Cyclooxygenase-2 and Reactive Oxygen Species mediated regulation of MDR1 expression in HepG2 and RAW 246.7 cells: Experimental and *in silico* analysis

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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Enrolment No. 03LAPH06


Dedicated
to
my parents & sister

University of Hyderabad
(A Central University established in 1974 by act of parliament)
HYDERABAD- 500 046, INDIA

DECLARATION

I hereby declare that the work embodied in this thesis entitled "**Cyclooxygenase-2 and Reactive Oxygen Species mediated regulation of MDR1 expression in HepG2 and RAW 246.7 cells: Experimental and *in silico* analysis"** has been carried out by me under the supervision of Prof. P. Reddanna and this has not been submitted for any degree or diploma of any other university earlier.

Prof. P. Reddanna
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CERTIFICATE

This is to certify that **Mr. K. Raja Ram Mohan Roy** has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph.D. ordinance of this University. We recommend his thesis "**Cyclooxygenase-2 and Reactive Oxygen Species mediated regulation of MDR1 expression in HepG2 and RAW 246.7 cells: Experimental and *in silico* analysis"** for submission for the degree of Doctor of Philosophy of this University.

Prof. P. Reddanna _____
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…..✍ ***Roy***

CONTENTS

LIST OF FIGURES

ABBREVIATIONS

µM	**:**	**micro molar**
^{0}C	**:**	**degree centigrade/ degree celsius**
AA	**:**	**arachidonic acid**
ATP	**:**	**adenosine triphosphate**
COX	**:**	**cyclooxygenase**
DNA	**:**	**deoxy ribonucleic acid**
EDTA	**:**	**ethylene diamine tetra acetic acid**
FACS	**:**	**fluorescence activated cell sorter**
FBS	**:**	**fetal bovine serum**
g	**:**	**gram**
h	**:**	**hour(s)**
kDa	**:**	**kilodalton**
l	**:**	**litre**
mg	**:**	**milligram**
min	**:**	**minutes**
ml	**:**	**milliliter**
mM	**:**	**millimolar**
MTT	**:**	**3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide**
NBT	**:**	**nitroblue tetrazolium**
nm	**:**	**nanometers**
NSAIDs	**:**	**non-steroidal anti-inflammatory drugs**
OD	**:**	**optical density**
PAGE	**:**	**polyacrylamide gel electrophoresis**
PBS	**:**	**phosphate buffered saline**
PCR	**:**	**polymerase chain reaction**

SDS	**:**	**sodium dodecyl sulfate**
TEMED	**:**	**N,N,N',N'-tetramethylene diamine**
Tris	**:**	**tris-(Hydroxymethyl) aminoethane**
UV	**:**	**ultraviolet**
ROS	**:**	**reactive oxygen species**
SOD	**:**	**superoxide dismutase**
CuZnSOD	**:**	**copper, zinc-superoxide dismutase (SOD-1)**
MnSOD	**:**	**manganese-superoxide dismutase (SOD-2)**
FeSOD	**:**	**iron-superoxide dismutase**
ECSOD	**:**	**extracellular superoxide dismutase (SOD-3)**

I. Cancer

A major feature of all higher eukaryotes is the defined life span of the organism, a property that extends to the individual somatic cell, whose growth and division are highly regulated. A notable exception is provided by cancer cells, which arise as variants that have lost their usual growth control. These cancer cells form a mass of tissue called tumor. The cells in malignant (cancerous) tumors are abnormal and divide without control or order. They can invade and damage nearby tissues. Also, cancer cells can break away from a malignant tumor and spread to other parts of the body by a process called metastasis. Genetic alterations in two types of genes can contribute to the cancer process. Proto-oncogenes are normal genes that are involved in cell growth and division. Change in regulation of these genes lead to the development of oncogenes, which can promote excessive cell growth and division. Tumor suppressor genes are involved in controlling cell division. When tumor suppressor genes dysfunction, cells grow and divide abnormally, which leads to tumor growth. De regulation of genes or genetic changes that are not corrected by the cell can lead to the production of abnormal proteins. Damaged proteins may not respond to normal signals, may over-respond to normal signals, or otherwise fail to carry out their normal functions. These malfunctions of proteins lead to disruption of normal crosstalk

between the signaling components of cell division machinery. Normal cell growth and division are largely under the control of a network of chemical and molecular signals. Disruption of the signaling process results in abnormal growth and division of cells. This condition of abnormal growth and uncontrolled division of cells is called cancer, which is one of the major causes of death world wide, including India.

In India 9.5 % deaths are due to cancer and with an estimated increase to 14.7% by 2030 (Fig. 1).

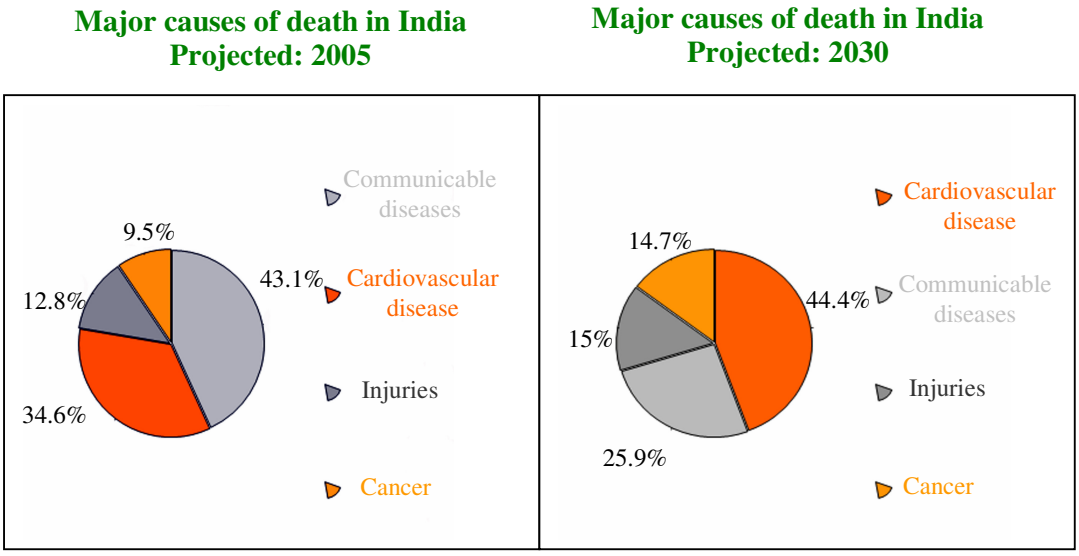


Fig. 1. Major causes of death in India (*Source: www.who.int*)

In 2005, 7.6 million people died of cancer out of 58 million deaths worldwide. More than 70% of all cancer deaths occur in low and middle income countries, where resources available for prevention, diagnosis and treatment of cancer are limited or nonexistent. Based on projections, cancer deaths will continue to rise with an estimated 9 million people dying from cancer in 2015, and 11.4 million dying in 2030.

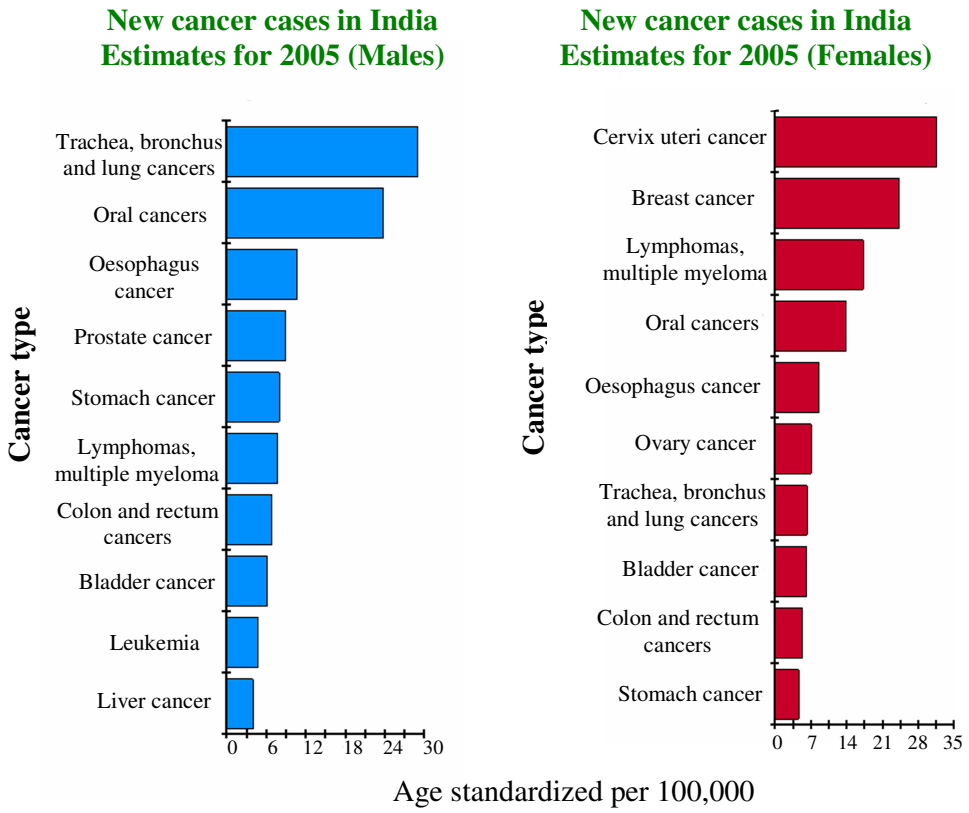


Fig. 2. Estimates of new cancer cases in India (*Source: www.who.int*)

In India, lung and oral cancers are most common types of cancer occurring in males where as uteri and breast cancer are common in females (Fig. 2).

II. Cancer types

Histologically there are hundreds of different cancers, which are grouped into five major categories: carcinoma, sarcoma, myeloma, leukemia, and lymphoma. In addition, there are also some cancers of mixed types.

Carcinoma refers cancer of epithelial origin or of the internal or external lining of the body. Carcinomas account for 80 to 90 percent of all cancer cases.

Sarcoma originates in supportive and connective tissues such as bone, tendon, cartilage, muscle, and fat. Sarcoma tumors usually resemble the tissue in which they grow.

Myeloma is cancer that originates in the plasma cells of bone marrow. Leukemias ("liquid cancers" or "blood cancers") are cancers of the bone marrow (the site of blood cell production).

Lymphomas develop in the glands or nodes of the lymphatic system, a network of vessels, nodes, and organs (specifically the spleen, tonsils, and thymus) that purify bodily fluids and produce infection-fighting white blood cells, or lymphocytes. Unlike the leukemias which are sometimes called "liquid

cancers," lymphomas are "solid cancers." Lymphomas may also occur in specific organs such as the stomach, breast or brain.

III. Cancer and treatment

Treatment for cancer may involve chemotherapy, radiation therapy, surgery, hormonal therapy, biological therapy or some combination of these. Chemotherapy is the use of anti-cancer drugs that destroy cancer cells by stopping growth or multiplication at some point in their life cycles. Chemotherapy is often given in cycles of alternating treatment and rest periods. Radiation therapy is with ionizing radiation, which destroys cells or the genetic material of cells in the area being treated, thereby making it impossible for these cells to grow. Surgery involves removal of the tumor. Sometimes, surrounding tissue and lymph nodes are also removed. Hormone therapy is the use of hormones, to change the way hormones help cancers to grow in the body. Biological therapy (Immunotherapy) makes use of the body's immune system, either directly or indirectly, to fight cancer. The most advanced forms of treatment may produce a 5-year survival rate of 75% or more for certain types of cancer, e.g. cancer of the uterine corpus, breast, testis, and melanoma. By contrast, survival rates in cancer of the pancreas, liver, stomach, and lung are generally less than 15%.

IV. Chemotherapy

Chemotherapy is the most effective and widely used form of cancer treatment till date. The most well studied and effective chemotherapy agents are Cisplatin, Doxorubicin, Etoposide, Hydroxyurea, Imitinab, Methotrexate, Paclitaxel and Vinblastine. Most anti-cancer drugs act by inhibiting DNA synthesis or some other process in the cell cycle. While chemotherapy can be quite effective in treating cancer, these agents do not differentiate normal healthy cells from cancer cells and as a result leading to various side effects. The way in which the other cells are affected determines the side-effects of the individual drugs. Other cells affected include blood cells, hair follicles and cells that line the digestive tract. As a result, side effects may include loss of hair, poor appetite, nausea and vomiting, diarrhea, or mouth and lip sores. Moreover, continuous exposure to these chemotherapeutic agents leads to the development of drug resistance, because of which the patients fail to respond to chemotherapy.

V. Chemotherapy and Drug resistance

Drug resistance in cells may be due to three major mechanisms: first, decreased uptake of drugs; second, various changes in the cells that effect the capacity of cytotoxic drugs to kill cells, including alterations in cell cycle, increased repair of DNA damage, reduced apoptosis and altered metabolism of

drugs; and third, increased energy-dependent efflux of drugs. Of these mechanisms, the one that is most commonly encountered in drug resistant conditions is the increased efflux of a broad class of cytotoxic drugs that is mediated by a family of energy dependent transporters, known as ATP-binding cassette (ABC) transporters.

VI. ABC Transporters

ATP binding cassette (ABC) family transporters are characterized by the presence of an ATP-binding site, ABC signature motif and requirement of ATP hydrolysis for their transport action. The human genome contains 48 genes that encode ABC transporters, which have been divided into seven sub families labeled A to G. Diverse substrates are translocated by ABC transporters, ranging from chemotherapeutic drugs to naturally occurring biological compounds. Although several members of the transporters family have dedicated functions involving the transport of substrates, it is becoming evident that the complex physiological network of ABC transporters has a pivotal role to play in host detoxification and protection of body against xenobiotics. This role is revealed by the tissue distribution of ABC transporters, which are highly expressed in pharmacological barriers, such as the brush border membrane of intestinal cells,

the biliary canalicular membrane of hepatocytes, the luminal membrane in the proximal tubules of the kidney and the epithelium of the blood-brain barrier.

VII. MDR1

Among mammalian ABC transporters, P-glycoprotein (P-gp) family and multidrug resistance associated protein (MRP) family have a major role in drug transport. They can generate profound drug resistance through reduced accumulation of substrates (drugs). P-glycoprotein family consists of two classes: Class I (MDR1 in humans, MDR1a and MDR1b in rodents) and class II (MDR 2 or 3 in humans and MDR 2 in rodents). Class I P-gps are present in various normal tissues such as liver, brain, kidney and intestine, where as class II P-gps are essentially expressed in the liver on the canicular membrane of hepatocytes.

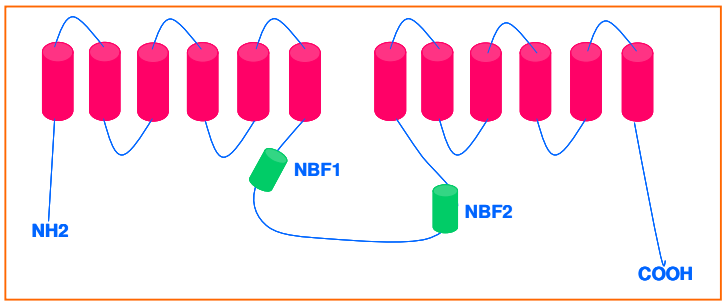


Fig. 3. Domain organization of MDR1

(NBF- Nuclear binding fold; Barrels indicate twelve transmembrane domains)

MRP family consists of at least six members, known as MRP1-MRP6. Typically MRP's are larger (190-200 kDa) than P-gp (170 kDa), containing 250 additional amino acids at the amino - terminal region. MDR1 contains twelve transmembrane domains and two nucleotide binding domains.

Expression of multidrug resistance genes seen in most of the organs that have a role in absorption and elimination, signifies their role in the efflux of drugs and xenobiotics. Expression of MDR1 was shown in the apical membranes of the liver, kidney, gut and at the blood-brain barrier (Thiebaut *et al.,* 1987; Cordo-nCardo *et al.,* 1989). MDR1 and MDR3 mRNA expression was observed in the gastrointestinal tract, cerebral cortex, cerebellum, kidney, lung, and liver of rodents (Brady *et al.,* 2002). Transcripts of multidrug resistance genes were observed in a wide variety of conditions and malignancies. Over expression of MDR1 mRNA in isolated hepatocytes of endotoxin-treated rats (Vos *et al.,* 1998) and in adenocarcinomas derived from adrenal, kidney, liver and bowel (Fojo *et al.,* 1987), breast (Filipits *et al.,* 1996) and in prostate (Bhangal *et al.,* 2000) was observed.

Multidrug resistance is the primary impediment in the cancer chemotherapy. MDR1 transports drugs that are central to most chemotherapeutic regimens, including doxorubicin, daunorubicin, vincristine, actinomycin-D,

paclitaxel, docetaxel, etoposide, teniposide, bisantrene etc. Due to wide range of drugs transported by MDR1, the expression of MDR1 is one of the major factors explaining chemotherapy failure in cancer patients.

VIII. MDR1 inhibitors

Due to the significance of MDR1 inhibition in successful chemotherapy, a plethora of agents have been developed that modify, modulate, or reverse the MDR phenotype. Many natural and synthetic products of various structures, including calcium channel blockers [e.g., verapamil, nifedipine], calmodulin antagonists (e.g., trifluoperazine, chlorpromazine), various steroids (e.g., progesterone, tamoxifen), quinolines (e.g., chloroquine, quinidine), immunosuppressive drugs (e.g., cyclosporine, rapamycin), antibiotics (e.g., rifapicin, tetracyclines), surfactants (e.g., Tween 80, Cremophor-EL), and alkaloids (e.g., reserpine, yohimbine) have been shown to block the function of MDR1. New, more potent MDR1 inhibitors such as PSC388, GF120918, dexverapamil and XR9576 are now being evaluated in clinical trials. In most cases MDR reversal agent may expose the patient to unacceptable side effects or toxicity at doses required for effectiveness (Ross *et al.,* 1994; Malayeri *et al.,* 1996). These limitations have spurred efforts to search for new approaches and more effective compounds.

IX. Regulation of MDR1 expression

The complex regulation of MDR1, the important member of ABC drug transporters, has been studied to an extent but our understanding of the MDR1 transcription may still be in its infancy. Recently it became evident that the altered expression of several growth and death controlling proteins can adversely affect drug therapy. The first evidence that tumor suppressor protein could influence the expression of drug resistance gene came from the observation that wild type p53 repressed the transcription of MDR1 gene (Chin *et al.,* 1992). Elevated levels of c-Fos have been demonstrated in a number of drug resistant cell lines when compared to their drug-sensitive counterparts (Bhushan *et al.,* 1992). The MDR1 gene also is target of the ras/raf-signaling pathway. Given the role of MDR1 in the protection against environmental adversity, MDR1 is highly responsive to stress signals. MDR1 inducers include heat shock, cytokines, oxygen free radicals, partial hepatectomy, inflammation, exposure to carcinogens including chemotherapeutics, hypoxia and UV and X- irradiation. In many cell lines and metastatic sarcomas it is generally accepted that MDR1 expression is increased through the upregulation of MDR1 mRNA levels (Abolhoda *et al.,* 1999). Several transcription factors including, GC, HSF1, Sp1, AP-1, NF-IL6, NF-Y, NF-κB, EGR1, YB-1 and MEF-1 up-regulate MDR1 gene transcription.

To date, efforts to combat the overexpression of MDR1 have involved the use of functional modulators or reversal agents that block the MDR1 mediated efflux of anti cancer drugs. Intervention to prevent the transcription of drug transporters rather than block their function subsequent to their overexpression is important. Nevertheless studies on the transcription of MDR1 may provide a therapeutic target in our quest to prevent resistance to cancer therapeutics.

Among many different regulators of MDR1 transcription, Reactive Oxygen Species (ROS) and Cyclooxygenase-2 (COX-2) were shown to be key players, but the mechanisms underlying are not well elucidated. Using experimental and *in silico* analysis, an attempt was made in the present study to elucidate the role of ROS and COX-2 in the regulation of MDR1 expression and study effect of antioxidant (C-Phycocyanin) and COX-2 inhibitor (Celecoxib) treatment on the regulation of MDR1 expression.

CHAPTER - 1

(COX-2 and Multidrug resistance)

1.1. Introduction

1.1.1. Cyclooxygenases

Cyclooxygenase (COX) or prostaglandin H_2 synthase (PGHS) is the enzyme that catalyzes the biosynthesis of prostaglandins (PGs) from the substrate arachidonic acid (AA). Cyclooxygenase is a membrane-bound enzyme catalyzing the first two steps in prostaglandin (PG), thromboxane and prostacyclin biosynthesis. The stress and cytokine-inducible COX-2 isoform and constitutively expressed COX-1 isoform, catalyze the reactive oxygen species-dependent conversion of arachidonic acid into prostaglandin H_2 (PGH_2), which is subsequently converted to prostanoids, including prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2) and prostaglandin $F_2\alpha$ ($PGF_{2\alpha}$), thromboxanes and prostacyclin (Marnett *et al.,* 1999). Both COX-1 and COX-2 are homodimeric, glucosylated, heme containing proteins with two catalytic sites. Both isoforms have high structural identity but are different in substrate and inhibitor selectivity (Smith *et al.,* 1996), also in their intracellular localization. Unlike COX-1 enzyme, COX-2 has valine at position 523 instead of isolueucine. The difference between valine and isoleucine is of a single methyl group. This substitution allows COX-2 inhibitors to access the secondary internal side pocket of the

molecule that is obstructed by isoleucine in the COX-1 isoform (Kurumbail *et al.,* 1996).

COX-1 is constitutively expressed in most cells at physiological conditions, although there is mild increase (2-4 fold) in response to hormonal or growth factor stimulation. Unlike COX-1, COX-2 expression is minimal in most tissues under basal conditions, but is dramatically up regulated upto 80 fold in inflamed tissues with cytokines, growth factors, phorbol esters, bacterial lipopolysaccharides (LPS), ligands of G-protein-coupled receptors and reactive oxygen intermediaries. COX-2 gene features are those of an 'immediate early gene' that is not always present but is highly upregulated during inflammation or pathological processes. Initially it was thought that COX-2 was not constitutively expressed in any tissue. However, recent work has demonstrated constitutive expression of COX-2 in a variety of non-inflammatory tissues, including kidney, brain, pancreatic islets, bone, testis, tracheal epithelium and ovary (DuBios *et al.,* 1998; Neeraja *et al.,* 2003).

1.1.2. COX-2 and Cancer

Several studies have demonstrated elevated expression of COX-2 but not COX-1, in different types of human cancer, suggesting the presence of COX-2

correlates with cancer development (Eberhart *et al.,* 1994; Ristimaki *et al.,* 1997; Zimmermann *et al.,* 1999; Wolff *et al.,* 1998; Tucker *et al.,* 1999). COX-2 expression in colon cancer cells has been found to promote angiogenesis of co-cultured endothelial cells by stimulating the production of angiogenic factors (Tsujii *et al.,* 1998). Human gastric and breast tumors express higher levels of COX-2 than the surrounding normal tissue (Brown and Lippman, 2000). The importance of PGs in tumorigenesis is evidenced by the data demonstrating the ability of NSAIDs to inhibit growth and metastasis of tumors *in vivo* (Snyderman *et al.,* 1995) and mostly of colon cancer (Rao *et al.,* 1991; Sheng *et al.,* 1997).

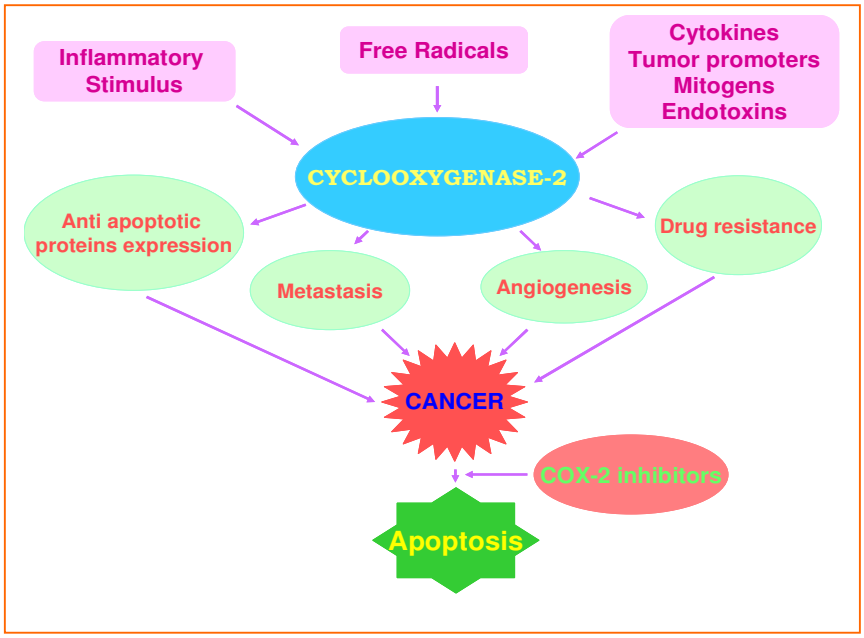


Fig. 4. Role of Cyclooxygenase-2 in Cancer

COX-2 overexpression in tumor cells seems to be associated with increase in angiogenesis, tumor invasion and suppression of host immunity. Recent studies indicated COX-2 overexpression in several malignancies such as colorectal and breast cancers (Denkert *et al.,* 2003) and also leukemia (Nakanishi *et al.,* 2001). High levels of prostaglandins, possibly reflecting the overexpression of the COX-2 enzyme and playing important roles in proliferation and differentiation of various cancer cell lines, have been detected in different tumor types (Sheng *et al.,* 2001). Inflammation causes an increased synthesis of COX-2 dependent prostaglandins and specific COX-2 inhibitors have emerged as important pharmacological tools for treatment of pain and arthritis.

1.1.3. NSAIDs

In 1860, salicylic acid was chemically synthesized and used as external antiseptic, antipyretic and in the treatment of rheumatism. In 1899, Felix Hoffman synthesized acetyl salicylic acid or aspirin. After the development of aspirin numerous other drugs were discovered and used as a group and were known as "aspirin like drugs". As these drugs were clearly distinct from steroidal glucocorticoids, they were categorized as nonsteroidal anti-inflammatory drugs (NSAIDs). NSAIDs are a heterogeneous group of chemically unrelated

compounds with similar therapeutic effects i.e. anti-inflammatory, anti-pyretic, analgesic and anti-thrombotic properties. John Vane elucidated the mechanism of action of NSAIDs in 1971 by demonstrating that NSAIDs inhibit the prostaglandin biosynthesis *via* inhibition of cyclooxygenase enzyme.

1.1.4. COX-2 inhibitors

Drugs that demonstrate consistent COX-2 inhibition but have no effect on COX-1 throughout their dosage ranges are called COX-2 selective inhibitors (Dewitt *et al.,* 1993). Structure activity studies indicated that a cis-stilbene moiety containing a 4-methylsulfonyl or sulfonamide substitution in one of the pendent phenyl rings is required for COX-2 specificity (Talley *et al.,* 1999). Computer aided drug design (CADD) is being used to design highly selective COX-2 inhibitors (Reddy *et al.,* 2007). Highly selective COX-2 inhibitors, with greater than 1000 fold selectivity for COX-2 over COX-1 have been developed and are at various stages of development. Celecoxib and Rofecoxib are two such agents and are now clinically available in many countries.

1.1.5. Celecoxib

Celecoxib (Celebrex also known as SC-58635) (4-[5-(4-methylphenyl)-3trifluoromethyl} 1 H-pyrazol-1-yl) benzene sulfonamide) is a 1, 5 substituted pyrazole (Fig. 4). The empirical formula for celecoxib is $C_{17}H_{14}F_3N_3O_2S$ (MW 381.38). This was the first COX-2 inhibitor approved for the use in U.S, for relief of signs and symptoms of rheumatoid arthritis and osteoporosis in adults. In addition to analgesic, antipyretic and anti-inflammatory activity, it has chemopreventive properties against colon cancer.

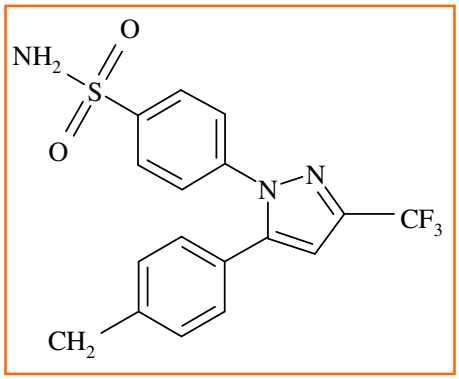


Fig. 5. Chemical structure of celecoxib

1.1.6. COX-2 and multidrug resistance

Epidemiological studies have shown that individuals' regularly taking asprin or other NSAIDs have reduced incidence of colon cancer than those that do not (Dubois *et al.,* 1998). In the intestinal epithelial cells COX-2 expression correlated well with the resistance to apoptosis (Tsujii *et al.,* 1995). Inhibition of COX-2 activity with specific inhibitors in medullary intestinal cells increased their susceptibility towards apoptosis (Hao *et al.,* 1999). COX-2 selective inhibitors have induced apoptosis in colon, prostate, leukemia and esophagus cancer cell lines (Hale *et al.,* 1996; Hsu *et al.,* 2000; Subhashini *et al.,* 2005; Li *et al.,* 2001). Increased synthesis of PG was shown to be associated with poor disease prognosis in humans (Khan *et al.,* 1982).

Intrinsic up-regulation of MDR1 gene expression has been observed in untreated hepatocellular carcinoma of human and rodent origin, associated with primary resistance to certain antineoplastic compounds (Bradley *et al.,* 1992; Huang *et al.,* 1992). Also MDR1 gene expression was observed during inflammation-associated processes such as cholestasis (Schrenk *et al.,* 1993) and during liver regeneration (Arino *et al.,* 1990). However, hepatic mechanisms and mediators of intrinsic MDR1 gene activation are poorly understood. Simultaneous over expression of the heme protein COX-2 and MDR1 reported in the

regenerative nodules of cirrhotic livers and in well-differentiated hepatocellular carcinoma (Nagasue *et al.,* 1995; Koga *et al.,* 1999) suggests a possible correlation between the two.

Similar regulation of MDR1 by COX-2 was recently reported in human breast tumors through immunohistochemical analysis. In drug resistant cell lines that over-express MDR1 also there was significant expression of COX-2 (Ratnasinghe *et al.,* 2001). Recently it was shown that human multidrug resistance protein MRP4 function as a prostaglandin efflux transporter and is inhibited by non-steroidal anti-inflammatory drugs (Reid *et al.,* 2003). In another study it was shown that regulation of MDR1 expression was dependent on COX-2 activity (Vimal *et al.,* 2002). Similarly role of NSAIDs to enhance the cytotoxic effects of doxorubicin and vincristine was reported in T98G human malignant glioma cells (Roller *et al.,* 1999). These studies indicate a possible role for COX-2 in multidrug resistance and the use of COX-2 inhibitors to enhance the anti tumor activity of cancer chemotherapeutic agents.

Recent studies have demonstrated overexpression of COX-2 in regenerative nodules of cirrhotic livers and in well-differentiated hepatocellular carcinoma (Koga *et al.,* 1999; Shiota *et al.,* 1999). The endogenous overexpression of MDR1 was also characterized in these pathological conditions

(Huang *et al.,* 1992; Nagasue *et al.,* 1995; Takanishi *et al.,* 1997). Parallel upregulation of COX-2 and MDR1 during pathophysiological changes and chemopreventive effects of COX-2 inhibitors suggest that the activation of the cyclooxygenase system might be critical event in the development of MDR1 mediated drug resistance. To date only few studies (Clinical studies) were taken up to study the correlation between multidrug resistance phenotype and COX-2 expression.

With this background, a study was taken on HepG2 cells to unravel the link between COX-2 and MDR1 with the following specific objectives:

1.1.7. Objectives:

- To study the association between COX-2 and multidrug resistance.
- To understand the molecular mechanisms involved in the regulation of MDR1 expression by COX-2.

1.2. Materials and methods

1.2.1. Chemicals

PBS, RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco BRL (California, USA). Nitrocellulose membrane was from Millipore (Bangalore, India). TMB/H_2O_2, phosphatase inhibitor cocktail 1 and 2 and β-actin antibodies were purchased from Sigma-Aldrich (Bangalore, India). Polyclonal antibodies to MDR1 and monoclonal antibodies to JNK and p-JNK were from Santa Cruz Biotechnology (California, USA). Single step RT-PCR kit was from AB Gene Technologies (Surrey, UK). Monoclonal COX-2 anti body, PGE_2 estimation kit and PGE_2 were from Cayman Chemical Co. USA. Celecoxib was a generous gift from Unichem Laboratories, India. Doxorubicin was a generous gift from Dabur Pharma, India. All the other chemicals and reagents were purchased from local companies and are of molecular biology grade.

1.2.2. HepG2 cell line

These are human hepatocellular carcinoma cells. They are adherent in nature and grow as clumps. Their origin is from hepatocytes. The hepatocellular carcinoma cells express both COX-2 and MDR1 constitutively.

1.2.3. Cell culture and treatment

Hepatocellular carcinoma cell line (HepG2) were grown in RPMI 1640 medium supplemented with 10% heat inactivated 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 ^{o}C. The cultured cells were sub-cultured twice each week and the exponentially growing cells were used for all treatments. Celecoxib dissolved in DMSO and doxorubicin dissolved in PBS were used for the treatments. 40 mM stock of celecoxib and 5 mM stock of doxorubicin were employed in this study. At the time of treatment working solutions were diluted accordingly in RPMI 1640 medium. The drugs were added to the cells, 6 h after the subculture. Stock of celecoxib was freshly prepared before every treatment.

The final concentration of the vehicle (DMSO) never exceeded 0.1%. HepG2 cells exposed to 0.1% DMSO served as controls.

1.2.4. Effect of celecoxib or doxorubicin on proliferation of HepG2 cells

Cell proliferation was determined by MTT [3-{4,5- Dimethylthiazol-2yl}-2,5-diphenyltetrazolium bromide] assay (Campling *et al.,* 1988). HepG2 cells (5 x 10^3 cells/well) were seeded to 96 well culture plate in the presence or absence of celecoxib (1µM, 10 µM, 25 µM, 50 µM and 100 µM) or doxorubicin (100 nM,

500 nM, 1µM, 10 µM, 25 µM and 50 µM) for 12, 24 and 48 h in a final volume of 100 µl. After treatment, the medium was removed and 20 µl of MTT (5 mg/ml of PBS) was added to the fresh medium. After 2 h incubation at 37 ^{o}C, 100 µl of DMSO was added to each well and plates were agitated for 1 min. Absorbance was read at 570 nm on a multi well plate reader. Percent inhibition of proliferation was calculated as a fraction of control (control was without celecoxib or doxorubicin).

1.2.5. Synergistic effect of celecoxib and doxorubicin on proliferation of HepG2 cells

HepG2 cells (5 x 10^3 cells/well) were seeded to 96 well culture plate. Doxorubicin (100 nM, 500 nM, 1µM, 10 µM, 25 µM and 50 µM) was added in the presence or absence of celecoxib (25 µM) for 24 h in a final volume of 100 µl. After treatment the medium was removed and 20 µl of MTT (5mg/ml of PBS) was added to the fresh medium. After 2 h incubation at 37 ^{o}C, 100 µl of DMSO was added to each well and plates were agitated for 1 min. Absorbance was read at 570 nm on a multi well plate reader. Percent inhibition of proliferation was calculated as a fraction of control (control was without doxorubicin or celecoxib in cells treated with doxorubicin alone and was without doxorubicin and with 25 µM celecoxib in the cells treated with both doxorubicin and celecoxib).

1.2.6. Intracellular Drug accumulation assays

HepG2 cells (1 x 10^6 cells/well) were seeded into 6 well culture plates. Cells were incubated with celecoxib at concentrations of 10 µM and 25 µM for 24 h. To determine intracellular drug accumulation, 50 µM of doxorubicin (standard anti-cancer drug) was added and incubated for 2 h. Then the cells were trypsinized and the final doxorubicin accumulated in HepG2 cells was analyzed using flow cytometer (Chan *et al.,* 2000).

1.2.7. RT-PCR analysis

HepG2 cells were seeded at a density of 5 x 10^6 in 90 mm culture dishes. Cells were treated with celecoxib (1 µM, 10 µM and 25 µM) or PGE_2 (6 µg/ml) for 24 h. Cells were harvested and total RNA was extracted from control and treated HepG2 cells using TRIzol reagent. Semi-quantitive RT-PCR was performed with 5 µg total RNA, isolated from HepG2 cells, using one step RT-PCR kit. Primers used were as follows: Human MDR1: forward, 5'-TGA CTA CCA GGC TCG CCA A-3', reverse, 5'-TAG CGA TCT TCC CAG CAC CTT-3'; which yields 252 bp product (Marroni *et al.,* 2003), Actin: forward, 5'-GTT TGA GAC CTT CAA CAC CCC-3', reverse, 5'-GTG GCC ATC TCC TGC TCG AAG TC-3'; which yields 318 bp product (Hanif *et al.,* 1996). Reverse transcription was performed at 47 ^oC for 30 min. Subsequent to inactivation of reverse

transcriptase (2 min, 94 ^{o}C), the samples were subjected to 25 amplification cycles, each consisting of 20 sec at 94 ^{o}C, 1 min at 54 ^{o}C, and 1 min at 72 ^{o}C, followed by 5 min at 72 ^{o}C after the last cycle. The products were analyzed on 1% agarose gel.

1.2.8. Preparation of whole cell extracts and immunoblot analysis

HepG2 cells at a density of 5 x 10^6 were seeded in 90 mm culture dishes. They were incubated with celecoxib (1µM, 10 µM and 25 µM) and PGE_2 (6 µg/ml). Cells, harvested after 24 h 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 µg/ml leupeptin, 20 µg/ml aprotinin and phosphatase inhibitor cocktail 1 and 2 with 100 fold dilution). After 30 min of shaking at 4 ^{o}C, the mixtures were centrifuged (10,000 x 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 µg of protein from each treatment was resolved on 10% gels 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 [JNK and p-JNK (1:250 dilution), COX-2 and MDR1 (1:500 dilution)] in 10 ml of antibody-diluted buffer (1X Tris-buffered saline and 0.05% Tween-20 with 1% milk) with gentle shaking at 4 ^{o}C for 8-12 h and then incubated with peroxidase conjugated secondary antibodies. Signals were detected by using peroxidase substrate, TMB/H_2O_2. Equal protein loading was confirmed by reprobing the nitrocellulose membranes with β-actin antibodies (1:500 dilution).

1.2.9. Electrophoretic mobility shift assay (EMSA)

HepG2 cells at a density of 5 x 10^6 were seeded in 90 mm culture dishes. Cells were incubated with celecoxib (10 μM and 25 μM) for 6 h and PGE_2 (6 μg/ml) for 1, 2 and 6 h. Cells were harvested and then used for nuclear protein extraction. The cells were washed with PBS and 200 μl 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 sec each. The nuclei were then harvested by centrifugation at 16,000 rpm for 1 min. The nuclear pellet was resuspended in 40 μl of nuclear protein extraction buffer (420 mM

NaCl, 10 mM HEPES, 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM DTT and 25% glycerol) and incubated on ice for 30 min with intermittent vortexing of 10 sec each. The sample was then centrifuged at 13,000 rpm for 30 min at 4 ^oC. The supernatant collected was used for the mobility shift assay after protein estimation using Bradford assay (Bradford, 1976). Nuclear extracts (8 µg) were incubated with γ-^{32}P labeled double stranded oligonucleotide with specific AP-1 binding sequence (5'-CTG AAT CAA CTG CTT CAA-3') for 30 min at 37 ^oC. 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).

1.2.10. PGE_2 estimation

HepG2 cells at a density of 5 x 10^6 were seeded in 90 mm culture dishes. They were incubated with celecoxib (1µM, 10 µM & 25 µM) for 24 h. At the end of the treatment period, culture medium was collected to determine the amount of PGE_2 secreted by these cells and stored at -80 ^oC. The quantitative analysis of PGE_2 released into the medium was assessed by using PGE_2 immunoassay kit as per manufacturer's instructions (Cayman, USA).

1.2.11. *In silico* studies

Model Overview

The model was developed using Teranode's Biological Modeler, which uses MML (Mathematical Modeling Language, developed by the National Simulation Resource at the University of Washington) and allows creation of kinetic equations. The Teranode™ software was used to create and integrate the pathway with ODEs (ordinary differential equations) solved numerically by the Radau method (Hairer and Wanner, 1999). A schematic of the elements of the model and their connections are shown in figure 18.

Model Structure and Description

Celecoxib mediated regulation of MDR1 expression has been modeled as follows:

1. COX-2 catalyzes the conversion of arachidonic acid to prostanoids by a two-step process. In the first step, two molecules of O_2 are added to form a short-lived, unstable intermediate Prostaglandin G_2 (PGG_2). PGG_2 is then converted to PGH_2 by shedding of single oxygen (Vogel, 2000).
2. PGE_2 synthase catalyzes conversion of COX-derived PGH_2 to PGE_2 through an isomerization reaction (Xu *et al.,* 2006; Ouellet *et al.,* 2002; Thoren *et al.,* 2003).
3. PGE_2 bound prostaglandin (EP2/4) receptor (Kotani *et al.,* 1995; Kataoka *et al.,* 2005) mediates signal transduction by activating PKA pathway (Fujino *et al.,* 2005).

4. ERK1/2, p38MAPK and JNK in turn are activated by phosphorylation mediated by PKA (Gallagher *et al.,* 2002; Dalle *et al.,* 2004).

5. The phosphorylated MAPKs subsequently activate the protein c-Jun and c-Fos. Together these two proteins form an active AP-1 transcription complex (Chen and Hughes-Fulford, 2000; Roesler *et al.,* 1994).

6. AP-1 is the transcription factor regulating the expression of MDR1 (Sukhai and Piquette-Miller, 2000; Siverman and Schrenk, 1997; Silverman and Thorgeirsson 1995). MDR1 mRNA is translated to MDR1 protein in the cytoplasm.

7. Doxorubicin is a drug that acts at the level of Topoisomerase II. With the expression of MDR1 there is a rapid extrusion of the drug out of the cell and hence resistance to this chemotherapeutic agent (Park *et al.,* 1994).

8. Celecoxib selectively inhibits COX-2 thereby regulating MDR1 protein levels and increasing the overall doxorubicin retention in the cell (Gierse *et al.,* 2005).

Model assumptions

Model initial conditions without celecoxib have been calibrated to control levels from experimental data. Doxorubicin retention was also calibrated to control sample levels from experimental data. With celecoxib treatment, the model was calibrated to obtain the same MDR1 mRNA and protein levels as in

the experimental data and also similar intracellular doxorubicin levels. Celecoxib was modeled as a COX-2 inhibitor at the level of COX-2 activity. With 25 μM celecoxib treatment, the MDR1 mRNA and protein levels were calibrated to show similar reduction as in experimental data. Transcription and translation reactions for MDR1, COX-2 have been modeled simplistically and calibrated to the experimental levels. Ki values for celecoxib and doxorubicin were optimized to obtain the experimental fold differences in MDR1 mRNA and protein levels. This was used as the training data for the model to corroborate with the experimental data.

Simulation protocols

- Celecoxib was used at a concentration less than 35μM as in the experiments.
- Celecoxib was used with an arbitrary Ki of 3.89 μM.
- Doxorubicin was used at a concentration of 5 μM as in the experiments.
- Doxorubicin was used with an arbitrary Ki of 0.0025 μM.
- PGE_2 activator was used at a concentration of 1.5 μM and an arbitrary K act of 3 μM.
- The time run for the simulation was 86400 sec (24 h) as in the experimental set up.
- Levels of concentrations of most interacting species were chosen from values taken from literature.

1.2.12. Statistical analysis

Data reported as the mean +/- SE 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 significance.

1.3. Results

In order to understand the role of COX-2 in the regulation of the expression of MDR1, hepatocellular carcinoma cell line, HepG2, exposed to celecoxib was employed as model system.

1.3.1. Celecoxib and doxorubicin inhibited the proliferation of HepG2 cells

HepG2 cells were treated with celecoxib (1 µM to 100 µM) and doxorubicin (100 nM to 50 µM) for 12, 24 and 48 h and cell proliferation was

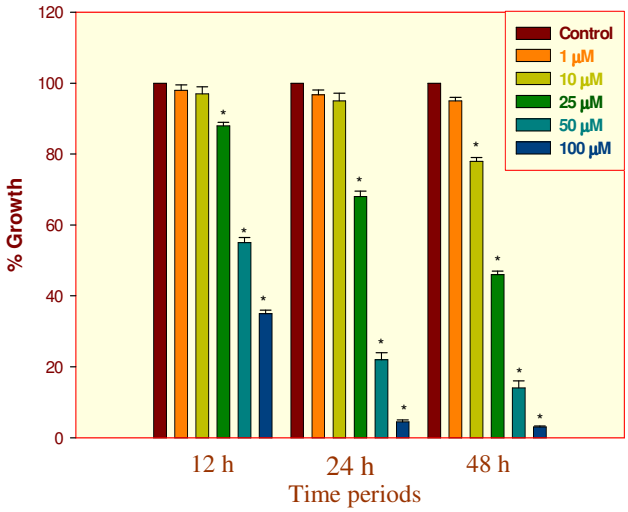


Fig. 6. Effect of celecoxib on the growth of HepG2 cells

HepG2 cells were treated with 1 µM, 10 µM, 25 µM, 50 µM and 100 µM of celecoxib for 12, 24 and 48 h. The percent viable cells were calculated in comparison to untreated cells. The number of cells in the control was taken as 100%. * Denotes statistical significance over control (P< 0.05).

determined by MTT assay. Under these experimental conditions a dose dependent decrease in proliferation of HepG2 cells was observed. 50% inhibition in the cell proliferation was observed at 35 µM concentration of celecoxib (Fig. 6) and 5 µM concentration of doxorubicin for 24 h (Fig. 7).

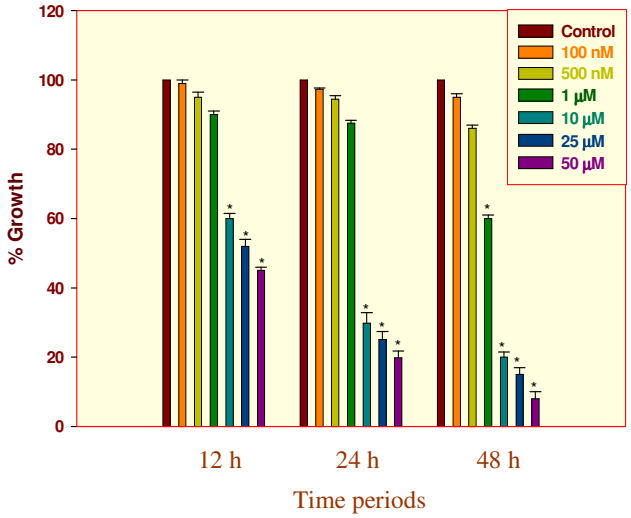


Fig. 7. Effect of doxorubicin on the growth of HepG2 cells

HepG2 cells were treated with 100 nM, 500 nM, 1 µM, 10 µM, 25 µM and 50 µM of doxorubicin for 12, 24 and 48 h. The percent viable cells were calculated in comparison to untreated cells. The number of cells in the control was taken as 100%. * Denotes statistical significance over control (P< 0.05).

1.3.2. Celecoxib and doxorubicin synergistically inhibited the proliferation of HepG2 cells

HepG2 cells treated with doxorubicin (100 nM, 500 nM, 1 µM, 10 µM, 25 µM and 50 µM), with or with out celecoxib (25 µM) for 24 h and cell proliferation was determined by MTT assay. In the presence of celecoxib, the percent inhibition in the growth of HepG2 cells was much higher than in the cells grown in the absence of 25 µM celecoxib at all the concentrations of doxorubicin studied (Fig. 8). As a result the IC-50 of doxorubicin for HepG2 cells was 5 µM in the absence of celecoxib and 500 nM in the presence of 25 µM celecoxib.

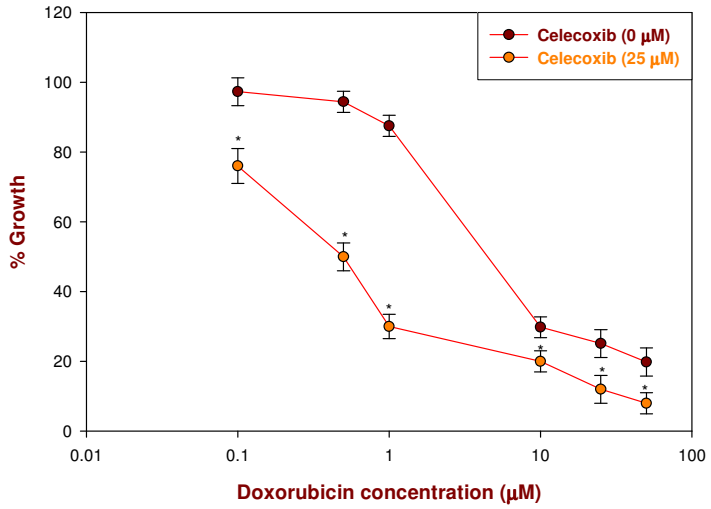


Fig. 8. Synergistic effect of celecoxib and doxorubicin on the growth of HepG2 cells

HepG2 cells were treated with doxorubicin (100 nM, 500 nM, 1 µM, 10 µM, 25 µM and 50 µM) and with or without celecoxib (25 µM) for 24 h. In case of doxorubicin and celecoxib treated cells, the number of cells in the 25 µM celecoxib alone treatment was taken as control (100%). * Denotes statistical significance over control (P< 0.05).

1.3.3. Celecoxib treatment increased the accumulation of doxorubicin in HepG2 cells

As celecoxib enhanced the chemotherapeutic potential of doxorubicin, further studies were undertaken to measure the doxorubicin accumulation in HepG2 cells in the presence and absence of celecoxib. HepG2 cells were treated with concentrations of celecoxib less than its IC-50 value. HepG2 cells were treated with 10 and 25 µM celecoxib for 24 h and then incubated with 50 µM doxorubicin for 2 h. Cells treated with celecoxib showed more accumulation of doxorubicin in them compared to the untreated controls.

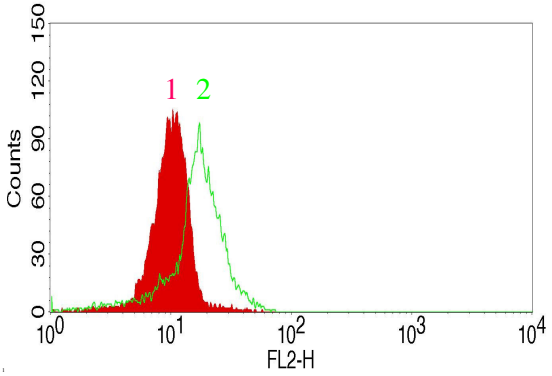


Fig. 9. Measurement of intracellular doxorubicin accumulation by flow cytometer

HepG2 cells were treated with 25 µM of celecoxib for 24 h and then incubated with 50 µM doxorubicin for 2 h. The fluorescence of doxorubicin retained in HepG2 cells was measured with FL2 filter. Histogram 1 (doxorubicin retained in control cells) overlaid with histogram 2 (doxorubicin retained in cells treated with 25 µM of celecoxib). Greater the FL2-Height, greater is the drug retention.

HepG2 cells treated with 10 µM and 25 µM celecoxib showed 33.3% and 56.7% (Fig. 9) more doxorubicin accumulation compared to untreated cells. *In silico* analysis also showed dose dependent increase in doxorubicin accumulation with celecoxib treatment (Fig. 10).

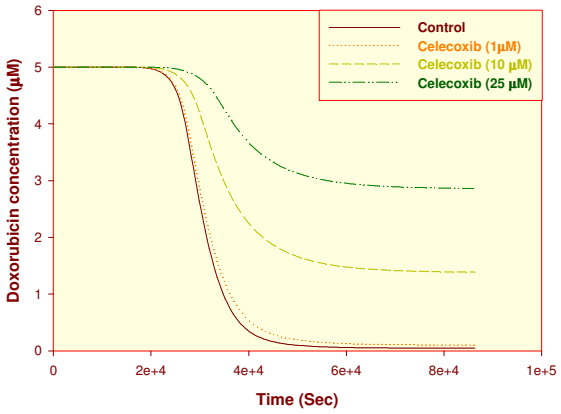


Fig. 10. Line plot showing simulation data of doxorubicin accumulation with celecoxib treatment

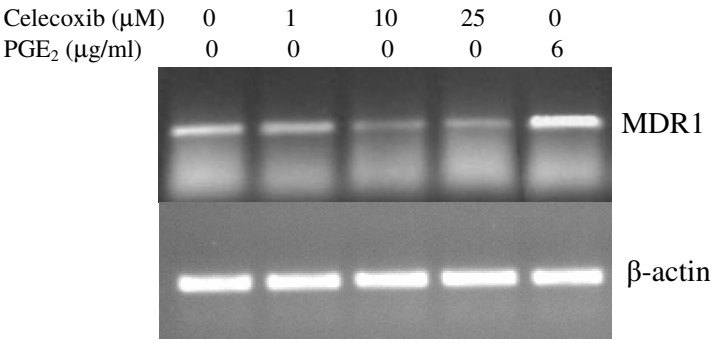
Concentration of doxorubicin accumulated in µM on Y-axis against time (sec) on X-axis. This was obtained by running the simulation model with celecoxib concentrations of 0 µM, 1 µM, 10 µM & 25 µM.

1.3.4. Celecoxib inhibited MDR1 mRNA expression

Treatment of HepG2 cells with celecoxib (1 µM, 10 µM and 25 µM) for 24 h resulted in a dose dependent inhibition of MDR1 transcription. Addition of PGE_2 (6 µg/ml) to the medium induced the expression of MDR1 mRNA

compared to the untreated controls (Fig. 11 A). *In silico* studies also showed similar trends compared to experimental results (Fig. 11 B).

A)



B)

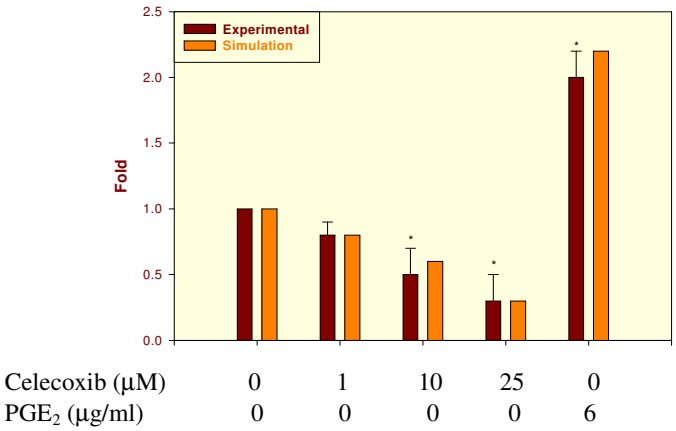


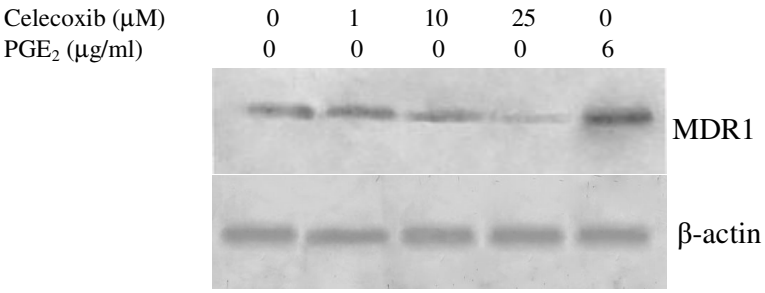
Fig. 11. Effect of celecoxib and PGE_2 on MDR1 mRNA expression in HepG2 cells

(A) RT-PCR analysis of celecoxib and PGE_2 mediated effect on MDR1 mRNA expression in HepG2 cells. (B) Bar graphs showing the fold difference in the expression levels of MDR1 mRNA obtained by experimental and simulation data. * Denotes statistical significance over control (P< 0.05)

1.3.5. Celecoxib inhibited MDR1 protein expression

Celecoxib inhibited the expression of MDR1 at protein level. Treatment of HepG2 cells with celecoxib (1 µM, 10 µM and 25 µM) for 24 h resulted in a dose dependent inhibition of MDR1 protein. Addition of PGE_2 (6 µg/ml) to the medium induced the expression of MDR1 protein compared to the untreated controls (Fig. 12 A).

A)



B)

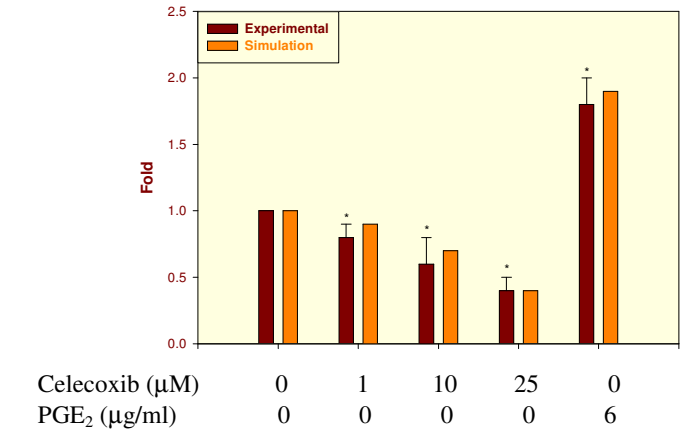


Fig. 12. Effect of celecoxib and PGE_2 on MDR1 protein expression in HepG2 cells

(A) Western blot analysis of celecoxib and PGE_2 mediated effect on MDR1 protein expression in HepG2 cells. (B) Bar graphs showing the fold difference in the expression levels of MDR1 protein obtained by experimental and simulation data. * Denotes statistical significance over control (P< 0.05).

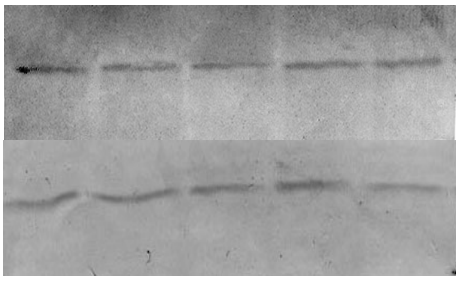
In silico simulation studies also showed inhibition in the MDR1 protein expression by celecoxib and the trend followed the experimental results (Fig. 12 B).

1.3.6. Effect of celecoxib on expression of COX-2 in HepG2 cells

To verify whether the regulation of MDR1 by celecoxib is mediated by the inhibition of COX-2 expression, the expression of COX-2 was analyzed in the presence and absence of celecoxib. Treatment with celecoxib (1 µM, 10 µM and 25 µM) for 24 h did not show any effect on the expression of COX-2. Addition of PGE_2 (6 µg/ml) to the medium also did not show any effect on COX-2 expression, when compared to the untreated controls (Fig. 13 A). *In silico* simulation studies also did not show any change in COX-2 expression with celecoxib treatment (Fig. 13 B).

A)

Celecoxib (µM)	0	1	10	25	0
PGE_2 (µg/ml)	0	0	0	0	6



B)

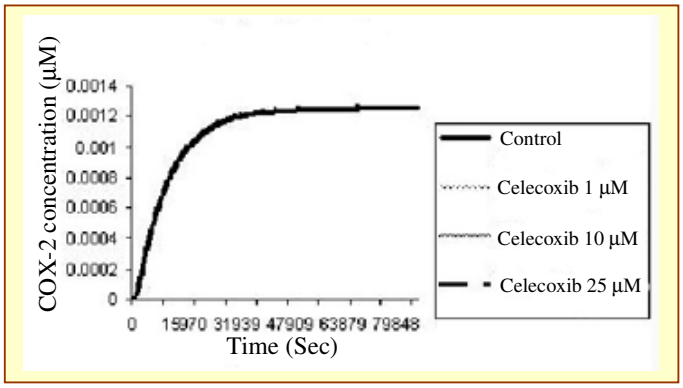


Fig. 13. Effect of celecoxib on COX-2 protein expression in HepG2 cells

(A) Western blot analysis of celecoxib and PGE_2 mediated effect on COX-2 protein expression in HepG2 cells. (B) Line Plot showing simulation data of COX-2 concentration in µM on Y-axis against time (sec) on X-axis with celecoxib treatment. This was obtained by running the simulation model with celecoxib concentrations of 0 µM, 1 µM, 10 µM & 25 µM.

1.3.7. Celecoxib inhibited PGE_2 release in HepG2 cells

To verify whether the regulation of MDR1 by celecoxib is by inhibition of COX-2 activity, we estimated the amount of PGE_2 release into the medium in the presence and absence of celecoxib in HepG2 cells. Treatment with celecoxib (1 µM, 10 µM and 25 µM) for 24 h showed a dose dependent decrease in the release of PGE_2 compared to the untreated cells (Fig. 14). Simulation results of PGE_2 release with celecoxib treatment aligns with the experimental data (Fig. 14).

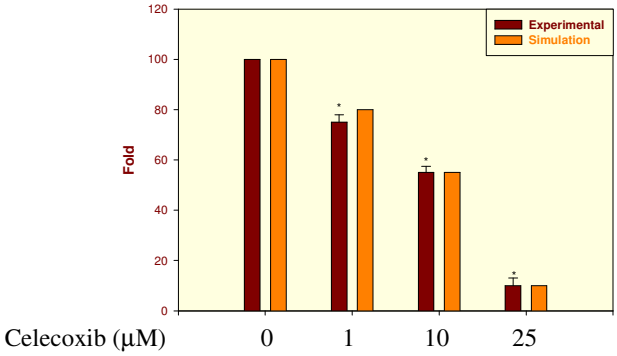


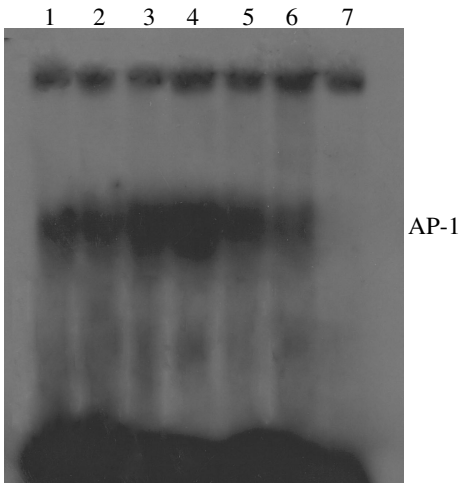
Fig. 14. Effect of celecoxib on PGE_2 release in HepG2 cells

Bar graphs showing the fold difference in the release of PGE_2 in HepG2 cells with celecoxib treatment (1 µM, 10 µM and 25 µM) obtained by experimental and simulation data. * Denotes statistical significance over control (P< 0.05)

1.3.8. Celecoxib inhibited the translocation of AP-1 in HepG2 cells

To identify the elements playing a role in the inhibition of MDR1 by celecoxib, studies were taken up on the formation of AP-1, a positive regulator of MDR1 expression, in the presence and absence of celecoxib in HepG2 cells. Treatment with celecoxib (10 µM and 25 µM) for 6 h inhibited the formation of AP-1 in a dose dependent manner and treatment with PGE_2 (6 µg/ml) for 1, 2 and 6 h showed a time dependent increase in the formation of AP-1 in HepG2 cells (Fig. 15 A). Simulation data of AP-1 translocation correlates well with the experimental data (Fig. 15 B).

A)



B)

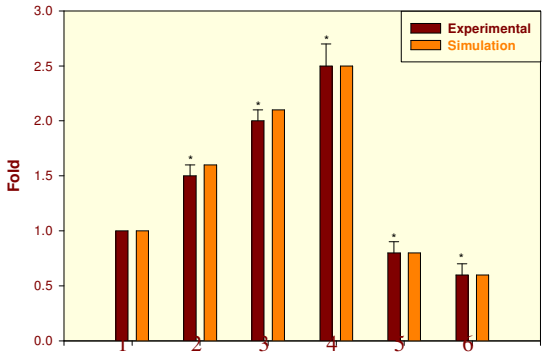


Fig. 15. Effect of celecoxib on nuclear translocation of AP-1 in HepG2 cells

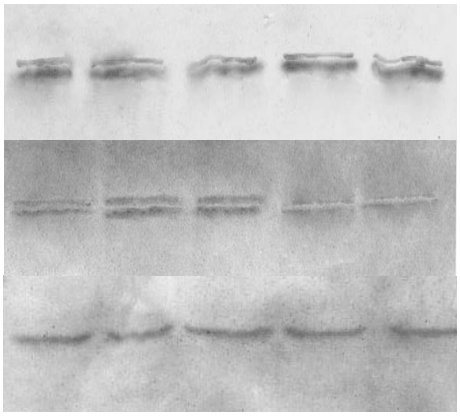
(A) Electro mobility shift assay (EMSA) of nuclear extracts using specific AP-1 binding probe in cells treated with celecoxib and PGE_2. Lane 1: Control; lane 2: PGE_2 (6 µg/ml) 1 h; lane 3: PGE_2 (6 µg/ml) 2 h; lane 4: PGE_2 (6 µg/ml) 6 h; lane 5: celecoxib (10 µM) 6 h; lane 6: celecoxib (25 µM) 6 h; lane 7: Cold competition. (B) Bar graphs showing the fold difference in the AP-1 translocation obtained by experimental and simulation data. * Denotes statistical significance over control ($P< 0.05$).

1.3.9. Effect of celecoxib and PGE_2 on the expression of p-JNK

As c-Jun and c-Fos are required for the formation of active AP-1 and as p-JNK plays a role in the upstream (phosphorylation of c-Jun) of AP-1 formation, we checked for the expression of p-JNK in the presence and absence of celecoxib. Treatment with celecoxib (10 µM and 25 µM) for 24 h inhibited the phosphorylation of JNK and treatment with PGE_2 (6 µg/ml) for 2 and 6 h showed a time dependent increase in the phosphorylation of JNK (Fig. 16A and 16B). Expression levels of non phosphorylated form of JNK, on the other hand, were unaltered in the presence and absence of celecoxib and also with PGE_2 treatment (Fig. 16 A). *In silico* studies showed a similar decrease in p-JNK levels with celecoxib treatment (Fig. 17A).

A)

Celecoxib (µM, h)	0	0	0	10, 24	25, 24
PGE_2 (µg/ml, h)	0	6, 2	6, 6	0	0



B)

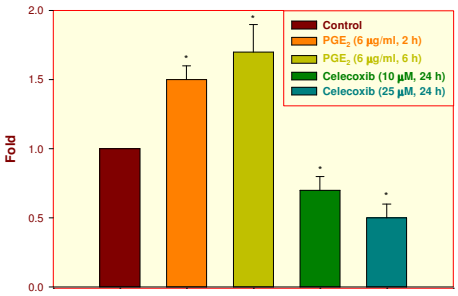


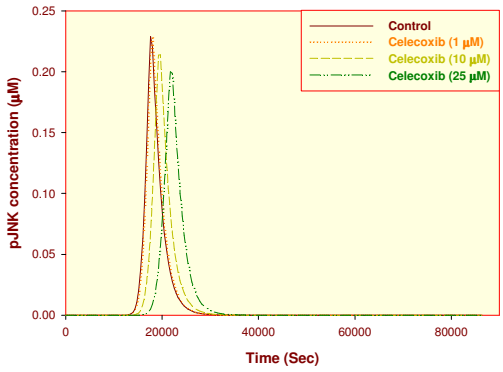
Fig. 16. Effect of celecoxib and PGE_2 on JNK phosphorylation in HepG2 cells

(A) Western blot analysis of celecoxib and PGE_2 mediated effect on JNK phosphorylation in HepG2 cells. (B) Bar graphs showing the expression levels of phosphorylated JNK obtained by experimental data. * Denotes statistical significance over control (P< 0.05)

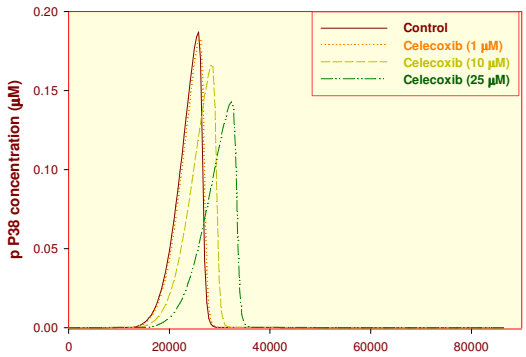
1.3.10. Effect of celecoxib on phosphorylation of JNK, ERK and p38

In silico studies showed a dose dependent decrease in phosphorylated levels of JNK, ERK and p38 with celecoxib treatment with respect to untreated controls (Fig. 17).

A)



B)



C)

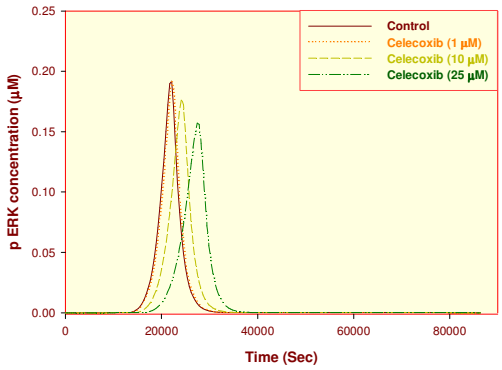


Fig. 17. *In silico* simulation data on the effect of celecoxib on phosphorylation of JNK, ERK and p38 in HepG2 cells

Line Plot showing simulation data of p-JNK (A), pP38 (B) and p-ERK (C) concentration in µM on Y-axis against time (sec) on X-axis with celecoxib treatment. This was obtained by running the simulation model with celecoxib concentrations of 0 µM, 1 µM, 10 µM & 25 µM. At 25 µM of celecoxib concentration, the levels of phosphorylated forms of JNK, ERK, p38 were decreased by ~1.3 folds

1.4. Discussion

The role of COX-2 in the cell has been the focus of intense investigation since the discovery of this enzyme and uncovering of the mechanisms of COX-2 in prosurvival activities. This prosurvival role of COX-2 could have profound effect on the therapeutic strategies for cancer and inflammatory disorders.

COX-2 overexpression in tumor cells seems to be associated with increase in angiogenesis, tumor invasion, down regulation of apoptosis and suppression of host immunity. Recent studies indicated COX-2 overexpression in several malignancies such as colorectal and breast cancers (Denkert *et al.,* 2003) and also leukemia (Nakanishi *et al.,* 2001). High levels of prostaglandins, possibly reflecting the overexpression of the COX-2 enzyme, may be playing important role in proliferation and differentiation of various cancer cell lines (Sheng *et al.,* 2001). COX-2 inhibitors have received increasing attention for their potential use as chemopreventive and therapeutic agents in cancer. Selective COX-2 inhibitors have been shown to have strong chemopreventive actions against colon carcinogenesis in rats (Kawamori *et al.,* 1998). Studies showing that drugs that inhibit COX-2 reduce the number of colorectal adenomas in animals and patients with familial adenomatous polyposis (Bertagnolli *et al.,* 2006). Very strong association between COX-2 expression and reduced susceptibility to

chemotherapy and poor outcome in a large series of advanced ovarian and cervical cancer patients has been established (Ferrandina *et al.,* 2002a, 2002b). The role of MDR1 in protecting cells from apoptosis has been studied in several cellular systems (Johnstone *et al.,* 2000). Few studies have shown that the activation of the cyclooxygenase system might be critical event in the development of MDR1 mediated drug resistance. The role of COX-2 in regulation of MDR1 expression and specific COX-2 inhibitors in overcoming multidrug resistance is not clear. Hence, a study was planned using hepatocellular carcinoma cell line (HepG2) to understand the role of cyclooxygenases, especially COX-2 in multidrug resistance and to test the efficacy of celecoxib, a specific COX-2 inhibitor, in multidrug resistance.

1.4.1. Celecoxib, a selective COX-2 inhibitor enhances the accumulation of doxorubicin in HepG2 cells

COX-2 selective inhibitors were shown to inhibit the proliferation of colon, prostate, leukemia and esophagus cancer cell lines (Hale *et al.,* 1996; Hsu *et al.,* 2000; Subhashini *et al.,* 2005; Li *et al.,* 2001). In the present study, when HepG2 cells were treated with celecoxib, 50% inhibition in the cell proliferation was observed at 35 µM concentration of celecoxib. Treatment of HepG2 cells with doxorubicin inhibited 50% of cell proliferation at 5 µM concentration. As

COX-2 inhibitors were shown to enhance the chemotherapeutic potential of anti-cancer drugs, it is hypothesized that COX-2 inhibitors might enhance the accumulation of the drug in the cells. Hence, doxorubicin accumulation in HepG2 cells was estimated in the presence and absence of celecoxib. HepG2 cells were treated with concentrations of celecoxib less than its IC-50 value. HepG2 cells treated with 10 µM and 25 µM celecoxib for 24 h showed 33.3% and 56.7% more doxorubicin accumulation compared to untreated cells. Similar results were obtained when rat glomerular mesangial cells were treated with NS- 398, a selective COX-2 inhibitor. These cells showed increased accumulation of Rhodamine 123 when treated with NS 398 (Vimal *et al.,* 2002).

1.4.2. Celecoxib enhances the sensitivity of HepG2 cells to doxorubicin

As celecoxib enhanced the accumulation of doxorubicin, synergistic effects of doxorubicin and celecoxib were studied on the proliferation of HepG2 cells. IC-50 of doxorubicin on proliferation of HepG2 cells was decreased from 5 µM in the absence of celecoxib to 500 nM in the presence of 25 µM celecoxib. These results suggest that the synergism of doxorubicin and celecoxib on proliferation of HepG2 cells is due to increased accumulation of doxorubicin in the presence of celecoxib as observed in the present study. In the light of above observation it is hypothesized that increase in doxorubicin accumulation might be

due to the decreased efflux of doxorubicin by the drug transporters, which might be due to the decrease in the expression or activity of the drug transporters.

1.4.3. Celecoxib mediated down regulation of MDR1 expression enhances the sensitivity of HepG2 cells to doxorubicin

In order to test the above hypothesis, the expression of MDR1, a major drug transporter protein, at both mRNA and protein level was analyzed. Treatment with celecoxib resulted in a dose dependent inhibition of MDR1 expression both at mRNA and protein level. Addition of PGE_2 (6 µg/ml), a key product of COX-2, to the medium induced the expression of MDR1 at mRNA and protein level compared to the untreated controls. These results clearly demonstrate that COX-2 plays a major role on MDR1 expression in HepG2 cells. Similar observations were reported in rat glomerular mesangial cells, where in the transfection of COX-2 expression vector resulted in increased expression of MDR1 and its expression decreased with NS-398 treatment (Vimal *et al.,* 2002). It was also shown that PGE_2 addition to the culture medium of rat primary hepatocytes upregulated MDR1b mRNA expression and MDR1 dependent transporter activity (Ziemann *et al.,* 2002). Furthermore, structurally different cyclooxygenase inhibitors (Indomethacin, Meloxicam, NS-398) mediated inhibition of EGF-induced MDR1 mRNA overexpression, resulting in enhanced

intracellular accumulation of MDR1 substrate, rhodamine 123 in rat primary hepatocytes cultures (Ziemann *et al.,* 2002).

1.4.4. Celecoxib mediated downregulation of MDR1 expression is mediated by the inhibition of COX-2 but not its expression

To verify whether the regulation of MDR1 expression by celecoxib is by inhibition of COX-2, further studies were taken up on the expression of COX-2 and the formation of its product, PGE_2. Treatment with celecoxib did not show any effect on the expression of COX-2. PGE_2 levels, however, were reduced in celecoxib treated cells compared to the untreated cells in a dose dependent manner. Also addition of PGE_2 (6 µg/ml) to the medium did not show any change in COX-2 expression compared to the untreated controls. These studies clearly demonstrate that celecoxib mediated inhibition of COX-2 and thus PGE_2 release down regulates MDR1 expression in HepG2 cells.

1.4.5. Celecoxib-induced downregulation of MDR1 expression is mediated by the inhibition of AP-1 complex

To further understand the regulation of MDR1 expression by COX-2, studies were taken up on transcriptional regulators of MDR1. There are some indirect evidences indicating that AP-1 complex may be involved in the transcription of several drug transporters. AP-1 is the general term for

transcription factor complexes composed of the Fos and Jun oncogene families (Shaulian and Karin, 2001). Elevated levels of c-Fos have been demonstrated in a number of drug resistant cell lines when compared to their drug- sensitive counterparts (Bhushan *et al.,* 1992). Putative nonconsensus AP-1 binding sites have been reported in human and rodent class I Pgp promoters. While AP-1 site in the murine homologue mediates the repression of this gene (Ikeguchi *et al.,* 1991), the AP-1 binding elements in the promoter of hamster (155 to -49) (Teeter *et al.,* 1991) and human genes (-121 to -115) (Daschner *et al.,* 1999) are involved in transcriptional activation. In view of AP-1's role in transcription of drug transporters, further studies were taken up on the formation of AP-1 complex, a positive regulator of MDR1 expression, in the presence and absence of celecoxib in HepG2 cells. Treatment with celecoxib inhibited the formation of AP-1 in a dose dependent manner and treatment with PGE_2 (6 µg/ml) for 1, 2 and 6 h showed a time dependent increase in the formation of AP-1 in HepG2 cells.

1.4.6. Celecoxib-induced inhibition in the AP-1 complex is mediated by decreased phosphorylation of JNK (c-Jun NH2-terminal protein kinase)

As c-Jun and c-Fos are required for the formation of active AP-1 and as c-Jun NH2-terminal protein kinase (JNK) plays a critical role in the upstream (phosphorylation of c-Jun) of AP-1 formation, the expression of p-JNK in the

presence and absence of celecoxib was studied. Treatment with celecoxib inhibited the phosphorylation of JNK and treatment with PGE_2 (6 µg/ml) for 2 and 6 h showed a time dependent increase in the phosphorylation of JNK. Expression levels of non phosphorylated form of JNK were unaltered in the presence and absence of celecoxib and also with PGE_2 treatment. A similar line of circumstantial evidence comes from studies on KB-3 cells, where adriamycin, vinblastine and etoposide activate JNK and this was found to be associated with an increase in MDR1 expression (Osborne and Chambers, 1996). Two multidrug resistant variants of KB-3 cells, KB-A1 and KB-V1, showed increased basal levels of JNK activity when compared to the KB-3 parental cell line. These results indicate that JNK activity might be critical in MDR1 expression and this MDR1 expression can be inhibited by interfering at the level of JNK activation.

1.4.7. Celecoxib-induced downregulation of MDR1 expression appears to be mediated by the inactivation of signal transduction pathways involving ERK, JNK and p38

One of the reasons for up-regulated expression of MDR1 in many of the cancer cells could be the constitutive activation of signal transduction pathways which control the transcription of MDR1 gene. Since a number of transcription factors are known to positively up regulate the expression of MDR1 and are known to be phosphorylated by MAP kinases, the activation of MAP kinases may

be playing a role in the transcriptional regulation of MDR1. Hence in the present study the activation of ERK, JNK and p38 protein kinases was studied using *in silico* analysis. Treatment with celecoxib (25 µM) reduced the activation of ERK, JNK and p38 by ~1.3 folds compared to the untreated cells. This decrease in the activation of protein kinases might also be the reason, to certain extent, for the decrease in MDR1 expression in celecoxib treated cells.

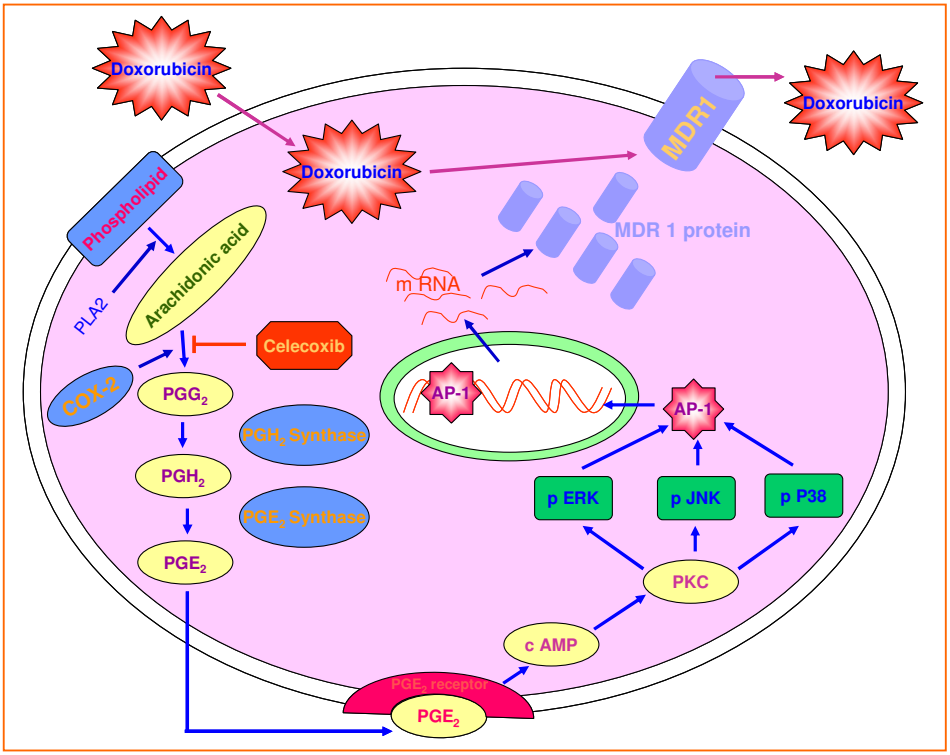


Fig.18. Schematic representation of the model showing the regulation of MDR1 expression by COX-2 and site of interference by celecoxib

1.4.8. *In silico* analysis correlated with our experimental results

The *in silico* experiments were performed using mathematical modeling language developed at University of Washington. The kinetics of interaction between components of the pathway was integrated with differential equations and solved. Initial levels of components of the pathway were calibrated to match the levels obtained via experimental data.

The levels of MDR1, COX-2, pJNK, AP-1 thus obtained by *in silico* simulation experiments with celecoxib and PGE_2 treatment matched to the experimental results. A gradual increase in doxorubicin retention with increase in celecoxib concentration was observed both *in silico* and experimental studies. Additionally the phosphorylation status of MAP kinases, ERK1/2, JNK, p38 with celecoxib treatment were obtained with *in silico* studies. Altogether, this *in silico* data supported our experimental approach and results.

Available experimental and *in silico* data thus strongly suggest that COX-2 may function as a prosurvival factor by protecting the HepG2 cells. Celecoxib, the selective COX-2 inhibitor, enhanced the sensitivity of HepG2 cells to doxorubicin by downregulating MDR1 expression. Further studies indicate that celecoxib by inhibiting COX-2 and PGE_2 formation downregulates MDR1 expression by inhibiting the formation of AP-1, phosphorylation of JNK, ERK

and p38. The schematic representation of the model showing the regulation of MDR1 expression by COX-2 and site of interference by celecoxib is shown in Fig. 18. In conclusion the forgoing studies clearly demonstrate the role of COX-2 in development of drug resistance and usefulness of COX-2 inhibitor, celecoxib, in overcoming drug resistance.

1.5. Summary

In order to understand the role of COX-2 in the regulation of MDR1 expression, hepatocellular carcinoma cell line, HepG2, was employed as a model system. In the above system celecoxib, a selective inhibitor of COX-2, increased the accumulation of doxorubicin in HepG2 cells and thus leading to enhanced sensitivity of the cells to doxorubicin. This enhanced sensitivity of HepG2 cells to doxorubicin by celecoxib appears to be mediated by the down regulation of MDR1. To further understand the pathway a computational model was created for the PGE_2–p-JNK-AP-1 pathway. These experimental and *in silico* studies collectively suggest that celecoxib inhibits MDR1 expression by interfering at the level AP-1 translocation and inactivation of signal transduction pathways involving ERK, JNK and p38. These studies thus demonstrate the role of COX-2 in the development of drug resistance and the usefulness of COX-2 inhibitors in overcoming drug resistance in HepG2 cells

CHAPTER - 2

(ROS and Multidrug resistance)

2.1. Introduction

2.1.1. Reactive oxygen species

Reactive oxygen species (ROS) is a collective term used for all those species that contain chemically reactive oxygen moieties and ROS encompasses a variety of partially reduced metabolites of oxygen [e.g., superoxide anions ($O_2^{\cdot-}$)], hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2), possessing higher reactivities than molecular oxygen (Thannickal and Fanburg, 2000). They are generated intracellularly through a variety of processes, for example as by products of normal aerobic metabolism or as second messengers in various signal transduction pathways. They can also be derived from exogenous sources, either being taken up directly by cells from the extracellular milieu or produced as a consequence of the cell's exposure to some environmental insult. Transient fluctuations in ROS serve important regulatory functions, but when present at high and/or sustained levels, ROS can cause severe damage to DNA, proteins and lipids. The cell is endowed with an extensive antioxidant systems to combat ROS, either directly by interception or indirectly through reversal of oxidative damage. When ROS overcomes the cellular defense systems and redox homeostasis is altered, the result is oxidative stress. Oxidative stress is implicated in the pathogenesis of a wide variety of diseases including atherosclerosis, diabetes,

cancer, pulmonary fibrosis, neurodegenerative disorders, arthritis and is believed to be a major factor in aging (Finkel and Holbrook, 2000). ROS can be generated at many different organelles in response to various stimuli. Major sources of ROS production include the mitochondrion, endoplasmic reticulum, plasma membrane and cytosol.

2.1.2. Anti oxidant defenses

Antioxidants are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. The primary antioxidant defense system that prevents oxidative damage directly by intercepting ROS before they can damage intracellular targets consists of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase and thirodoxin reductase. Four classes of SOD have been identified that include Mn-SOD, Cu-Zn-SOD, Ni-SOD and extracellular SOD. All four SOD enzymes destroy the free radical superoxide by converting it to H_2O_2. H_2O_2 is one of the major ROS in the cell. The primary defense mechanisms against H_2O_2 are catalase (Michiels *et al.,* 1994) and GPx through the glutathione (GSH) redox cycle (Reed, 1990). Catalase disintegrates H_2O_2 to water and molecular oxygen. Overexpression of catalase in cytosolic or mitochondrial compartments has been demonstrated to protect cells against oxidative injury (Bai *et al.,* 1999). The GSH

system is probably the most rapid and abundant weapon against ROS accumulation and GSH (γ-glu-cys-gly) regulates the redox state of many other cellular substances. The system consists of GSH, glutathione peroxidase and glutathione reductase. Glutathione peroxidase catalyses the reduction of H_2O_2 and other peroxides and converts GSH to its oxidized form, GSSG. GSSG is then reduced back to GSH by glutathione reductase.

Although there are several enzyme systems within the body that scavenge free radicals, the principle micronutrient (vitamin) antioxidants are vitamin E, beta-carotene, and vitamin C. Additionally, Selenium, a trace metal that is required for proper function of one of the body's antioxidant enzyme systems, is sometimes included in this category. The body cannot manufacture these micronutrients so they must be supplied in the diet.

2.1.3. C-Phycocyanin

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. Spirulina with 62% protein content is the richest

source of Vitamin B-12. It is also rich in beta-carotene and other carotenoids, Vitamin E, minerals (e.g. Manganese, Copper, Iron, Zinc and Selenium), trace elements (e.g. Selenium) and essential fatty acid, gamma-linolenic acid. Recent studies have demonstrated anti oxidant (Miranda *et al.,* 1998), antimutagenic (Chamorro *et al.,* 1996), antiviral (Ayehunie *et al.,* 1998; Hayanshi *et al.,* 1996), anticancer (Mishima *et al.,* 1998; Chen and Zhang, 1995; Schwartz *et al.,* 1988, Schwartz and Shklar, 1987), anti-allergic (kim *et al.,* 1998; Yang *et al.,* 1997), immune enhancing (Hayashi *et al.,* 1994; 1998), hepato-protective (Torres-duran *et al.,* 1998; Gonzalez de rivera *et al* ., 1993), blood vessel relaxing (Paredes-carbajal *et al.,* 1997) and blood lipid lowering effects (Iwata *et al.,* 1990) of Spirulina extracts. The biological and pharmacological properties of Spirulina are attributed mainly to calcium-spirulina and C-Phycocyanin (C-PC) (Hayashi *et al.,* 1996). However, C-PC gained more attention because of its anti-cancer activity and is believed that it might enhance the body's immunity to fight against diseases.

C-PC, the water soluble non-toxic biliprotein pigment, isolated from *Spirulina platensis* has significant anti-oxidant activity (Romay *et al.,* 1998; Romay and Gonzalez, 2000). Phycocyanin was shown to inhibit inflammation in mouse ears (Romay *et al.,* 1998) and prevent acetic acid induced colitis in rats

(Gonzalez *et al.,* 1999). C-PC is used for the treatment of diseases such as Alzheimer's and Parkinson's (Rimbau *et al.,* 1999; 2001). Previous studies from this laboratory showed that C-PC is a specific cyclooxygenase-2 (COX-2) inhibitor (Reddy *et al.,* 2000), hepato-protective (Sathyasaikumar *et al.,* 2007), induces apoptosis in doxorubicin resistant human hepatocellular carcinoma cell line (Roy *et al.,* 2007), human chronic myeloid leukemia cell line-K562 (Subhashini *et al.,* 2004) and also in LPS-stimulated RAW 264.7 cells (Reddy *et al.,* 2003). C-PC was shown to be a peroxyl radical scavenger both *in vivo* and *in vitro* (Bhat *et al.,* 2000), inhibitor of CCl_4 induced lipid peroxidation (Vadiraja *et al.,* 1998) and inhibitor of $ONOO^-$ mediated deleterious biological effects (Bhat *et al.,* 2001).

2.1.4. ROS: Role in diseases

There is growing awareness that oxidative stress plays a role in various clinical conditions such as malignant diseases, diabetes, atherosclerosis, chronic inflammation, viral infection, and ischemia-reperfusion injury (Behrend *et al.,* 2003; Apel *et al.,* 2004; Bergamini *et al.,* 2004; Reddy and Clark, 2004; Shah and Channon, 2004; Willner, 2004.) ROS can cause oxidative DNA and protein damage, damage to tumor suppressor genes and enhanced expression of proto-oncogenes (Wei, 1992; Cerutti, 1994; Bohr and Dianov , 1999) and oxidative

stress has been shown to induce malignant transformation of cells in culture (Weitzman and Gordon, 1990). Diseases associated with oxidative stress such as diabetes mellitus and cancer show a pro-oxidative shift in the redox state and impaired glucose clearance suggesting that muscle mitochondria is the major site of elevated ROS production. This condition may be referred to as 'mitochondrial oxidative stress'. Cancer patients commonly have decreased glucose clearance capacity, high glycolytic activity and lactate production. It is, therefore, suggested that the observed pro-oxidative shift is mediated by an increased availability of mitochondrial energy substrate. The 'inflammatory oxidative conditions' are typically associated with an excessive stimulation of NADPH oxidase by cytokines and other factors. The increased ROS production or changes in intracellular glutathione levels are often involved with pathological changes indicative of a dysregulation of signal cascades or gene expression (Droge *et al.,* 2002).

2.1.5. ROS: Role in multidrug resistance

ROS is produced in the cells as a result of various signaling pathways such as receptor tyrosine kinases (RTKs) which become activated by growth factors – epidermal growth factor, platelet derived growth factor, fibroblast growth factor as well as cytokines (tumor necrosis factor, γ-interferon and interleukins) leading

to an intracellular tyrosine phosphorylation cascade (Behrend *et al.,* 2003). Reactive oxygen species may be also formed in response to xenobiotic exposure. Ligand (xenobiotic) receptor binding has been shown to induce the production of ROS (Finkel, 1998). The ROS activated signal transduction pathways are regulated by two distinct protein families – the Mitogen Activated Protein Kinase (MAPK) and the redox sensitive kinases. Redox-sensitive signaling factors regulate multiple processes including proliferation, cell cycle and anti-apoptotic signaling pathways. The modification of gene expression by reactive oxygen species has direct effects on cell proliferation and apoptosis through the activation of transcription factors including AP-1 and NF-κB pathways. Likewise, reactive oxygen species function as second messengers involved in activation of NF-κB by tumor necrosis factor and cytokines. Alteration in the redox potential of the cells exposed to xenobiotics may affect ROS responsive signaling pathways.

Exposure to xenobiotics and carcinogens alters the physiological expression of multidrug resistance genes. Aflotoxin B1 (Burt and Thorgeirsson, 1988), phenothiazine (Burt and Thorgeirsson, 1988), 2-acetylaminofluorene (Burt and Thorgeirsson, 1998), methyl sulfonate (Fardel *et al.,* 1996), 3-methylcholanthrene (Fardel *et al.,* 1998) and mitoxantrone (Schrenk *et al.,* 1996)

were shown to induce the expression of MDR1. The hepatocarcinogen, 2-AAF activates the expression of MDR1 through the generation of ROS and activation of NF-κB in rat hepatoma cells (Lei *et al.,* 2001). ROS activates signal transduction pathways that enhance NF-κB translocation to nucleus (Kabe *et al.,* 2005). Numerous reports indirectly support the notion that intracellular ROS lead to the activation of NF-κB (Brash *et al.,* 1991; Hollstein *et al.,* 1991; Reid and Loeb, 1993). Akt/Protein kinase B (PKB), a cell survival protein, was also shown to be phosphorylated in response to 2-AAF treatment, which further activates NF-κB pathway. Upregulation of the activity and expression of Akt by ROS was reported (Zhang *et al*., 2005). It is known that 2-AAF induces the generation of ROS in rat hepatoma cells (Lei *et al*., 2001) and interaction of ROS and PI_3K further activates the NF-κB dependent pathway (Lee *et al.,* 2005). Akt/PKB activation increases resistance to apoptosis and enhances cell survival. ROS activate signal transduction pathways that enhance NF-κB, a positive regulator of MDR1 expression, translocation to nucleus which may be critical in regulation of MDR1 expression. The fact that natural antioxidants such as taxifolin (Wang *et al.,* 2005) and synthetic compound (3E)-4-(2-hydroxyphenyl) but-3-en-2-one (Tsao *et al*., 2005) suppress the generation of intracellular ROS and in turn inhibit

the activation of NF-κB, suggests their possible involvement in the regulation of MDR1.

In the light of the above scenario, the present study is undertaken to evaluate the role of ROS in the regulation of MDR1 expression and analyze the effect of natural antioxidants. These studies were taken up in mouse macrophage cell line (RAW 264.7) exposed to 2-AAF alone or in combination with C-PC, a natural antioxidant isolated from *Spirulina platensis.* Additionally computational modeling and *in silico* approaches were employed to further understand the underlying mechanisms.

2.1.6. Objectives

- ❖ To study the association between reactive oxygen species (ROS) and multidrug resistance.
- ❖ Elucidate the role of antioxidants (C-Phycocyanin) in overcoming drug resistance.
- ❖ To understand the molecular mechanisms involved in the regulation of MDR1 expression by ROS and antioxidants.

2.2. Materials and Methods

2.2.1. Chemicals

PBS, RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco BRL (California, USA). Nitrocellulose membrane was from Millipore (Bangalore, India). 2-AAF, TMB/H_2O_2, Acetyl-CoA, DCFH-DA, DPI, phosphatase inhibitor cocktail 1 and 2 and β-actin antibodies were purchased from Sigma-Aldrich (Bangalore, India). Polyclonal antibodies to Akt and MDR1 were from Santa Cruz Biotechnology (California, USA) and phospho-Akt (p-Akt) was from Upstate (Charlottesville, VA, USA). p50 and p65 antibodies were from Oncogene research products (California, USA). Single step RT-PCR kit was from AB Gene Technologies (Surrey, UK). Akt inhibitor IV was obtained from Calbiochem (Darmstadt, Germany). ECL detection kit was from Amersham Biosciences (Buckinghamshire, UK). FAST CAT Chloramphenicol acetyl transferase assay kit was from Molecular Probes (California, USA). Transfection reagent (Gene Juice) was from Novagen (Darmstadt, Germany). CAT expression plasmids containing 5'deletion fragments of mdr1 promoter were kindly provided by Dr. Martine Raymond, Institut de Recherches Cliniques de Montreal, Montreal, Canada. C-PC was a generous gift from Prof. K. M. Madyastha, Indian

Institute of Science (I. I. Sc.), India. All the other chemicals and reagents were purchased from local companies and are of molecular biology grade.

2.2.2. Cell culture and treatment

Mouse macrophage cells (RAW 264.7) were grown in RPMI 1640 medium supplemented with 10% heat inactivated 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 ^{o}C. The cultured cells were sub-cultured twice each week. Exponentially growing cells were used for all treatments. C-PC dissolved in PBS was used for the treatments. 20 mM stock of 2-AAF, dissolved in absolute alcohol was employed in this study. The final concentration of the vehicle (ethanol) never exceeded 0.1%. RAW 264.7 cells exposed to 0.1% ethanol served as controls.

2.2.3. Northern blot analysis of MDR1 mRNA expression

RAW 264.7 cells were seeded at a density of 5 x 10^6 in 90 mm culture dishes. Cells were first pre-incubated with C-PC (5 µM, 10 µM, 25 µM & 50 µM) for 4 h and DPI (10 µM), Akt inhibitor IV (10 µM) for 30 min and then 2-AAF was added at a final concentration of 100 µM. Cells were harvested after 6 h incubation with 2-AAF. Total RNA was extracted from control and treated RAW 264.7 cells using TRIzol reagent. The isolated RNA was quantified

spectrophotometrically and the integrity was checked on agarose formaldehyde gels. 30 µg of total RNA was separated by electrophoresis on 1.5% agarose gel containing 0.66 M formaldehyde and transferred to a nylon membrane. After cross linking of RNA to membrane using UV (245 nm) for 1 min at 1.5 joules/cm^2, the membranes were incubated in pre-hybridization solution (6 X SSC, 5 X Denhardt's reagent, 0.5% SDS, 50% Formamide and 100 µg/ml Salmon sperm DNA) for 1-2 h at 42 ^oC. The membranes were then transferred to hybridization solution containing cDNA probes at 55 ^oC for 20 h. cDNA probes for MDR1 and GAPDH were obtained by RT-PCR. RT-PCR was performed with 5 µg total RNA, isolated from RAW 264.7 cells, using one step RT-PCR kit. Primers used were as follows: mouse MDR1: forward, 5'-TGCTTATGGATCCCAGAGTGAC-3', reverse, 5'-TTGGTGAGGATCTCT CCGGCT-3'; which yields 435 bp product, GAPDH: forward, 5'-CTCATG ACCACAGTCCATGCCATC-3', reverse, 5'-CTGCTTCACCACCTTCTTGAT GTC-3'; which yields 272 bp product (Jason and Li , 2002). Reverse transcription was performed at 47 ^oC for 30 min. Subsequent to inactivation of reverse transcriptase (2 min, 94 ^oC), the samples were subjected to 30 amplification cycles, each consisting of 20 sec at 94 ^oC, 1 min at 54 ^oC, and 1 min at 72 ^oC, followed by 5 min at 72 ^oC after the last cycle. The products were analyzed on 1%

agarose gel and then extracted by QIA quick gel extraction kit (Qiagen). Each probe was labeled with α-^{32}P deoxy adenosine triphosphate, using random priming reaction (Fermentas). After incubation in hybridization solution, membranes were washed three times at 55 ^{o}C for 10 min with wash buffer containing 2 X SSC and 0.1% SDS. Other two washes were with 1 X and 0.5 X SSC along with 0.1% SDS. Membranes were then exposed to phosphoimager screen (Amersham) for 4 hours. The screen was later visualized on Amersham typhoon variable mode imager 9410.

2.2.4. Measurement of ROS

ROS production in control and treated RAW 264.7 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 ROS. Cells seeded at a density of 2 x 10^6 in 60 mm culture dishes were first pre-incubated with C-PC (25 µM & 50 µM) for 4 h and DPI (10 µM), Akt inhibitor IV (10 µM) for 30 min and then 2-AAF was added at a final concentration of 100 µM for 45 min. Cells were harvested after 10 min incubation with DCFH-DA (10 µM) and washed with PBS. ROS 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: λ_{exc} = 488 nm, λ_{em} = 525 nm. 20,000 cells were analyzed per sample.

Superoxide generation upon 2-AAF treatment was measured according to the procedure described elsewhere (De Mendez *et al.,* 1994). RAW 264.7 cells were seeded at a density of 5 x 10^5 per well in 100 µl of Hank's balanced salt solution without Ca^{2+} and Mg^{2+} containing 150 µM ferricytochrome C and 50 µM and 100 µM concentrations of 2-AAF. A control reaction contained 0.1 µg of superoxide dismutase (SOD). After incubation at 37^o C for 60 min, the absorbance of cell suspension at 550 nm was determined. Extension coefficient used for superoxide determination was 21 mM^{-1} cm^{-1}.

2.2.5. Electrophoretic mobility shift assay (EMSA)

RAW 264.7 cells at a density of 5 x 10^6 were seeded in 90 mm culture dishes. Cells were first pre-incubated with C-PC (5 µM, 10 µM, 25 µM & 50 µM) for 4 h and DPI (10 µM), Akt inhibitor IV (10 µM) for 30 min and then 2-AAF was added at a final concentration of 100 µM. Cells were harvested after 2 h incubation with 2-AAF and were used for nuclear protein extraction. The cells were washed with PBS and harvested by centrifugation at 2000 rpm for 3 min. 200 µl 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 sec each. The nuclei were then harvested by centrifugation at 16,000 rpm for 1 min. The nuclear pellet was resuspended in 40 µl of nuclear protein extraction buffer (420 mM NaCl, 10 mM HEPES, 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM DTT and 25% glycerol) and incubated on ice for 30 min with intermittent vortexing of 10 sec each. The sample was then centrifuged at 13,000 rpm for 30 min at 4 ^oC. The supernatant collected was used for the mobility shift assay after protein estimation using Bradford assay (Bradford, 1976). Nuclear extracts (8 µg) were incubated with γ-^{32}P labeled double stranded oligonucleotide with specific NF-κB binding sequence (5'-AGT TGA GGG GAC TTT CCC AGG C-3') for 30 min at 37 ^oC. For supershift assay nuclear extract of 2-AAF treated cells were incubated with antibodies against p50 or p65 of NF-κB for 20 min at 37 ^oC. 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 also examined by competition with unlabeled oligonucleotide (cold competition).

2.2.6. Preparation of whole cell extracts and immunoblot analysis

RAW 264.7 cells at a density of 5 x 10^6 were seeded in 90 mm culture dishes. They were first pre-incubated with C-PC (5 µM, 10 µM, 25 µM & 50 µM) for 4 h and DPI (10 µM), Akt inhibitor IV (10 µM) for 30 min and then 2-AAF was added at a final concentration of 100 µM. Cells, harvested after 90 min (Akt and p-Akt) and 6 h (MDR1) incubation with 2-AAF, were used for preparation of whole cell extract. The harvested, control and treated, RAW 264.7 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 µg/ml leupeptin, 20 µg/ml aprotinin and phosphatase inhibitor cocktail 1 and 2 with 100 fold dilution). After 30 min of shaking at 4 ^{o}C, the mixtures were centrifuged (10,000 x 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 µg of protein from each treatment was resolved on 10% (Akt and p-Akt) and 7% (MDR1) SDS-PAGE gels 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 [Akt (1:250

dilution), p-Akt (1:500 dilution) and MDR1 (1:500 dilution)] in 10 ml of antibody-diluted buffer (1X Tris-buffered saline and 0.05% Tween-20 with 1% milk) with gentle shaking at 4 ^{o}C for 8-12 h and then incubated with peroxidase conjugated secondary antibodies. Signals were detected by using peroxidase substrate, TMB/H_2O_2 or ECL kit. Equal protein loading was confirmed by reprobing the nitrocellulose membranes with β-actin antibodies (1:500 dilution).

2.2.7. Transient transfection and preparation of cell extracts

RAW 264.7 cells were seeded at a density of 6 x 10 ^{5} cells in 60 mm culture dishes and transfected with 5 µg plasmid DNA (p141 or p245) using transfection reagent in the serum free medium according to manufacturers' instructions. After 5 h of incubation cells were washed twice with PBS and supplied with serum containing medium. Plasmid, pGFP, was co-transfected and it was used as a transfection control. After 20 h of growth in the normal medium, cells were pre-incubated with C-PC (25 µM & 50 µM) for 4 h and then 2-AAF was added at a final concentration of 100 µM. Cells were harvested after 4 h incubation with 2-AAF and washed with PBS. Cells were re-suspended in 100 µl of 0.25 M Tris-HCl, pH 7.4 and subjected to freeze thaw cycles by altering the suspensions between ethanol bath and a 37 ^{o}C water bath with intermittent vortexing. The lysed suspension was centrifuged at 12,000 rpm at 4 ^{o}C for 5 min.

The extracts were heated at 65 ^{o}C for 10 min to inactivate endogenous acetylating enzymes. The quantity of protein was estimated by Bradford assay (Bradford, 1976).

2.2.8. Chloramphenicol acetyl transferase (CAT) enzyme assay

Protein (4 µg/60 µl) from the extracts of cells transfected with p141 and p245 was mixed with 10 µl of CAT substrate solution and incubated at 37 ^{o}C for 5 min. To this 10 µl of 9 mM acetyl CoA was added and further incubated for another 2 h. CAT reaction was stopped by adding 1 ml of ice-cold ethyl acetate. The samples were then centrifuged at 10,000 rpm for 3 min, 900 µl of the top ethyl acetate layer was taken to a fresh tube, evaporated to dryness and the contents were redissolved in 20 µl of ethyl acetate. The samples and mono acetyl chloramphenicol reference standard were analyzed on TLC. Chloroform and methanol (85:15 v/v) mixture was used as eluant. The TLC plates were analyzed on Amersham typhoon variable mode imager 9410. Later acetylated CAT substrate spot (mono acetyl chloramphenicol) from TLC plates was scraped, extracted into methanol and fluorescence signals were estimated using fluorometer at 504 nm.

2.2.9. Confocal analysis

RAW 264.7 cells (1 x 10^5) were exposed to C-PC (50 µM) for 24 h. Cells after treatment were observed for C-PC entry into RAW 264.7 cells under confocal microscope.

2.2.10. *In silico* modeling

Model Overview

The model was developed using Teranode's Biological Modeler, which uses MML (Mathematical Modeling Language, developed by the National Simulation Resource at the University of Washington) to simulate the 2-AAF-induced MDR1 expression via a ROS mediated pathway. Rates of change of the concentration of each component with time are modeled as differential equations that are solved by the Radau method (Hairer and Wanner, 1999). A schematic representation of the elements of the model and their connections are shown in figure 36.

Model structure and description

AAF induced ROS-Akt-NF-κB pathway has been modeled as follows:

1. AAF causes Ca^{2+} elevation in the cytoplasm causing activation of NADPH oxidase (Lefebvre *et al.,* 1992). NADPH oxidase transports electrons from intracellular NADPH via FAD and its two heme groups to oxygen,

leading to formation of superoxide radical which is then converted to H_2O_2 (Banfi *et al.,* 2004).

2. ROS (H_2O_2) promotes tyrosine phosphorylation by activating protein kinases (Rhee, 2006). ROS in this system helps in the autophosphorylation and activation of the tyrosine kinase Src (Giannoni *et al.,* 2005; Rosado *et al*., 2004).

3. Activated Src via activation of PI_3K and formation of PIP_3, activates RAC by converting RAC GDP to RAC GTP.

4. The activated RAC GTP in turn further activates NADPH oxidase which causes further production of ROS and it goes into a postive feedback loop through Src (Seshiah *et al* ., 2002). This leads to a biphasic production of ROS with 2-AAF treatment, with a slow initial rise and once the feedback loop sets in, a subsequent exponential rise in ROS (Seshiah *et al* ., 2002).

5. Activated Src activates PDK1 (Prasad et al., 2000), that in turn activates Akt (Taniyama *et al*., 2004). This leads to the activation of NF-κB.

6. NF-κB is the transcription factor regulating MDR1 expression. MDR1 mRNA is translated to MDR1 protein in the cytoplasm.

Model assumptions

Model initial conditions without 2-AAF activation have been calibrated to control sample levels from experimental data. There is a basal level of ROS being produced from NDAPH oxidase, producing the control level of MDR1

mRNA and protein expression as in the experimental control sample. With 2-AAF treatment the model is calibrated to obtain the same ROS levels as in experimental data and also obtain similar MDR1 mRNA and MDR1 protein levels. With 50 μM C-PC treatments, the ROS levels in the model were calibrated to show similar reduction as in experimental data. Also, Ki values for DPI and Akt inhibitor IV were optimized to obtain the experimental fold difference in MDR1 protein levels. This was used as the training data for the model to match the experimental setup.

Simulation protocols

1. AAF was used at a concentration of 100 μM as in the experiments.
2. NADPH oxidase inhibitor, DPI, was used at a concentration of 10 μM and an arbitrary Ki of 1.01 μM.
3. Akt inhibitor IV was used at a concentration of 10 μM with an arbitrary Ki of 25 nM.
4. C-PC was modeled as a peroxyl radical scavenger, which form water and O_2 with an arbitrary Kf.
5. Levels of initial concentrations of most species were chosen from values obtained in literature

2.2.11. Statistical analysis

Data reported as the mean +/- SE of three independent experiments. Statistical analysis of differences was carried out by one-way analysis of variance (ANOVA). A P-value of less than 0.05 was considered to indicate significance.

2.3. Results

In order to understand the role of ROS in the regulation of MDR1 expression, mouse macrophage cell line, RAW 264.7 cells, exposed to 2-AAF was employed as a model system. RAW 264.7 cells exposed to 100 µM AAF resulted in significant induction of MDR1 at mRNA and protein levels and the increase in the MDR1 levels were decreased upon C-PC treatment.

2.3.1. C-PC inhibited the 2-AAF-induced transcription of MDR1

To elucidate whether C-PC inhibited the 2-AAF induced expression of MDR1 mRNA, Northern blot analysis was performed. Concentration and time dependent activation of MDR1 expression by 2-AAF was standardized. Prior incubation of RAW 264.7 cells with C-PC for 4 h at concentrations of 5 µM, 10 µM, 25 µM and 50 µM inhibited the 2-AAF induced expression of MDR1 mRNA in a dose dependent manner (Fig. 19). Pre-incubation of cells with DPI (10 µM) and Akt inhibitor IV (10 µM) for 30 min markedly diminished the transcription of MDR1, though not to the same extent of C-PC (Fig. 19). Unaltered levels of GAPDH transcripts in all treatments confirm specific inhibition of MDR1 expression by C-PC. *In silico* studies also showed similar trends when compared to the experimental data (Fig. 20).

C-PC (µM)	0	0	5	10	25	50	0	0
2-AAF (µM)	0	100	100	100	100	100	100	100
DPI (µM)	0	0	0	0	0	0	10	0
Akt inhibitor IV (µM)	0	0	0	0	0	0	0	10

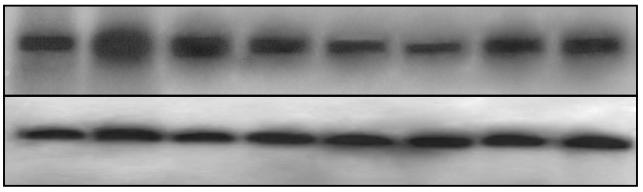
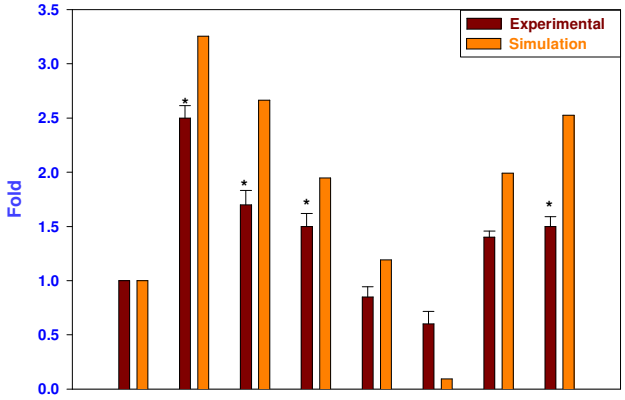


Fig.19. Northern blot analysis of C-PC, DPI and Akt inhibitor IV mediated effect on MDR1 mRNA expression in RAW 264.7 cells.



C-PC (µM)	0	0	5	10	25	50	0	0
2-AAF (µM)	0	100	100	100	100	100	100	100
DPI (µM)	0	0	0	0	0	0	10	0
Akt inhibitor IV (µM)	0	0	0	0	0	0	0	10

Fig. 20. Bar graphs showing the fold difference in the expression levels of MDR1 mRNA obtained by experimental and simulation data.

*** Denotes statistical significance over control (P< 0.05).**

2.3.2. C-PC inhibited the 2-AAF-induced MDR1 protein expression

To elucidate whether C-PC inhibited the 2-AAF induced expression of MDR1 protein, Western blot analysis was performed. Prior incubation of RAW 264.7 cells with C-PC for 4 h at concentrations of 5 µM, 10 µM, 25 µM and 50 µM inhibited the 2-AAF induced expression of MDR1 in a dose dependent manner (Fig. 21). DPI (10 µM) and Akt inhibitor IV (10 µM) treatment also diminished the expression of MDR1. Unaltered levels of β-actin in all treatments confirm specific inhibition of MDR1 by C-PC. *In silico* studies showed similar inhibition of 2-AAF induced MDR1 protein levels (Fig. 22).

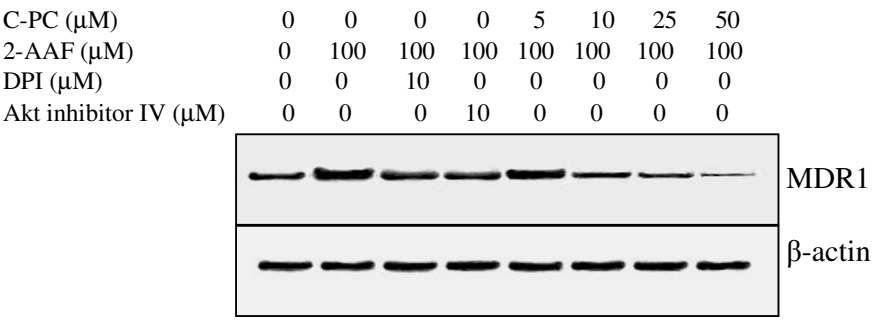
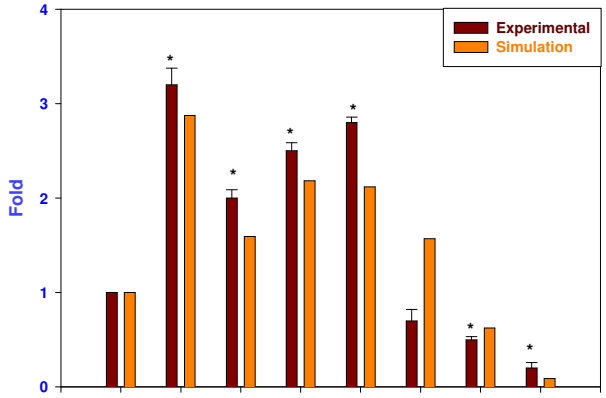


Fig. 21. Western blot analysis of C-PC, DPI, Akt inhibitor IV mediated effect on MDR1 protein expression in RAW 264.7 cells.



C-PC (µM)	0	0	0	0	5	10	25	50
2-AAF (µM)	0	100	100	100	100	100	100	100
DPI (µM)	0	0	10	0	0	0	0	0
Akt inhibitor IV (µM)	0	0	0	10	0	0	0	0

Fig. 22. Bar graphs showing the fold difference in the expression levels of MDR1 protein obtained by experimental and simulation data.

*** Denotes statistical significance over control (P< 0.05).**

2.3.3. C-PC decreased the 2-AAF-induced generation of ROS

In the context of anti-oxidant properties of C-PC, further studies were undertaken for analyzing the potential of C-PC as a ROS scavenger. C-PC was tested for its effect on 2-AAF-induced generation of ROS in RAW 264.7 cells. Generation of ROS in the cells was standardized with 2-AAF (100 µM) treatment at various time points using DCFH-DA by FACS analysis. Compared to control, 5.5 folds enhancement of ROS generation was observed at 100 µM concentration

of 2-AAF in 45 min time interval. Cells pre-incubated with C-PC showed a dose dependent decrease in the generation of ROS.

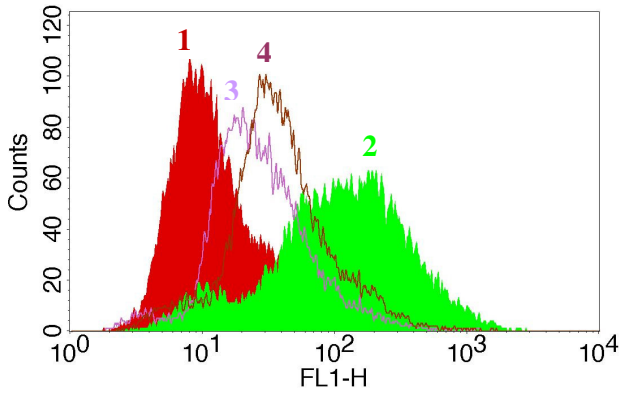


Fig. 23. FACS analysis on generation of ROS using DCFH-DA in cells exposed to 2-AAF with or without C-PC pretreatment

1, Control; 2, 2-AAF; 3, 2-AAF + C-PC (25 µM); 4, 2-AAF + C-PC (50 µM)

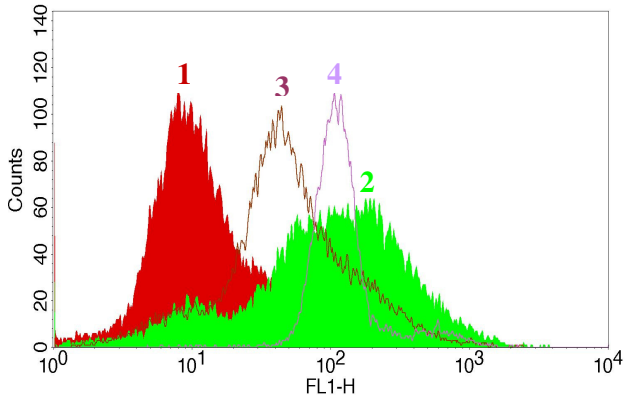


Fig. 24. FACS analysis on generation of ROS using DCFH-DA in cells exposed to 2-AAF with or without DPI and Akt inhibitor IV pretreatment.

1, Control; 2, 2-AAF; 3, 2-AAF + DPI (10 µM); 4, 2-AAF + Akt inhibitor IV (10 µM).

Fig. 23 shows 60% and 45% decrease in the generation of ROS in cells pre-incubated with C-PC at 50 µM and 25 µM compared to that of enhanced ROS generation (5.5 folds) in cells treated with 2-AAF alone.

Cells treated with DPI (10 µM) showed 40% decrease in the generation of ROS, where as treatment with Akt inhibitor IV (10 µM) did not alter generation of ROS (Fig. 24). Production of superoxide anion by NADPH Oxidase increased 1.5 and 2.5 folds in response to 2-AAF (50 µM and 100 µM) treatment (Fig. 25).

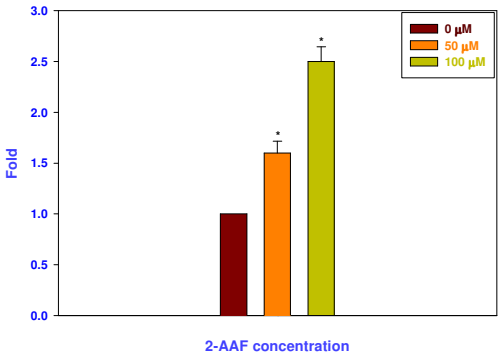


Fig. 25. Bar graph showing the generation of superoxide in cells after 60 min of 2-AAF treatment

*** Denotes statistical significance over control (P< 0.05)**

Biphasic ROS generation in response to 2-AAF treatment was observed *in silico* model (Fig. 26) and ROS levels measured at 45 min and 2.5 h time period with C-PC, DPI and Akt inhibitor IV using *in silico* model were shown in Table 1. In the biphasic ROS generation, first phase is due to ROS generated by

NADPH oxidase upon 2-AAF treatment and the second phase is due to the positive feedback loop of ROS generation due to Src activation (Fig. 36).

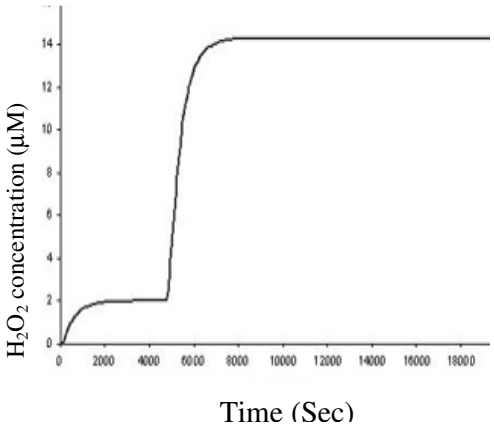


Fig. 26. Line Plot showing concentration of H_2O_2 being formed in µM on Y axis against time (sec) on X axis.

This was obtained by running simulation model with a trigger of 100 µM 2-AAF for 20,000 sec without any inhibitor. ROS levels obtained at 2700 sec were in the first phase of ROS generation by 2-AAF.

Treatment	ROS levels (µM) at 45 min	ROS levels (µM) at 2.5 h
Control	0.324	0.3225
AAF (100 µM)	1.952	14.24
2-AAF + C-PC (5 µM)	1.142	13.21
2-AAF + C-PC (10 µM)	0.788	5.59
2-AAF + C-PC (25 µM)	0.399	4.037
2-AAF + C-PC (50 µM)	0.217	0.236
2-AAF + DPI (10 µM)	0.9025	6.57
2-AAF + Akt inhibitor IV (10 µM)	1.952	14.24

Table 1. ROS levels with different treatments at 45 min and 2.5 h.

The model was simulated with 2-AAF individually and in combination with inhibitors (C-PC, DPI and Akt inhibitor IV)

2.3.4. C-PC inhibited the activation of Akt

Akt, a cell survival protein is activated by various growth and survival factors. Akt signaling pathway is involved in 2-AAF-induced expression of MDR1. In addition to the anti-oxidant properties of C-PC, it was investigated whether C-PC has any effect on the activation of Akt in 2-AAF treated RAW 264.7 cells. Treatment of cells with 2-AAF at a concentration of 100 µM for 90 min resulted in maximum increase in the phosphorylation of Akt.

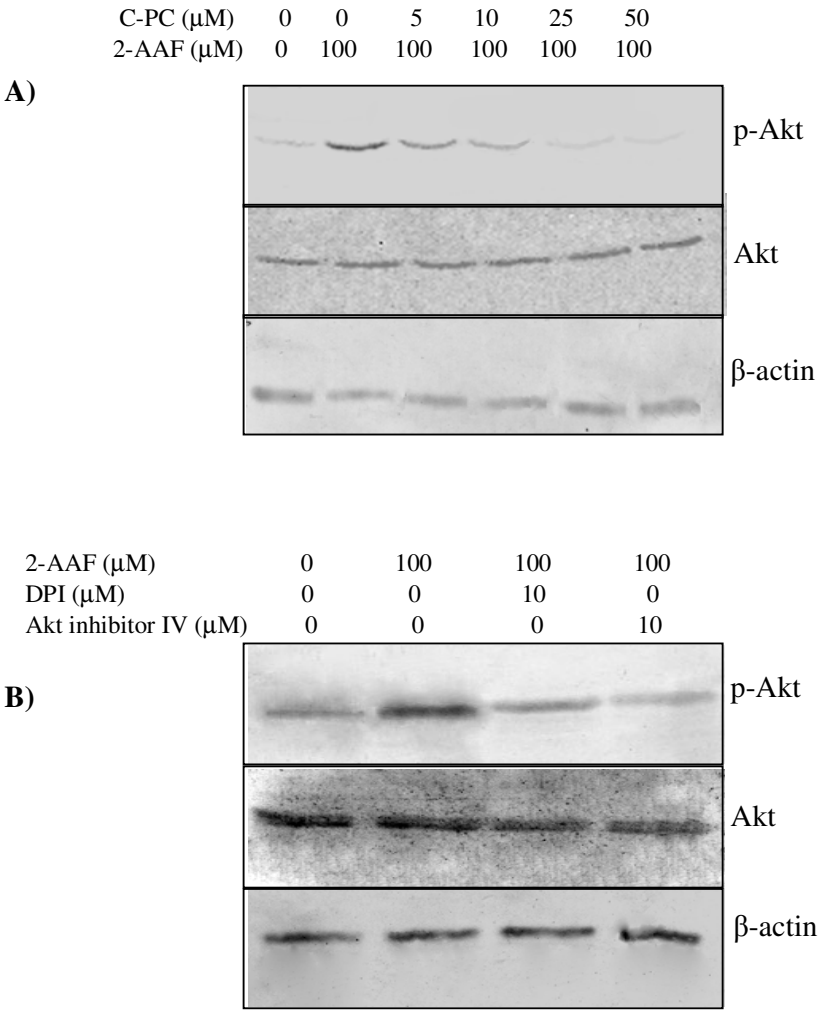
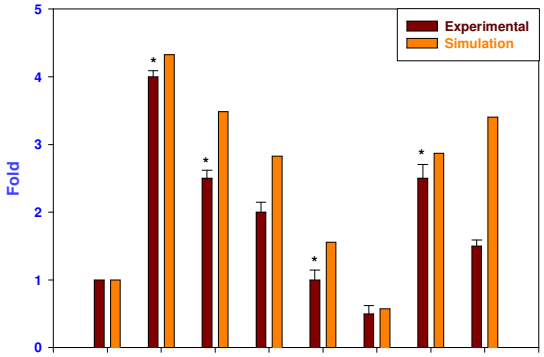


Fig. 27. Western blot analysis of on Akt and p-Akt protein expression in RAW 264.7 cells under the influence of C-PC (A), DPI and Akt inhibitor IV (B)



C-PC (µM)	0	0	5	10	25	50	0	0
2-AAF (µM)	0	100	100	100	100	100	100	100
DPI (µM)	0	0	0	0	0	0	10	0
Akt inhibitor IV (µM)	0	0	0	0	0	0	0	10

Fig. 28. Bar graphs showing the fold difference in the expression levels of p-Akt protein obtained by experimental and simulation data

*** Denotes statistical significance over control (P< 0.05)**

Fig. 27A shows the Western blot analysis of Akt and p-Akt in C-PC treated cells. The level of phosphorylated Akt increased in 2-AAF (100 µM) alone treated cells compared to control cells and decreased in concentration dependent manner in cells pre-incubated with C-PC. Pre-incubation of cells with DPI (10 µM) and Akt inhibitor IV (10 µM) for 30 min also resulted in inhibition of Akt phosphorylation induced by 2-AAF (Fig. 27B). The levels of Akt, on the other hand, were un-altered irrespective of treatments compared to control. The simulation data for Akt inhibition by C-PC, DPI and Akt inhibitor IV aligned quite well with the experimental data (Fig. 28).

2.3.5. C-PC prevented 2-AAF-induced translocation of NF-κB into nucleus

Activation of Akt stimulates IKKα that ultimately leads to the increase in the translocation of NF-κB from cytoplasm to nucleus. In the present study an increase in the translocation of NF-κB from cytoplasm to nucleus was observed in the 2-AAF (100 µM) treated RAW 264.7 cells compared to the control cells (Fig. 29). To investigate the effect of C-PC on the 2-AAF-induced translocation of NF-κB to the nucleus, the nuclear extracts of cells pre-incubated with C-PC were analyzed.

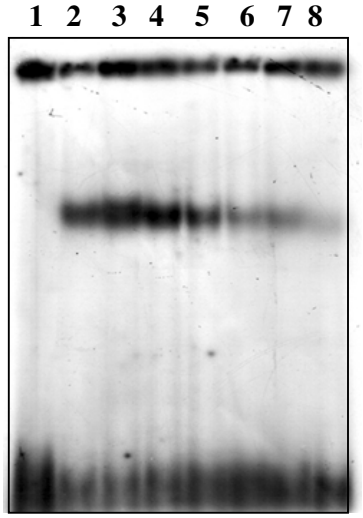


Fig. 29. Effect of C-PC on nuclear translocation of NF-κB in RAW 264.7 cells

Electro mobility shift assay of nuclear extracts using specific NF-κB binding probe in cells exposed to 2-AAF (100 µM) with or without C-PC. Lane 1: Free probe; lane 2: Control; lane 3: 2-AAF; lane 4: 2-AAF + C-PC (5 µM); lane 5: 2-AAF + C-PC (10 µM); lane 6: 2-AAF + C-PC (25 µM); lane 7: 2-AAF + C-PC (50 µM); lane 8: Cold competition

These studies revealed decrease in the translocation of NF-κB in a concentration dependent manner in C-PC treated cells compared to the 2-AAF alone treated cells (Fig. 29).

DPI and Akt inhibitor IV treatment also inhibited NF-κB translocation to nucleus (Fig. 30A). Incubation of nuclear extract of 2-AAF treated cells with antibody either to p50 or p65 subunit of NF-κB shifted the band, suggesting the specificity towards NF-κB (Fig. 30B).

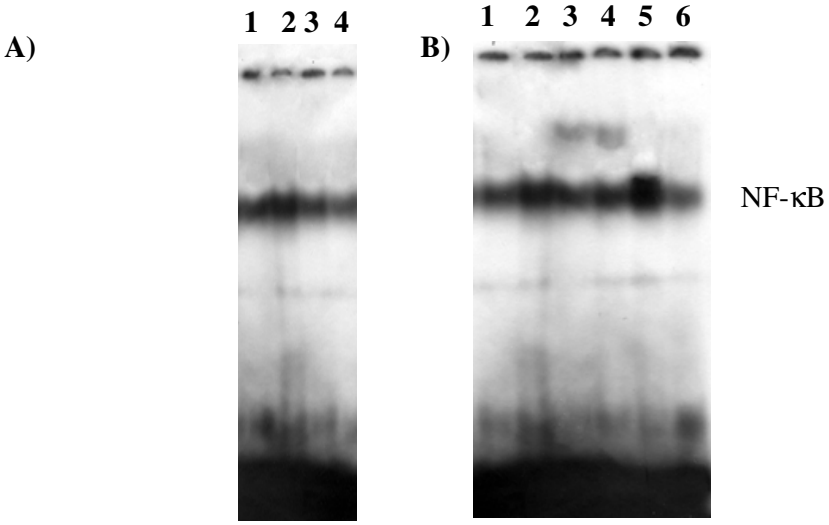
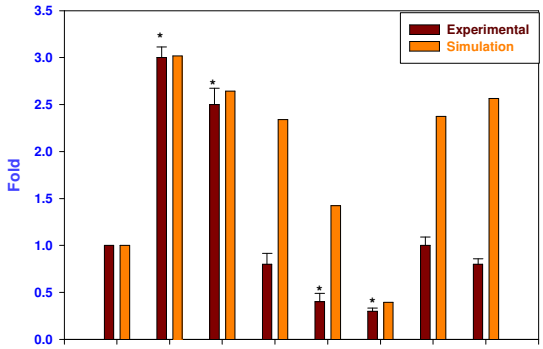


Fig. 30. Effect of DPI and Akt inhibitor IV on nuclear translocation of NF-κB in RAW 264.7 cells

Electro mobility shift assay of nuclear extracts using specific NF-κB binding probe in cells exposed to 2-AAF (100 µM) with or without DPI and Akt inhibitor IV pretreatment (A) Lane 1: Control; lane 2: 2-AAF; lane 3: 2-AAF + DPI (10 µM); lane 4: 2-AAF + Akt inhibitor IV (10 µM) (B) Super shift assay of nuclear extracts using specific antibody against p50 and p65 in cells exposed to 2-AAF (100 µM) Lane 1: Control; lane 2: 2-AAF treated; lane 3: 2-AAF + Anti-p50; lane 4: 2-AAF + Anti-p65; lane 5: TNFα (5 nM); lane 6: Cold competition

Treatment with TNFα (5 nM) induced the NF-κB translocation and served as positive control. Lack of signal in the cold competition sample (excess of unlabelled oligo) shows the specificity of the NF-κB binding probe. Simulation data of NF-κB translocation correlated well with the experimental data (Fig. 31).



C-PC (μM)	0	0	5	10	25	50	0	0
2-AAF (μM)	0	100	100	100	100	100	100	100
DPI (μM)	0	0	0	0	0	0	10	0
Akt inhibitor IV (μM)	0	0	0	0	0	0	0	10

Fig. 31. Bar graphs showing the fold difference in the NF-κB translocation obtained by experimental and simulation data

*** Denotes statistical significance over control (P< 0.05)**

2.3.6. C-PC inhibited NF-κB dependent induction of mdr1 promoter by 2-AAF

Presence of putative NF-κB binding site (position -163 to -157) was indicated in the promoter of mdr1 (Mathieu *et al.,* 2001). To evaluate the role of

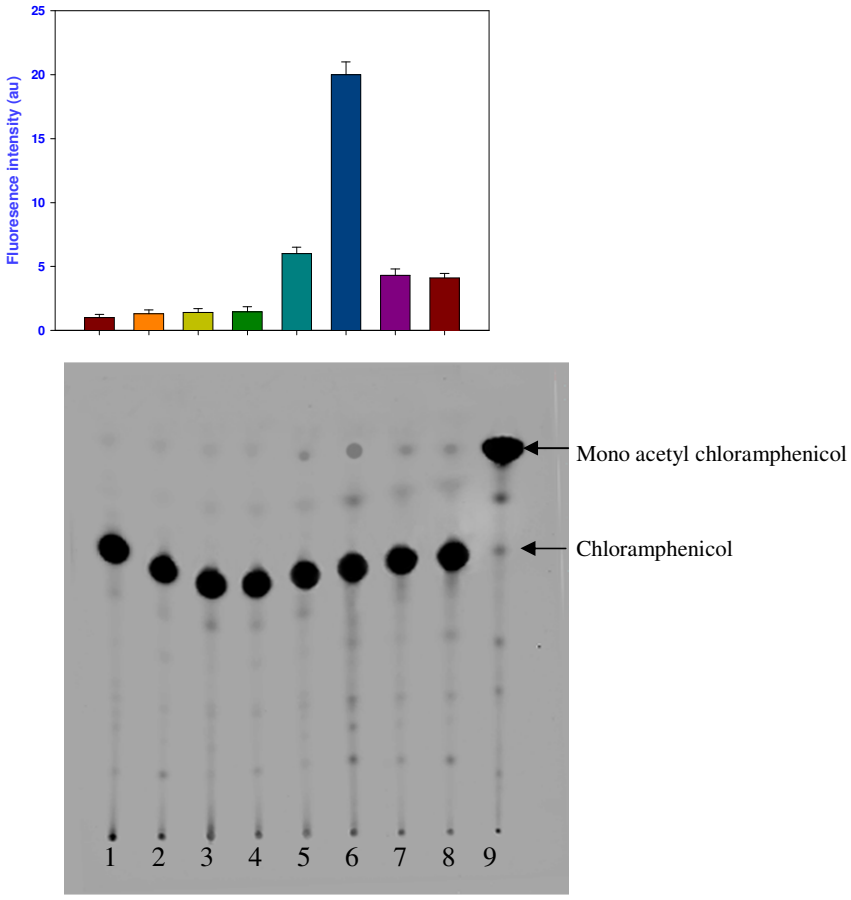


Fig. 32. Transient transfection analysis using plasmids containing CAT gene under the promoter of mouse mdr1 in RAW 264.7 cells

TLC analysis and relative fluorescence intensities of CAT reaction products (Monoacetyl chloramphenicol) obtained using the lysates of the cells exposed to 2-AAF (100 µM) with C-PC (3, 4 & 7, 8) and without C-PC pretreatment (1, 2 & 5, 6) and transfected with the plasmid (p141) lacking putative NF-κB binding element (1-4) and plasmid (p245) with putative NF-κB binding element (5-8). 1: Control; 2: 2-AAF; 3: 2-AAF + C-PC (25 µM); 4: 2-AAF + C-PC (50 µM); 5: Control; 6: 2-AAF; 7: 2-AAF + C-PC (25 µM); 8: 2-AAF + C-PC (50 µM). The relative fluorescence intensity of the products is shown in the bar diagram

putative NF-κB binding site and to support NF-κB as the regulatory element in the induction of mdr1 upon 2-AAF treatment and to analyze the role of C-PC on NF-κB activation, transient transfection analysis was carried out.

Minimal induction of CAT, i.e. formation of mono acetyl chloramphenicol in the control cells and those treated with 100 μM 2-AAF and C-PC along with 2-AAF was observed in the cells transfected with plasmid (p141) lacking putative NF-κB binding element (-163 to –157) in the promoter region of CAT. Increase in the induction of CAT i.e. increase in formation of mono acetyl chloramphenicol in 2-AAF (100 μM) treated cells and a decrease in the induction of CAT upon prior incubation of cells with C-PC was observed in cells transfected with plasmid (p245) containing the putative NF-κB binding element in the promoter region of CAT. Relative fluorescence signal intensities of mono acetyl chloramphenicol formed were shown in Fig. 32 in the form of bar diagrams

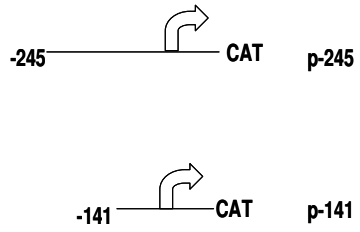


Fig. 33. Schematic representation of 5' deletion fragments of mdr1 promoter fused with CAT reporter gene

Arrows indicate transcription start site of mdr1

Schematic representation of 5'deletion fragments of mouse mdr1 promoter fused to the CAT reporter gene was shown in Fig. 33.

2.3.7. C-PC inhibited the activation of Src

In silico studies showed an increase in phosphorylated Src levels with 2-AAF treatment and a dose dependent decrease in phosphorylated Src with C-PC treatment (Fig. 34).

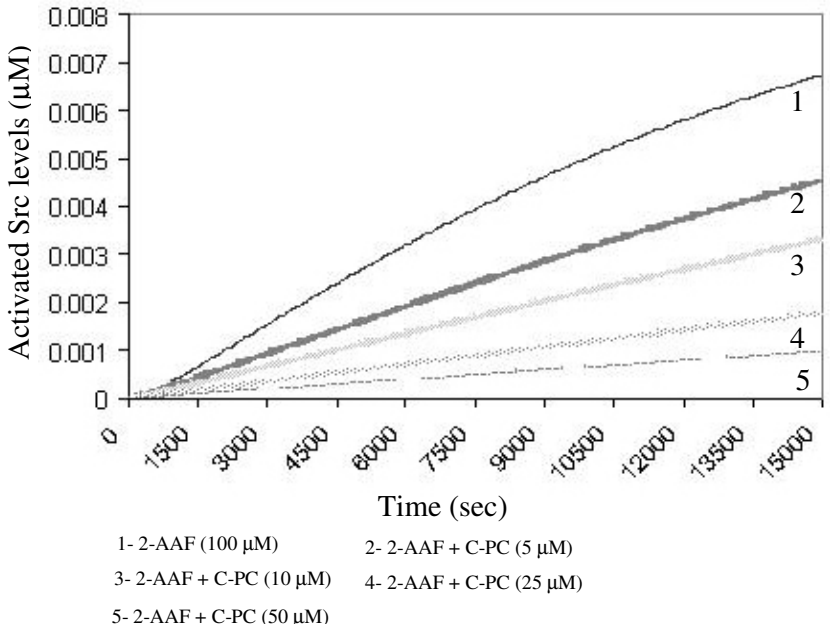
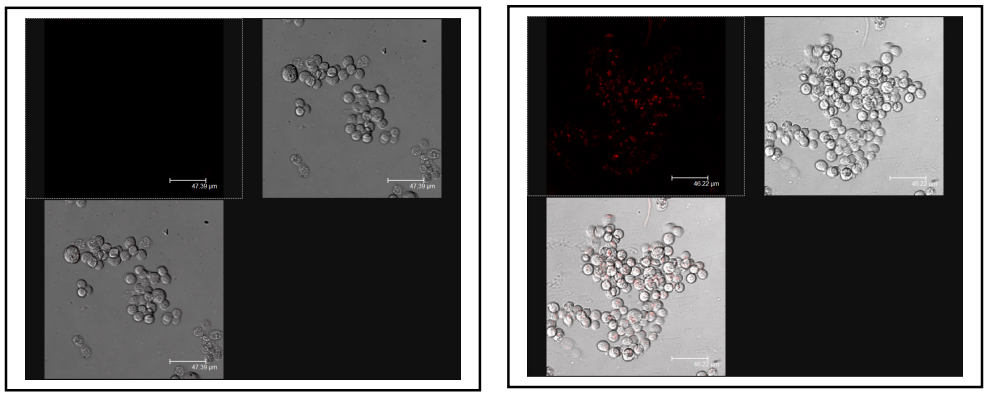


Fig. 34. Graph showing simulation data of phosphorylated Src levels with C-PC treatment

The model was simulated in a loop run with 100 µM 2-AAF and increasing concentrations of C-PC (5, 10, 25 and 50 µM)

2.3.8. Confocal analysis

The entry of C-PC into RAW264.7 cells was determined using laser scanning confocal microscopy. These studies showed strong fluorescence signal in the cells treated with C-PC, which was absent in untreated control cells (Fig. 35). These results indicate that C-PC might be entering the cells or binding to cell surface receptors.



A **B**

Fig. 35. Confocal microscopic images

Confocal images of the RAW 264.7 cells incubated with (B) and without (A) C-PC (50 µM) 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

2.4. Discussion

The use of natural products as drugs is gaining significance rapidly. Although their current use as drugs is at miniscule, with the rise of side effects of chemotherapeutic drugs, research on natural products is being promoted to develop alternative therapies. Drugs derived from natural products constitute a wide range; from antibiotics & anti-oxidants to anti-cancer compounds. One such compound with growing attention is C-PC, a soluble biliprotein present in *S. platensis*. Previous studies from this laboratory indicate that C-PC is a selective COX-2 inhibitor (Reddy *et al.,* 2000), induces apoptosis in doxorubicin resistant human hepatocellular carcinoma cell line (Roy *et al.,* 2007), chronic myeloid leukemia cells (Subhashini *et al.,* 2004) and in RAW 264.7 cells stimulated with LPS (Reddy *et al.,* 2003). Other studies have revealed anti-proliferative (Li *et al.,* 2006), hypocholesterolemic (Nagaoka *et al.,* 2005), hepatoprotective (Satyasaikumar *et al.,* 2007; Vadiraja *et al.,* 1998) and anti-oxidant (Romay and Gonzalez, 2000) properties of C-PC. As 2-AAF-induced expression of MDR1 is through generation of ROS, in the present study the potential of C-PC, a potent anti-oxidant, in counteracting the effects of 2-AAF in mouse macrophage cell line (RAW 264.7) was evaluated.

2.4.1. C-PC inhibited 2-AAF induced expression of MDR1 both at transcriptional and translational level

MDR1, which is induced by many external stimuli, is considered to be a stress inducible gene. Expression of MDR1 mRNA increases when the cells were treated with anti-tumor agents and xenobiotics. The present study also showed increased expression of MDR1 gene upon treatment with 2-AAF. Similar induced expression of MDR1 by 2-AAF was reported in rat hepatoma cells (Lei *et al.,* 2001) and rat primary hepatocyte cultures (Gant *et al.,* 1991). Preincubation of cells with C-PC, on the other hand, inhibited the expression of MDR1. These results suggest the possible involvement of ROS in the 2-AAF–induced MDR1 expression and the blocking of the same by the antioxidant C-PC. This assumption is supported by the decrease in 2-AAF induced expression of MDR1 by DPI, the inhibitor of NADPH oxidase. In order to test this, further studies were undertaken to measure the ROS generated in the cells exposed to 2-AAF alone and in combination with C-PC.

2.4.2. C-PC mediated decrease in MDR1 expression is by inhibition of ROS generation

Reactive oxygen species may be formed during physiological processes or in response to xenobiotic exposure. Ligand (xenobiotic) receptor binding has been

shown to induce the production of ROS (Finkel, 1998) thereby participating in the receptor mediated biological responses. In the present study hepatocarcinogen, 2-AAF, induced the oxidative stress by excessive generation of ROS in RAW 264.7 cells, which was effectively decreased by C-PC. This is in accordance with earlier studies where C-PC was shown to inhibit the generation of reactive oxygen and nitrogen species *in vivo* and *in vitro* (Bhat and Madyastha, 2000). *In silico* analysis revealed biphasic generation of ROS in response to 2-AAF treatment (Fig. 26). These studies thus demonstrate generation of ROS by 2-AAF and the blocking of the same by C-PC in RAW 264.7 cells.

The *in silico* analysis also was employed to determine the ROS levels in the calibrated model with DPI and Akt IV inhibitor as well. Although the cells were incubated with these inhibitors for only 30 min, they showed a reduction in MDR1 expression. The reduction, however, is not as prominent as C-PC (50 μM) treatment. This may be due to the ROS levels not being reduced as much by these treatments, as

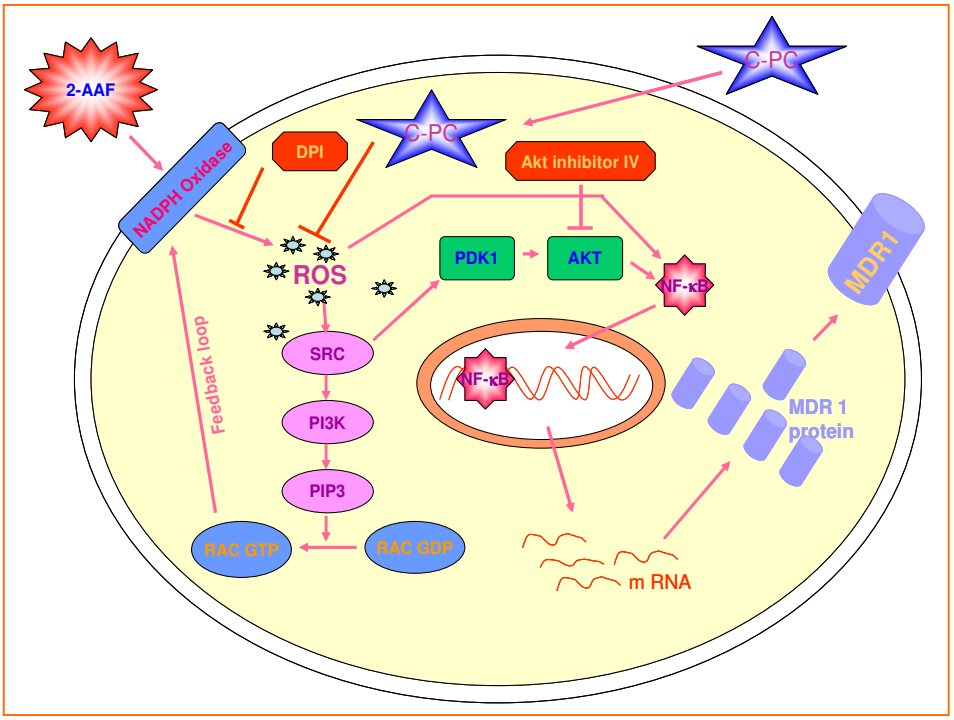


Fig. 36. Schematic representation of the model showing 2-AAF-induced MDR1 expression through ROS generation and possible sites of interference by C-PC, DPI and Akt inhibitor IV

observed in the present study (Table 1) and with the positive feedback initiated by 2-AAF treatment for ROS production (Fig. 36). These results suggest that ROS scavenging by C-PC is more effective than inhibiting a pathway intermediate such as Akt (Fig. 36). Also NADPH oxidase inhibition, by DPI may not be completely eliminating ROS production. *In silico* studies also showed a decrease in

phosphorylated Src levels with C-PC treatment (Fig. 34), which indicates that C-PC stops the positive feedback by scavenging ROS, ultimately leading to effective reduction of ROS and thus leading to inhibition of MDR1 expression (Fig. 36).

2.4.3. C-PC mediated decrease in ROS inturn inhibits Akt phosphorylation

Alteration in the redox potential of the cells exposed to 2-AAF may affect ROS responsive signaling pathways. One such type of signaling pathway is Akt/PKB cell survival pathway. Akt/PKB is a serine threonine kinase and its phosphorylation is a critical event in cell survival. Akt can be activated by a wide variety of growth stimuli, including platelet-derived growth factor, epidermal growth factor and this activated Akt acts as a survival signal that protects cells from apoptosis induced by various stress conditions. C-PC was shown as ROS scavenger but its ability to inhibit phosphorylation of Akt has not been investigated. In the present study, Western blot analysis of Akt and its phosphorylated form showed increased phosphorylation of Akt in 2-AAF treated cells. The reported upregulation of the activity and expression of Akt by ROS (Zhang *et al.,* 2005) supports the possible involvement of ROS in the phosphorylation of Akt in cells exposed to 2-AAF. This 2-AAF-induced phosphorylation of Akt, however, was decreased effectively by C-PC treatment. This may be due to the decrease in generation of ROS or C-PC might have

interfered with the phosphorylation of Akt at the cell membrane. Consistently, treatment of cells with DPI also inhibited Akt phosphorylation. Similar inhibition of the phosphorylation of Akt by various natural anti-oxidants was reported (Huang *et al.,* 2005; Chen *et al.,* 2005). *In silico* model shows that Akt is activated by Src and expression of Src is decreased upon C-PC treatment (Fig. 34). Reducing the ROS levels by C-PC reduced Src activation and there by Akt activation. Akt inhibitor IV, on the other hand, reduced the activation of Akt but not eliminated Src activation by ROS. This may be responsible for the lack of total inhibition of MDR1 expression by Akt inhibitor IV. In the light of the above, it can be suggested that C-PC may be regulating MDR1 expression by mechanisms other than Akt activation. One such mechanism may be by inhibition of COX-2. Increase in COX-2 activity was previously shown to induce MDR1 (Vimal *et al.,* 2002). Our previous studies have shown that C-PC inhibits COX-2 activity (Reddy *et al.,* 2000).

Akt was shown to be involved in the 2-AAF mediated activation of NF-κB (Kuo *et al.,* 2002). ROS generated by hypolipidomic drugs belonging to peroxisomal proliferator family (Becuwe *et al.,* 2005) was also shown to be involved in the up regulation of IKKα activity and activation of NF-κB pathway. In order to test the involvement of ROS and Akt in the activation of NF-κB,

further studies were taken up on NF-κB, which is a positive regulator in 2-AAF induced MDR1 expression.

2.4.4. C-PC mediated decrease in ROS generation inhibits NF-κB translocation

NF-κB is considered to be the most important regulatory element that controls the expression of stress inducible genes. Sequence analysis indicated the presence of putative NF-κB binding sequence in the mouse MDR1 promoter (position –163 to –157) (Mathieu *et al.,* 2001). The observed decrease in the generation of ROS and phosphorylation of Akt in RAW 264.7 cells treated with C-PC suggests that these observed changes may affect the translocation of NF-κB to the nucleus. The present studies clearly demonstrate the increased translocation of NF-κB to the nucleus in response to 2-AAF treatment and its decrease upon treatment with C-PC in a dose dependent manner. As expected inhibitors of NADPH Oxidase (DPI) and Akt phosphorylation (Akt inhibitor IV) inhibited the translocation of NF-κB to the nucleus. These studies support the involvement of ROS and Akt in the activation of NF-κB. Studies on Isovitexin, a food phytochemical with ROS scavenging activity, also showed a similar decrease in NF-κB activity (Lin *et al.,* 2005). Simulation studies also revealed similar decrease in 2-AAF induced NF-κB translocation upon C-PC treatment.

In conclusion, the experimental and simulation studies demonstrate that 2-AAF-induced expression of MDR1 in mouse macrophage cells (RAW 264.7) is mediated through the generation of ROS, phosphorylation of Akt and activation of NF-κB. C-PC, a known free radical scavenger and a potent antioxidant, down regulates the 2-AAF-induced expression of MDR1 by interfering at the level of ROS generation, phosphorylation of Akt and activation of NF-κB (Fig. 36). This down regulation of MDR1 expression by C-PC, induced by xenobiotics such as 2-AAF, suggests usefulness of C-PC in overcoming the drug resistance in cellular systems.

2.5. Summary

In the present study the effects of C-Phycocyanin (C-PC), a biliprotein from *Spirulina platensis,* on the 2-acetylaminofluorene (2-AAF)-induced expression of MDR1 in mouse macrophage cell line (RAW 264.7) were analyzed. The experimental and *in silico* studies revealed a significant inhibition of 2-AAF-induced expression of MDR1 protein in C-PC treated mouse macrophage cell line. MDR1 induction by 2-AAF was found to be dependent on ROS (reactive oxygen species)-Akt (protein kinase B)-NF-κB (Nuclear factor kappa B) signaling pathway. Generation of ROS, phosphorylation of Akt and corresponding nuclear translocation of NF-κB, the events that play a major role in the induction of MDR1 expression, were decreased significantly in C-PC treated cells. NADPH oxidase inhibitor, DPI (Diphenyl iodide), and pharmacological inhibitor of Akt, Akt inhibitor IV, also showed a reduction in MDR1 expression, although not to the same extent as C-PC mediated inhibition of MDR1 expression. To further understand the mechanism a computational model of the detailed ROS-Akt-NF-κB pathway was created. C-PC was modeled purely as a ROS scavenger and this representation matched the experimental trends accurately. Also the ROS levels with the different treatments were determined through *in silico* investigation which showed C-PC as more effective in reduction

of MDR1 expression than inhibitors of NADPH oxidase and Akt. The experimental and *in silico* studies thus collectively suggest that 2-AAF induces MDR1 by ROS dependent pathway and C-PC is a potential negative regulator of MDR1 expression. This down regulation of MDR1 expression by C-PC, induced by xenobiotics such as 2-AAF, suggests it's usefulness in overcoming the drug resistance in cellular systems.

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List of Publications

1. **Roy KR**, Arunasree KM, Dhoot A, Aparna R, Reddy GV, Vali S, Reddanna P (2007). 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. *Archives of Biochemistry and Biophysics* .459:169-77.

2. **Roy KR,** Arunasree KM, Reddy NP, Dheeraj B, Reddy GV, Reddanna P (2007). Alteration of mitochondrial membrane potential by C-Phycocyanin induces apoptosis in doxorubicin resistant human hepatocellular carcinoma cell line-HepG2. ***Biotechnology and Applied Biochemistry*** (PMID 17274761, In press).

3. **Roy KR,** Reddy NP, Reddy GV, Reddanna P (2007). C-Phycocyanin ameliorates 2-acetylaminofluorene induced oxidative stress and MDR1 expression in albino mice. *Hepatology Research* (Communicated).

4. Mahipal SVK, Subhashini J, Reddy MC. Reddy MM, Anilkumar K, **Roy KR**, Reddy GV, Reddanna P (2007). Effect of 15-Lipoxygenase-2 Metabolite, 15-(*S*)-H(P)ETE on Chronic Myelogenous Leukemia Cell Line K-562: Reactive Oxygen Species (ROS) Mediates Caspase-dependent Apoptosis. *Biochemical Pharmacology* (In press).

5. Sathyasaikumar KV, Swapna I, Reddy PV, Murthy ChR, **Roy KR**, Dutta Gupta A, Senthilkumaran B, Reddanna P (2007). Co-administration of C-Phycocyanin ameliorates thioacetamide induced hepatic encephalopathy in Wistar rats. *Journal of the Neurological Sciences* 252: 67-75.

6. Sreekanth D, Aruansree MK, **Roy KR**, Reddy TC, Reddy GV, Reddanna P (2007). Betanin, a betacyanin pigment isolated from Opuntia ficus indica induces apoptosis in human chronic myeloid leukemia cell line – K562 *Phytomedicine* (PMID 17482444, In press).

7. Reddy MM, Mahipal SV, Subhashini J, Reddy MC, **Roy KR**, Reddy GV, Reddy PR, Reddanna P (2006). Impaired steroidogenesis and spermatogenesis during acute endotoxemia in rats: Role of inflammatory mediators and oxidative stress. ***Reproductive Toxicology*** 22: 493-500.

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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

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Abstract

We studied the effects of C-Phycocyanin (C-PC), a biliprotein from *Spirulina platensis* on the 2-acetylaminofluorene (2-AAF)-induced expression of MDR1, encoded by the multidrug resistance (MDR1) gene, in mouse macrophage cell line (RAW 264.7). Our experimental and *In silico* studies revealed a significant inhibition of 2-AAF-induced expression of MDR1 protein in C-PC treated mouse macrophage cell line. MDR1 induction by 2-AAF was dependent on ROS (reactive oxygen species)-Akt (protein kinase B)-NF-κB (Nuclear factor kappa B) signaling pathway. Generation of ROS, phosphorylation of Akt and corresponding nuclear translocation of NF-κB, the events that play a major role in the induction of MDR1 expression, were decreased significantly in C-PC treated cells. NADPH oxidase inhibitor, DPI (Diphenyl iodide), and pharmacological inhibitor of Akt, Akt inhibitor IV, also showed a reduction in MDR1 expression, although not to the same extent as C-PC mediated inhibition of MDR1 expression. To further understand the mechanism, we created a computational model of the detailed ROS-Akt-NF-κB pathway. C-PC was modeled purely as a ROS scavenger and this representation matched the experimental trends accurately. Also the ROS levels determined through *In silico* investigation showed that C-PC was more effective in reduction of MDR1 expression than inhibitors of NADPH oxidase and Akt. Our experimental and *In silico* studies collectively suggest that 2-AAF induces MDR1 by ROS dependent pathway and C-PC is a potential negative regulator of MDR1 expression. This down regulation of MDR1 expression, induced by xenobiotics such as 2-AAF, suggests C-PC's usefulness in overcoming the drug resistance in cellular systems.

Keywords: 2-Acetylaminofluorene; MDR1; NF-κB; Simulation; *In silico*; C-Phycocyanin; RAW 264.7 cells

Multidrug resistance (MDR)[1] is the primary impediment in the cancer chemotherapy. Cancer cells develop resistance to various drugs primarily by the over expression of MDR1, a membrane P-glycoprotein (P-gp) of 170 kDa [1]. Over expression of MDR1 expels drugs and xenobiotics in an energy dependent manner, thus reducing their intracellular accumulation. MDR1 in humans and MDR1a/MDR3 and MDR1b/MDR1 in rodents function as drug transporters. Expression of multidrug resistance genes seen in most of the organs that have a role in absorption and elimination signifies their role in the efflux of drugs and xenobiotics. Expression of MDR1 was shown in the apical membranes of the liver, kidney, gut and at the blood-brain barrier [2,3]. MDR1 and MDR3 mRNA expression was observed in the gastrointestinal tract, cerebral cortex, cerebellum, kidney, lung, and liver of rodents [4]. Transcripts of multidrug resistance genes were observed in a wide variety of conditions

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[1] *Abbreviations used:* au, arbitrary units; FACS, fluorescence activated cell sorter; MDR, multidrug resistance.

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and malignancies. Over expression of MDR1 mRNA in isolated hepatocytes of endotoxin-treated rats [5] and in adenocarcinomas derived from adrenal, kidney, liver and bowel [6], breast cancer [7] and in prostate cancer [8] was observed.

Exposure to xenobiotics and carcinogens alters the physiological expression of multidrug resistance genes. Aflotoxin B1 [9], phenothiazine [9], 2-acetylaminofluorene [9], methyl sulfonate [10], 3-methylcholanthrene [11] and mitoxantrone [12] were shown to induce the expression of MDR1. The hepatocarcinogen, 2-AAF activates the expression of MDR1 through the generation of ROS and activation of NF-κB in rat hepatoma cells [13]. ROS activates signal transduction pathways that enhance NF-κB translocation to nucleus [14]. Akt/Protein kinase B (PKB), a cell survival protein, was also shown to be phosphorylated in response to 2-AAF treatment, which further activates NF-κB pathway. It is known that 2-AAF induces the generation of ROS in rat hepatoma cells [13] and interaction of ROS and PI_3K further activates the NF-κB dependent pathway [15]. Akt/PKB activation increases resistance to apoptosis and enhances cell survival. Natural antioxidants such as taxifolin [16] and synthetic compound (3E)-4-(2-hydroxyphenyl) but-3-en-2-one [17] suppressed the generation of intracellular ROS and in turn inhibited the activation of NF-κB.

Spirulina is non-nitrogen fixing blue-green algae with over 30 years long history of safe human consumption. The pharmacological properties of *Spirulina* were mainly due to calcium-spirulan and C-Phycocyanin (C-PC). Our previous studies showed that C-PC, a water-soluble bili-protein from *Spirulina platensis* is a specific cyclooxygenase-2 (COX-2) inhibitor [18], induces apoptosis in human chronic myeloid leukemia cell line-K562 [19] and also in LPS-stimulated RAW 264.7 cells [20]. C-PC was shown to be a peroxyl radical scavenger both *in vivo* and *in vitro* [21], inhibitor of CCl_4 induced lipid peroxidation [22] and inhibitor of $ONOO^-$ mediated deleterious biological effects [23]. As 2-AAF induced expression of MDR1 is dependent on ROS generation and NF-κB activation [13] and C-PC being a natural antioxidant, we hypothesized that C-PC might inactivate ROS mediated MDR1 expression. Hence the present study is undertaken to evaluate the role of C-PC on 2-AAF-induced expression of MDR1 in mouse macrophage cell line (RAW 264.7). Additionally computational modeling and *In silico* analysis was used to further understand the underlying mechanisms.

Materials and methods

Chemicals

PBS, RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco BRL (California, USA). Nitrocellulose membrane was from Millipore (Bangalore, India). 2-AAF, TMB/H_2O_2, Acetyl-CoA, DCFH-DA, DPI, phosphatase inhibitor cocktail 1 and 2 and β-actin antibodies were purchased from Sigma-Aldrich (Bangalore, India). Polyclonal antibodies to Akt and MDR1 were from Santa Cruz Biotechnology (California, USA) and phospho-Akt (p-Akt) was from Upstate (Charlottesville, VA, USA). Antibodies (p50 and p65) were from Oncogene Research Products (California, USA). Single step RT-PCR kit was from AB Gene Technologies (Surrey, UK). Akt inhibitor IV was obtained from Calbiochem (Darmstadt, Germany). ECL detection kit was from Amersham Biosciences (Buckinghamshire, UK). C-PC was a generous gift from Prof. K. M. Madyastha, Indian Institute of Science (I. I. Sc.), India. All the other chemicals and reagents were purchased from local companies and are of molecular biology grade.

Cell culture and treatment

Mouse macrophage cells (RAW 264.7) were grown in RPMI 1640 medium supplemented with 10% heat inactivated 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 sub-cultured twice each week. Exponentially growing cells were used for all treatments. C-PC dissolved in PBS was used for the treatments. 20 mM stock of 2-AAF, dissolved in absolute alcohol was employed in this study. The final concentration of the vehicle (ethanol) never exceeded 0.1%. RAW 264.7 cells exposed to 0.1% ethanol served as controls.

Northern blot analysis of MDR1 mRNA expression

RAW 264.7 cells were seeded at a density of 5×10^6 in 90 mm culture dishes. Cells were first pre-incubated with C-PC (5, 10, 25 and 50 μM) for 4 h and DPI (10 μM), Akt inhibitor IV (10 μM) for 30 min and then 2-AAF was added at a final concentration of 100 μM. Cells were harvested after 6 h incubation with 2-AAF. Total RNA was extracted from control and treated RAW 264.7 cells using TRIzol reagent. The isolated RNA was quantified spectrophotometrically and the integrity was checked on agarose formaldehyde gels. 30 μg of total RNA was separated by electrophoresis on 1.5% agarose gel containing 0.66 M formaldehyde and transferred to a nylon membrane. After cross linking of RNA to membrane using UV (245 nm) for 1 min at 1.5 joules/cm^2, the membranes were incubated in prehybridization solution (6× SSC, 5× Denhardt's reagent, 0.5% SDS, 50% Formamide and 100 μg/ml Salmon sperm DNA) for 1–2 h at 42 °C. The membranes were then transferred to hybridization solution containing cDNA probes at 55 °C for 20 h. cDNA probes for MDR1 and GAPDH were obtained by RT-PCR. RT-PCR was performed with 5 μg total RNA, isolated from RAW 264.7 cells, using one step RT-PCR kit. Primers used were as follows: mouse MDR1: forward, 5′-TGCTTATGGATCCCA GAGTGAC-3′, reverse, 5′-TTGGTGAGGATCTCTCCGGCT-3′; which yields 435 bp product, GAPDH: forward, 5′-CTCATGACCA CAGTCCATGCCATC-3′, reverse, 5′-CTGCTTCACCACCTTCT TGATGTC-3′; which yields 272 bp product [24]. Reverse transcription was performed at 47 °C for 30 min. Subsequent to inactivation of reverse transcriptase (2 min, 94 °C), the samples were subjected to 30 amplification cycles, each consisting of 20 s at 94 °C, 1 min at 54 °C, and 1 min at 72 °C, followed by 5 min at 72 °C after the last cycle. The products were analyzed on 1% agarose gel and then extracted by QIA quick gel extraction kit (Qiagen). Each probe was labeled with α-^{32}P deoxy adenine triphosphate, using random priming reaction (Fermentas). After incubation in hybridization solution, membranes were washed three times at 55 °C for 10 min with wash buffer containing 2× SSC and 0.1% SDS. Other two washes were with 1× and 0.5× SSC along with 0.1% SDS. Membranes were then exposed to phosphoimager screen (Amersham) for 4 h. The screen was later visualized on Amersham typhoon variable mode imager 9410.

Measurement of ROS

ROS production in control and treated RAW 264.7 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 ROS. Cells seeded at a density of 2×10^6 in 60 mm culture dishes were first pre-incubated with C-PC (25 μM & 50 μM) for 4 h and DPI (10 μM), Akt

inhibitor IV (10 μM) for 30 min and then 2-AAF was added at a final concentration of 100 μM for 45 min. Cells were harvested after 10 min incubation with DCFH-DA (10 μM) and washed with PBS. ROS 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.

Superoxide generation upon 2-AAF treatment was measured according to the procedure described elsewhere [25]. RAW 264.7 cells were seeded at a density of 5×10^5 per well in 100 μl of Hank's balanced salt solution without Ca^{2+} and Mg^{2+} containing 150 μM ferricytochrome C and 50 and 100 μM concentrations of 2-AAF. A control reaction contained 0.1 μg of superoxide dismutase (SOD). After incubation at 37 °C for 60 min, the absorbance of cell suspension at 550 nm was determined. Extinction coefficient used for superoxide determination was 21 $mM^{-1}\,cm^{-1}$.

Electrophoretic mobility shift assay (EMSA)

RAW 264.7 cells at a density of 5×10^6 were seeded in 90 mm culture dishes. Cells were first pre-incubated with C-PC (5, 10, 25 and 50 μM) for 4 h and DPI (10 μM), Akt inhibitor IV (10 μM) for 30 min and then 2-AAF was added at a final concentration of 100 μM. Cells were harvested after 2 h incubation with 2-AAF and were used for nuclear protein extraction. The cells were washed with PBS and harvested by centrifugation at 2000 rpm for 3 min. 200 μl 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 μl of nuclear protein extraction buffer (420 mM NaCl, 10 mM Hepes, 10 mM $MgCl_2$, 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 [26]. Nuclear extracts (8 μg) were incubated with γ-^{32}P labeled double stranded oligonucleotide with specific NF-κB binding sequence (5'-AGT TGA GGG GAC TTT CCC AGG C-3') for 30 min at 37 °C. For supershift assay nuclear extract of 2-AAF treated cells were incubated with antibodies against p50 or p65 of NF-κB for 20 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 also examined by competition with unlabeled oligonucleotide (cold competition).

Preparation of whole cell extracts and immunoblot analysis

RAW 264.7 cells at a density of 5×10^6 were seeded in 90 mm culture dishes. They were first pre-incubated with C-PC (5, 10, 25 and 50 μM) for 4 h and DPI (10 μM), Akt inhibitor IV (10 μM) for 30 min and then 2-AAF was added at a final concentration of 100 μM. Cells, harvested after 90 min (Akt and p-Akt) and 6 h (MDR1) incubation with 2-AAF, were used for preparation of whole cell extract. The harvested, control and treated, RAW 264.7 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 μg/ml leupeptin, 20 μg/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 [26]. 100 μg of protein from each treatment was resolved on 10% (Akt and p-Akt) and 7% (MDR1) SDS-PAGE gels 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 [Akt (1:250 dilution), p-Akt (1:500 dilution) and MDR1 (1:500 dilution)] in 10 ml of antibody-diluted buffer (1× 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 peroxidase substrate, TMB/H_2O_2 or ECL kit. Equal protein loading was confirmed by reprobing the nitrocellulose membranes with β-actin antibodies (1:500 dilution).

In silico modeling

Model overview

The model was developed using Teranode's Biological Modeler, which uses MML (Mathematical Modeling Language, developed by the National Simulation Resource at the University of Washington) to simulate the 2-AAF-induced MDR1 expression via a ROS mediated pathway. Rates of change of the concentration of each component with time are modeled as differential equations that are solved by the Radau method [27]. A schematic of the elements of the model and their connections are shown in Fig. 7.

Model structure and description

AAF induced ROS-Akt-NF-κB pathway has been modeled as follows:

1. AAF causes Ca^{2+} elevation in the cytoplasm causing activation of NADPH oxidase [28]. NADPH oxidase transports electrons from intracellular NADPH via FAD and its two heme groups to oxygen, leading to formation of superoxide radical which is then converted to H_2O_2 (ROS) [29].
2. ROS (H_2O_2) promotes tyrosine phosphorylation by activating protein kinases [30]. ROS in this system helps in the autophosphorylation and activation of the tyrosine kinase Src [31,32].
3. Activated Src via activation of PI_3K and formation of PIP_3, activates RAC by converting RAC GDP to RAC GTP.
4. The activated RAC GTP in turn further activates NADPH oxidase which causes further production of ROS and it goes into a postive feedback loop through Src [33]. This leads to a biphasic production of ROS with 2-AAF treatment, with a slow initial rise and once the feedback loop sets in, a subsequent exponential rise in ROS [33].
5. Activated Src activates PDK1 [34], that in turn activates Akt [35]. This leads to the activation of NF-κB.
6. NF-κB is the transcription factor regulating MDR1 expression. MDR1 mRNA is translated to MDR1 protein in the cytoplasm.

Model assumptions

Model initial conditions without 2-AAF activation have been calibrated to control sample levels from experimental data. There is a basal level of ROS being produced from NADPH oxidase, producing the control level of MDR1 mRNA and protein expression as in the experimental control sample. With 2-AAF treatment the model is calibrated to obtain the same ROS levels as in experimental data and also obtain similar MDR1 mRNA and MDR1 protein levels. With 50 μM C-PC treatments, the ROS levels in the model were calibrated to show similar reduction as in experimental data. Also, Ki values for DPI and Akt inhibitor IV were optimized to obtain the experimental fold difference in MDR1 protein levels. This was used as the training data for the model to match the experimental setup.

Simulation protocols

1. AAF was used at a concentration of 100 μM as in the experiments.
2. NADPH oxidase inhibitor, DPI, was used at a concentration of 10 μM and an arbitrary Ki of 1.01 μM.
3. Akt inhibitor IV was used at a concentration of 10 μM with an arbitrary Ki of 25 nM.
4. C-PC was modeled as a peroxyl radical scavenger, which form water and O_2 with an arbitrary Kf.
5. Levels of initial concentrations of most species were chosen from values obtained in literature.

Statistical analysis

Data reported as the mean ± SE of three independent experiments. Statistical analysis of differences was carried out by one-way analysis of variance (ANOVA). A *P*-value of less than 0.05 was considered to indicate significance.

Results

C-PC inhibited the 2-AAF-induced transcription of MDR1

To elucidate whether C-PC inhibited the 2-AAF induced expression of MDR1 mRNA, Northern blot analysis was performed. Concentration and time dependent activation of MDR1 expression by 2-AAF was standardized. Prior incubation of RAW 264.7 cells with C-PC for 4 h at concentrations of 5, 10, 25 and 50 μM inhibited the 2-AAF (100 μM) induced expression of MDR1 mRNA in a dose dependent manner (Fig. 1a). Pre-incubation of cells with DPI (10 μM) and Akt inhibitor IV (10 μM) for 30 min markedly diminished the transcription of MDR1, though not to the same extent of C-PC (Fig. 1a). Unaltered levels of GAPDH transcripts in all treatments confirm specific inhibition of MDR1 expression by C-PC. *In silico* studies also showed similar trends when compared to the experimental data (Fig. 1b).

C-PC inhibited the 2-AAF-induced MDR1 protein expression

To elucidate whether C-PC inhibited the 2-AAF induced protein expression of MDR1, Western blot analysis was performed. Prior incubation of RAW 264.7 cells with C-PC for 4 h at concentrations of 5, 10, 25 and 50 μM inhibited the 2-AAF induced expression of MDR1 protein in a dose dependent manner (Fig. 2a). DPI (10 μM) and Akt inhibitor IV (10 μM) treatment also diminished the expression of MDR1 protein. Unaltered levels of β-actin in all treatments confirm specific inhibition of MDR1 by C-PC. *In silico* studies showed similar inhibition of 2-AAF induced MDR1 protein levels (Fig. 2b).

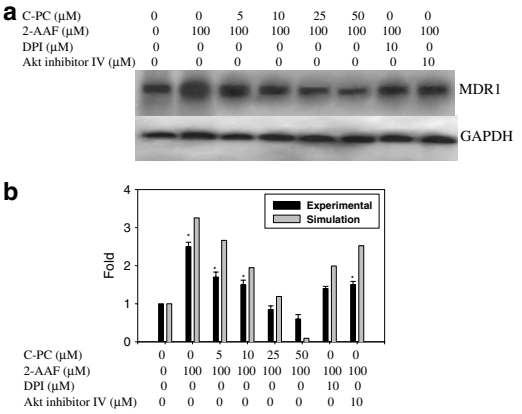

Fig. 1. (a) Northern blot analysis MDR1 mRNA expression in RAW 264.7 cells showing the effect of C-PC, DPI and Akt inhibitor IV (b) Bar graphs showing the fold difference in the expression levels of MDR1 mRNA obtained by experimental and simulation data. *Statistical significant ($P < 0.05$).

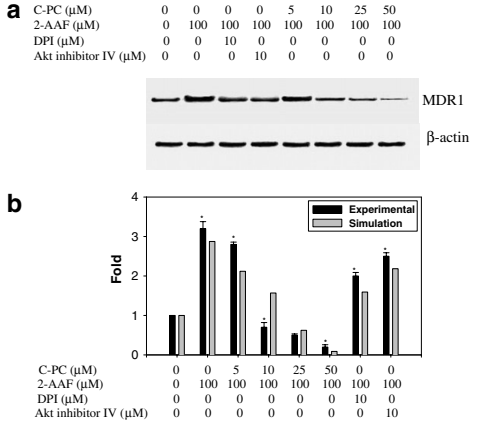

Fig. 2. (a) Western blot analysis of C-PC, DPI, Akt inhibitor IV mediated effect on MDR1 protein expression in RAW 264.7 cells. (b) Bar graphs showing the fold difference in the expression levels of MDR1 protein obtained by experimental and simulation data. *Statistical significant ($P < 0.05$).

C-PC decreased the 2-AAF-induced generation of ROS

In the context of anti-oxidant properties of C-PC, further studies were undertaken for analyzing the potential of C-PC as a ROS scavenger. C-PC was tested for its effect on 2-AAF-induced generation of ROS in RAW 264.7 cells. Generation of ROS in the cells was standardized with 2-AAF (100 μM) treatment at various time points using DCFH-DA by FACS analysis. Compared to control, 5.5-fold enhancement of ROS generation was observed at 100 μM concentration of 2-AAF in 45 min time interval. Cells pre-incubated with C-PC showed a dose dependent decrease in the generation of ROS. Fig. 3a shows that there was 60 and 45% decrease in the generation of ROS in cells pre-incubated with C-PC at 50 and 25 μM compared to that of enhanced ROS generation (5.5-fold) in cells treated with 2-AAF alone. Cells treated with DPI (10 μM) showed 40% decrease in the generation of ROS, where as treatment with Akt inhibitor (10 μM) did not alter generation of ROS (Fig. 3b). Production of superoxide anion by NADPH Oxidase increased 1.5 and 2.5-folds in response to 2-AAF (50 and 100 μM) treatment (Fig. 3d).

Biphasic ROS generation in response to 2-AAF treatment was observed in *In silico* model (Fig. 3c) and ROS levels measured at 45 min and 2.5 h time period with C-PC, DPI and Akt inhibitor using *In silico* model were shown in Table 1.

C-PC inhibited the activation of Akt

Akt, a cell survival protein is activated by various growth and survival factors. Akt signaling pathway is involved in 2-AAF-induced expression of MDR1. In addition to the anti-oxidant properties of C-PC, we investigated

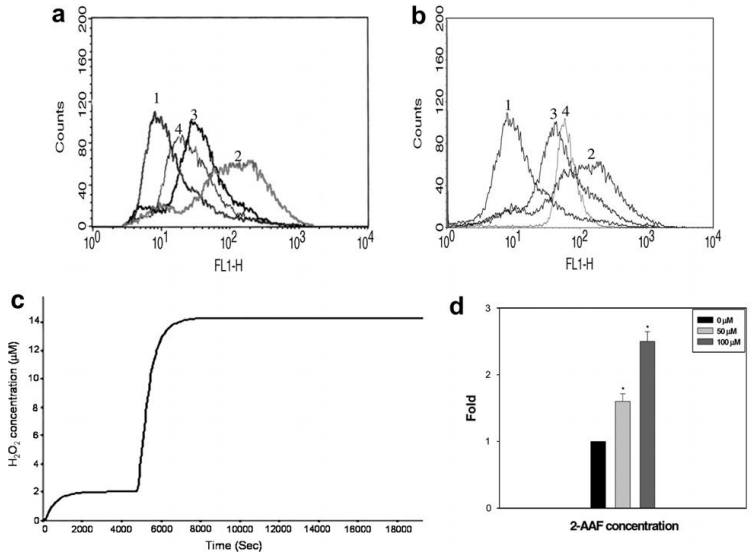

Fig. 3. FACS analysis of generation of ROS using DCFH-DA in cells exposed to 2-AAF (100 μM) with or without C-PC, DPI, Akt inhibitor IV pretreatment. (a) 1, Control; 2, 2-AAF; 3, 2-AAF + C-PC (25 μM); 4, 2-AAF + C-PC (50 μM). (b) 1, Control; 2, 2-AAF; 3, 2-AAF + DPI (10 μM); 4, 2-AAF + Akt inhibitor IV (10 μM). (c) Line Plot showing concentration of H_2O_2 being formed in μM on *Y* axis against time (s) on *X* axis. This was obtained by running simulation model with a trigger of 100 μM 2-AAF for 20,000 s without any inhibitor. ROS levels obtained at 2700 s were in the first phase of ROS generation by 2-AAF. (d) Bar graph showing the generation of superoxide in cells after 60 min of 2-AAF treatment. *Statistical significant ($P < 0.05$).

Table 1
ROS levels with different treatments at 45 min and 2.5 h

Treatment	ROS levels (μM) at 45 min	ROS levels (μM) at 2.5 h
Control	0.324	0.3225
AAF (100 μM)	1.952	14.24
2-AAF + C-PC (5 μM)	1.142	13.21
2-AAF + C-PC (10 μM)	0.788	5.59
2-AAF + C-PC (25 μM)	0.399	4.037
2-AAF + C-PC (50 μM)	0.217	0.236
2-AAF + DPI (10 μM)	0.9025	6.57
2-AAF + Akt inhibitor IV (10 μM)	1.952	14.24

The model was simulated with 2-AAF individually and in combination with inhibitors (C-PC, DPI and Akt inhibitor IV).

whether C-PC has any effect on the activation of Akt in 2-AAF treated RAW 264.7 cells. Treatment of cells with 2-AAF at a concentration of 100 μM for 90 min resulted in maximum increase in the phosphorylation of Akt. Fig. 4a shows the Western blot analysis of Akt and p-Akt in C-PC treated cells. The level of phosphorylated Akt increased in 2-AAF (100 μM) alone treated cells compared to control cells and decreased in concentration dependent manner in cells pre-incubated with C-PC. Pre-incubation of cells with DPI (10 μM) and Akt inhibitor IV (10 μM) for 30 min also resulted in inhibition of Akt phosphorylation (Fig. 4b). The levels of Akt, on the other hand, were un-altered irrespective of treatments compared to control. The simulation data for Akt inhibition aligns with the experimental data (Fig. 4c).

C-PC prevented 2-AAF-induced translocation of NF-κB into nucleus

Activation of Akt stimulates IKKα that ultimately leads to the increase in the translocation of NF-κB from cytoplasm to nucleus. We noticed increase in the translocation of NF-κB in the 2-AAF (100 μM) treated RAW 264.7 cells compared to the control cells (Fig. 5a). To investigate the effect of C-PC on the 2-AAF-induced translocation of NF-κB to the nucleus, we analyzed the nuclear extracts of cells pre-incubated with C-PC. These studies revealed decrease in the translocation of NF-κB in a concentration dependent manner in C-PC treated cells compared to the 2-AAF alone treated cells (Fig. 5a). DPI and Akt inhibitor IV treatment also inhibited NF-κB translocation to nucleus (Fig. 5b). Incubation of nuclear extract of 2-AAF treated cells with antibody either to p50 or p65 subunit of NF-κB shifted the band, suggesting the specificity towards NF-κB (Fig. 5c). Treatment with TNFα (5 nM) induced the NF-κB translocation and served as positive control. Lack of signal in the cold competition sample (excess of unlabelled oligo) shows the specificity of the NF-κB binding probe. Simulation data of NF-κB translocation correlated with the experimental data (Fig. 5d).

C-PC inhibited the activation of Src

In silico studies showed an increase in phosphorylated Src levels with 2-AAF treatment and a dose dependent decrease in phosphorylated Src with C-PC treatment (Fig. 6).

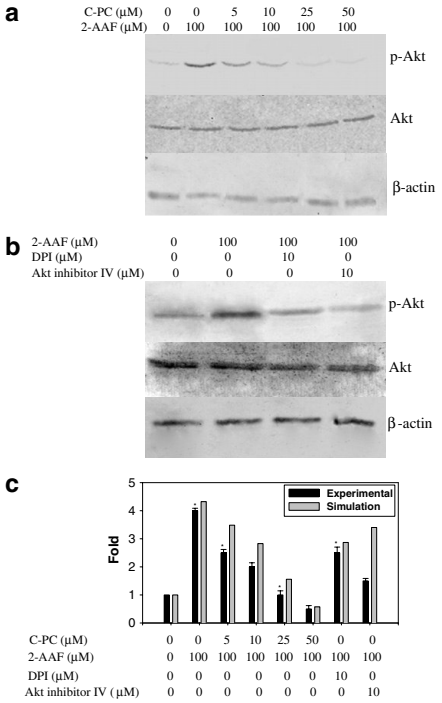

Fig. 4. Western blot analysis of C-PC (a), DPI (b), Akt inhibitor IV (b) mediated effects on Akt and p-Akt protein expression in RAW 264.7 cells. (c) Bar graphs showing the fold difference in the expression levels of p-Akt protein obtained by experimental and simulation data. *Statistical significant ($P < 0.05$).

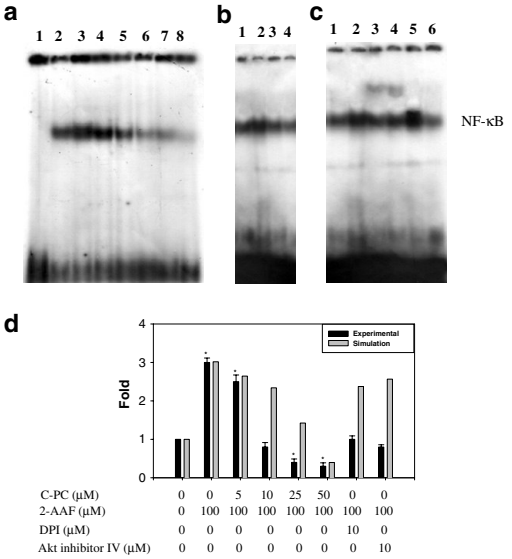

Fig. 5. Effect of C-PC on nuclear translocation of NF-κB in RAW 264.7 cells. Electro mobility shift assay of nuclear extracts using specific NF-κB binding probe in cells exposed to 2-AAF (100 μM) with or without C-PC, DPI, Akt inhibitor IV pretreatment. (a) Lane 1: Free probe; lane 2: Control; lane 3: 2-AAF; lane 4: 2-AAF + C-PC (5 μM); lane 5: 2-AAF + C-PC (10 μM); lane 6: 2-AAF + C-PC (25 μM); lane 7: 2-AAF + C-PC (50 μM); lane 8: Cold competition. (b) Lane 1: Control; lane 2: 2-AAF; lane 3: 2-AAF + DPI (10 μM); lane 4: 2-AAF + Akt inhibitor IV (10 μM). (c) Super shift assay of nuclear extracts using specific antibody against p50 and p65 in cells exposed to 2-AAF (100 μM). Lane 1: Control; lane 2: 2-AAF; lane 3: 2-AAF + Anti-p50; lane 4: 2-AAF + Anti-p65; lane 5: TNFα (5 nM); lane 6: Cold competition. (d) Bar graphs showing the fold difference in the NF-κB translocation obtained by experimental and simulation data. *Statistical significant ($P < 0.05$).

Discussion

The use of natural products as drugs is gaining significance rapidly. Although their current use as drugs is at minimal, with the rise of side effects of chemotherapeutic drugs, research on natural products is being promoted to develop alternative therapies. Drugs derived from natural products constitute a wide range; from antibiotics and anti-oxidants to anti-cancer compounds. One such compound with growing attention is C-PC, a soluble biliprotein present in *S. platensis*. Our previous studies indicate that C-PC is a selective COX-2 inhibitor [18], induces apoptosis in chronic myeloid leukemia cells [19] and in RAW 264.7 cells stimulated with LPS [20]. Other studies have revealed anti-proliferative [36], hypocholesterolemic [37], hepatoprotective [22] and anti-oxidant [38] properties of C-PC. As 2-AAF-induced expression of MDR1 is through generation of ROS, we evaluated the potential of C-PC, a potent anti-oxidant, in counteracting the effects of 2-AAF in mouse macrophage cell line (RAW 264.7).

MDR1, which is induced by many external stimuli, is considered to be a stress inducible gene. Expression of MDR1 mRNA increases when the cells were treated with anti-tumor agents and xenobiotics. Our results also showed increased expression of MDR1 gene upon treatment with 2-AAF. Similar induced expression of MDR1 by 2-AAF was reported in rat hepatoma cells [13] and rat primary hepatocyte cultures [39]. Preincubation of cells with C-PC, on the other hand, inhibited the expression of MDR1. These results suggest the possible involvement of ROS in the 2-AAF–induced MDR1 expression and the blocking of the same by the antioxidant C-PC. This assumption is supported by the decrease in 2-AAF induced expression of MDR1 by NADPH oxidase inhibitor DPI. In order to test this, further studies were undertaken to measure the ROS generated in the cells exposed to 2-AAF alone and in combination with C-PC.

Reactive oxygen species may be formed during physiological processes or in response to xenobiotic exposure. Ligand (xenobiotic) receptor binding has been shown to induce the production of ROS [40] thereby participating in the receptor mediated biological responses. In the present

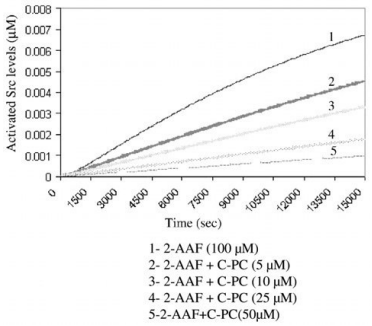

Fig. 6. Graph showing simulation data of phosphorylated Src levels with C-PC treatment. The model was simulated in a loop run with 100 μM 2-AAF and increasing concentrations of C-PC (5, 10, 25 and 50 μM).

study hepatocarcinogen, 2-AAF, induced the oxidative stress by excessive generation of ROS in RAW 264.7 cells, which was effectively decreased by C-PC. This is in accordance with earlier studies where C-PC was shown to inhibit the generation of reactive oxygen and nitrogen species *in vivo* and *in vitro* [21]. *In silico* analysis reveals biphasic generation of ROS in response to 2-AAF treatment (Fig. 3c). These studies thus demonstrate generation of ROS by 2-AAF and the blocking of the same by C-PC in RAW 264.7 cells.

The *In silico* analysis of the mechanism allowed us to determine the ROS levels in the calibrated model with DPI and Akt IV inhibitor as well. Although the cells were incubated with these inhibitors for only 30 min and they do show a reduction in MDR1 expression, the reduction is not as prominent as C-PC (50 μM) treatment. This may be due to the ROS levels not being reduced as much by these treatments (Table 1) and with the positive feedback initiated by 2-AAF treatment for ROS production. ROS scavenging by C-PC is more effective than inhibiting a pathway intermediate such as Akt. Regarding NADPH oxidase inhibition, the inhibition by DPI does not completely eliminate ROS production. *In silico* studies also showed a decrease in phosphorylated Src levels with C-PC treatment (Fig. 6). As a result, C-PC may stop the positive feedback by scavenging ROS. C-PC, is thus more effective in reducing ROS levels and further reduce MDR1 expression.

Alteration in the redox potential of the cells exposed to 2-AAF may affect ROS responsive signaling pathways. One such type of signaling pathway is Akt/PKB cell survival pathway. Akt/PKB is a serine threonine kinase and its phosphorylation is a critical event in cell survival. Akt can be activated by a wide variety of growth stimuli, including platelet-derived growth factor, epidermal growth factor and this activated Akt acts as a survival signal that protects cells from apoptosis induced by various stress conditions. C-PC was shown as ROS scavenger but its ability to inhibit phosphorylation of Akt has not been investigated. In our study, Western blot analysis of Akt and its phosphorylated form showed increased phosphorylation of Akt in 2-AAF treated cells. The reported upregulation of the activity and expression of Akt by ROS [41] supports the possible involvement of ROS in the phosphorylation of Akt in cells exposed to 2-AAF. This 2-AAF-induced phosphorylation of Akt was effectively decreased by C-PC treatment in a dose dependent manner. This may be due to the decrease in generation of ROS or C-PC might have interfered with the phosphorylation of Akt at the cell membrane. Treatment of cells with DPI also inhibited Akt phosphorylation. Similar inhibition of the phosphorylation of Akt by various natural anti-oxidants was reported [42,43]. *In silico* model showed Akt activation by Src and its decrease upon C-PC treatment (Fig. 6). Reducing the ROS levels by C-PC reduced Src activation and there by Akt activation. Akt inhibitor IV on the other hand reduced the activation of Akt but might have not eliminated Src activation by ROS generated by 2-AAF treatment. This may be responsible for the lack of total inhibition of MDR1 expression by Akt inhibitor IV. In the light of above, C-PC may be regulating MDR1 expression by mechanisms other than Akt activation. One such mechanism may be by inhibition of Cyclooxygenase-2 (COX-2). Increase in COX-2 activity was previously shown to induce MDR1 [44]. Our previous studies have shown that C-PC inhibits COX-2 activity [18]. Akt was shown to be involved in the 2-AAF mediated activation of NF-κB [45]. ROS generated by hypolipidomic drugs belonging to peroxisomal proliferator family [46] was also shown to be involved in the up regulation of IKKα activity and activation of NF-κB pathway. In order to test the involvement of ROS and Akt in the activation of NF-κB, further studies were taken up on NF-κB, which is a positive regulator in 2-AAF induced MDR1 expression.

NF-κB is considered to be the most important regulatory element that controls the expression of stress inducible genes. Sequence analysis indicated the presence of putative NF-κB binding sequence in the mouse MDR1 promoter (position −163 to −157) [47]. The observed decrease in the generation of ROS and phosphorylation of Akt in RAW 264.7 cells treated with C-PC made us to presume that these observed changes may affect the

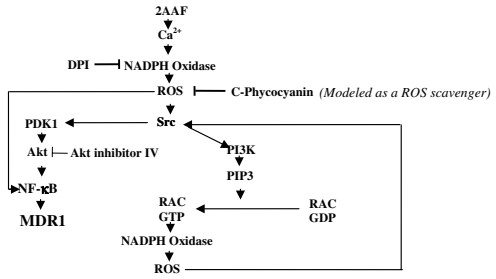

Fig. 7. Schematic representation of the simulation model showing 2-AAF-induced MDR1 expression through ROS generation and possible sites of interference by C-PC, DPI and Akt inhibitor IV.

translocation of NF-κB to the nucleus. Our studies clearly demonstrate that treatment of cells with 2-AAF increased the translocation of NF-κB to the nucleus and this translocation is dose dependently decreased upon treatment with C-PC. As expected inhibitors of NADPH Oxidase (DPI) and Akt phosphorylation (Akt inhibitor IV) inhibited the translocation of NF-κB to the nucleus. These studies support the involvement of ROS and Akt in the 2-AAF-induced activation of NF-κB. NF-κB was also shown to be activated by ROS independent of Akt activation [48,49]. So in the presence of Akt inhibitor IV, ROS generated by 2-AAF treatment might activate NF-κB, independent of Akt. Studies on Isovitexin, a food phytochemical with ROS scavenging activity, also showed a similar decrease in NF-κB activity [50]. Simulation studies also revealed similar decrease in NF-κB translocation upon C-PC treatment. Due to the diverse mechanisms of C-PC action and due to its potent anti-oxidant capacity, C-PC is more effective than DPI or Akt inhibitor IV in inhibiting the expression of MDR1.

In conclusion, our experimental and simulation studies demonstrate that 2-AAF-induced expression of MDR1 in mouse macrophage cells (RAW 264.7) is mediated through the generation of ROS, phosphorylation of Akt and activation of NF-κB. C-PC, a known free radical scavenger and a potent antioxidant, down regulates the 2-AAF-induced expression of MDR1 by interfering at the level of ROS generation, phosphorylation of Akt and activation of NF-κB (Fig. 7). This down regulation of MDR1 expression induced by xenobiotics such as 2-AAF by C-PC, suggests its usefulness in overcoming the drug resistance in cellular systems.

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