. Electro mobility shift assay of nuclear extracts using specific NF- κ B (Fig. A) and AP-1 (Fig. B) binding probe in cells treated with C-PC, DPI and PGE₂. Lane 1: Free probe; Lane 2: Control; Lane 3: C-PC (5 μ M) 12 h; Lane 4: C-PC (25 μ M) 12 h; Lane 5: C-PC (50 μ M) 12 h; Lane 6: DPI (10 μ M) 6 h; Lane 7: PGE₂ (6 μ g/ml) 6 h; Lane 8: Cold competition. Bar graph represents densitometric values of NF- κ B and AP-1 levels. * Denotes statistical significance over control ($P < 0.05$).

been reported to play a key role in the regulation of MDR. A recent study has shown the use of COX-2 inhibitors in overcoming the p-glycoprotein mediated drug resistance in epileptic brain of rats (Van Vliet et al., 2010). Recently we have shown that celecoxib, a selective COX-2 inhibitor, enhanced the accumulation of doxorubicin and down regulation of MDR1 in HepG2 cells in dose dependent manner (Roy et al., 2010).

Many synthetic MDR modulators like reversins 121 and 205 (Sharom et al., 1999) and the cyclosporin D analog Valspodar (PSC 833) (Sikic, 1999) effectively reverse the MDR phenotype *in vitro*. However, the efficacy of these compounds in animal studies and clinical trials has been unsatisfactory due to dose restrictive toxicity. In view of that, there has been an increased interest in the use of natural compounds for the treatment of cancer patients, who constitutively express P-glycoprotein and are resistant to many synthetic chemotherapeutic agents. Although their current use as drugs is at nominal, with the rise of undesirable side effects of synthetic chemotherapeutic drugs, research on natural products is being promoted to combat MDR. Drugs derived from natural products constitute a wide range, from antibiotics and antioxidants to anti-cancer agents. It has been reported that natural products from curcumin, rosemary, *Mangifera Indica* and *Anrodia*

camphorate, well known for their antioxidant and anti inflammogenic properties, have been proven as effective MDR modulators (Anuchapreeda et al., 2002; Plouzek et al., 1999; Chieli et al., 2009; Chang et al., 2008). In view of this background we have selected C-Phycocyanin, the main component of *S. platensis*, for the present study. C-Phycocyanin is extensively used in various medicinal applications, due to its antioxidant (Wang et al., 2007), anti-inflammatory (Romay et al., 1998) and pharmacological (Nagaoka et al., 2005) activities. Earlier investigations from our laboratory indicate that C-PC is a selective COX-2 inhibitor with anti-inflammatory and anti-cancer properties (Reddy et al., 2000, 2003; Roy et al., 2007b; Sathyaikumar et al., 2007; Subhashini et al., 2004; Pardhasaradhi et al., 2003). C-PC has also shown much promise in the prevention of hepatocarcinogen induced oxidative stress and multidrug resistance (Roy et al., 2007a, 2008). The present study demonstrates the possibility of using C-PC in cancer chemotherapy to overcome MDR.

In the present study treatment of HepG2 cells with C-PC showed an increase in the accumulation of doxorubicin in a concentration dependent manner. As a result of this, the sensitivity of HepG2 cells to doxorubicin was enhanced by 5 folds. Similar increase in the accumulation of rhodamine 123 and doxorubicin with other natural antioxidants was shown earlier in human cervical and breast cancer

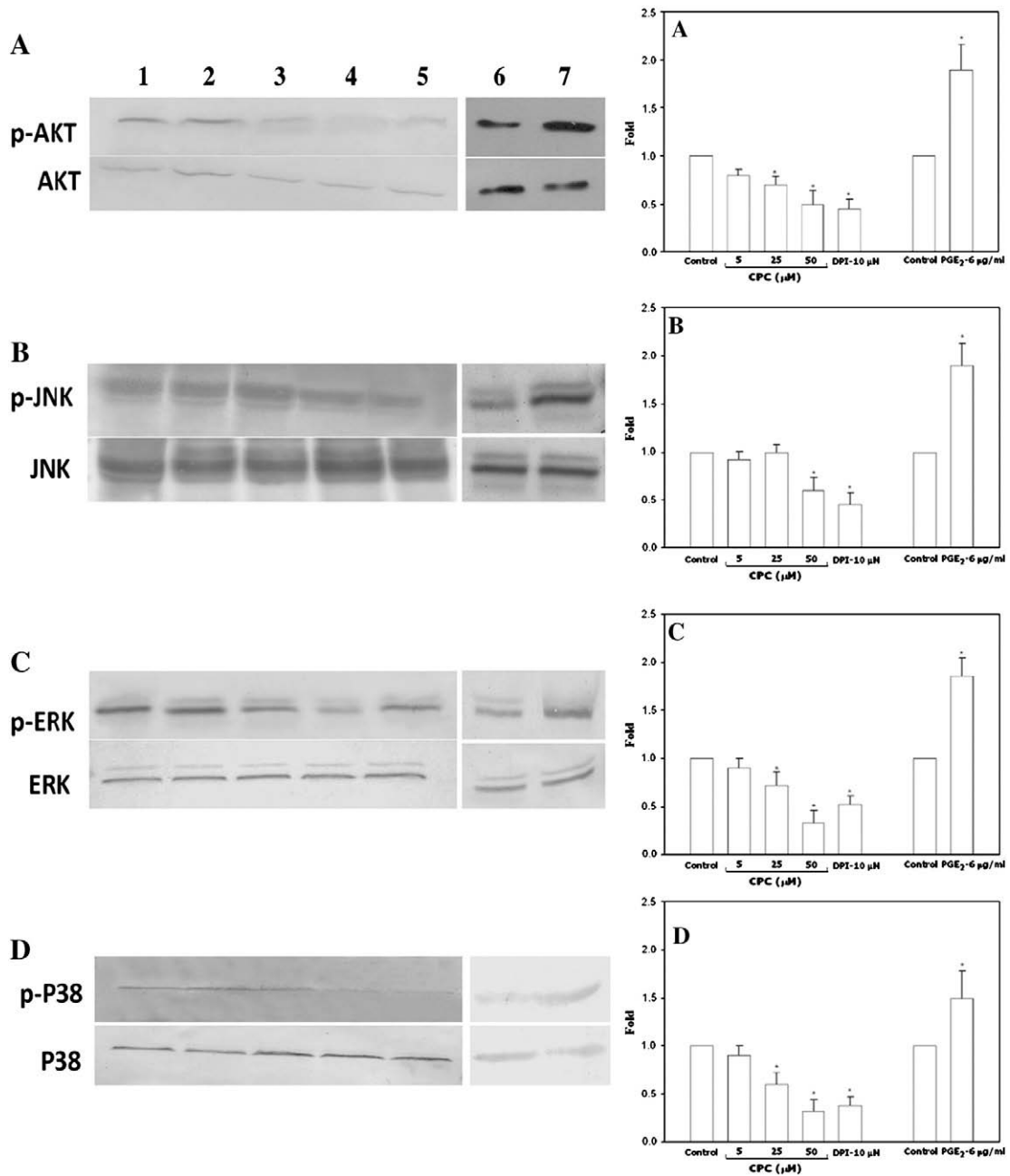


Fig. 8. Effect of C-PC on phosphorylation of Akt, JNK, ERK and p38 in HepG2 cells. Cells were treated with C-PC (5, 25 and 50 μM), DPI (10 μM) and PGE₂ (6 $\mu\text{g}/\text{ml}$) for 24 h. Lane 1: Control; Lane 2: C-PC (5 μM); Lane 3: C-PC (25 μM); Lane 4: C-PC (50 μM); Lane 5: DPI (10 μM); Lane 6: control; Lane 7: PGE₂ (6 $\mu\text{g}/\text{ml}$); Aliquots of cell lysates were resolved by SDS-PAGE and analyzed for Akt, p-Akt, JNK, p-JNK, ERK, p-ERK, P38 and p-P38 protein expression by western blotting. Bar graph represents densitometric values of respective protein levels. * Denotes statistical significance over control ($P < 0.05$).

cell lines (Plouzek et al., 1999; Anuchapreeda et al., 2002). The present study demonstrates a dose dependent down regulation of MDR1 expression in HepG2 cells, when treated with C-PC. This down regulation of MDR1 by C-PC appears to be dependent on reactive oxygen species and COX-2. While DPI, a known NADPH oxidase inhibitor, decreased the expression of MDR1, PGE₂, a product of COX-2, up regulated MDR1 expression, both at mRNA and protein levels. This data confirms the role of reactive oxygen species and COX-2 in MDR1 regulation. Similar down regulation of MDR1 by DPI and up regulation by PGE₂ was reported in macrophages and hepatocellular carcinoma cells, respectively (Roy et al., 2007a, 2010; Patel et al., 2002). Additionally, the specific role of COX-2 in MDR1 expression was further confirmed by COX-2 knockdown experiments.

The signaling pathways that govern cell proliferation, survival and oncogenesis are of prime concern in cancers. Most of the signals for cell survival trigger growth factor receptors which activate MAPK and PI3K-Akt pathways and promote cell growth (Hu et al., 2005). Given the role of MAPK and Akt in the development of multi drug resistance (Guan et al., 2004; Huang and Hung, 2009), the effect of C-PC on these signaling mediators was checked. The data presented here show that C-PC and DPI inhibit MDR1 in parallel with the inactivation of MAPK and Akt pathways in HepG2 cells. The treatments with PGE₂, on the other hand, increased the MAPK and Akt levels, followed by the induction of MDR1. These MAP kinases are known to activate c-fos and c-jun, promoting the formation of AP-1. Activation of Akt is known to stimulate IKK α that ultimately leads to the increase in the translocation of NF- κ B from

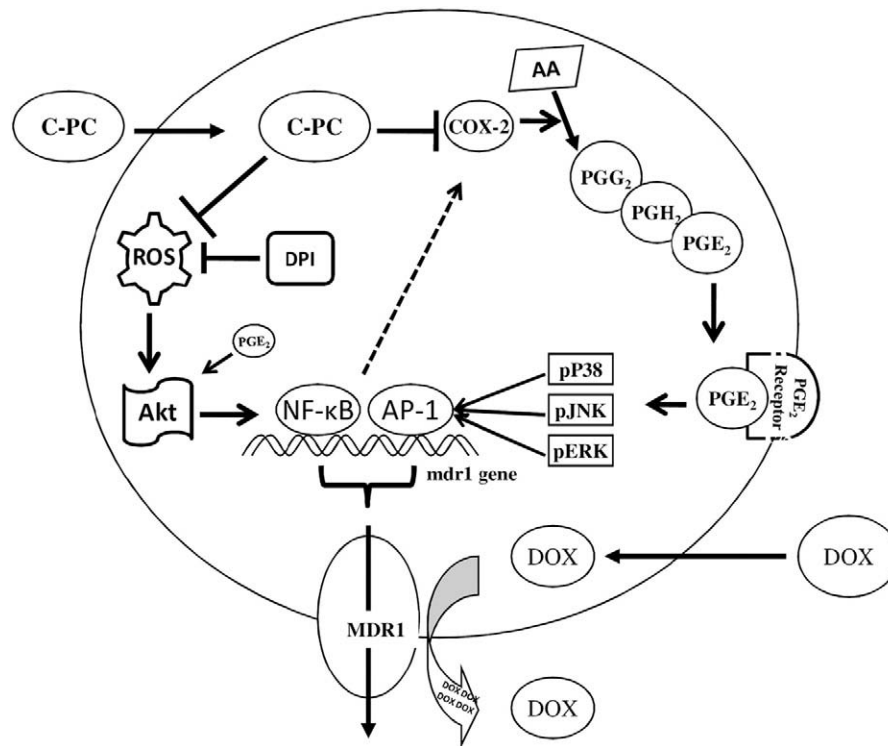


Fig. 9. Schematic representation showing the proposed mechanism involved in the regulation of MDR1 expression by reactive oxygen species and COX-2 and site of interference by C-Phycocyanin.

cytoplasm to nucleus. The observed decrease in the reactive oxygen species, COX-2, phosphorylation of ERK, p38, JNK and Akt in HepG2 cells treated with C-PC may have effect on the translocation of NF-κB and AP-1 to the nucleus. Hence further studies were taken up on NF-κB and AP-1, the key MDR1 regulators. Treatment of HepG2 cells with C-PC reduced the nuclear levels of NF-κB and AP-1 in a concentration dependent manner and similar down regulation was observed with the treatment of DPI (10 μM). PGE₂ treatment, on the other side, showed an increase in the nuclear translocation of NF-κB and AP-1. Similar down regulation of MAPK/AP-1 and Akt/NF-κB activity in MDR expressing cancer cell lines by natural antioxidants with anti-inflammatory properties was reported (Gopalakrishnan et al., 2006; Choi et al., 2008).

In summary, present study unravels the signaling pathways involved in the reactive oxygen species and COX-2 dependent regulation of MDR1. The experimental data presented in this study strongly suggest that C-PC regulates the expression of MDR1 in a reactive oxygen species and COX-2 dependent manner and potentiates the effects of doxorubicin in HepG2 cells. Further, NF-κB and AP-1 mediated signal transduction pathways involved in the regulation of MDR1 expression are inhibited by C-PC treatment. The regulation of MDR1 expression by reactive oxygen species and COX-2 and the site of interference by C-PC is shown in Fig. 9. In conclusion, the foregoing studies clearly demonstrate the role of reactive oxygen species/redox signaling and COX-2 in the development of MDR1 mediated drug resistance and usefulness of C-Phycocyanin in overcoming doxorubicin resistance in hepatocellular carcinoma cell line, HepG2.

Conflict of interest

The authors state no conflict of interest.

Acknowledgements

This work was supported by research grants from Council of Scientific and Industrial Research (CSIR) (Grant No. 37(1360)/08/EMR-II) and from Department of Biotechnology (Grant No. BT/PR-6106/

PID/20/237/05) Govt. of India. We duly acknowledge CSIR, for providing Senior Research Fellowship to Reddy P. Nishanth; Department of Science and Technology, for providing UOH-PURSE fellowship to Karnati R. Roy; University Grants Commission, New Delhi for providing Dr. D.S. Kothari Post Doctoral Fellowship to Gorla V. Reddy. We thank Dr. CM. Habibullah, Director, Centre for Liver Research & Diagnostics (CLRD), Deccan College of Medical Sciences, Hyderabad, India, for providing human primary hepatocytes for our studies.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ejphar.2010.09.011.

References

- Anuchapreeda, S., Leechanachai, P., Smith, M.M., Ambudkar, S.V., Limtrakul, P.N., 2002. Modulation of P-glycoprotein expression and function by curcumin in multidrug-resistant human KB cells. *Biochem. Pharmacol.* 64, 573–582.
- Bhargal, G., Halford, S., Wang, J., Roylance, R., Shah, R., Waxman, J., 2000. Expression of the multidrug resistance gene in human prostate cancer. *Urol. Oncol.* 5, 118–121.
- Bosch, F.X., Ribes, J., Cléries, R., Diaz, M., 2005. Epidemiology of hepatocellular carcinoma. *Clin. Liver Dis.* 9, 191–211.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
- Bradley, G., Sharma, R., Rajalakshmi, S., Ling, V., 1992. P-glycoprotein expression during tumor progression in the rat liver. *Cancer Res.* 52, 5154–5161.
- Campling, B.G., Pym, J., Galbraith, P.R., Cole, S.P., 1988. Use of the MTT assay for rapid determination of chemosensitivity of human leukemic blast cells. *Leuk. Res.* 12, 823–831.
- Chang, C.Y., Huang, Z.N., Yu, H.H., Chang, L.H., Li, S.L., Chen, Y.P., Lee, K.Y., Chuu, J.J., 2008. The adjuvant effects of *Antrodia Camphorata* extracts combined with anti-tumor agents on multidrug resistant human hepatoma cells. *J. Ethnopharmacol.* 118, 387–395.
- Chieli, E., Romiti, N., Rodeiro, I., Garrido, G., 2009. *In vitro* effects of *Mangifera indica* and polyphenols derived on ABCB1/P-glycoprotein activity. *Food Chem. Toxicol.* 47, 2703–2710.
- Choi, B.H., Kim, C.G., Lim, Y., Shin, S.Y., Lee, Y.H., 2008. Curcumin down-regulates the multidrug-resistance *mdr1b* gene by inhibiting the PI3K/Akt/NF kappa B pathway. *Cancer Lett.* 259, 111–118.

- Cordon-Cardo, C., O'Brien, J.P., Casals, D., Rittman-Grauer, L., Biedler, J.L., Melamed, M.R., Bertino, J.R., 1989. Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc. Natl. Acad. Sci. U. S. A.* 86, 695–698.
- Filipits, M., Suchomel, R.W., Dekan, G., Haider, K., Valdimarsson, G., Depisch, D., Pirker, R., 1996. MRP and MDR1 gene expression in primary breast carcinomas. *Clin. Cancer Res.* 2, 1231–1237.
- Fojo, A.T., Ueda, K., Slamon, D.J., Poplack, D.G., Gottesman, M.M., Pastan, I., 1987. Expression of a multidrug-resistance gene in human tumors and tissues. *Proc. Natl. Acad. Sci. U. S. A.* 84, 265–269.
- Gillet, J.P., Efferth, T., Remacle, J., 2007. Chemotherapy-induced resistance by ATP-binding cassette transporter genes. *Biochim. Biophys. Acta* 1775, 237–262.
- Gopalakrishnan, A., Xu, C.J., Nair, S.S., Chen, C., Hebbar, V., Kong, A.N., 2006. Modulation of activator protein-1 (AP-1) and MAPK pathway by flavonoids in human prostate cancer PC3 cells. *Arch. Pharm. Res.* 29, 633–644.
- Guan, J., Chen, X.P., Zhu, H., Luo, S.F., Cao, B., Ding, L., 2004. Involvement of extracellular signal-regulated kinase/mitogen-activated protein kinase pathway in multidrug-resistance induced by HBx in hepatoma cell line. *World J. Gastroenterol.* 10, 3522–3527.
- Hu, H., Jiang, C., Li, G., Lu, J., 2005. PKB / AKT and ERK regulation of caspase-mediated apoptosis by methylseleninic acid in LNCaP prostate cancer cells. *Carcinogenesis* 26, 1374–1381.
- Huang, W.C., Hung, M.C., 2009. Induction of Akt activity by chemotherapy confers acquired resistance. *J. Formos. Med. Assoc.* 108, 180–194.
- Huang, C., Wu, M., Xu, G., Li, D., Cheng, H., Tu, Z.X., Jiang, H.Q., Gu, J.R., 1992. Overexpression of the mdr1 gene and P-glycoprotein in human hepatocellular carcinoma. *J. Natl. Cancer Inst.* 84, 262–264.
- Juliano, R.L., Ling, V., 1976. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim. Biophys. Acta* 455, 152–162.
- Koga, H., Sakisaka, S., Ohishi, M., Kawaguchi, T., Taniguchi, E., Sasatomi, K., Harada, M., Kusaba, T., Tanaka, M., Kimura, R., Nakashima, Y., Nakashima, O., Kojiro, M., Kurohiji, T., Sata, M., 1999. Expression of Cyclooxygenase-2 in human hepatocellular carcinoma: relevance to tumor differentiation. *Hepatology* 29, 688–696.
- Kuo, M.T., 2009. Redox regulation of multidrug resistance in cancer chemotherapy: molecular mechanisms and therapeutic opportunities. *Antioxid. Redox Signal.* 11, 99–133.
- Nagaoka, S., Shimizu, K., Kaneko, H., Shibayama, F., Morikawa, K., Kanamaru, Y., Otsuka, A., Hirahashi, T., Kato, T., 2005. A novel protein C-phycocyanin plays a crucial role in the hypocholesterolemic action of *Spirulina platensis* concentrate in rats. *J. Nutr.* 135, 2425–2430.
- Nagasue, N., Dhar, D.K., Makino, Y., Yoshimura, H., Nakamura, T., 1995. Overexpression of P-glycoprotein in adenomatous hyperplasia of human liver with cirrhosis. *J. Hepatol.* 22, 197–201.
- Pardhasaradhi, B.V., Ali, A.M., Kumari, A.L., Reddanna, P., Khar, A., 2003. Phycocyanin-mediated apoptosis in AK-5 tumor cells involves down-regulation of Bcl-2 and generation of ROS. *Mol. Cancer Ther.* 2, 1165–1170.
- Parkin, D.M., Bray, F., Ferlay, J., Pisani, P., 2005. Global cancer statistics, 2002. *CA Cancer J. Clin.* 55, 74–108.
- Patel, V.A., Dunn, M.J., Sorokin, A., 2002. Regulation of MDR-1 (P-glycoprotein) by cyclooxygenase-2. *J. Biol. Chem.* 277, 38915–38920.
- Plouzek, C.A., Ciolino, H.P., Clarke, R., Yeh, G.C., 1999. Inhibition of P-glycoprotein activity and reversal of multidrug resistance *in vitro* by rosemary extract. *Eur. J. Cancer* 35, 1541–1545.
- Reddy, C.M., Bhat, V.B., Kiranmai, G., Reddy, M.N., Reddanna, P., Madyastha, K.M., 2000. Selective inhibition of cyclooxygenase-2 by C-phycocyanin, a biliprotein from *Spirulina platensis*. *Biochem. Biophys. Res. Commun.* 277, 599–603.
- Reddy, M.C., Subhashini, J., Mahipal, S.V., Bhat, V.B., Srinivas Reddy, P., Kiranmai, G., Madyastha, K.M., Reddanna, P., 2003. C-Phycocyanin, a selective cyclooxygenase-2 inhibitor, induces apoptosis in lipopolysaccharide-stimulated RAW 264.7 macrophages. *Biochem. Biophys. Res. Commun.* 304, 385–392.
- Romay, C., Ledón, N., González, R., 1998. Further studies on anti-inflammatory activity of phycocyanin in some animal models of inflammation. *Inflamm. Res.* 47, 334–338.
- Roy, K.R., Arunasree, K.M., Dooth, A., Aparna, R., Reddy, G.V., Vali, S., Reddanna, P., 2007a. C-Phycocyanin inhibits 2-acetylaminofluorene-induced expression of MDR1 in mouse macrophage cells: ROS mediated pathway determined via combination of experimental and In silico analysis. *Arch. Biochem. Biophys.* 459, 169–177.
- Roy, K.R., Arunasree, K.M., Reddy, N.P., Dheeraj, B., Reddy, G.V., Reddanna, P., 2007b. Alteration of mitochondrial membrane potential by *Spirulina platensis* C-phycocyanin induces apoptosis in the doxorubicin resistant human hepatocellular carcinoma cell line HepG2. *Biotechnol. Appl. Biochem.* 47, 159–167.
- Roy, K.R., Nishanth, R.P., Sreekanth, D., Reddy, G.V., Reddanna, P., 2008. C-Phycocyanin ameliorates 2-acetylaminofluorene induced oxidative stress and MDR1 expression in the liver of albino mice. *Hepatol. Res.* 5, 511–520.
- Roy, K.R., Reddy, G.V., Maitreyi, L., Agarwal, S., Achari, C., Vali, S., Reddanna, P., 2010. Celecoxib inhibits MDR1 expression through COX-2-dependent mechanism in human hepatocellular carcinoma (HepG2) cell line. *Cancer Chemother. Pharmacol.* 65, 903–911.
- Sathyasaikumar, K.V., Swapna, I., Reddy, P.V., Murthy, Ch.R., Roy, K.R., Dutta Gupta, A., Senthilkumaran, B., Reddanna, P., 2007. Co-administration of C-Phycocyanin ameliorates thioacetamide-induced hepatic encephalopathy in Wistar rats. *J. Neurol. Sci.* 252, 67–75.
- Sharom, F.J., Yu, X., Lu, P., Liu, R., Chu, J.W., Szabó, K., Müller, M., Hose, C.D., Monks, A., Váradi, A., Seprödi, J., Sarkadi, B., 1999. Interaction of the P-glycoprotein multidrug transporter (MDR1) with high affinity peptide chemosensitizers in isolated membranes, reconstituted systems, and intact cells. *Biochem. Pharmacol.* 58, 571–586.
- Sikic, B.L., 1999. Modulation of multidrug resistance: a paradigm for translational clinical research. *Oncology* 3, 183–187.
- Subhashini, J., Mahipal, S.V., Reddy, M.C., Mallikarjuna Reddy, M., Rachamalla, A., Reddanna, P., 2004. Molecular mechanisms in C-Phycocyanin induced apoptosis in human chronic myeloid leukemia cell line-K562. *Biochem. Pharmacol.* 68, 453–462.
- Szatrowski, T.P., Nathan, C.F., 1991. Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res.* 51, 794–798.
- Tan, B., Piwnicka-Worms, D., Ratner, L., 2000. Multidrug resistance transporters and modulation. *Curr. Opin. Oncol.* 5, 450–458.
- Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M.M., Pastan, I., Willingham, M.C., 1987. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc. Natl. Acad. Sci. U. S. A.* 84, 7735–7738.
- Van Vliet, E.A., Zibell, G., Pekcec, A., Schlichtiger, J., Edelbroek, P.M., Holtman, L., Aronica, E., Gorter, J.A., Potschka, H., 2010. COX-2 inhibition controls P-glycoprotein expression and promotes brain delivery of phenytoin in chronic epileptic rats. *Neuropharmacology* 58, 404–412.
- Vos, T.A., Hoiveld, G.J., Koning, H., Childs, S., Meijer, D.K., Moshage, H., Jansen, P.L., Muller, M., 1998. up regulation of the multidrug resistance genes, Mrp1 and Mdr1b, and down-regulation of the organic anion transporter, Mrp2, and the bile salt transporter, Sgpp, in endotoxemic rat liver. *Hepatology* 28, 1637–1644.
- Wang, H., Liu, Y., Gao, X., Carter, C.L., Liu, Z.R., 2007. The recombinant beta subunit of C-phycocyanin inhibits cell proliferation and induces apoptosis. *Cancer Lett.* 247, 150–158.
- Wartenberg, M., Ling, F.C., Schallenberg, M., Baumer, A.T., Petrat, K., Hescheler, J., Sauer, H., 2001. Down-regulation of intrinsic P-glycoprotein expression in multicellular prostate tumor spheroids by reactive oxygen species. *J. Biol. Chem.* 276, 17420–17428.