C-PHYCOCYANIN AND ARACHIDONIC ACID CASCADE: ROLE IN APOPTOSIS OF LIPOPOLYSACCHARIDE STIMULATED MOUSE MACROPHAGE CELL LINE, RAW 264.7

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

C. MADHAVA REDDY

DEPARTMENT OF ANIMAL SCIENCES
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD-500 **046, INDIA**

DECEMBER 2001

ENROLMENT No. 98LAPH05

Dedicated to

My Parents

&

My Mentor

Prof. P. Reddanna

UNIVERSITY OF HYDERABAD

(A Central University Established in 1974 by an act of Parliament)

HYDERABAD-500 **046, INDIA**

DECLARATION

I hereby declare that the work embodied in this thesis entitled "C-Phycocyanin and Arachidonic Acid Cascade: Role in Apoptosis of Lipopolysaccharide Stimulated Mouse Macrophage Cell Line, RAW 264.7" has been carried out by me under the supervision of Prof. P. Reddanna and that this has not been submitted for any degree or diploma of any other university earlier.

29/12/01

(Prof. P. Reddanna)

Research Supervisor

(C. Madhava Reddy)

Research Scholar

UNIVERSITY OF HYDERABAD

(A Central University Established in 1974 by an act of Parliament)

HYDERABAD-500 046, INDIA

CERTIFICATE

This is to certify that ***Mr. C Madhava Reddy*** *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 "* ***C-Phycocyanin and Arachidonic Acid Cascade: Role in Apoptosis of Lipopolysaccharide Stimulated Mouse Macrophage Cell Line, RAW 264.7*** *"for submission for the degree of (Doctor of Philosophy of this university.*

Prof. P. Reddanna
Supervisor

Head
Department of Animal Sciences

HEAD
Deptt. of Animal Sciences

Dean
School of Life Sciences

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ABBREVIATIONS

AA	: arachidonic acid
BCIP	: 5-bromo-4-chloro-3-indolyl phosphate
COX	: cyclooxygenase
DDC	: diethyldithiocarbomate
DNA	: deoxy ribonucleic acid
FACS	: fluorescence activated cell sorter
FBS	: fetal bovine serum
g	: gram
h	: hour (s)
HETE	: hydroxyeicosatetraenoic acid
HPETE	: hydroperoxyeicosatetraenoic acid
HPLC	: high performance liquid chromatography
kDa	: kilodalton
l	: litre
LOX	: lipoxygenase
LPS	: lipopolysaccharide
LT(s)	: leukotriene(s)
mg	: milligram
min	: minute(s)
ml	: milliliter
mM	: milli molar

MTT	: 3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyltetrazolium bromide
NBT	: nitroblue tetrazolium
nm	: nanometers
NMMA	: N^G-monomethyl-L-arginine
NSAIDs	: nonsteroidal anti-inflammatory drugs
PARP	: poly (ADP-ribose) polymerase
PBS	: phosphate buffered saline
PG(s)	: prostaglandin(s)
rpm	: revolutions per minute
µM	: micro molar
°C	: degree centigrade/ degree Celsius

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- C. Madhava Reddy

Introduction

Many of the lipids involved as second messengers in cell signaling pathways arise from arachidonic acid pathways. Arachidonic acid (all-cis-5, 8,11,14-eicosatetraenoic acid) is the most abundant polyunsaturated fatty acid found in mammalian tissues, esterified at *sn2* position of the glycerol backbone of cell membrane phospholipids. The release of AA is the critical step in the biosynthesis of eicosanoids, the oxygenated metabolites of arachidonic acid. The release of AA from the *sn2* position of cellular phospholipids is mediated by either phospholipase A_2 or by the combined action of phospholipase C and diacylglycerol lipase (Fig. 1). The two most studied mammalian forms of PLA_2 are type II, a 14 kDa protein known to exist both as an extra-cellular and cell associated forms, and a cytosolic 85 kDa protein. The coexistence of both the cytosolic PLA_2 ($cPLA_2$) and cell associated type II PLA_2 has been reported (Marshall and Roshak, 1993). The $cPLA_2$ primarily supports the formation of prostaglandins (PGs) and the enzymes other than $cPLA_2$ possibly provide the substrate for the formation of leukotrienes (LTs) (Marshall *et al,* 1997).

The metabolic fate of arachidonic acid depends on the cell type and its specific complement of enzymes. These include Cytochrome P-450 pathway, Lipoxygenase pathway and Cyclooxygenase pathway (Fig. 2).

1.1 Cytochrome P-450 Pathway

Cytochrome P-450, the epoxygenase enzymes are ubiquitous and involved in the oxygenation reactions of AA in addition to their involvement in xenobiotic metabolism (Capdevila *et al,* 1981). Multiple isoforms of cytochrome P-450 metabolize AA to three types of eicosanoid products. Allylic oxidation forms several mid chain conjugated dienols, ω-terminal

Fig. 1: Specificity of phospholipases in the release of arachidonic acid

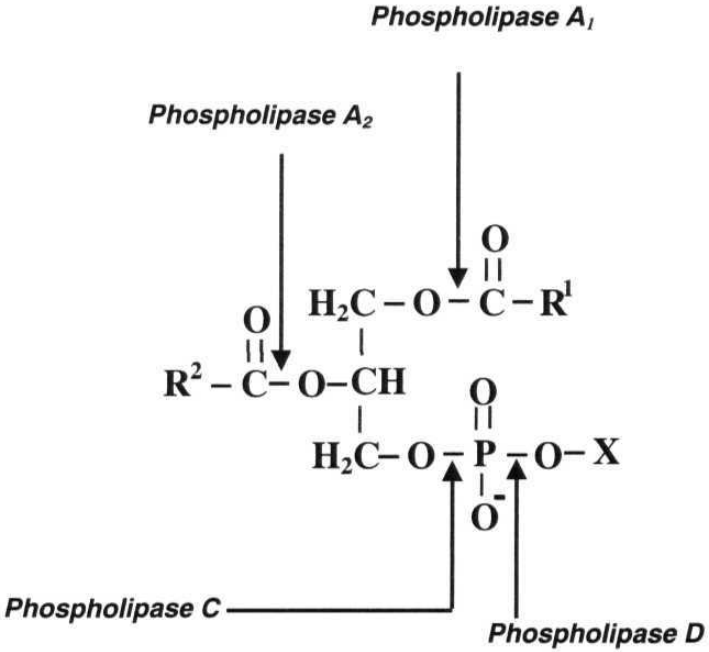
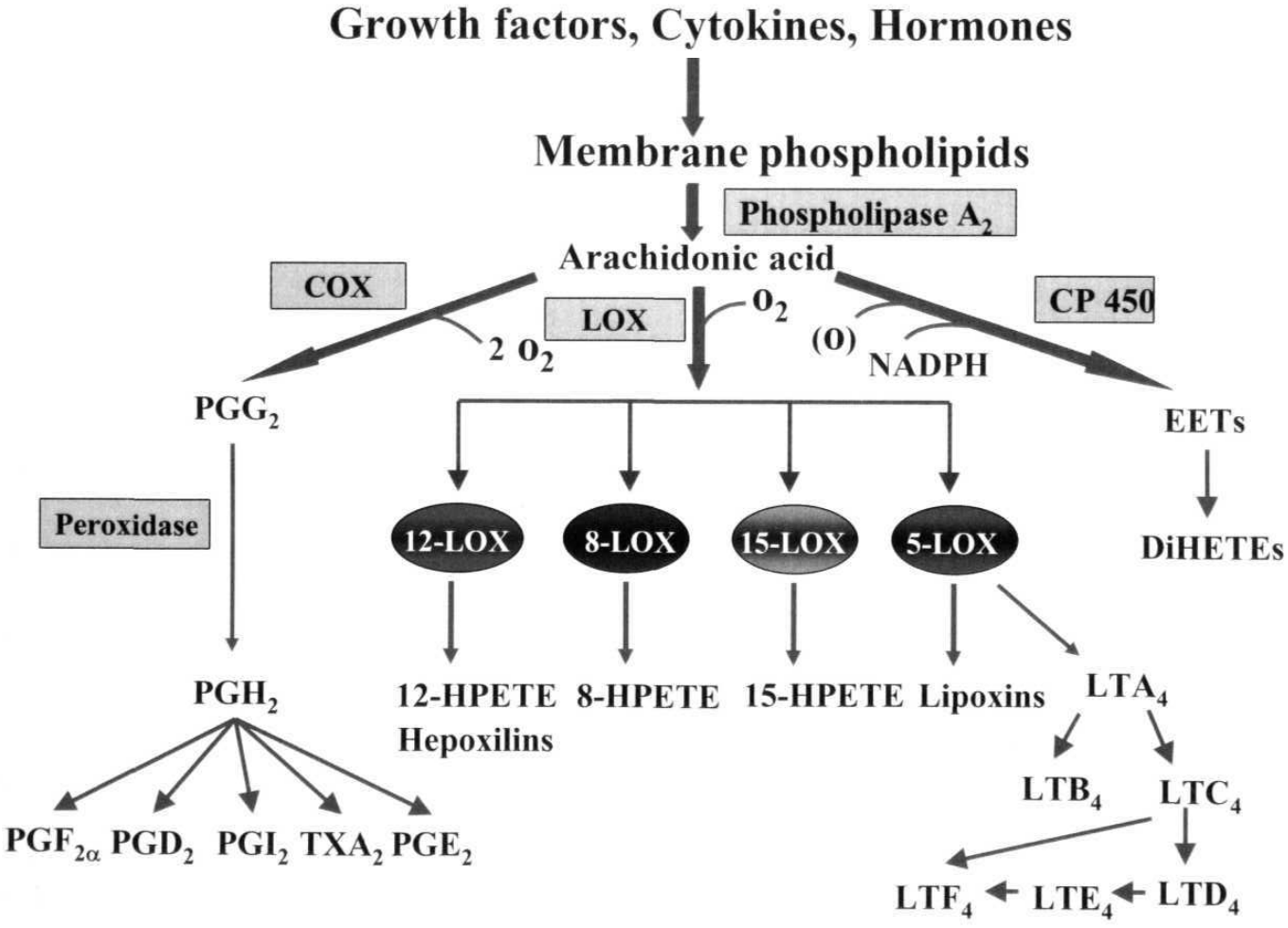


Fig. 2: Major oxygenation pathways of arachidonic acid

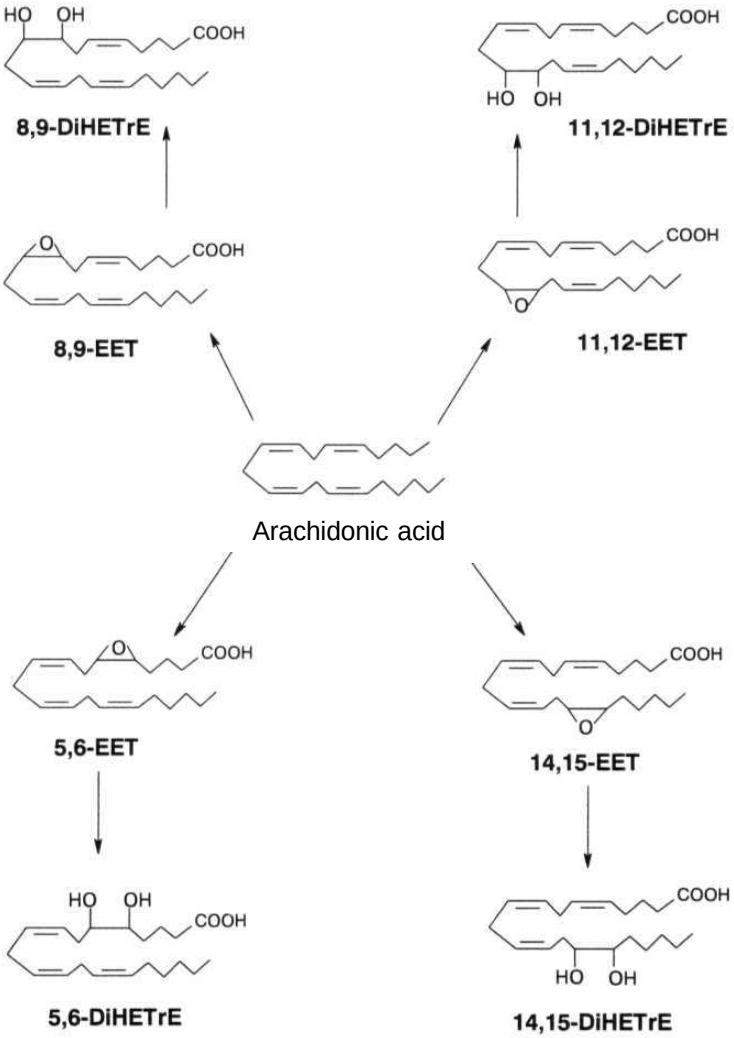


hydroxylation forms C16 to C20 alcohols of AA and olefin epoxidation results in the production of four cis-epoxyeicosatrienoic acids (14,15-; 11,12-; 8,9-; and 5,6-EETs) (Fig.3)

Biological Role of Cytochrome P-450 Pathway

Several studies have demonstrated that P450 epoxygenase-derived eicosanoids have a multitude of potent biological activities. For example EETs have been shown to have potent effects on peptide hormone secretion (Falck ef *al,* 1983; Cashman *et al,* 1987; Synder *et al,* 1989), vascular and bronchial smooth muscle tone (Proctor *et al*, 1987; Campbell *et al,* 1996; Zou *et al*, 1996), and ionic transport (Harris *et al,* 1990; Romero *et al,* 1991; Pascual *et al,* 1998). The EETs have also been shown to play critical roles in regulating cellular proliferation (Harris *et al,* 1990; Sheu *et al,* 1995; Chen ef *al,* 1998), inflammation (Node *et al,* 1999), hemostasis (Node ef *al,* 2001), and a variety of intracellular signaling pathways (Chen ef *al,* 1998; 2000). Epoxides of linoleic acid are toxic, where as epoxides of arachidonic acid have a wide range of biological effects. The 5,6-EET of arachidonic acid provides an unorthodox route to oxidized prostanoids (Serhan and Oliw, 2001). 5,6 EET is an excellent substrate for COX and thromboxane synthase, which transform 5,6 EET to 5,6-epoxyprostaglandins and 5,6-epoxythromboxanes (Oliw, 1984;Balazy, 1991). Vascular and renal effects of 5,6 EET can be blocked by COX inhibitors (Carroll ef a/, 1993), which suggests that the pharmacological effects of 5,6-EET can result from its transformation by COX. 20-HETE probably plays an important role as secondary messenger in the regulation

Fig. 3: Epoxygenase pathway of arachidonic acid



of renal tubuloglomerular feedback and sodium transport (Roman and Alonso-Galicia, 1999).

1.2 Lipoxygenase Pathway

Lipoxygenase pathway leads to the formation of hydro (pero) xyeicosatetraenoic acids [H (P) ETEs], leukotrienes (LTs) and lipoxins from AA. Among these, LTs are the most potent biologically active compounds. Lipoxygenases (LOX) comprise a family of non-heme, iron containing dioxygenase enzymes which incorporate molecular oxygen into polyunsaturated fatty acids with 1-cis, 4-cis-pentadiene structures such as arachidonic acid and linoleic acid, which is transformed into 1-hydroperoxy-2, 4-trans, cis-pentadiene product, generating the corresponding hydroperoxy derivative of the fatty acid. About 70 years ago a lipoxygenase (earlier referred to as lipoxidase) was found in soybeans (Andre and Hou, 1932) and this plant enzyme has been extensively investigated (Tappel, 1963). For long time it was believed that, there were no LOXs in animal tissues. However, along with the discovery of bioactive thromboxane in 1974, Hamberg and Samuelsson found the production of 12-HETE upon incubation of human platelets with exogenous arachidonic acid (Hamberg and Samuelsson, 1974). The 12-HETE production suggested the presence of a LOX enzyme, which oxygenated at position 12 of arachidonic acid. This discovery marked the starting point of animal LOX research and over the following years more and more LOX isoforms were discovered. LOXs have also been detected in lower marine organisms (Hawkins and Brash, 1987; Brash *et al,* 1991; 1996; Hada *et al,* 1997), but they are not expressed in bacteria or yeast.

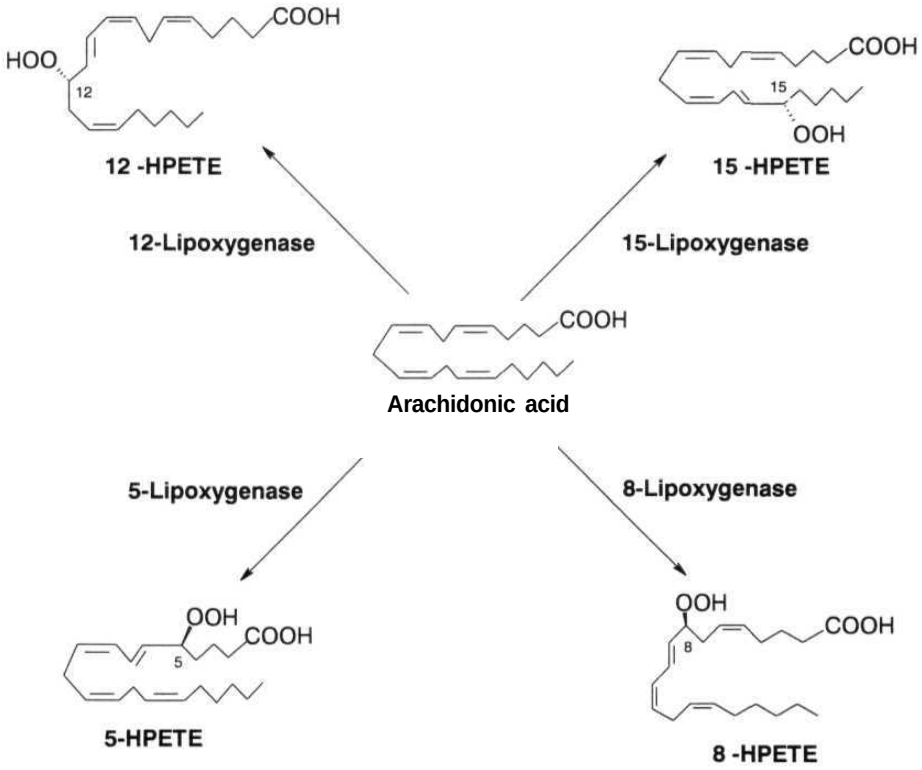
1.2.1 Classification of Lipoxygenases

LOXs have been classified according to their positional specificity of arachidonate oxygenation (Yamamoto, 1992). For example, 15-LOX introduces molecular oxygen at carbon atom 15 of arachidonic acid (formation of 15-HPETE) where as arachidonate 12- lipoxygenase introduces at 12-position to generate 12-HPETE. These enzymes are now referred to as 12-, 15-, 5- and 8-lipoxygenases (Fig. 4). Most of the animal LOXs are cytosolic and require Ca^{2+} and ATP for their maximal activity (Rouzer *et al*, 1986; Hogaboom *et al,* 1986; Veera Reddy *et al*, 2000).

1.2.1.1 12-Lipoxygenases

12-lipoxygenase was discovered in human (Hamberg and Samuelsson, 1974) and bovine platelets (Nugteren, 1975) as the first lipoxygenase in animal kingdom. Two subtypes of 12-LOX exist, based on biochemical, immunological and molecular biological criteria: human platelet type 12-LOX, referred to as P-12-LOX, which metabolizes arachidonic acid into 12(S) HETE (Funk *et al,* 1990); the leukocyte type, or L-12-LOX, converts arachidonic acid or linoleic acid into 12(S) HETE and also produces small quantities of 15 (S)-HETE (Hada *et al,* 1991). 12-LOX metabolites, have been found to play a central role in various stages of the metastatic process in tumors and are, therefore, potential targets for anticancer drug development. The platelet type has been cloned from human platelets and the megakaryocytic cell line, HEL (Funk *et al,* 1990; Izumi *et al,* 1990). The other 12-LOX, namely the leukocyte type has been detected in porcine leukocytes (Yoshimoto *et al,* 1990), pituitary (Ueda *et al,* 1990), vascular smooth cells (Natarajan *et al,* 1993) and also in

Fig. 4: Mammalian lipoxygenases

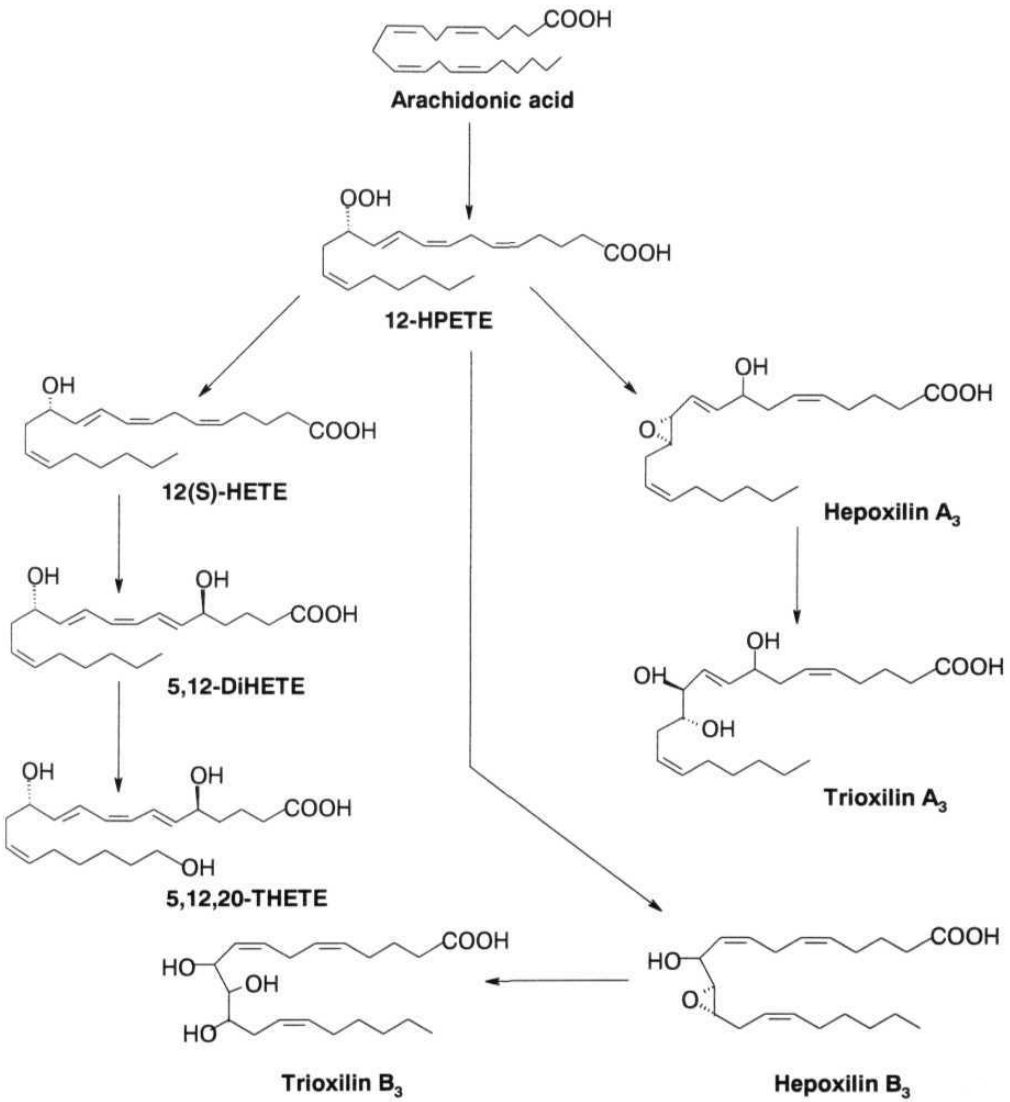


human adrenal glomerulosa cells (Gu *et al,* 1994), human monocytes, endothelial and vascular smooth muscle cells (Kim *et al,* 1995). The porcine leukocyte 12-lipoxygenase is only 65% homologous to the human platelet 12-lipoxygenase (Funk *et al,* 1990; Izumi *et al,* 1990;Yoshimoto *et al,* 1990) whereas it is 87% homologous to human 15-LOX (Sigal *et al,* 1988; Yoshimoto *et al,* 1990). 12-Lipoxygenases of either type have been implicated in the biosynthesis of hepoxilins and lipoxins (Fig. 5). Hepoxilins may be involved in the regulation of ion transport across biomembranes. Lipoxins can be synthesized unicellularly in leukocytes via concerted action of 5- and 12/15-LOXs or transcellularly by the leukocyte 5-LOX. The platelet 12-LOX appears to be involved in the regulation of immunological and haemodynamic processes. The leukocyte type 12-LOX is closely related to mammalian 15-LOX at the molecular level (Yamamoto, 1992).

Biological Role of 12-Lipoxygenases

Porcine 12-LOX in vascular smooth muscle cells was upregulated by platelet-derived growth factor (Natarajan ef *al,* 1996) and by cytokines such as interleukin-1, -4 and -8 (Natarajan *et al,* 1997). The 12-LOX product, 12 (s)-HETE has been shown to play a role in the growth promoting effects of angiotensin II in vascular smooth muscle and adrenal cells (Natarajan *et al,* 1992; 1994), in the chemotactic effects of platelet derived growth factor (Natarajan *et al,* 1996), and in mediating several major steps of the process of hematogenous metastasis of cancer cells (Honn *et al,* 1991; 1994). 12-HETE was shown to increase tumor cell motility and invasive potential (Honn *et al,* 1991; 1994). Liu *et al* (1994) demonstrated that greater amounts of 12-HETE was produced by highly metastatic tumor cells than

Fig. 5: 12-Lipoxygenase pathway of arachidonic acid



the low metastatic tumor cells, suggesting that biosynthesis of 12-HETE by tumor cells is a determinant of their metastatic potential. N/N1003A cells, like primary cultured human lens epithelial cells or neonatal rat lenses, require 12-LOX activity for EGF dependent growth (Haque *et al,* 1999). Increased levels of 12-HETE were reported in psoriatic lesion of human skin (Hammarstrom *et al,* 1975).

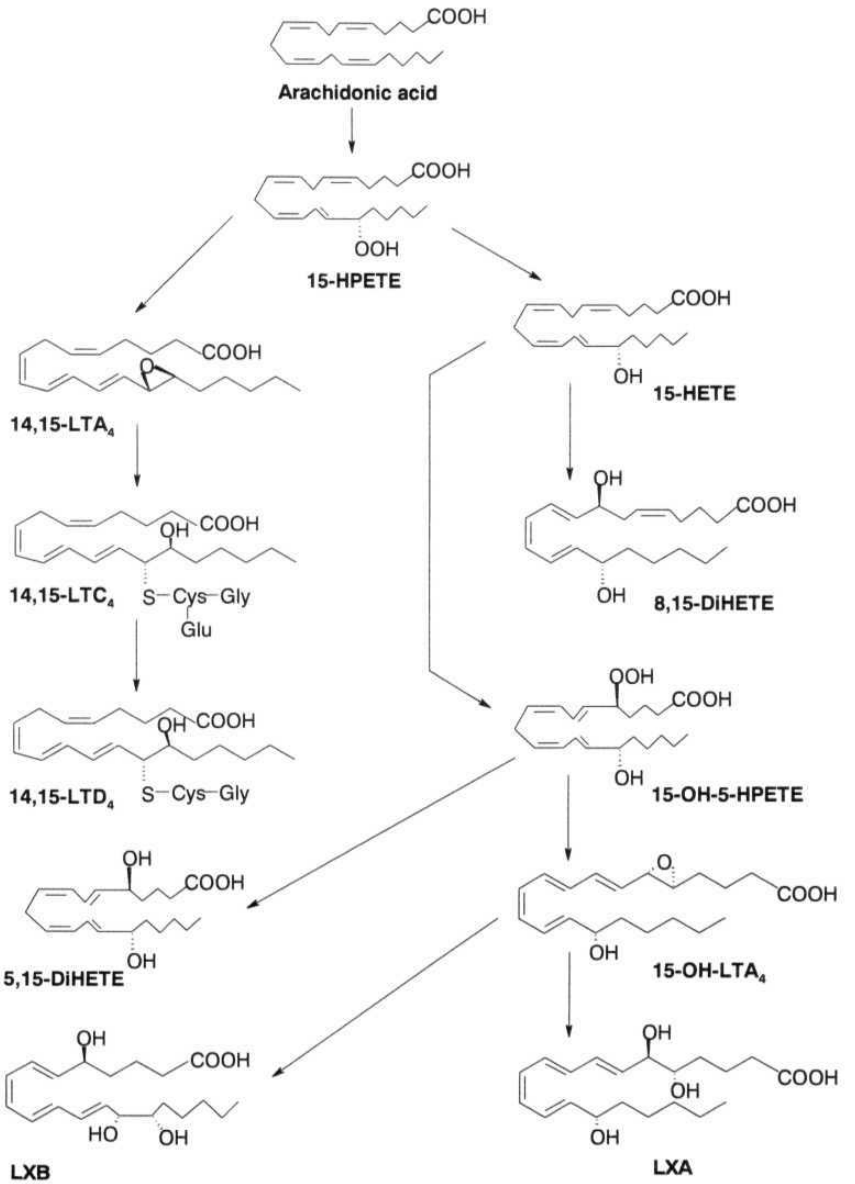
1.2.1.2 15-Lipoxygenases

15-LOX is involved in inflammation (Samuelsson *et al,* 1987; Vanderhoek, 1988), reticulocyte differentiation (Rapoport *et al,* 1979; Nadel *et al,* 1991; van Leyen *et al,* 1998; Schewe and Kuhn, 1991) and atheroma formation (Feinmark and Cornicelli, 1997; Kuhn and chan, 1997; Kuhn ef *al,* 1997). 15-LOX was detected and purified to homogeneity in rabbit reticulocytes (Rapoport ef *al,* 1979) and well characterized with respect to its protein, chemical and enzymatic properties (Schewe *et al,* 1986). 15-LOX exists in two forms; reticulocyte type 15-LOX has been termed 15-LOX-1, whereas the newly discovered 15-LOX is called 15-LOX-2 (Brash ef *al,* 1997). Reticulocytes (Rapoport *et al,* 1979), human eosinophils (Turk *et al,* 1982; Nadel *et al*, 1991) and airway epithelial cells (Hunter *et al,* 1985; Sigal ef *al,* 1992) express large amounts of 15-LOX, produces 15(s)-HPETE, which is converted to 15(s)-HETE (Fig.6). However, only rabbit reticulocytes are suitable for large scale enzyme preparation. 15-lipoxygenase has been cloned from human reticulocytes (Sigal *et al*, 1988).

Biological Role of 15-Lipoxygenases

15-LOX is capable of oxidizing phospholipids and biomembranes (Schewe ef *al,* 1975). The oxidation products of LDL may play a vital role in

Fig. 6: 15-Lipoxygenase **pathway of arachidonic acid**



the primary stages of atherosclerosis (Kuhn and Chan, 1997; Feinmark and Cornicelli, 1997). Thus, the 15-LOX may constitute a pharmacological target for the development of antiatherosclerotic drugs. The reticulocyte type 15-LOXs have been implicated in cell maturation and differentiation (Rapoport *et al,* 1990; van Leyen *et al,* 1998). 15-LOX-1 was found to be over expressed in coiorectai cancers and was implicated in carcinogenesis (Ikawa *et al.,* 1999). Van Leyen *et al* (1998) reported that arachidonate 15-LOXs might be involved in organeile degradation during differentiation of the eye lens cell. 13-HODE, the product of 15-lipoxygenase pathway of linoleic acid occurs in neutrophils, alveolar macrophages and scaly skin of psoriasis patients. Epidermal growth factor can stimulate 13-hydroxyoctadecadienoic acid (13-HODE) formation in BT-20 breast cancer cells by a 15-LOX dependent mechanism (Reddy *et al,* 1997). 12-and 15-HETEs have angiogenic and mitogenic effects on endothelial cells (Setty *et al,* 1987).

1.2.1.3 8-Lipoxygenase

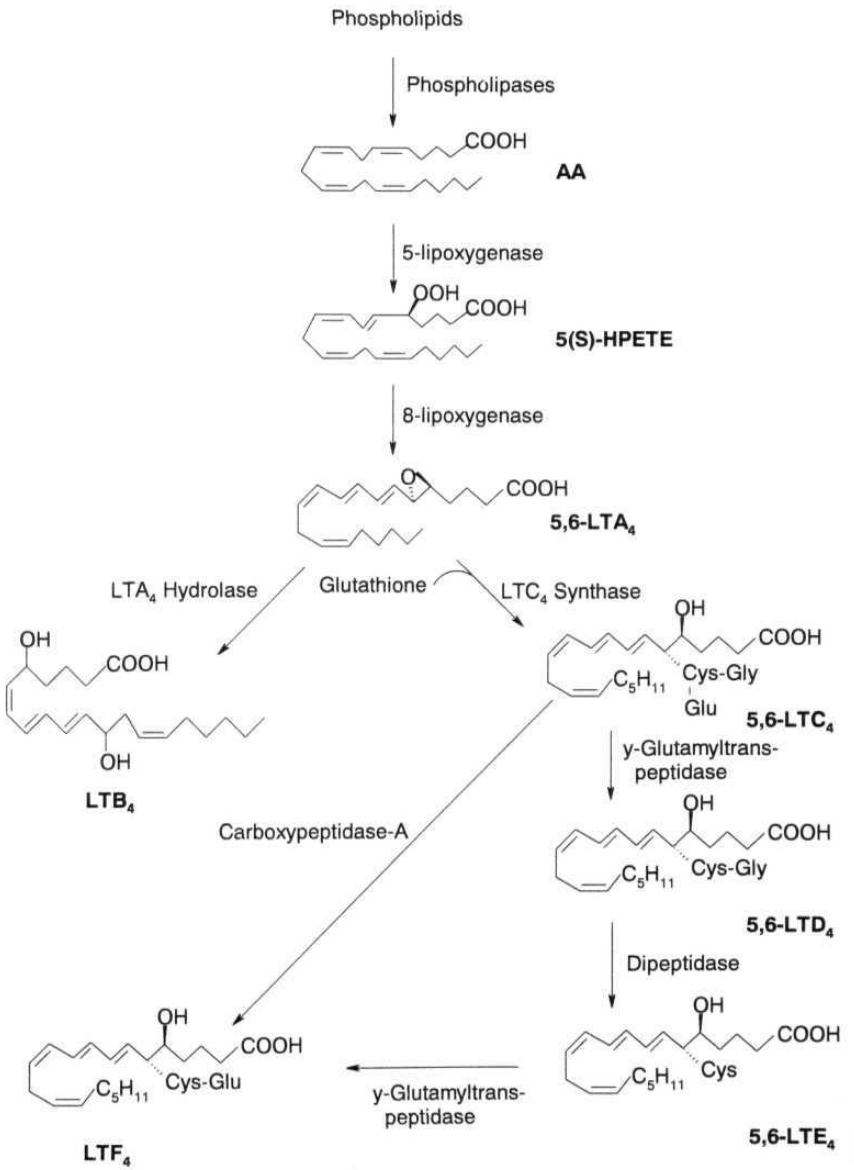
Very little is known about 8-lipoxygenases. The 8-LOX product, 8(s)-HETE, isolated from epidermal cells, has been thought to activate G-protein coupled receptors and nuclear hormone receptors, which regulate pathways involved in lipid homeostasis and inflammation processes (Gillmor *et al,* 1997).

1.2.1.4 5-Lipoxygenase

5-LOX is involved in the biosynthesis of leukotrienes, proinflammatory mediators participating in various forms of acute and subacute inflammation. In addition to human allergic asthma, LTs

contribute to the pathologic changes during colitis (Zipser *et al,* 1987), psoriasis (Chan *et al,* 1987), glomerulonephritis (Wu *et al,* 1993) and endotoxima (Keppler *et al,* 1987). 5-LOX in the presence of FLAP catalyzes the oxygenation of arachidonic acid at C-5 into 5-hydroperoxyeicosatetraenoic acid (5-HPETE) (Samuelsson *et al,* 1987), followed by a second reaction in which 5-HPETE is dehydrated to form the unstable allylic epoxide, LTA4 by the same enzyme. Once formed, LTA4 is further metabolized to either LTB_4 *via* stereoselective hydration by LTA4 hydrolase or to LTC_4 through glutathione conjugation catalyzed by LTC_4 synthase. The LTA4, is hydrolyzed by LTA4 hydrolase to form biologically active dihydroxy acid, LTB_4 (Borgeat and Samuelsson, 1979). Another pathway is the formation of LTC_4 from LTA4 by an enzymatic conjugation with glutathione at C-6 position, a reaction catalyzed by LTC_4 synthase (Jakschik *et al,* 1982). Successive elimination of glutamic acid from LTC_4 by γ -glutamyl transpeptidase (Orning *et al,* 1980) and glycine by dipeptidase gives LTD_4 and LTE_4 respectively (Sok *et al,* 1981). LTE_4 on further action by γ -glutamyl transpeptidase results in the formation of LTF_4 (Anderson *et al,* 1982). LTF_4 can be formed directly from LTC_4 by the action of carboxypeptidase A (Reddanna *et al,* 1988) (Fig.7). These three sulfidopeptide LTs are commonly referred to as the slow-reacting substances of anaphylaxis (Piper, 1985). 5-HPETE is also converted to a dihydroperoxyeicosatetraenoic acid (diHPETE), which may lead to the formation of trihydroxyeicosatetraenoic acid, termed as lipoxins (Rokach and Fitzsimmons, 1988). 5-LOX protein expression and activity is mainly observed in myeloid cells. Granulocytes, monocytes/macrophages, mast

Fig. 7: 5-Lipoxygenase **pathway of arachidonic acid**



cells and B-lymphocytes express 5-LOX, while platelets, endothelial cells, T-cells and erythrocytes are 5-LOX negative (Borgeat and Samuelsson, 1979; Murphy *et al,* 1979; Fels *et al*, 1982; Fitzpatrick *et al,* 1984; Claesson and Haeggstrom, 1988; Jakobsson *et al,* 1992). cDNA has been cloned for human and rat 5-LOX (Balcarek *et al,* 1988; Dixon *et al,* 1988; Matsumoto *et al,* 1988), which are almost identical (92%). The human 5-LOX gene contains 14 exons and span more than 82kb. The promoter contains several GC boxes, but lacked TATA and CCAAT sequences (Funk ef *al,* 1989). The human 5-LOX gene was mapped to chromosome 10 (Funk *et al*, 1992).

Biological Role of 5-Lipoxygenase

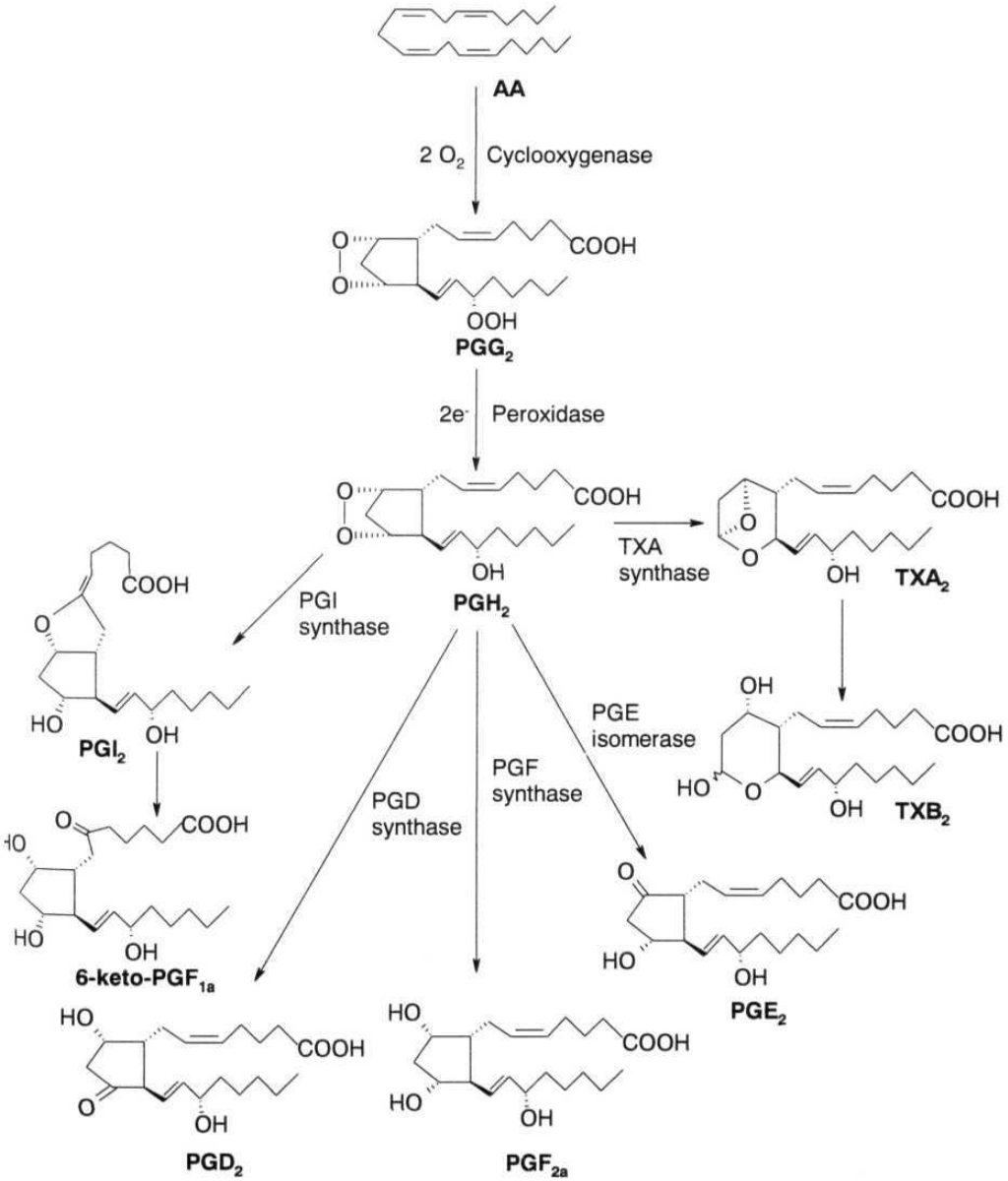
The cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4) released from the lung tissue of asthmatic patients on exposure to allergens play a pathophysiological role in immediate hypersensitivity reactions by increasing the vascular permeability in post capillary venules and stimulating mucus secretion (Bisgaard, 1984). The dihydroxy leukotriene, LTB_4 displays chemokinetic and chemotactic activity towards granulocytes and induces granulocyte activation which includes adherence of neutrophils to vessel walls and release of lysosomal enzymes. LTC_4 was demonstrated to be present in the hypothalamus is implicated in the release of leutinizing hormone releasing hormone (Hulting ef *al,* 1985). Lipoxins, trihydroxy eicosanoids with a conjugated tetraene formed by the combined action of 5- and 15-LOX, inhibit natural killer cell cytotoxicity (Samuelsson *et al,* 1987). Lipoxins act as both immunologic and haemodynamic regulators. Leukotrienes are also implicated in insulin

release (Pek and Walsh, 1984), steroidogenesis (Dix *et al,* 1984) and ovulation (Reich *et al,* 1985). Cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4) are identified as important mediators of bronchoconstriction and hypersensitivity reactions.

1.3 Cyclooxygenase **Pathway**

Cyclooxygenase pathway leads to the formation of PGs, thromboxanes and prostacyclin- a family of autocrine and paracrine mediators that contribute to many physiological and pathophysiological responses. Cyclooxygenase (COX; Prostaglandin endoperoxide synthase, EC 1.14.99.1) catalyzes two separate enzyme reactions; i). the bis-oxygenation of arachidonic acid at carbons 11 and 15 (Cyclooxygenase activity) to form hydroperoxy endoperoxide PGG_2 and ii). the subsequent bi-electron reduction at 15-hydroperoxy group of PGG_2 (peroxidase activity) to form another endoperoxide derivative, PGH_2 (Fig.8) (Smith *et al,* 1996). These two reactions occur at distinct but structurally and functionally interconnected sites. The endoperoxides, PGG_2 and PGH_2 are very unstable with a half-life of 4-5 min. PGH_2 is transformed to different primary products, such as PGD_2, PGE_2 $PGF_{2\alpha}$, prostacyclin and thromboxanes, collectively known as prostanoids. The array of PGs produced varies depending on the downstream enzymatic machinery present in a particular cell type. For example, endothelial cells primarily produce PGI_2, whereas platelets mainly produce TXA_2 from PGH_2. Prostaglandins are found in animals as primitive as the coelenterates and in a wide variety of human tissues (Mead *et al*, 1986). PGs not only play a central role in inflammation, but also regulate other critical physiological responses. In humans, PGs are

Fig. 8: Cyclooxygenase pathway of arachidonic acid



involved in diverse functions, including blood clotting, ovulation, initiation of labor, bone metabolism, nerve growth and development, wound healing, kidney function, blood vessel tone and immune responses (Table 1).

1.3.1 Discovery of Two Isoforms of Cyclooxygenase

In the early 1990s, evidence emerged, with regard to the existence of two distinct cyclooxygenases in mammals (Raz *et al,* 1989; Xie *et al,* 1991; Kujubu *et al,* 1991). Investigators studying cell growth discovered a new gene product that was induced *in vitro,* with great similarity to COX. At the same time other investigators discovered that COX activity could be induced by cytokines such as interleukin-1 and inhibited by corticosteroids. Corticosteroids inhibited the interleukin-1 induced COX activity but not basal COX activity. These observations led to the hypothesis that there are two COX isoenzymes. One of the COX enzyme (COX-1) was theorized to be constitutiveiy expressed and responsible for basal PGs production, while the second COX enzyme (COX-2) was induced by inflammatory stimuli such as interleukin-1 (IL-1) and suppressed by glucocorticoids. Initially it was thought that COX-2 was not constitutiveiy 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 (DuBois *et al,* 1998). Furthermore, the constitutive expression of COX-2 in colon carcinoma has been shown to represent a casual factor in the transformation of this tissue (Oshima *et al*, 1996).

Table 1: Physiological functions of prostaglandins

Prostaglandin(s) involved	Physiological function
PGE_2, $PGF_{2\alpha}$, PGI_2	Relax vascular smooth muscle
TXA_2	Promote platelet aggregation
PGI_2	Inhibit platelet aggregation, Relax uterine smooth muscle
PGE_2, PGI_2	Relax bronchial smooth muscle
$PGF_{2\alpha}$	Contract bronchial smooth muscle Contract uterine myometrium
PGE_2, PGI_2	Protect gastric mucosa, Increase renal blood flow and provoke diuresis and natriuresis
PGE_2, $PGF_{2\alpha}$	Contract uterine smooth muscle
PGD_2	Promotion of sleep
PGE_2	Awakens from sleep, Inflammation, renal function

1.3.2 Comparison of COX-1 and COX-2

Both COX-1 and COX-2 are homo-dimeric, glycosylated, heme containing proteins with 2 catalytic sites. These enzymes have similar turnover number and K_m value for AA and O_2. Both isoforms have high structural identity but are different in substrate and inhibitor selectivity (Smith ef *al,* 1996), and also in their intracellular localization. COX-2 accepts a wider range of fatty acids as substrates when compared to COX-1. Thus, although both enzymes can utilize AA and dihomo-γ-linolenate equally well, COX-2 oxygenates other fatty acid substrates such as eicosapentaenoic acid, γ-linolenic acid, α-linolenic acid and linoleic acid more efficiently than does COX-1. COX-2 acetylated by aspirin on serine 530 will still oxidize AA to 15-HETE, whereas similarly acetylated COX-1 will not oxidize AA at all. Unlike COX-1 enzyme, COX-2 has valine at position 523 instead of isoleucine. The difference between valine and isoleucine is 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; Vane, 1998). The COX-2 active site is about 20% larger and has a slightly different shape than that of COX-1 (Luong *et al,* 1996). This difference in active site size and shape is due to three amino acid differences between COX-1 and COX-2: isoleucine 523 to valine 523 in the first shell of the active site, and isoleucine 434 to valine 434 and histidine 513 to arginine 513 in the surrounding second shell. 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 it is dramatically upregulated up to 80 fold in inflamed tissues (macrophages, fibroblasts, chondrocytes, epithelial, endothelial cells etc) with cytokines, growth factors, human chorionic gonadotropin, phorbol esters, bacterial lipopolysaccharides (LPS), ligands of G-protein-coupled receptors and reactive oxygen intermediates; cyclic adenosine monophosphate and serum. For example COX-2 expression and PGE_2 production are greatly enhanced in rheumatoid synovium compared to the less inflamed osteoarthritic synovium, and in animal models of inflammatory arthritis (Crofford ef *al*, 1994; Anderson *et* a*l*, 1996). This is the result of excessive production of interleukin-1, tumor necrosis factor and growth factors in the rheumatoid joint. This provides for a constant level of enzyme in most cell types to synthesize PGs responsible for homeostatic functions. In contrast, the features of COX-2 gene are those of an "immediate-early" gene that is not always present but is highly regulated and upregulated during inflammation or pathological process. Both the isoforms have a molecular weight of 71 kDa and are almost identical in length, with just over 600 amino acids, of which 60%-65% sequence is identical between COX-1 and COX-2 from the same species and 85%-90% identity among individual isoforms from different species (Shimokawa and Smith, 1992). However, the human COX-2 gene at 8.3 kb is a small immediate early gene, whereas human COX-1 originates from a much larger 22kb gene. The gene product also differs, with the mRNA for the inducible enzyme being approximately 4.5 kb and that of constitutive enzyme being 2.8 kb. COX-2 mRNA contains long 3' untranslated regions (3' UTR) containing several different

polyadenylation signals and multiple 5'-AUUUA-3' instability sequences that act to mediate rapid degradation of the transcript. These features differentiate the gene for COX-1 into a gene consistent with rapid transcription and mRNA processing for processing a continuously transcribed stable message. COX-1 and COX-2 proteins are encoded by separate genes that diverged well before birds and mammals (Reed *et al,* 1996). The genes for COX-1 and COX-2 are located on separate chromosomes, with COX-1 on chromosome 9 and COX-2 on chromosome 1. The promoter region of the COX-2 gene contains a TATA sequence and transcription factor response elements which are sensitive to inflammatory mediators accounting for its rapid inducibility. The gene for COX-1 lacks the TATA sequence and immediate early response elements (Table 2).

1.3.3 Physiological and Pathophysiological Functions of COX-1 and COX-2

COX-1 is implicated in many homeostatic physiological functions, whereas COX-2 is implicated in physiological as well as pathological conditions.

i. **Stomach**

In most species, including humans, cytoprotective PGs (prostacyclin, PGI_2) are synthesized in the stomach mainly by COX-1, although small quantity of COX-2 is expressed constitutively (Kargman *et al,* 1996) due to their vasodilating properties, enhancing mucosal blood flow. In the stomach, COX-1 stimulates mucus and bicarbonate secretion. COX-2 levels are raised in gastrointestinal infections (DeWitt *et al,* 1993) and human gastric adenocarcinoma (Lim *et al*, 2000).

Table 2: Comparison of cyclooxygenase enzymes

Property	COX-1	COX-2
Cellular level expression	**Constitutive** in most tissues, in particular, stomach, kidney, monocyte /macrophage, platelet.	**Inducible** in endothelial cells, synoviocyte, chondrocyte, fibroblast, smooth muscle, reproductive tract. **Constitutive** in CNS, kidney, pancreas, testis.
Range of induced gene expression	2-4 fold	10-80 fold
Rate of gene activation	24 hrs	0.5 -4 hrs
Molecular mass	72 kDa (599 amino acids)	72 kDa (604 amino acids)
Subcellular localization	ER membrane	ER & Nuclear membrane.
Chromosome location	Human chromosome-9q32-33.3	Human chromosome-1q25.2-25.3
Relative size of active site	< COX-2	> COX-1
Size of Gene	22kb, 11 exons	8.3kb, 10 exons
Size of mRNA	2.7kb	4.2kb
Effect of glucocorticoids	Little or None	Inhibits expression
Endogenous inducers		Pro-inflammatory cytokines, growth factors, Oncogene expression
Endogenous inhibitors	. . .	Glucocorticosteroids, IL-1
Amino acid homology	60% identity to COX-2 75% similar to COX-2	60% identity to COX-1 75% similar to COX-1
Substrate utilization	AA and dihomo-γ-linolenate	AA and dihomo-γ-linolenate, eicosapentaenoic acid, γ-linolenic acid, α-linolenic acid and linoleic acid
Patho/physiological activity	Cyto-protective	Inflammatory

ii. Kidney

COX-1, expressed in the vasculature, glomeruli and collecting ducts of the kidney, appears to be important in producing the vasodilating PGs, which maintain renal plasma flow and glomerular filtration rate during conditions of systemic vasoconstriction. Though COX-1 is the primary isozyme, COX-2 expression is enhanced in macula densa of the juxtaglomerular apparatus (Harris *et al,* 1994) and medullary interstitial cells. COX-2 "knocked out" mice show severe disruption of kidney development (Dinchuk *et al,* 1995; Morham *et al,* 1995; Langenbach *et al,* 1995).

iii. Platelets

In platelets, the only isoform detectable is COX-1. Loss of platelet aggregation is not only a well established side effect of NSAIDs treatment (due to loss of Thromboxane formation), but also therapeutic aim of half an aspirin a day prophylaxis against thromboembolic disease.

iv. Central Nervous System (CNS)

COX-1 is distributed in neurons throughout the brain, but COX-2 isoform has also been observed under basal conditions in rat brain in the hippocampus, pyramidal cells and amygdala (Breder *et al,* 1996; DuBois *et al,* 1998). COX-2 is upregulated by normal and abnormal nerve activity (Yamagata *et al,* 1993) and neurons expressing COX-2 show evidence towards apoptosis (Tocco *et al,* 1997). COX-2 is expressed constitutively in neurons of Alzheimer's patients. Epidemiological studies have demonstrated that individuals taking selective COX-2 inhibitors have a

lower risk of Alzheimer's disease without damaging the stomach mucosa (Stewart *et al,* 1997).

v. Inflammation, Arthritis & Pain

COX-2 induction has been observed in both human osteoarthritis-affected cartilage (Amin *et al,* 1997) as well as in synovial tissue taken from patients afflicted with rheumatoid arthritis (Kang *et al,* 1996; Siegle *et al,* 1998). In human polymorphonucleocytes exposed to several cytokines, increased expression of COX-2 and PGE_2 levels. There was no increased expression in human polymorphonucleocytes not exposed to these cytokines (Maloney *et al,* 1998). Increased PGs are produced by increased expression of COX-2 in cartilage cells (Anderson *et al,* 1996; Amin *et al,* 1997). These may contribute to joint pain and joint destruction in both osteoarthritis and rheumatoid arthritis. COX-2 expression is increased in spinal cord neurons following peripheral inflammation (Goppelt-Struebe and Beiche, 1997).

vi. Bone

PGs are potent regulators of skeletal metabolism and have variable effects in bone tissue. They are thought to be involved in the coupled intracellular crosstalk between osteoblasts and osteoclasts, which determines metabolic bone turnover in diseases such as osteoporosis. PGs affect the balance between the bone loss and formation and COX-2 induction in osteoblasts seems to be essential for bone remodeling (Pilbeam *et al,* 1997).

vii. Cancer

Several studies have demonstrated elevated expression of COX-2 and not COX-1, in different types of human cancer, suggesting that the presence of COX-2 correlates with cancer development (Eberhart *et al,* 1994; Ristimaki *et al,* 1997; Zimmermann ef *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). The importance of PGs in tumorogenesis is evidenced by the data demonstrating the ability of the NSAIDs to inhibit growth and metastasis of tumors *in vivo* (Snyderman *et al,* 1995) and most notably, for colon cancer (Rao *et al,* 1991; Sheng *et al,* 1997). Human gastric (Ritsimiki *et al,* 1996) and breast (Brown and Lippman, 2000) tumors express higher levels of COX-2 than surrounding normal tissue. Several population based studies have detected a 40-50% decrease in relative risk for colorectal cancer in persons who regularly use NSAIDs (Thun *et al,* 1993; Smalley and DuBois, 1997). The cancer preventive actions of COX-2 inhibitors may involve inhibition of angiogenesis (Tsujii *et al,* 1998) or modulation of mitosis and apoptosis (Watson, 1998).

viii. Apoptosis

Apoptosis, programmed cell death, is a natural event in many cells. Cell culture models have shown that COX-2 expression contributes significantly to the tumorigenic potential of epithelial cells by increasing the adhesion to extra cellular matrix and making them resistant to apoptosis (Tsujii and DuBois, 1995). PGE_2 potently induces apoptosis in T and B

cells (Mastino *et al,* 1992; Brown *et al,* 1992) and is able to anatogonize activation of inducers of apoptosis in T cells.

1.4 NSAIDs Overview and Cyclooxygenase Inhibitors

Dating as far back as ancient Greece and Egypt, salicylic acid and salicylates extracted from myrtle leaves, willow tree bark and other plants were used as therapeutic agents to treat fever, pain and swelling. In 1860, salicylic acid was chemically synthesized in Germany and used as external antiseptic and antipyretic and in the treatment for "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 became known as " Aspirin like drugs". And because these medications were clearly distinct from steroidal glucocorticoids, they were categorized as nonsteroidal anti-inflammatory drugs (NSAIDs). The term NSAIDs was first applied to phenylbutazone after its introduction into clinical practice in 1949. NSAIDs are a heterogenous group of chemically unrelated compounds with similar therapeutic effects i.e. anti-inflammatory, anti-pyretic, analgesic and anti-thrombotic properties. They also share side effects, such as gastrointestinal erosions, decreased renal function, ulceration and inhibit platelet function. Still NSAIDs constitute one of the largest groups of Pharmaceuticals, with a world market in excess of $13 billion per annum (Brooks, 2000). A great deal of research in this area has followed Vane's critical discovery in 1971 that the mechanism of action of aspirin-like drugs or NSAIDs was mediated through their inhibition of prostaglandin biosynthesis *via* Cyclooxygenase enzyme. The cyclooxygenase enzyme was first isolated in 1976. However, in 1991 it was discovered that there are actually two distinct cyclooxygenases in

mammals: COX-1 is a "constitutive" enzyme, whereas COX-2 is "inducible" and short lived. COX-1 is generally responsible for production of PGs and thromboxane synthesis in many different cell types (except red cells) in their basal states including the gastro-intestinal tract, liver, kidney and blood platelets, suggesting that low levels of prostanoids are important in serving critical physiological (homeostatic) functions (So called "housekeeping enzyme") (Vane, 1994). PGs generated in the gastro-intestinal tract are cytoprotective for the gastric mucosa, PGs in liver and kidney regulate regional blood flow and generation of thromboxane in platelets increase the tendency for platelets to clump and form a clot (thrombus). COX-2 plays a major role in PGs biosynthesis in inflammatory cells (i.e. monocytes/macrophages), in Alzheimer's and in cancer. PGs synthesis in these sites contributes to the development of inflammation and hyperplasia. Currently available NSAIDs in the market are known to inhibit these two isoforms to different extents and this feature accounts for their shared therapeutic properties and side effects (Vane ef a/, 1998). Accumulating evidence indicates that the unwanted side effects, especially gastroduodenal erosion and disruption of normal renal function, appears to be caused by the inhibition of COX-1, whereas the therapeutic, anti-inflammatory effects result from the inhibition of COX-2. The different tissue distribution of the COX enzymes has provided a rational for the development of COX-2 selective inhibitors as nonulcerogenic, antipyretic, anti-inflammatory and analgesic agents without the gastrointestinal ulceration and hematological side effects that plague all currently available

NSAIDs (Xie *et al*, 1992; DeWitt *et al*, 1993; Engelhardt *et al,* 1996; Seibert *et al,* 1997; Needleman and Isakson, 1997).

Recent developments on the crystal structure and mechanism of action of COX-2 have led to the use of molecular modeling approaches to develop COX-2 specific inhibitors. Based on biochemical, pharmacological and clinical studies, the following four categories of COX inhibitors have been proposed (Lazer *et al*, 1997; Lipsky *et al*, 1998).

i. COX-1 Selective Inhibitors

These agents target COX-1 activity without any measurable effect on COX-2 activity. So far, low dose aspirin is the only drug in this category (Patrono, 1994).

ii. COX- Nonselective Inhibitors

These agents demonstrate no meaningful biological or clinical differences in the inhibition of COX-1 versus COX-2 activity. Such compounds include commonly used NSAIDs such as ibuprofen, naproxen sodium and indomethacin.

iii. COX-2 Preferential Inhibitors

NSAIDs such as etodolac (Lodine®) and nabumetone (Relafen®) demonstrate some COX-2 specificity at lower doses and are more COX-1 specific at higher anti-inflammatory doses. As a result, these NSAIDs have been referred to as COX-2 "preferential". In biochemical assays, these agents usually demonstrate a 2- to 10- fold difference in the concentration required to inhibit recombinant COX-2 versus COX-1 *in vitro.* Meloxicam (Noble and Balfour, 1996), etodolac, nabumetone (Cipollone *et al,* 1995) and nimesulide (Davis and Brogden, 1994) are the only agents that clearly

fall into this category. Nabumetone appears to be a more effective inhibitor in some experimental systems of COX-2 than COX-1 (Roth, 1996). Etodolac also inhibits COX-2 isoform more than COX-1 (10 to 1 ratio) (Patrignani *et al* 1994; Glaser *et* a/, 1995)

iv. COX-2 Selective Inhibitors

Drugs that demonstrate consistent COX-2 specific inhibition but have no effect on COX-1 throughout their dose ranges are called COX-2 "selective" (Dewitt ef *al,* 1993; Simon and Smith, 1998). Biochemical assays usually reveal more than a 100-fold difference in the concentration that inhibits recombinant COX-2 as compared with COX-1, depending on the test system. Other highly selective NSAIDs that have greater than 1000 fold selectivity for COX-2 over COX-1, such as valdecoxib, parecoxib and deracoxib, are in various stages of clinical development. Structure activity studies indicate that a cis-stilbene moiety containing a 4-methylsulfonyl or sulfonamide substitutuent in one of the pendent phenyl rings is required for COX-2 specificity (Talley *et al,* 1999). Celecoxib (Celebrex) and Rofecoxib (Vioxx) are two such agents and are now clinically available in many countries. COX-2 inhibitors delayed the healing of gastric ulcers in mice (Mizuno *et al,* 1997) and rats (Schmassmann *et al,* 1998) and inhibited angiogenesis, epithelial cell proliferation and maturation of the granulation tissue in chronic gastric ulcers (Schmassmann *et al*, 1998). Specific COX-2 inhibitors may also have a role in the management of such widely differing conditions as chronic pain associated with conditions other than musculoskeletal disorders, Alzheimer's disease and colorectal cancer (DuBois *et al*, 1998).

Two selective COX-2 inhibitors, Celecoxib (Celebrex ®) and Rofecoxib (Vioxx ®), have received approval from the Food and Drug Administration (FDA) in the United States. Table 3 Summarizes pharmacokinetics of the COX-2 specific inhibitors.

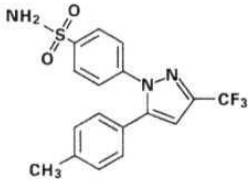
Celecoxib

Celecoxib (Celebrex ®, also known as SC-58635) (4-[5-(4-methylphenyl)-3-trifluoromethyl] 1 H-pyrazoyl-1-yl) benzene sulfonamide) is a 1,5-diaryl substituted pyrazole (Fig.9) (Penning *etal,* 1997). 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 use in U.S, for relief of signs and symptoms of rheumatoid arthritis and osteoarthritis, but not for analgesia in adults. In addition to the analgesic, antipyretic and anti-inflammatory activity, it has chemoprotective properties against colon cancer. Peak plasma concentrations occur approximately three hours after an oral dose and, when taken with a high-fat meal, peak concentration were delayed for about 1-2 h, with an increase in total absorption of 10-20%. Celecoxib is extensively protein bound primarily to plasma albumin (>97%). The apparent volume of distribution (V/F), determined after oral administration is approximately 400 l in humans (5.7 l/kg) (Karim *et al*, 1997) which is larger than expected V/F when compared to other NSAIDs and probably relates to the lipophilic nature of celecoxib. Celecoxib is hepatically metabolized by the cytochrome P450 enzyme, CYP2C9, into three inactive metabolites and eliminated predominantly by the liver with little (<3%) unchanged drug recovered in the feces and urine. Celecoxib inhibits CYP2C9 enzymes and thus may cause elevation of plasma concentrations

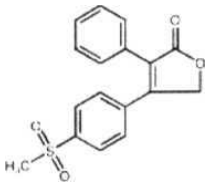
Table 3: Pharmacokinetics of COX-2 specific inhibitors

Factor	Celecoxib (Celebrex ®)	Rofecoxib (Vioxx®)
Composition	1,5-diaryl pyrazole-based compound	Methylsuphonylphenyl derivative
COX-2 selectivity	375 fold	> 800 fold
Tmax	3h	2-3 h
Half-life	12 h	17h
Distribution	97% plasma protein-bound; volume of distribution 350-450 l.	85% plasma protein bound; not dialyzable; volume of distribution 80-100 l (dose dependent); cross placenta and blood brain barrier.
Metabolism	Cytochrome P-450 2C9; inactivate metabolites	Reduction by cytosolic enzymes in liver; inactivate metabolites.
Route of administration	Oral	Oral
Excretion	Primarily by hepatic metabolism; excreted in urine and stool.	Excreted in urine (72%) and stool (14%) excreted in breast milk.
Therapeutic index		>300
Approved by FDA for	rheumatoid arthritis (RA) and osteoarthritis (OA)	Osteoarthritis (OA) and acute pain and primary dysmenorrhea.
Recommended dose		
osteoarthritis (OA)	200 mg/ day	12.5 mg/day
rheumatoid arthritis (RA)	100-200 mg twice/day	Not approved
acute pain and primary dysmenorrhea	Not approved	50 mg/day, orally

Fig. 9: Chemical structures of celecoxib and rofecoxib



Celecoxib



Rofecoxib

of celecoxib metabolized by this isoenzyme, such as some β-blockers, antidepressants and antipsychotics. Celecoxib is well tolerated, with an adverse effect profile similar to placebo. The most commonly reported adverse effects include headache, diarrhea, rhinitis, nausea, sinusitis, dyspepsia and abdominal pain. Celecoxib is reported to be approximately 375 times more selective for COX-2 than COX-1. The recommended dosage of celecoxib for treatment of osteoarthritis is 200 mg once daily or 100 mg twice daily. The dosage for rheumatoid arthritis is higher, 100-200 mg daily up to twice daily. Higher than recommended doses of celecoxib (600 mg bid for 7 days) have no effect on platelet aggregation and bleeding time. A single dose of 800 mg of celecoxib did not inhibit COX-1 dependent platelet aggregation.

Rofecoxib

Rofecoxib (Vioxx ®, also known as MK-0966) (4-[4-(methylsulfonyl) phenyl]-3-phenyl-2 [5H] -furanone) is structurally similar to celecoxib (Fig.9). Rofecoxib was approved for the use in osteoarthritis and for acute pain in adults and treatment of primary dysmenorrhea. Rofecoxib does not have approval for rheumatoid arthritis treatment. Treatment with rofecoxib was well tolerated and similar to placebo. The most frequent adverse events are upper respiratory infection, diarrhea, nausea and headache. Rofecoxib is known to cross the placenta and blood-brain barrier in rats and extensively protein bound in plasma primarily to albumin (~ 87%) and has an apparent volume of distribution of 90 l in humans (1.3 l/kg). The larger than expected V/F when compared to other traditional NSAIDs may relate to the lipophilic nature of rofecoxib (Scott and Lamb, 1999). The

hepatic cytochrome P450 plays a minor role in the metabolism of rofecoxib, which is mediated primarily through reduction of cytosolic enzymes in the liver, with less than 1% excreted unchanged in urine (Scott and Lamb, 1999). Rofecoxib does not inhibit CP450 (CYP2C9) enzyme system and has fewer potential metabolic interactions. Rofecoxib has a longer half-life than celecoxib and is suitable for daily-once dosage. Rofecoxib has approximately an 800 fold selectivity for COX-2 in human enzyme systems. The recommended starting dosage of rofecoxib for treatment of osteoarthritis is 12.5 mg daily up to maximum dosage of 25 mg daily. For management of acute pain or treatment of primary dysmenorrhea, the recommended starting dosage is 50 mg daily for a maximum of five days.

1.4.1 NSAIDs and Apoptosis

Two distinct patterns of cell death, named apoptosis and necrosis, have been identified based on the morphology of dying cells (Wyllie *et al*, 1980) (Table 4). For decades it has been recognized that growth and development, as well as the maintenance of appropriate cell numbers in adult multicellular organisms, is dependent not only on proper cell division but also on a carefully regulated programme of cell death (apoptosis). The apoptotic pathway can be triggered by extracellular agents, pathological processes and also occurs during normal development and tissue remodeling and not all cells necessarily will die in response to the same stimulus. Apoptosis differs from necrosis in that the apoptotic cell kills itself in a manner that does not harm neighboring cells nor elicits an inflammatory response. Activation of apoptosis in tumors has thus been a target for some chemotherapies. Apoptosis probably contributes to many

Table 4. Difference between apoptosis and necrosis

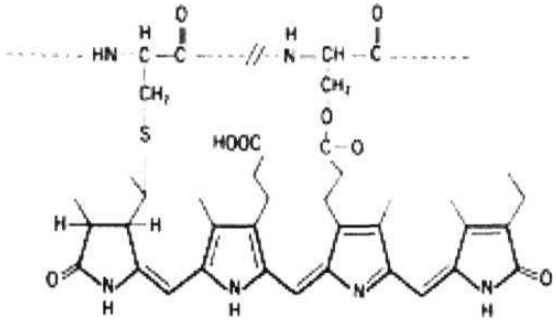
Apoptosis	Necrosis
Morphological features	
Membrane blebbing, but no loss of integrity Aqqregation of chromatin at the nuclear membrane	Loss of membrane integrity
Begins with shrinking of cytoplasm and condensation of nucleus	Begins with swelling of cytoplasm and mitochondria
Ends with fragmentation of cell into smaller bodies	Ends with total cell lysis
Formation of membrane bound vesicles (apoptotic bodies)	No vesicle formation, complete lysis
Mitochondria become leaky due to pore formation involving proteins of the Bcl-2 family	Disintegration (swelling) of organelles
Biochemical features	
Tightly regulated process involving activation and enzymatic steps.	Loss of regulation of ion homeostasis
Energy (ATP) dependent (active process, does not occur at 4°C)	No energy requirement (passive process, also occurs at 4°C)
Non-random mono- and oligonucleosomal length fragmentation of DNA (ladder pattern after agarose gel electrophoresis)	Random digestion of DNA (smear of DNA after agarose gel electrophoresis)
Prelytic DNA fragmentation	Postlytic **DNA** fragmentation
Physiological significance	
Affects individual cells	Affects group of contiguous cells
Induced by physiological stimuli	Evoked by non-physiological disturbances
Phagocytosis by neighboring cells or macrophages	Phagocytosis by macrophages
No inflammatory response	Significant inflammatory response

chronic degenerative processes, including cancer, Alzheimer's disease and Parkinson's disease and heart failure. Cells undergoing apoptosis show characteristic morphological and biochemical features including cell shrinkage, chromatin condensation and formation of apoptotic bodies. Although NSAIDs can both inhibit PG synthesis and induce apoptosis, there is recent evidence that the apoptosis effects is likely not due to COX inhibition.

1.5 C-Phycocyanin

Phycocyanin, allophycocyanin, phycoerythrin and phycoerythrocyanin are the principal phycobiliproteins. Phycobiliproteins are water soluble bile pigment- apoprotein complexes that constitute the major light harvesting antennae for oxygenic photosynthesis in cyanobacteria and red algae (Glazer, 1989). It consists of a- and p- subunit polypeptides to which one or more linear tetrapyrrole chromophores are covalently attached (Fig. 10). Phycocyanobilin is the chromophore of allophycocyanin and phycocyanin. In both proteins the chromophores are attached by cysteinyl thioether linkages at α-84 and β-84 positions; in phycocyanin a second Phycocyanobilin is found attached to cysteine at β-155 in a loop not found in allophycocyanin. Phycobiliproteins are stable at 2-5°C as ammonium sulfate precipitates. Purified biliproteins may disassociate into subunits under acidic or basic conditions, but are relatively stable at room temperature at neutral pH. Phycobiliproteins have been used in a variety of immunological assays and as fluorescent labels for cell sorting (Jung and Dailey, 1989). In addition, because of the high molar absorbtivity of these proteins at visible wavelength, they are

Fig. 10: Structure of C-Phycocyanin



convenient markers in such applications as gel electrophoresis, isoelectric focussing and gel exclusion chromatography.

C-phycocyanin is a non-toxic phycobiliprotein pigment isolated from the natural source namely *Spirulina platensis* (now it is called as *Arthrospira platensis),* which is an unicellular filamentous blue green algae. The cyanobacterium *Spirulina platensis* contains only two phycobiliproteins, allophycocyanin and C-phycocyanin (Boussiba and Richmond, 1979). C-phycocyanin is the major light harvesting pigment protein present in the antenna rods of S. *platensis,* whereas allophycocyanin is a minor component present only at the core. *Spirulina,* is a traditional food of some Mexican African and Asian peoples, who consume it as staple food for more than thousand years. Spirulina with 62% protein content and is the world's richest natural source of Vitamin B-12. It is also rich in beta-carotene and other carotenoids, Vitamin E, minerals (e.g., manganese, copper, iron, zinc, selenium), trace minerals (e.g., selenium) and essential fatty acid (e.g., gamma-linolenic acid). Phycocyanin helps preventing most of degenerative organ diseases by increasing general immunity. Spirulina has a soft cell wall made of complex sugars and protein and is different from most other algae (cell made up of cellulose) in that it is easily digested. C-phycocyanin has the following remarkable properties:

- hepatoprotective (Vadiraja *et al*, 1998)
- antioxidant (Romay *et al,* 1998 a; Romay and Gonzalez, 2000),
- cytoprotective (Rimbau ef *al,* 2001)
- radical scavenging properties (Vadiraja and Madyastha, 2000),
- antiarthritic (Remirez *et al,* 1999)

- anti-inflammatory activity in various *in vitro* and *in vivo* experimental models (Romay *et al*, 1998 a,b; Gonzalez ef *al,* 1999)
- treatment of oxidative stress-induced neuronal injury in neurodegerative diseases, such as Alzheimer's and Parkinson's (Rimbau *et al,* 1999; 2001).
- protective effect of renal failure (Fukino ef *al,* 1990)
- prevention of experimental oral and skin cancers. Once administered phycocyanin is selectively taken up by cancer cells and upon subsequent irradiation destruction of cancer cells occur (Morcos and Henry, 1992).
- enhances cytotoxic effect of laser therapy (Morcos *et al*,1988)
- used as a natural pigment in the food, drug and cosmetics industries to replace the currently used synthetic pigments, which are carcinogenic. It is used in anti-wrinkle cream, anti-pimple lotions and facemasks.

1.6 Purpose of the Study

In view of the involvement of lipoxygenase and cyclooxygenases in the mediation of allergy, asthma, inflammatory disorders, cancer and Alzheimer's disease, these enzymes have become natural targets for the development of drugs against several disorders, starting from a skinned knee to cancer. As pointed above C-phycocyanin has many therapeutic roles including anti-inflammatory, antiarthritic, anticancer and against other degenerative disorders. However, little is known about the mechanism of action of C-phycocyanin. Since arachidonic acid cascade is involved in the mediation of several degenerative disorders and C-phycocyanin is known to play therapeutic role, in the present study an attempt is made to

understand the role of C-phycocyanin in the regulation of arachidonic acid metabolism. Also the involvement of C-phycocyanin in cell growth and multiplication was undertaken on mouse macrophage cell lines, RAW 264.7. The molecular mechanism involved in C-phycocyanin induced cell death was elucidated.

Methodology

2.1 Materials

The mouse monocyte/macrophage cell line, RAW 264.7, was provided by National Centre for Cell Science (NCCS), Pune, India.

Celecoxib and Rofecoxib were generous gift from Unichem Laboratories Ltd., Mumbai, India. C-phycocyanin from *Spirulina platensis* was kindly provided by Prof. K.M. Madyastha, Indian Institute of Science, Bangalore, India.

RPMI-1640 medium, Fetal Bovine Serum (FBS), Phenylmethylsulfonyl fluoride (PMSF), Leupeptin, Aprotinin, Pepstatin A, Trypsin, Tween-20, Triton X-100, Sodium Chloride, Lipopolysaccharide (*Escherichia coli* 026: B6), Propidium Iodide, Ethidium Bromide, Trypan Blue, 3-[4,5-Dimethylthiazol-2-yl]- 2,5-diphenyltetrazolium bromide (MTT), Hematin, N, N, N', N'-tetramethyl-p-phenylene diamine, Arachidonic Acid, Ponceau S, Igepal CA-630, Boric Acid, Sodium Orthovanadate, Sodium Bicarbonate, Diethyldithiocarbomate, phosphotidyl Choline, EDTA and Calcium Chloride were purchased from Sigma Chemical Company (St. Louis, USA).

Penicillin, Streptomycin, gentamycin, Phosphate buffered saline, LB medium and Skim milk powder were purchased from HiMedia Laboratories Limited.

Ribonuclease A (DNase free), 100 bp DNA ladder, Protein A-peroxidase conjugate, Goat anti rabbit IgG- alkaline phosphatase conjugate, Rabbit anti-goat IgG- peroxidase conjugate, TMB/H_2O_2 and protein molecular weight marker were purchased from Bangalore Genei Pvt. Ltd.

Acrylamide, N, N' -Methylene-bis-acrylamide, Sodium Dodecyl Sulfate, Ammonium Persulfate, β-Mercaptoethanol and bromophenol blue were purchased from Bio-Rad Laboratories (Richmond, USA).

PGE_2 EIA kit was purchased from Assay Designs, Inc. Ann Arbor, USA. 25 cm^2 flask, 75 cm^2 flask, 6 well and 96 well multidishes and serological pipettes were purchased from Nalge Nunc International.

Hybond ECL Nitrocellulose membrane, Adenosine 5'- Triphosphate (ATP) and Isopropyl-β-D-thio galactopyranoside (IPTG) were purchased from Amersham Life Science. Agarose, Grace's Insect culture medium were purchased from Gibco BRL.

Affinity purified Goat Anti-PARP, affinity purified rabbit anti-human Bcl-2 and apoTACS-Basic *In Situ* apoptosis detection kit purchased from R& D systems Inc, USA. Affinity purified goat polyclonal anti-COX-1, COX-2, iNOS from Santa Cruz Biotechnology, Inc. Apoptotic DNA ladder kit purchased from Roche Molecular Biochemicals, USA.

All other chemicals, which have not been mentioned here, were procured from the local companies and were of high quality.

2.2 Methods

2.2.1 Purification of Cyclooxygenase-1

Ram seminal vesicles were collected from local slaughterhouse and stored at -80°C until used. Few hours before experimentation, the ram seminal vesicles were removed from the freezer and stored at 4°C for thawing. The tissue was weighed, minced into small pieces and homogenized in the buffer containing 100 mM Tris-HCl pH 8.0, 5 mM EDTA, 5 mM DDC. The homogenization was done in a blender initially and

later in a Potter-Elvehjam homogenizer. The homogenate was filtered through two layers of cheesecloth to remove fat and waste material. The filtrate was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was filtered through glass wool or cheesecloth to remove debris and the supernatant was analyzed for the presence of protein and its activity. The supernatant obtained above was again centrifuged at 33,000 rpm for 1 h 10 min at 4°C to obtain microsomal pellet. The microsomal pellet obtained above was suspended in minimum volume of solubilization buffer, containing 100 mM Tris-HCI buffer pH 8.0, 5 mM EDTA, 5 mM DDC and 1% Triton X-100, with slow stirring at 4°C for 30 min. The sample was centrifuged again at 42,000 rpm for 1 h 10 min at 4°C. The supernatant obtained was dialyzed against the dialyzing buffer (25 mM Tris-HCI pH 8.0, 1 mM EDTA and 1mM GSH) with four changes, with gradual decrease in the concentration of detergent from 1% to 0% at the end. The dialysate was later centrifuged at 10,000 rpm for 20 min at 4°C and the resulting supernatant was used as the enzyme source for assays and for enzyme purification. The dialysate obtained above was loaded on to DE-52 anion exchange column, which was equilibrated with 25 mM Tris-HCI pH 8.0 buffer containing 1 mM EDTA. The column was washed with wash buffer (25 mM Tris-HCI pH 8.0 and 1 mM EDTA) and 3 ml fractions were collected. Each fraction was analyzed for the presence of protein and enzyme activity. The active fractions were pooled, concentrated and used for further studies as the enzyme source.

2.2.2 Expression and Extraction of Recombinant Human Cyclooxygenase-2

Spodoptera frugiperda (*Sf*9) cells were maintained at 28°C in Grace's Insect culture medium supplemented with 10% Fetal bovine serum, 3.3 g/l yeastolate, 3.3 g/l lactalbumin hydrolysate and 0.25 mg/ ml amphotercin B, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells at 60% confluency, were infected with baculovirus containing human COX-2 (h COX-2 cDNA sequence cloned into BamH1 site of the baculovirus expression vector pVL 941). After 72 h of infection, the cells were collected by centrifugation at 2000 rpm for 5 min at 4°C. The pellet was suspended in minimum volume of Tris-HCl buffer (50 mM, pH 7.2) containing 5 mM EDTA, 300 mM Sucrose, 5 mM Diethyldithiocarbomate, 1 μg/ml pepstatin and 1 mM phenol and sonicated for 3 min. The cell lysate was subjected to centrifugation (100,000 g for 1 hr) at 4°C and the microsomal pellet obtained was suspended in Tris-HCl buffer (25 mM, pH 7.2) containing 0.5% glycerol, 0.8%Tween 20 and 1 mM Phenol. This microsomal fraction was used for further studies.

2.2.3 Expression and Extraction of Recombinant Human 5-Lipoxygenase

E. coli containing human 5-LOX was grown in LB medium containing 100 μg/ml ampicillin under shaking conditions (200 rpm) at 37°C. IPTG (I mM) was added after 6 h and the culture was continued overnight at 30°C. Cells were harvested by centrifugation at 4000 rpm for 10 min at 4°C and suspended in 50 mM KH_2PO_4 pH 7.4 containing 3 mM EDTA, 1 mM PMSF and 0.1% Triton X-100 and kept on ice for 30 min and sonicated. The sample was centrifuged at 10,000 rpm for 30 min at 4°C and the

supernatant was pooled. The supernatant pool was subjected to 15-60% ammonium sulfate fractionation at 4°C. The sample was centrifuged at 10,000 rpm for 30 min at 4°C and the protein pellet was dispersed in minimum amount of 50 mM KH_2PO_4 pH 7.4, dialyzed against same buffer until free of ammonium sulfate. The dialysate sample was applied to DE-52 column previously equilibrated with 50 mM KH_2PO_4 pH 7.4. The protein was eluted with a linear gradient of 0-0.5 M KCl in 50 mM KH_2PO_4 pH 7.4 and 1 ml fractions were collected. The active fractions were pooled, concentrated and used as the enzyme source.

2.2.4 Purification of Rabbit Reticulocyte 15-Lipoxygnease

The 15-LOX enzyme was extracted and purified from rabbit reticulocytes according to the method of Rapoport *et al* (1979) with slight modifications by inducing experimental anemia by either daily bleeding of rabbits for 5 days or daily injection of neutralized 1-acetyl-2-phenylhydrazine solution (11, 8.8, 6.6, 7.7, 8.8 mg/kg body weight for 5 days respectively). The reticulocyte rich blood was collected from the rabbits, starting from 6th day, by ear bleedings, in a tube containing anticoagulant. The cells were washed 3 times with isotonic saline and haemolyzed in twice the volume of distilled water. The haemolysate was cautiously adjusted to pH 6.0 by the addition of 1 N HCl. The stroma was removed by centrifugation at 20,000 X g for 20 min at 4°C. The stroma-free supernatant was subjected to ammonium sulfate fractionation and the precipitate at 55% saturation was dissolved in minimum volume of water and dialyzed against 0.01 M potassium phosphate buffer, pH 7.4. The dialyzed enzyme was later subjected to DEAE cellulose column

chromatography as per the standard protocols. The active fractions obtained were pooled, concentrated and used as the enzyme source.

2.3 C-Phycocyanin

The C-phycocyanin used in this study was purified from *Spirulina platensis* and homogeneous as judged by electrophoresis and absorption spectra. SDS-PAGE showed that the C-phycocyanin yielded two bands with molecular masses of 20 kDA and 19 kDa (Fig. 11 B). In Native PAGE it showed single band of 39 kDa (Fig. 11 A). The purity was measured by the ratio (>4) of absorbance at 620 nm (chromophore) and 280 nm (protein). The pigment has single visible absorbance maximum between 615 and 620 nm (Fig. 12) and a fluorescence emission maximum at around 640 nm.

2.4 *In vitro* Cyclooxygenases **and Lipoxygenases** Assays

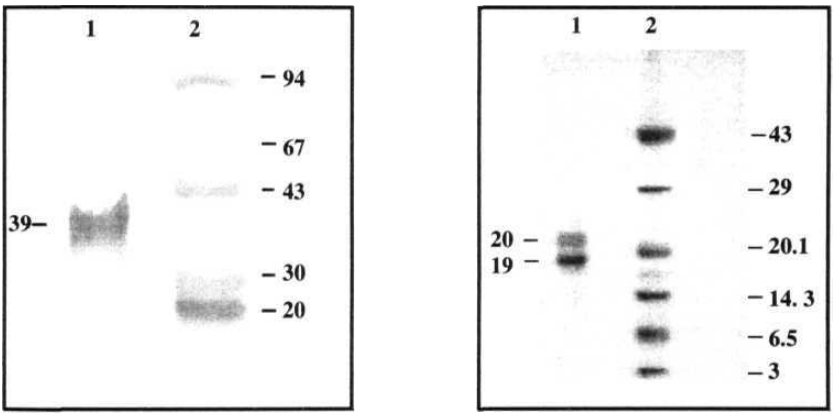
Several methods have been described for the determination of Cyclooxygenase and Lipoxygenase activity. The following methods are employed to assay Cyclooxygenase and Lipoxygenase activities.

i. **Spectrophotometric** Assay

Enzymatic activities of COX-1 and COX-2 were measured according to the method of Copeiand ef *al* (1994) with slight modifications using a chromogenic assay based on the oxidation of N, N, N', N'-tetramethyl-p-phenylene diamine (TMPD) during the reduction of PGG_2 to PGH_2. The assay mixture contained Tris-HCl buffer (100 mM, pH 8.0), hematin (15 μM), EDTA (3 μM) enzyme (100 μg, COX-1 or COX-2) and test compound. The mixture was preincubated at 25°C for 15 min and then the reaction was initiated by the addition of arachidonic acid and TMPD in total volume

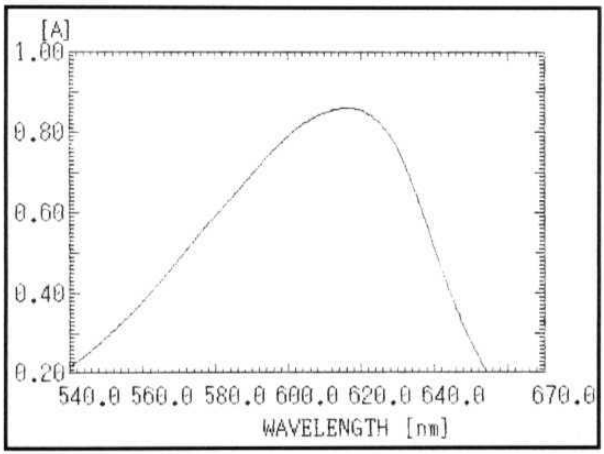
Fig. 11: Native PAGE and SDS-PAGE of C-Phycocyanin

A. Native PAGE **B. SDS-PAGE**



Lane 1: 5 μg protein
Lane 2: Molecular weight markers

Fig. 12: Absorbance spectrum of C-Phycocyanin from *Spirulina platensis*



of 1.0 ml. The enzyme activity was measured by estimating the initial velocity of TMPD oxidation for the first 25 sec of the reaction by following the increase in absorbance at 603 nm. A low rate of nonenzymatic oxidation observed in the absence of COX-1 and COX-2 was subtracted from the experimental value while calculating the percent inhibition. The effect of different concentrations of C-phycocyanin, indomethacin, celecoxib and rofecoxib were examined under the same experimental conditions.

ii. Polarographic Method

Enzyme activity was measured polarographically by using a Clark's oxygen electrode on Gilson Model 5/6 oxygraph. The reaction mixture in a volume of 1.6 ml contained buffer (100 mM Tris-HCl pH 8.0, contained 2 mM EDTA, 5 μM L-tryptophan, 500 μM phenol, 1 μM hematin for Cyclooxygenases, 100 mM KH_2PO_4, pH 7.4 contained 3 mM ATP, 12 mM calcium chloride, 3 mM EDTA and 40 μg/ml phosphatidyl choline for rh-5-lipoxygenase and 100 mM KH_2PO_4, pH 7.4 contained 2% sodium cholate for rabbit reticulocyte 15-lipoxygenase), enzyme and ethanolic substrate arachidonic acid (100 μM) for cyclooxygenase and rh-5-lipoxygenase and linoleic acid (90 μM) for rabbit reticulocyte 15-lipoxygenase. Decrease in the concentration of oxygen was measured till the slope reduced and maximal slope of O_2 curve was used for the calculation of enzyme activity as per the following equation (Berkely and Galliard, 1976):

$$\text{Enzyme activity} = \frac{\text{Reaction vol. in ml} \times \text{O}_2\text{Conc. /ml (0.23 μ moles)} \times \text{Slope/min}}{\text{Volume of enzyme in ml} \times \text{Sensitivity}}$$

One unit of enzyme activity is defined as 1μ mole of oxygen consumed per min. Specific activity is expressed as units/mg protein.

iii. Human Whole Blood Assay for Cyclooxygenase-2 (ex-vivo assay)

The human whole blood assay is the best method, currently available to assess inhibition of COX-2 (Brideau *et al*, 1996). Human whole blood was collected from medial vein of a healthy volunteer into a sterile tube containing Heparin (10 III/ml), who has no inflammatory conditions and has not taken any NSAIDs for at least two days prior to blood collection. The blood in clean glass tubes (0.5 ml) was incubated with Lipopolysaccharide (LPS) from *Escherichia coli* (100 μg/ml) and different concentrations of celecoxib and C-phycocyanin for 24 h at 37°C. After incubation period, the plasma was collected by centrifugation at 8,000 rpm for 10 min at room temperature and then equal volume of ethanol: water (4:1) and acetic acid (10 μl/ml) were added. The samples were incubated for 15 min at room temperature. The mixture was then centrifuged at 10,000 rpm for 15 min at room temperature. The supernatant was passed through pre-equilibrated (5 ml hexane, 5 ml ethyl acetate containing 1% methanol, 5 ml ethanol and with 5 ml of ultra pure water) C-18 cartridges. The cartridges were washed with 5 ml of ultra pure water followed by 5 ml HPLC grade hexane and the adsorbed PGE_2 was eluted with 5 ml of ethyl acetate containing 1% methanol. The PGE_2 in organic solvent was dried under a stream of nitrogen and later dissolved in EIA buffer supplied with PGE_2 EIA kit. The levels of PGE_2 were measured using EIA kit as per the manufacturer's instructions (Assay Designs, Inc).

iv. Estimation of Prostaglandin E_2 Production in LPS Stimulated RAW 264.7 cells in the presence of C-Phycocyanin by Enzyme Immunoassay

RAW 264.7 cells were cultured in 6 well culture plates. Following an adherence period cells were treated with LPS (1 μg/ml), NMMA (1mM) and 5 to 100 μM concentrations of C-phycocyanin. The incubation was continued for 24, in a humidified chamber at 37°C. At the end of the treatment period, the culture medium was collected to determine the amounts of PGE_2 secreted spontaneously by these cells and stored at -80°C. The quantitative analysis of prostaglandin E_2 released into the medium was assayed by using the PGE_2 immunoassay kit as per manufacturer's instructions (Assay Designs, Inc. Ann Arbor, USA). Detection limit was 39 pg/ml. An antibody to PGE_2 had 70% cross-reactivity to PGE_1, 0.6% cross-reactivity to 6-keto$PGE_{1\alpha}$ and less than <0.1% cross-reactivity to other prostaglandins.

v. HPLC Assay of COX-2 Activity

COX-2 assay on HPLC was performed by using the whole cell lysate from LPS stimulated RAW 264.7 cells in the presence / absence of aspirin by monitoring the formation of 15-HETE (Locompte *et al*, 1994). The protein from total cell lysate (0.8 mg of protein in 1 ml) was diluted 1:1 with reaction buffer (100 mM Tris-HCl, 10 mM $CaCl_2$) and incubated with 25 μM arachidonic acid at 37°C for 30 min. Eicosanoids were extracted from the incubation buffer by acidification to pH 3.5 with HCl and applied to a C18-PrepSep solid phase extraction column pretreated with methanol. The sample was then washed with acidified water, eluted with methanol,

evaporated to dryness, and reconstituted in HPLC solvent. The products were then separated on Shimadzu HPLC system using Waters μ bondapak C_{18} column. The elution was monitored at 235 nm. The solvent system consisted of methanol: water: acetic acid (80:20:0.1 v/v/v) and the flow rate was set at 1 ml/min. Peaks obtained were screened for the presence of conjugated diene spectrum. The product formed was identified basing on retention time and Co-Chromatography with the standard 15-HETE.

2.5 Cell Culture and Treatments

The mouse monocyte/macrophage cell line, RAW 264.7 was maintained in RPMI-1640 medium supplemented with 10% Fetal bovine serum, Sodium bicarbonate (2 g/l), 100 IU/ml penicillin, 100 μg/ml gentamycin and 100 μg/ml streptomycin. The cells were maintained at 37°C in a humidified atmosphere of 95% air, 5% CO_2 and were fed every 3-4 days. Cells were grown to approximately 60% confluency in culture flasks. Cells in the log phase were detached by using trypsin-EDTA (0.1%) and were split at a ratio of 1:2 to 1:6, and were routinely fed 24 h before harvest for experiments. Before each experiment, cells were washed twice with sterile Ca^{2+} and Mg^{2+} free phosphate buffered saline and then were resuspended in sterile PBS at a concentration of 10^6 cells/ml. Cell number and Viability were determined by 0.4% trypan blue using a hemocytometer with an anticipated accuracy of + 10-20%. Cell were incubated for different periods of time at 37°C in the presence of various concentrations of C-phycocyanin, LPS (1μg/ml) and NMMA (1mM). A stock solution of 1 mM of C-phycocyanin was prepared in PBS and diluted in standard growth

medium to a final concentration of 5 μM to 100 μM. RAW 264.7 cells in the maximal range of 20 passages were used for this study.

2.6 Cell Viability Assay

The *in vitro* effects of C-phycocyanin on the growth of C-phycocyanin on RAW 264.7 cells was determined by measuring 3-[4,5-Dimethylthiazol-2-yl]- 2,5-diphenyltetrazolium bromide (MTT) dye absorbance of living cells (Campling *et al*, 1988). RAW 264.7 cells were cultured in flat-bottomed 96 microtitre plate, at initial concentrations of 1 X 10^4 cells/ well. Following an adherence period, the medium was changed and cells were treated with LPS, NMMA and with or without C-phycocyanin (5, 10, 20 and 50 μM) for 0, 24, 48, 72 h at 37^0C. RAW 264.7 cells cultured without C-phycocyanin were used as control. At the end of each time point, 50μl of a sterile solution of MTT (4 mg/ml) in PBS was added to each well and the plates were incubated for an additional 4 h at 37°C, a purple-blue formazan precipitate was visualized in wells containing viable cells. MTT solution in the medium was aspirated off. To achieve solubilization of the formazan crystals formed in viable cells, 100 μl of dimethyl sulfoxide was added to each well. The plates were shaken for 30 min at room temperature and absorbance was read immediately at a wavelength of 570 nm on μ Quant Bio-Tek Instruments, Inc microtiter plate reader.

2.7 Preparation of Cell Lysate

For the determination of cyclooxygenases, inducible nitric oxide synthase, PARP, Bcl-2 and cytochrome *c* in RAW 264.7 macrophages, the extraction of total proteins was performed as per the method of Mitchell *et al,* (1994) with slight modifications. RAW 264.7 cells were cultured in 75

cm^2 flask. After reaching the confluence (60%), cells were treated with LPS, NMMA and C-phycocyanin (20 μM) and were incubated in humidified chamber at 37°C. After incubation, cell monolayers were harvested and washed with PBS and incubated with lysis buffer (Tris-HCl (50 mM, pH 7.5) containing 150 mM NaCl, 1 mM EDTA, 0.5% Igepal CA-630, 1% sodium deoxycholate, 1 mM sodium ortho-vanadate, 50 mM β-glycero phosphate, 50 mM sodium fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A and 1 mM phenylmethylsulfonyl fluoride). The samples were then placed on ice for 1 h and sonicated for 10 s and then centrifuged for 5 min at 14,000 rpm in Remi Cooling Microfuge centrifuge at 4°C to remove the particulate sediment. Cell supernatant was collected and stored for further analysis at -80^0C.

2.8 Protein Estimation

Protein content in the whole cell extract was determined by Bradford method (Bradford. 1976) which was measured at 595 nm (UV-1601 Shimadzu UV-Visible Spectrophotometer). BSA was employed as the standard.

Protein contents in all the chromatographic fractions were determined spectrophotometrically by Warburg and Christian method (1941). The concentration of the protein was calculated using the following formula:

mg protein/ml = [(A 280 X 1.55) - (A 260 X 0.76)] x dilution factor

2.9 SDS-PAGE

SDS-PAGE analysis of proteins was performed by Laemmli method (1970). Whole cell lysates were treated with 4X sample buffer containing

1% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue and 20% glycerol in 0.063 M Tris-HCl pH 6.8 for five min in boiling water bath at 100°C. Samples containing 100 μg of protein from whole-cell extracted of both control and treated cells were subjected to SDS-PAGE analysis using 7.5/10/15% separating gel with 5% stacking gel on 1 mm mini gels. Electrophoresis was carried out at a constant voltage (100 V) in a buffer containing 25 mM Tris-HCl, 192 mM glycine and 0.1% SDS pH 8.3 and was stopped when the tracking dye reached the bottom. Molecular weight marker proteins were also run simultaneously with the test samples.

2.10 Native PAGE

To confirm the native structure, the purified C-phycocyanin was electrophoresed on PAGE under non-denaturing conditions. The acrylamide and bisacrylamide ratio was 29.2: 0.8 and were polymerized in Tris-HCl pH 8.8 buffer to give a final acrylamide concentration of 10% in the gel. Sample buffer had no SDS and β-mercaptoethanol. Samples were directly loaded onto 4% stacking gel and separated at 100 v at 4°C in a buffer containing 25 mM Tris-HCl, 192 mM glycine pH 8.3 and was stopped when the tracking dye reached the bottom. After completion of the run, gel was removed and silver stained. Molecular weight marker proteins were also run simultaneously with the test samples.

2.11 Silver Staining

Silver staining of proteins separated on SDS-PAGE was carried out according to the procedure of Blum *et al* (1987) with slight modifications. The gels were incubated in fixative (50% methanol, 12% acetic acid and 50 μl of 37% formaldehyde/ 100 ml) for 1 h and were then treated with 50%

ethanol (3 X 20 min). This was followed by pre-treatment with sodium thiosulfate (20 mg/ 100 ml) for 1 min. The gels were rinsed with double distilled water (3 X 20 s) and impregnated in silver nitrate (0.5% silver nitrate, 187 µl of 37% formaldehyde) with gentle agitation on a shaker for 30 min. The gels were rinsed with double distilled water and developed with a solution containing 6% sodium carbonate and 50 µl of 37% formaldehyde. Finally, stained gels were thoroughly rinsed with double distilled water and stored in 50% methanol.

2.12 Western Blotting

Western blot analysis was carried out according to the procedure of Towbin *et al* (1979). The total cell lysate separated on SDS-PAGE were transferred on to the nitrocellulose membrane with a constant current of 50 V in a buffer containing 25 mM Tris-HCl, 192 mM glycine and 20% methanol for 6 h. The membrane was subsequently immersed in 0.5% Ponceau S in 1% acetic acid to stain the proteins and to verify that equal amounts of protein were loaded in each lane and transferred efficiently. After electrophoretic transfer of the proteins from the polyacrylamide gel to nitrocellulose membrane, nonspecific binding sites were blocked by incubating the membrane with 5% nonfat dry milk in Tris-buffered saline-Tween (TBST) (25 mM Tris (pH 8.0), 150 mM NaCl and 0.05% Tween 20 and 0.1% sodium azide) for over night at room temperature. After being washed three times in TBST (1X15 min, 2X 5 min), the membrane was probed with a polyclonal anti-goat cyclooxygenase-2, cyclooxygenase-1, nitric oxide synthase-2, PARP antibody, Bcl-2 antibody and cytochrome *c* diluted 1: 2000 (1:500 for cytochrome c) in 2% nonfat dry milk in TBST for

overnight at 4°C. The membrane was then washed three times with TBST (1X15 min, 2X 5 min). Each membrane was then incubated with the corresponding secondary antibody: a Protein A- peroxidase conjugate, anti-mouse- peroxidase conjugate, anti-goat IgG-peroxidase conjugated antibody diluted 1: 2000 in 2% nonfat dry milk TBST. The membrane was washed five times with TBST (1X15 min, 4X 5 min) and the proteins were visualized by incubating with colorigenic substrates, TMB/H_2O_2 for 15 min. The reaction was stopped by washing the membrane with PBS after which the membrane was dried and stored.

2.13 Confocal Microscopy Studies

RAW 264.7 cells cultured in 6 well plates were treated with LPS (1 fig/ml) and 20 μM C-phycocyanin for 24 h. The cells were trypsinized by trypsin-EDTA (0.1%), washed three times with phosphate buffer saline and then gradually fixed by adding 1 ml 70% ethanol and fixed overnight. The fixative was removed by centrifugation and washed twice with phosphate buffer saline, these cells were gently resuspended in 1 ml DNA staining reagent (phosphate buffer saline pH 7.4 containing 0.1% Triton X-100, 0.1 mM EDTA, 50 μg/ml of DNase-free RNase A and 50 μg/ml propidium iodide). The cells were then incubated in the dark for over one hour at room temperature. Intact and condensed nuclei visualized using plan-apocromat 63 X/1.4 oil objective under Zesis confocal microscope using Zesis LSM version –150 software (Scan zoom 3.1).

2.14 DNA Extraction and Gel Electrophoresis Analysis of DNA Fragmentation

Soluble (fragmented) DNA was extracted from both floating and attached cells after 48 h treatment with and without LPS and NMMA and C-

phycocyanin (10 and 20 μM), using Apoptotic DNA ladder kit as per the protocol given by the manufacturer (Roche Molecular Biochemicals). Electrophoresis of the resulting DNA was carried out in 2% horizontal agarose gel containing 0.05 mg/ml ethidium bromide. Electrophoresis was run for 3 h at room temperature at 5 V/cm in a buffer (pH 8.0) containing Tris-HCl (89 mM), Boric acid (89 mM) and EDTA (2 mM). The DNA bands (laddering) were viewed and photographed under ultraviolet transillumination.

2.15 *In situ* DNA nick end labeling (TUNEL Staining)

DNA strand breaks were identified by terminal deoxynucleotidyl transferase mediated UTP end labeling (TUNEL) technique using *in situ* apoTACS basic kit and is performed as per the protocol given by the manufacturer (R&D systems Inc, USA). Briefly, the control and C-phycocyanin treated cells for 24 h were fixed in 3.7% formaldehyde and spotted on glass microscopic slide and dried for 2 h on slide warmer at 45°C, rehydrated in 100%, 95%, and then 70% ethanol and PBS, incubated for 15 min at room temperature with 50 μl proteinase K and washed in dH_2O. Cells were incubated with terminal deoxynucleotidyl transferase (TdT) and bromo dexoyribinucleotide triphosphate (B-dNTP) for 1 h, washed with PBS and incubated with anti-BrdU for 1 h. After three washings in 0.05% Tween-20 in PBS, the cells were treated with streptavidin-HRP conjugate and incubated for 30 min. The cells were washed in 0.05% Tween-20 in PBS and treated with chromogen diaminobenzidine that turns the labeled DNA fragments brown. Cells were washed in dH_2O, counter stained with 0.3% methyl green in PBS, again

washed with dH_2O and ethanol and visualized under light microscope (Nikon).

2.16 Measurement of Apoptosis

DNA flow cytometry was performed according to the procedure of Nicoletti *et al* (1991) with slight modifications. RAW 264.7 cells cultured in 6 well plates were treated with LPS, NMMA and C-phycocyanin (5, 10 and 20 μM) for 24 h. The cells were trypsinized by trypsin-EDTA (0.1%), washed three times with phosphate buffer saline and then gradually fixed by adding 1 ml 70% ethanol and fixed overnight. The fixative was removed by centrifugation and washing twice with phosphate buffer saline, these cells were gently resuspended in 1 ml DNA staining reagent (phosphate buffer saline pH 7.4 containing 0.1% Triton X-100, 0.1 mM EDTA, 50 μg/ml of Dnase-free RNase A and 50 μg/ml propidium iodide). The cells were then incubated in the dark for over one hour at room temperature and analyzed within 24 h. Flowcytometric assay was performed using a FACS Vantage (Becton Dickinson). Ten thousand events were evaluated using the Cell Quest Program.

2.17 Isolation of Cytosolic Cytochrome c

After the cells were exposed to 20 μM C-phycocyanin for 4-24h, both floating and attached cells were collected, washed with PBS (pH 7.2) and buffer A containing 0.25M sucrose, 30 mM Tris-HCl (pH 7.9) and 1 mM EDTA and pelleted by brief centrifugation. The pellets were resuspended in buffer B (buffer A plus protease inhibitors 1 mM PMSF, 1 μg/ml leupeptin, 1 fig/ml pepstatin and 1μg/ml aprotinin) and homogenized with a glass dounce homogenizer with a B pestle (40 strokes). After the centrifugation at

14,000 rpm for 30 min, the supematants were collected and used to detect cytosolic cytochrome c release by Western blotting.

Results

3.1 Effect of C-Phycocyanin on Arachidonic Acid Cascade

3.1.1 Measurement of Lipoxygenase/Cyclooxygenase Activity: Polarographic Method

Lipoxygenase/Cyclooxygenase activity was measured by polarographic method using Clark's oxygen electrode. In a typical assay, the reaction was initiated by the addition of the substrate, arachidonic acid for 5-LOX and COX and linoleic acid for 15-LOX and the decrease in oxygen concentration in the reaction mixture was recorded on a graph paper. A typical oxygraphic recording of LOX/COX activity was presented in fig.13. The rate of decrease in oxygen concentration was taken for calculating the enzyme activity.

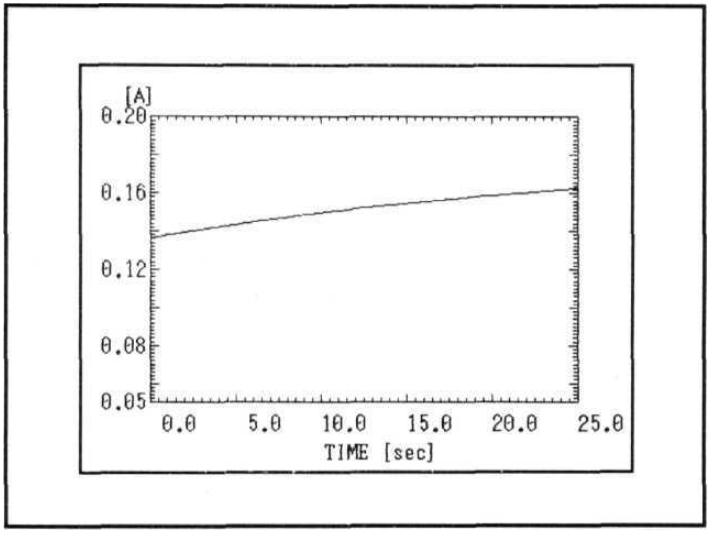
3.1.2 Measurement of COX-1/ COX-2 Activity: Spectrophotometric Method

The spectrophotometric assay of COX-1 and COX-2 is based on the oxidation of N, N, N', N'-tetramethyl-p-phenylene diamine (TMPD) during the reduction of PGG_2 to PGH_2. TMPD oxidation for the first 25 s of the reaction was monitored by following the increase in absorbance at 603 nm. The rate of increase in absorbance was taken for calculating the activity of the enzyme. A typical spectrophotometric recording of COX-1 / COX-2 activity was presented in fig. 14.

3.1.3 Measurement of COX-2 Activity: Whole Blood Assay

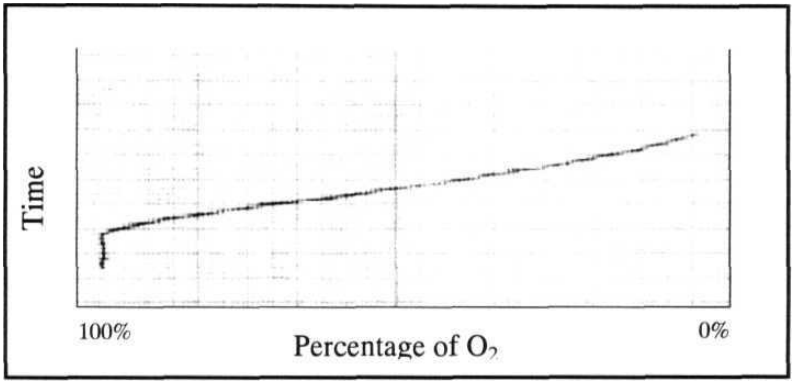
The activity levels of COX-2 were also measured in terms of PGE_2 levels in the plasma of freshly heparinized human whole blood incubated with LPS and different concentrations of C-phycocyanin and celecoxib for 24 h at 37°C was measured by employing enzyme immunoassay kit supplied by Assay Designs Inc.

Fig. 13: *In vitro* peroxidase assay of cyclooxygenase as determined on spectrophotometer



The typical assay mixture contained Tris-HCl buffer (100 mM, pH 8.0), hematin (15 μM), EDTA (3 μM) enzyme (100 μg, COX-1 or COX-2) and test compound in a volume of 1 ml. The reaction was initiated by the addition of arachidonic acid and TMPD and the increase in absorbance at 603 nm was monitored for 25 s. Rate of activity was determined based on the slope.

Fig. 14: Oxygraphic recording of lipoxygenase/ cyclooxygenase activity



The typical reaction mixture contained 100 mM Tris-HCl pH 8.0, 2 mM EDTA, 5 μM L-tryptophan, 500 μM phenol, 1 μM hematin for Cyclooxygenases; 100 mM KH_2PO_4, pH 7.4, 3 mM ATP, 12 mM calcium chloride, 3 mM EDTA and 40 μg/ml phosphatidyi choline for rh-5-lipoxygenase and 100 mM KH_2PO_4, pH 7.4, 2% sodium cholate for rabbit reticuiocyte 15-lipoxygenase in a volume of 1.6 ml. The reaction was initiated by the addition arachidonic acid for COX and 5-LOX and linoleic acid for 15-LOX and the decrease in O_2 concentration was monitored on an Oxygraph. The rate of decrease in O_2 concentration was taken into account for calculating activity.

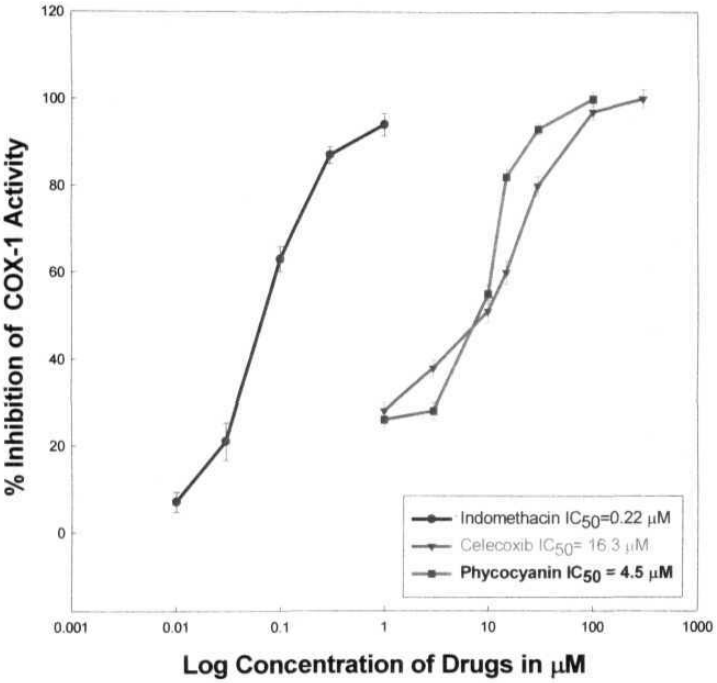
3.1.4 Effect of C-Phycocyanin on Activity of Lipoxygenases: Polarographic Method

The effect of C-phycocyanin on lipoxygenases was studied with recombinant human 5-lipoxygenase and rabbit reticulocyte 15-lipoxygenases using polarographic method. C-phycocyanin showed no inhibition on recombinant 5-lipoxygenase and rabbit reticulocyte 15-lipoxygenase activity levels, even upto 100 μM concentration. Hence no further studies were undertaken on LOX pathway.

3.1.5 Selective Inhibition of Cyclooxygenase-2 by C-Phycocyanin: Spectrophotometric Assay

Inhibition of COX-1 and COX-2 by C-phycocyanin was analyzed in both cell-free and whole blood assay systems. The partially purified enzyme from ram seminal vesicles served as the source of COX-1, while the human recombinant enzyme expressed in baculovirus infected *Sf*9 cells served as the source of COX-2. The inhibition of COX-1 by C-phycocyanin, celecoxib and indomethacin as measured by the spectrophotometric assay is shown in fig.15. From the fig. 15 it is evident that C-phycocyanin and celecoxib inhibited COX-1 activity almost completely at a concentration of 100 μM (~95%). However, much lower concentration of indomethacin (~1 μM) was required to exhibit the same effect. Indomethacin is the most potent inhibitor of COX-1 with IC_{50} value of 0.216 μM followed by C-phycocyanin (IC_{50}, 4.47 μM) and celecoxib (IC_{50}, 16.3 μM). The inhibition of COX-1 by C-phycocyanin was dose dependent, but independent of the period of preincubation with the enzyme. C-phycocyanin at a concentration of 1 μM inhibited COX-1 activity by 26% with or without preincubation of the enzyme with the inhibitor.

Fig. 15: Effect of C-Phycocyanin on cyclooxygenase-1 activity



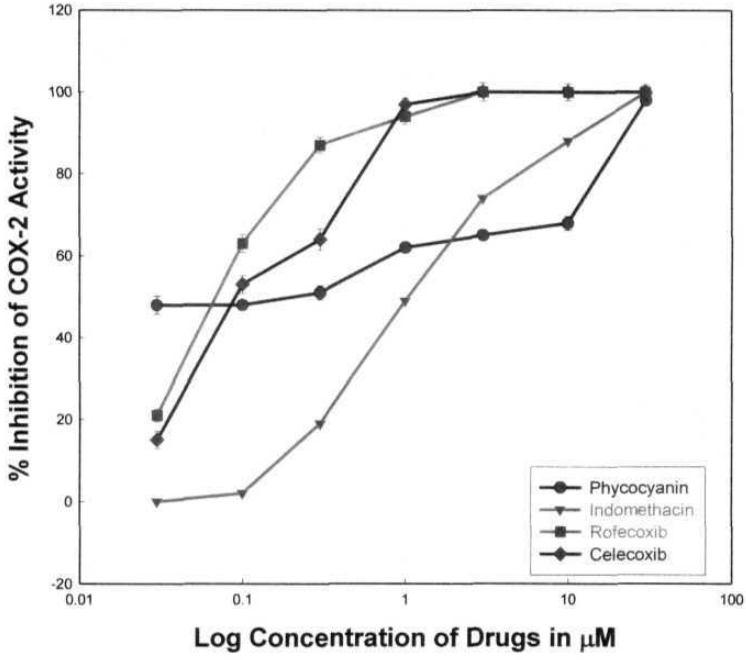
The inhibitory effect of Indomethacin, celecoxib and phycocyanin on ram seminal vesicle COX-1 activity was determined spectrophotometricaily basing on the oxidation of TMPD. A graph is plotted between log. Concentration of inhibitors and % inhibition of COX-1 activity Data represents the mean ± SD values from three separate experiments.

Similarly COX-2 activity was measured, with and without inhibitors, by the spectrophotometric assay and data is presented in fig. 16. The IC_{50} values calculated for different inhibitors are presented in table 5. The data presented revealed that C-phycocyanin is a potent inhibitor of human recombinant COX-2 with an IC_{50} value of 180 nM which is much lower than the values obtained for celecoxib (255 nM) and rofecoxib (401 nM), the known selective inhibitors of COX-2. It was noticed that at 0.03 μM concentration of the inhibitors, highest percentage of inhibition of COX-2 was recorded for phycocyanin (48%) as compared to rofecoxib (21 %) and celecoxib (15%) (Fig. 16). The extent of inhibition of COX-2 by C-phycocyanin was dependent on the preincubation period of the enzyme with inhibitor before the initiation of reaction with arachidonic acid (Fig. 17). Based on the IC_{50} values for COX-1 and COX-2, the relative ratios of IC_{50} COX-2/IC_{50} COX-1 were calculated and the data is shown in table 5. The COX-2/COX-1 ratio obtained for phycocyanin (0.04) is comparable to the values obtained for well-known selective COX-2 inhibitors such as celecoxib (0.015) and rofecoxib (0.0013). However, these values are significantly different from that obtained for indomethacin (7.9), a selective COX-1 inhibitor.

3.1.6 Inhibition of Cyclooxygenase-2 by C-Phycocyanin: Whole Blood Assay

COX-2 activity was also determined in human whole blood stimulated by LPS and measuring the release of prostaglandin E_2 (PGE_2) by EIA kit. C-phycocyanin at a concentration of 1 μM inhibited the release of PGE_2 to a significant extent (nearly 80% inhibition) with an IC_{50} value 80 nM. The data from fig. 18 also indicate that phycocyanin inhibits PGE_2

Fig. 16: Effect of C-Phycocyanin on cyclooxygenase-2 activity



The inhibitory effect of Indomethacin, celecoxib, rofecoxib and phycocyanin on human recombinant COX-2 activity was determined spectrophotometrically basing on the oxidation of TMPD. A graph is plotted between log concentration of inhibitors and % inhibition of COX-2 activity. Data represents the mean ± SD values from three separate experiments.

Fig. 17: Time dependent inhibition of Cyclooxygenase-2 by C-Phycocyanin

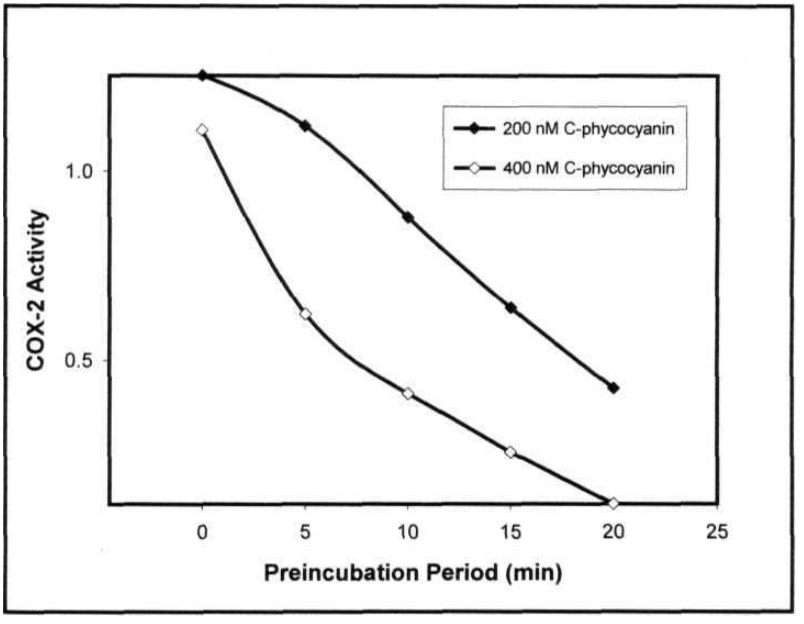
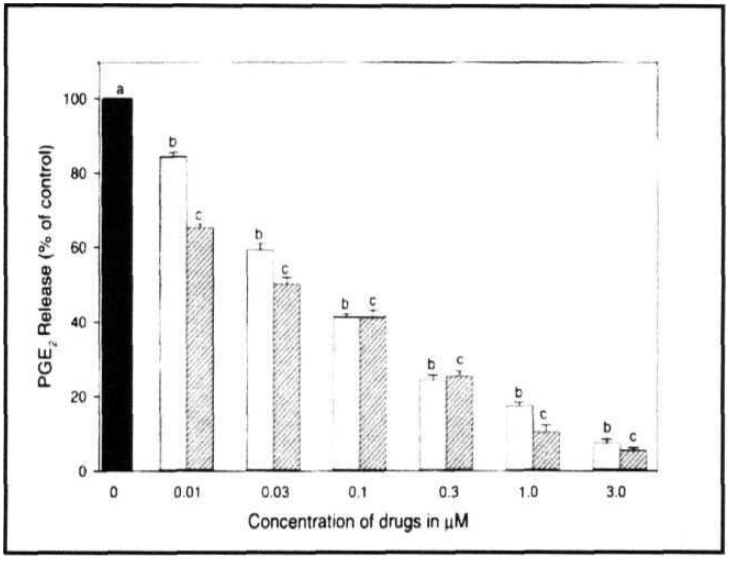


Fig. 18: Effect of C-Phycocyanin on cyclooxygenase-2 activity using Human Whole Blood Assay



Effect of phycocyanin and celecoxib on LPS stimulated PGE_2 synthesis in human whole blood assay. PGE_2 levels determined, without drug were taken as the maximal PGE_2 synthesis (100%), was used as control (a), with celecoxib (b) and with phycocyanin (c). The results are expressed as % of PGE_2 released into plasma. Each experiment was carried out with duplicate determinations and the data are the means of 3 experiments.

synthesis in a dose-dependent manner. The IC_{50} value determined for celecoxib in the whole blood assay was found to be 28 nM.

To understand the role of chromophore of C-phycocyanin in the inhibition of COX-1 and COX-2, these activities were measured in the presence of reduced phycocyanin as well as phycocyanobilin, the chromophore of phycocyanin. It was observed that both of these compounds significantly lost the selectivity towards COX-2 inhibition (Table 5). The IC_{50} value obtained for reduced phycocyanin was 5.6 μM and that of phycocyanobilin was 9.9 μM. However, there was not much change in the inhibition of COX-1 by these two compounds. As a result the relative ratios of IC50 COX-2/IC_{50} COX-1 for reduced phycocyanin and phycocyanobilin increased by several folds when compared to native phycocyanin.

The foregoing studies have clearly demonstrated that C-phycocyanin selectively inhibits COX-2. This may be one of the mechanisms by which C-phycocyanin is mediating its anti-inflammatory and anticancer effects. Many epidemiological studies have revealed the use of Aspirin or COX-2 inhibitors in reducing the risk of colon and other cancers (Thun *et al,* 1991 and 1993; Schreinemachers and Everson, 1994; Giovannucci *et al,* 1995). Evidence also suggests that increase in tumorigenic potential by COX-2 overexpression is associated with resistance to apoptosis (Tsujii and DuBois, 1995). Selective inhibitors of COX-2 have been demonstrated to induce apoptosis in a variety of cancer cells, including those of colon (Hara *et al,* 1997; Sheng *et al*, 1998; Erickson *et al,* 1999), stomach (Sawaoka *et al,* 1998), prostate (Liu *et al*, 1998) and

Table 5: The IC_{50} of C-Phycocyanin, in relation to known Inhibitors, towards COX-1 and COX-2.

Drugs	IC50 (μM)		COX-2/COX-1 ratio
	COX-1	COX-2	
Phycocyanin	4.5	0.18	0.04
Reduced Phycocyanin	5.6	9.7	1.73
Phycocyanobilin	9.9	39.0	3.93
Celecoxib	16.3	0.26	0.015
Rofecoxib	>300*	0.4	< 0.0013
Indomethacin	0.22	1.74	7.9

Maximum dissolved concentration.

IC50 values calculated basing on the activity levels of COX-1 / COX-2 measured in the presence/absence of inhibitors, by spectrophotometric assay.

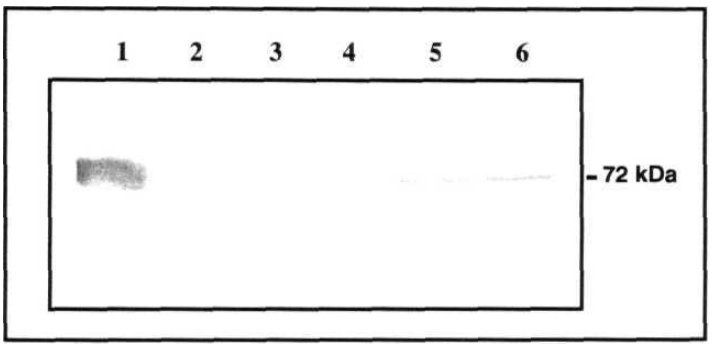
breast (Dempke *et al,* 2001). These observations are consistent with the COX-2 inhibitor being a chemopreventive agent that increases the susceptibility of cancer cell to apoptosis. In order to test this possibility, mouse macrophage cell line, RAW 264.7, expressing COX-2 in response to LPS treatment was selected for further studies on C-phycocyanin (Lee *et al,* 1992; Reddy and Herschman, 1994). This cell line was established from a tumor induced by abelson murine leukemia virus. Along with COX-2, another immediate early gene, the inducible isoform of nitric oxide synthase, is expressed in LPS stimulated RAW 264.7 macrophages. For these experiments, the nitric oxide synthase inhibitor L-NMMA is necessary to prevent endogenous NO generation that is known to initiate apoptotic cell death in macrophages (Sarith *et al,* 1993). Hence in the present study L-NMMA was employed along with LPS for all on mouse macrophage cell line, RAW 264.7.

3.2 Effect of C-Phycocyanin on Mouse Macrophage Cell Line, RAW 264.7

3.2.1 Effect of LPS on COX Expression in Mouse Macrophages

Mouse macrophage cell line, RAW 264.7, cultured in RPMI 1640 medium was incubated with LPS (1 μg) and L-NMMA (1 mM) and the expression of COX-1 and COX-2 was monitored by Western blot analysis. As shown in the fig. 25 A, COX-1 is constitutively expressed in the mouse macrophages. However, no expression of COX-2 was observed in these cells (Fig 23 A lane 2). In response to LPS treatment, RAW 264.7 cells expressed COX-2 protein within 6 h, with progressive increase upto 48 h (Fig. 19). From these results it is clear that mouse macrophage cell line

Fig. 19: Time-dependent changes in the levels of COX-2 in RAW 264.7 cells in response to LPS treatment



To determine the expression of COX-2 in response to LPS in RAW 264.7 cells, the protein from whole cell lysate (100 µg) was separated on 10% SDS-PAGE. After electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with affinity purified goat polyclonal anti-COX-2 antibodies.

Lane 1: COX-2 protein positive control (rh COX-2 expressed in baculovirus infected Sf9 cells)
Lane 2: RAW 264.7 cells without LPS
Lane 3 to 6: RAW cells with LPS for 6, 12, 24 and 48 h respectively

RAW 264.7 expresses COX-2 in response to LPS treatment. Hence mouse macrophages cell line RAW 264.7 was employed to test the effects of C-phycocyanin.

3.2.2 Effect of LPS on iNOS Expression in Mouse Macrophages

Effect of LPS on iNOS expression in mouse macrophage cell line, RAW 264.7 was monitored by Western blot analysis (Fig. 24 A). As shown in the figure no iNOS protein was detected in the cells without exposure to LPS (lane 1; Fig. 24 A). On exposure to LPS, iNOS induction was detected with in 8 h, reaching maximum by 12 h with a later decline. These studies indicate that iNOS is one of the early inducible genes in mouse macrophage in response to LPS treatment.

3.2.3 Decreased Cell Viability by C-Phycocyanin

Recent studies have shown the potential use of selective COX-2 inhibitors in the treatment and prevention of colon cancer (Fournier and Gordon, 2000; Dempke *et al,* 2001). Growth inhibition and apoptosis have been observed in several other cancer cell lines by COX-2 inhibitors (Everts *et al,* 2000; Buttar and Wang, 2000; Fournier and Gordon, 2000; Fosslien, 2000; Dempke *et al,* 2001). To test the effects of C-phycocyanin on growth and multiplication, RAW 264.7 cells were incubated with different concentrations of C-phycocyanin and the cell viability was examined by MTT assay. MTT assay is based on the principle that the actively growing cells generate reducing equivalents such as NADH that is necessary to meet energy requirement of living cells. In the assay NADH generated by living cells reduces the tetrazolium salt, MTT. RAW 264.7 cells treated with LPS, NMMA plus four different concentrations (5,10, 20 and 50 μM) of

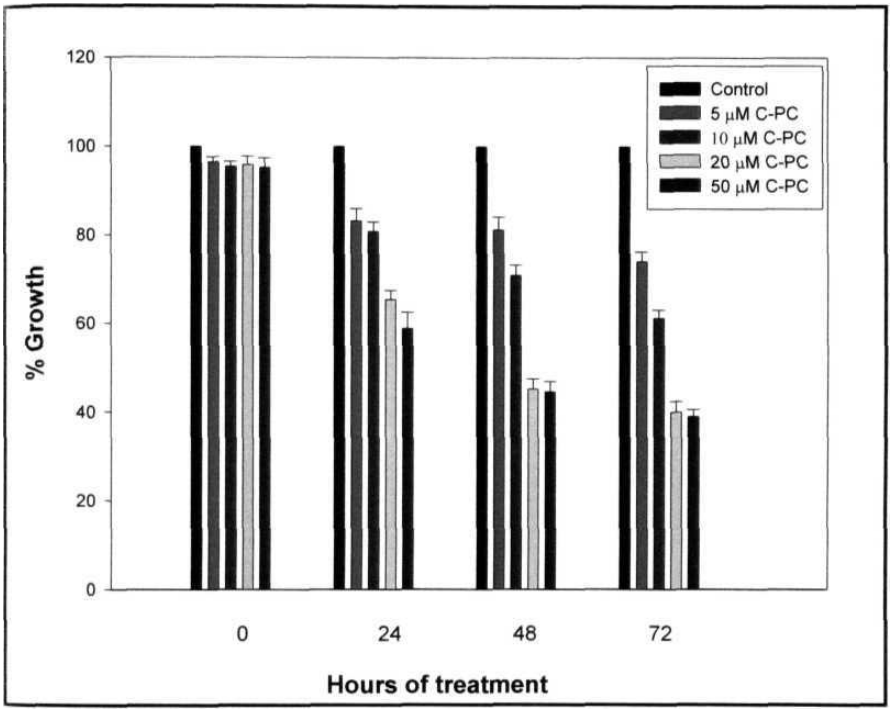
phycocyanin and the cell viability was examined after 0, 24, 48 and 72 h. The data presented in fig. 20 clearly shows 50% inhibition in the growth of RAW 264.7 cells at 20 μM concentration of phycocyanin. The extent of inhibition reached maximum by 48 h after exposure to C-phycocyanin, with a later stabilization. The decrease in the cell viability was dose dependent upto 20 μM C-phycocyanin.

Light microscopic analysis of the C-phycocyanin treated cells revealed pronounced morphological changes. Fig. 21 B shows the morphological alterations in RAW 264.7 cells after treatment with 20 μM C-phycocyanin for 48h. The cells became shrunken, round and detached from the plate. Also the number of cells decreased in response to C-phycocyanin treatment.

3.2.4 Confocal Microscopic Studies

A distinguishing feature of apoptosis is the condensation and fragmentation of nuclear chromatin, which can be monitored by confocal microscopy. Fig. 22 shows the confocal microscopic pictures of RAW 264.7 cells treated with or without C-phycocyanin. The pictures in fig. 22 show (a). fluorescence image, (b). phase contrast image, (c) overlay of fluorescence image with corresponding phase contrast image. Nuclear chromatin of control RAW 264.7 cells, permeabilized and stained with propidium iodide, showed normal chromatin with distinct large nuclei (Fig. 22 A). Phycocyanin treated cells, on the other hand, showed characteristic apoptotic nuclei, which are condensed and brightly fluoresced on confocal microscopy (Fig. 22 B).

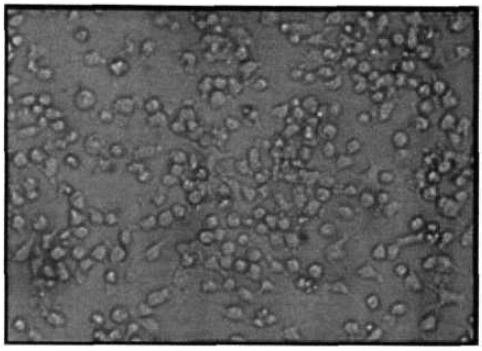
Fig. 20: Effect of C-Phycocyanin on cell growth in RAW 264.7 cells



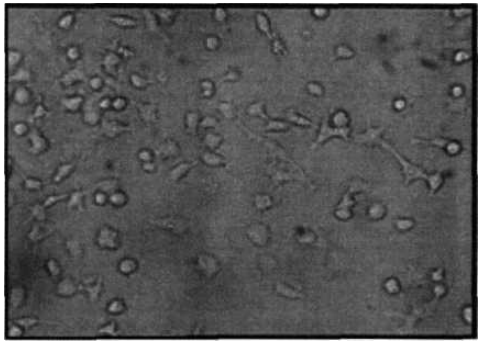
RAW cells stimulated with LPS (1μg/ml) were incubated with 5, 10, 20 and 50 μM concentrations of C-phycocyanin and the cell survival was determined after 0, 24, 48 and 72 h by MTT assay. The % viable cells were calculated in comparison to untreated cells. The number of cells in the control were taken as 100%. Each represent was carried out with triplicate and the data is the mean of 6 *experiments. Bars represent SD.*

Fig. 21: Phase contrast photomicrographs showing the effect of C-Phycocyanin in LPS stimulated RAW 264.7 mouse macrophages

A. Control



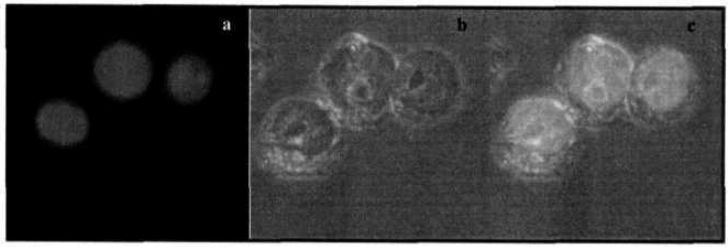
B. Treated



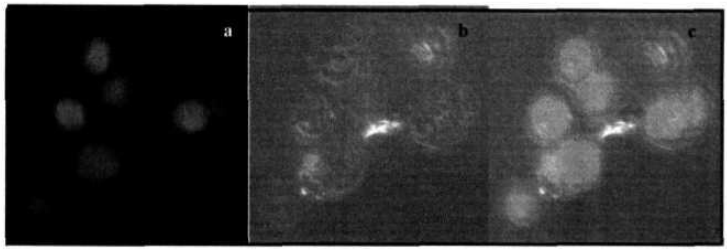
Morphology of mouse macrophage monolayres in cultures Phase contrast photomicrographs (20 X) were taken after 48 h exposure: A. Control cells exposed to LPS; B. cells exposed to LPS and 20 μM phycocyanin.

Fig. 22: Confocal microscopic studies showing C-Phycocyanin induced nuclear DNA condensation in RAW 264.7 cells stained with propidium iodide

A. RAW 264.7 cells with LPS



B. RAW 264.7 cells treated with LPS and 20 μM phycocyanin



Photomicrographs of RAW 264.7 cells, stained with propidium iodide to view nuclear condensation by confocal microscopy. Images were taken after 24 h exposure: A. Control cells treated with LPS; B. RAW 264.7 cells treated for 24 h with LPS and 20 μM phycocyanin.

a. fluorescence image.

b. phase contrast image.

c. overlay of fluorescence image with corresponding phase contrast image.

3.2.5 Effect of Phycocyanin on COX-1, COX-2, and iNOS Protein Expression

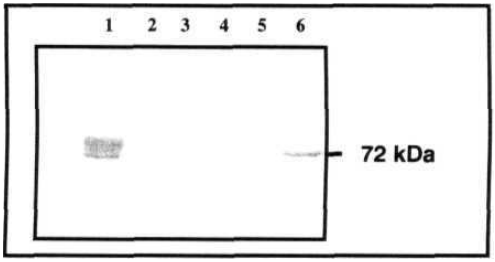
To investigate the effect of C-phycocyanin on the expression of COX-2, inducible nitric oxide synthase (iNOS) and COX-1, Western blot analysis was performed with whole cell lysate made from C-phycocyanin treated RAW 264.7 cells after challenging with LPS. The results presented in fig. 23, 24 &25 showed no significant changes in COX-2, iNOS and COX-1 protein levels in C-phycocyanin treated cells compared to LPS treated control cells. These results indicate no effect of C-phycocyanin on the protein levels of COX-1, COX-2 and iNOS, which are involved in mediating inflammation. Since PGE_2 is the main mediator of inflammation and phycocyanin is known to have anti-inflammatory effects, further studies were undertaken to estimate the levels of PGE_2 in phycocyanin treated macrophages by employing PGE_2 EIA kit.

3.2.6 Effect of C-Phycocyanin on PGE_2 Formation

The activity levels of COX-2 were also measured in terms of PGE_2 formed in mouse macrophage cells, stimulated with LPS. PGE_2 secreted into the medium was measured by employing enzyme immunoassay kit supplied by Assay Designs Inc. As shown in the fig. 26, C-phycocyanin decreased the levels of PGE_2 in a dose dependent manner reaching maximum inhibition at 100μM C-phycocyanin. These results indicate that the anti-inflammatory effects of C-phycocyanin are due to inhibition of COX-2 activity, without altering the expression of COX-2 protein.

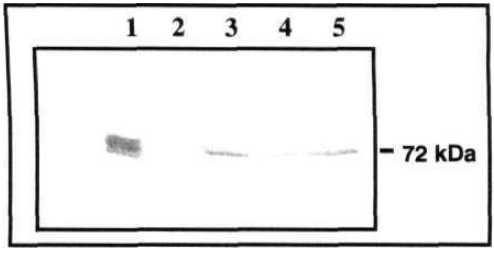
Fig. 23: Effect of C-Phycocyanin on COX-2 protein expression in LPS stimulated RAW 264.7 cells by immunoblot analysis

A. Time-dependent



Lane 1: COX-2 protein positive control (rh COX-2 expressed in baculovirus infected Sf9 cells)
Lane 2: RA W 264.7 cells without LPS
Lane 3 to 6: RAW 264.7 cells with LPS and 20 μM phycocyanin for 8, 12, 24 and 48 h respectively

B. Dose-dependent

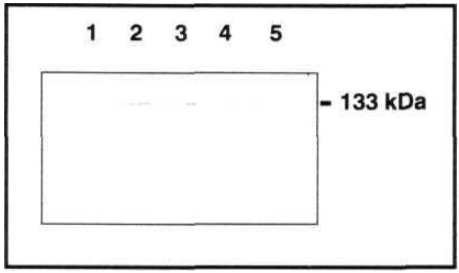


Lane 1: COX-2 positive control, (rh COX-2)
Lane 2: RAW 264.7 cells without LPS
Lane 3: RAW 264.7 cells with LPS and 25 μM phycocyanin 48 hrs
Lane 4: RA W 264.7 cells with LPS and 50 μM phycocyanin 48 hrs
Lane 5: RAW 264.7 cells with LPS and 100 μM phycocyanin 48 hrs

Whole cell lysates (100 μg) were separated on a 10% SDS-PAGE. After electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with affinity purified goat polyclonal anti-COX-2.

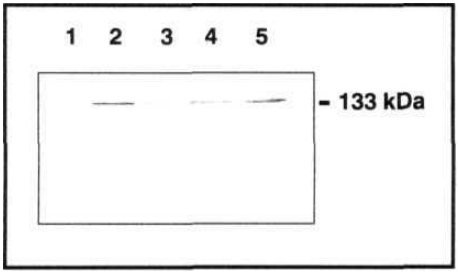
Fig. 24: Effect of C-Phycocyanin on iNOS protein expression in LPS stimulated RAW 264.7 cells by immunoblot analysis

A. Without phycocyanin



Lane 1: RAW 264.7 cells without LPS
Lane 2 to 5: RA W 264.7 cells with LPS for 8, 12, 24 and 48 h respectively

B. With phycocyanin

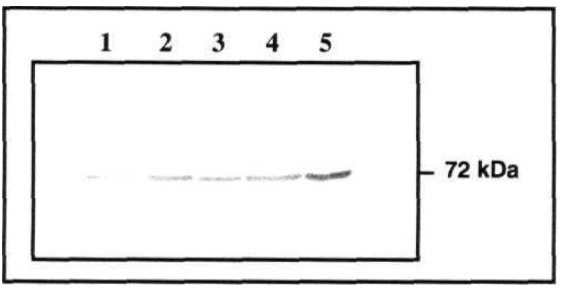


Lane 1: RAW 264.7 cells without LPS
Lane 2 to 5: RAW 264.7 cells with LPS and 20 μM phycocyanin for 8, 12, 24 and 48 h respectively

Whole cell lysates (100 μg) were separated on a 7.5% SDS-PAGE. After electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with affinity purified goat polyclonal anti-iNOS.

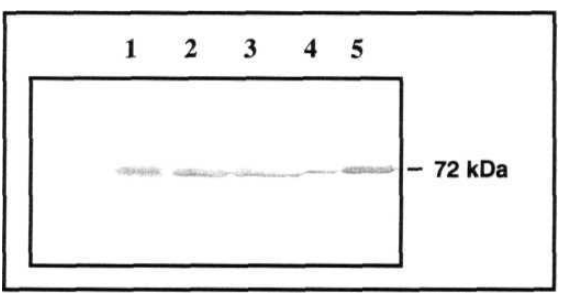
Fig. 25: Effect of C-Phycocyanin on COX-1 protein expression in LPS stimulated RAW 264.7 cells by immunoblot analysis

A. Without phycocyanin



Lane 1: COX-1 protein positive control (from ram seminal vesicles)
Lane 2 to 5: RA W 264.7 cells with LPS for 8, 12, 24 and 48 h respectively

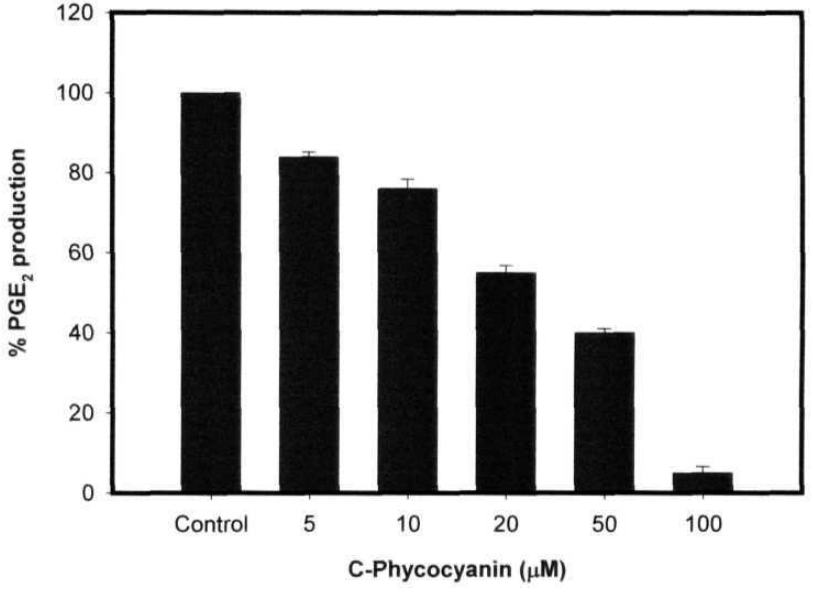
B. With phycocyanin



Lane 1: COX-1 protein positive control (from ram seminal vesicles)
Lane 2 to 5: RA W 264.7 cells with LPS and 20 μM phycocyanin for 8, 12, 24 and 48 h respectively

Whole cell lysates (100 μg) were separated on 10% SDS-PAGE, the proteins on the gel were transferred to nitrocellulose membrane and probed with affinity purified goat polyclonal anti-COX-1.

Fig. 26: Effect of C-phycocyanin on PGE_2 production in LPS stimulated RAW 264.7 cells



PGE_2 levels were measured in the medium taken from RAW 264.7 cells after 24 h of treatment with 5-100μMC-phycocyanin. PGE2 concentration was determined by EIA kit. Each experiment was carried out with duplicate determinations and the data is the mean of 2 experiments.

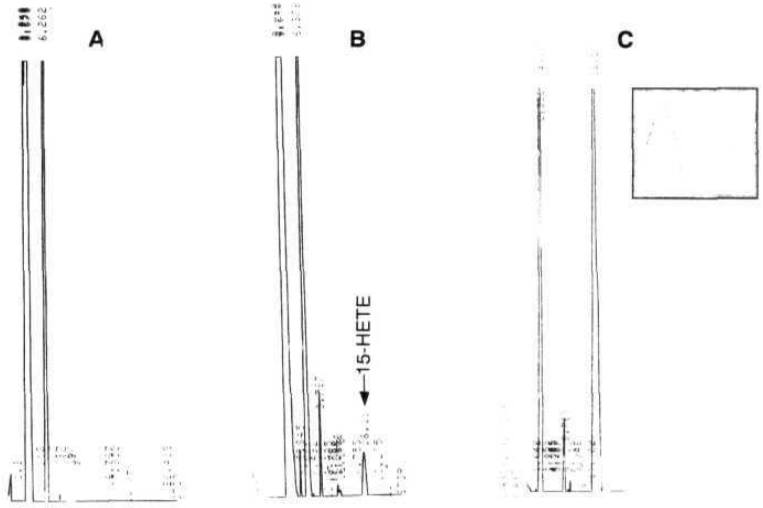
3.2.7 Measurement of COX-2 Activity: HPLC Assay

Both oxygraphic as well as spectrophotometric assays do not distinguish COX-1 from COX-2. In order to differentiate COX-2 from COX-1 and specifically measure COX-2 activity, HPLC based assay was employed. In the presence of aspirin, COX-2 is known to get partially inhibited by generating 15-HETE. COX-1, on the otherhand, gets totally inhibited by aspirin. This was exploited in the HPLC assay, where in the formation of 15-HETE in the presence of aspirin was monitored on HPLC. Fig. 27 shows the HPLC analysis of products generated in mouse macrophage cell line, RAW 264.7 treated with LPS, when incubated in the presence (Fig. 27 b) and absence (Fig. 27a) of aspirin. As shown in the fig. 27 b, the peak with retention time 12.2 min showed typical conjugated diene spectrum (inset) with absorption maximum of 235 nm. The peak was identified as 15-HETE, based on co-chromatography with standard 15-HETE. However, no such 15-HETE was formed in mouse macrophage cells without aspirin. The formation of 15-HETE thus demonstrates that the COX induced in response to LPS stimulation in mouse macrophages is COX-2.

3.2.8 Induction of Apoptosis by C-Phycocyanin

To investigate whether or not the loss of viability in response to C-phycocyanin correlates with a biochemical feature that discriminates between apoptosis and necrosis further studies were undertaken. The following techniques were used: confocal microscopy, internucleosomal DNA fragmentation by TUNEL assay and agarose gel electrophoresis, FACS analysis to determine subG_0/G_1 populations and PARP cleavage.

Fig. 27: HPLC assay of COX-2 activity in the presence/ absence of aspirin in LPS stimulated RAW 264.7 cells



A. RAW cells treated with LPS without aspirin

B. RAW cells treated with LPS and aspirin

C. Standard 15-HETE

Column : Waters μ bondapak C_{18}

Solvent : Methnol: water: acetic Acid 80:20:0.1

Flow Rate : 1 ml/min

Detection : 235 nm

3.2.8.1 Assay of DNA Fragmentation in C-Phycocyanin treated RAW 264.7 Cells

Biochemical hallmark of apoptosis is fragmentation of the genomic DNA, an irreversible event that commits the cell to die. DNA fragmentation can be visualized by agarose gel electrophoresis following DNA extraction and also by *in situ* DNA nick end labeling (TUNEL assay).

i. DNA Fragmentation by Agarose Gel Electrophoresis

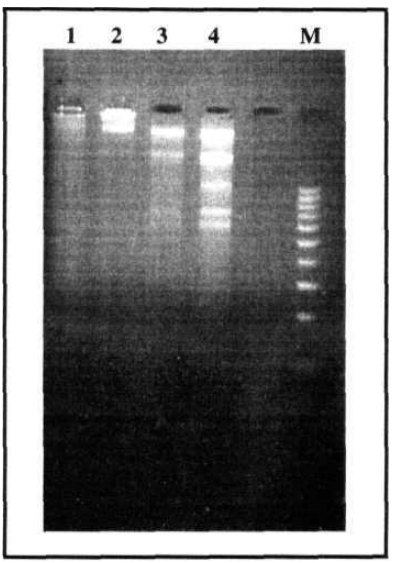
Detection of DNA fragmentation is widely considered as a biochemical hallmark of apoptosis. During later stages of apoptosis intemucleosomal cleavage of cellular DNA by endonucleases to 180 bp or oligomers of 180 bp fragments could be detected by extraction of nuclear DNA and agarose gel electrophoresis.

In the present study, C-phycocyanin induced apoptosis was examined with the DNA fragmentation assay. LPS stimulated RAW 264.7 cells, on exposure to 10/20 μM C-phycocyanin for 48 h showed generation of oligonucleosomal sized ladders of DNA fragmentation on agarose gels contained with ethidium bromide. The degree of nuclear DNA fragmentation was directly proportional to the concentration of C-phycocyanin. Treatment of RAW 264.7 cells without and with LPS did not induce the formation of any intemucleosomal DNA fragmentation (Fig. 28).

ii. *In situ* Detection of DNA Fragmentation by TUNEL Assay

The TUNEL assay remains the gold standard of detection of apoptosis within cells. In *in situ* DNA nick end labeling method, labeled nucleotides (BrdUTP) are incorporated into the free 3' hydroxyl ends of the fragmented DNA generated in response to apoptotic signals, using terminal deoxynucleotidyl transferase (TdT) enzyme. Incorporation of bromo

Fig. 28: Agarose gel electrophoresis showing internucleosomal DNA fragmentation induced by C-Phycocyanin in RAW 264.7 cells



RAW 264.7 cells were treated with and without LPS and combination of LPS and phycocyanin for 48 h. Both floating and adherent cells were collected and soluble DNA was extracted and electrophoresed on a 2% agarose gel containing 0.05 mg/ml ethidium bromide at 5 V/cm for 3 h. The gels were then photographed under UV illumination.

Lane 1: RAW 264.7 cells without LPS

Lane 2: RAW 264.7 cells with LPS

Lane 3: RAW 264.7 cells treated with LPS and 10 μM C-phycocyanin

Lane 4: RAW 264.7 cells treated with LPS and 20 μM C-phycocyanin

Lane M: 100 bp DNA ladder

dexoyribonucleotide triphosphate (BrdUTP) was detected using anti-BrdU antibody followed by streptavidin-horseradish peroxidase (HRP) conjugate and diaminobenzidine (DAB). DAB reacts with the labeled sample and generates an insoluble colored product at the site of DNA fragmentation. Counterstaining with methyl green aids in the morphological evaluation and characterization of normal and apoptotic cells, where the non-apoptotic cells get stained blue and the apoptotic cells stained brown.

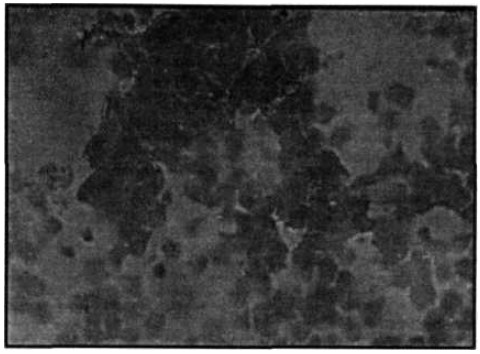
As shown in the fig. 29 B the nuclei of phycocyanin treated cells clearly showed a characteristic brown stains indicative of DNA fragmentation. Cells treated with LPS, on the other hand, did not show any brown staining (Fig. 29 A).

3.2.8.2 Flow Cytometric Analysis

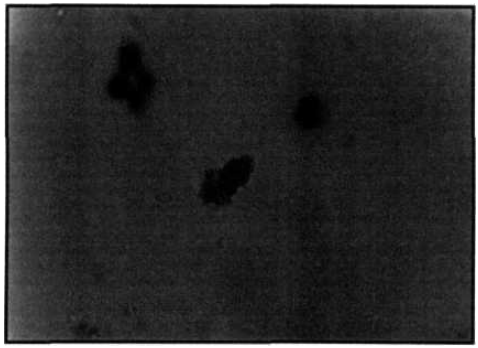
To determine whether cell death induced by C-phycocyanin is by necrosis or apoptosis, the distribution of DNA was examined by flow cytometry. Cells cultured for 24 h with LPS (1 μg/ml) and 5, 10 and 20 μM C-phycocyanin were taken for FACS analysis. Fig. 30 A shows the FACS analysis of control cells (cells treated with LPS), which shows prominent G_1, followed by S and G_2/M phases. Only 1.52% of these cells showed hypodiploid DNA (sub G0/G1 peaks). This control value of 1.52% hypodiploid DNA in control cells increased to 11.84%, 14.19% and 16.60% on exposure of cells to 5,10 and 20 μM C-phycocyanin, respectively. Cells undergoing apoptosis appeared to the left of the main G0/G1 peaks (i.e. sub G0/G1) in phycocyanin treated cells as compared to control untreated cells (Fig. 30 d).

Fig. 29: Phase contrast photomicrographs showing *in situ* detection of apoptotic RAW 264.7 cells, treated with C-Phycocyanin

A. Control

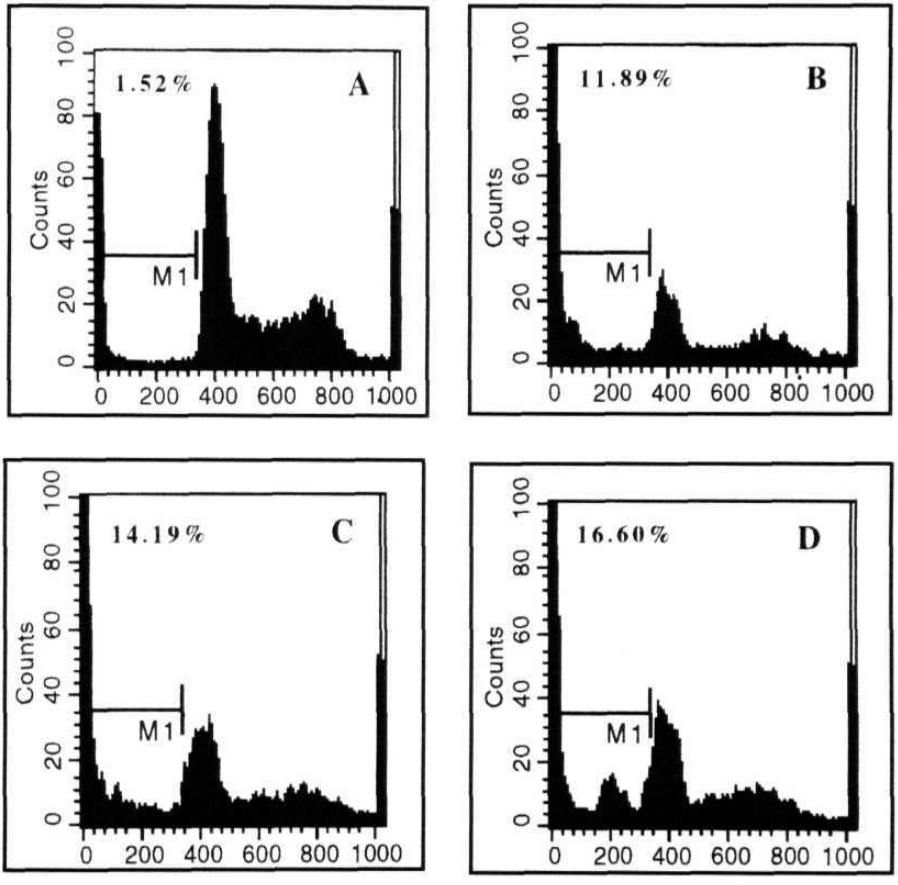


B. Treated



ApoTACS labeling of RAW 264.7 cells. Labeling was performed as per the protocol given by the manufacturer (R&D systems Inc). Phase contrast photomicrographs (100 X) were taken after 48 h exposure: A. Control cells exposed to LPS; B. cells exposed to LPS and 20 μM phycocyanin.

Fig. 30: Quantification of apoptosis in C-Phycocyanin induced RAW 264.7 cells by FACS



A:control; B: 5 μM C-phycocyanin;
C: 10 μM C-phycocyanin; D: 20 μM C-phycocyanin

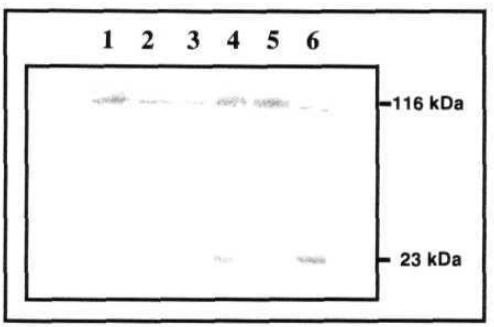
3.2.8.3 Cleavage of PARP

PARP, (poly- (ADP-ribose) polymerase, is a nuclear enzyme that is activated both during necrosis and apoptosis. However, the pattern of cleavage is variable in both modes of cell death with characteristic signature fragments of 85 and 23 kDa in apoptosis, 43 and 29 kDa in necrosis (Shah *et al*, 1996). During apoptosis, PARP (116 kDa) is cleaved between amino acids Asp^{214} and Gly^{215} to yield two fragments of 85 kDa and 23 kDa. PARP cleavage generally detected before DNA fragmentation. HRP-conjugated anti-PARP, antibody specifically recognizes the 23 kDa fragment of the cleaved PARP and uncleaved 116 kDa PARP. In the present study RAW 264.7 cells treated with 20 μM phycocyanin showed 23 kDa fragment along with the uncleaved 116 kDa PARP (Fig.31). In the control cells, however, no fragment of PARP was observed, except the uncleaved 116 kDa protein. This data clearly demonstrates the cleavage of PARP in C-phycocyanin treated RAW 264.7 cells.

3.2.8.4 Phycocyanin Induced Growth Suppression is Independent of Bcl-2 Expression

Bcl-2 family proteins play an important role in the regulation of cell death. In light of the recent reports that attributed COX-2 inhibitor-induced apoptosis to bcl-2 down regulation (Liu *et al,* 1998; Sheng *et al*, 1998), studies were undertaken to test whether Bcl-2 expression is affected after C-phycocyanin treatment in LPS stimulated RAW 264.7 cells. The results of Western blot analysis, employing Bcl-2 protein antibodies, revealed that C-phycocyanin did not affect the expression of Bcl-2 protein upto 48 h after treatment (Fig. 32). This result suggested that apoptosis induced by C-

Fig. 31: Western blot analysis showing the cleavage of PARP in cell extracts of control and C-Phycocyanin treated RAW 264.7 cells



Whole cell lysates from RAW 264.7 cells, incubated only with LPS and with LPS and phycocyanin, were fractionated on a 15% SDS-PAGE. After electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and the proteins were probed with anti-PARP antibodies. This antibody recognizes both uncleaved PARP (116kDa) and the cleaved fragment (23kDa).

Lane 1: RAW 264.7 cells treated with LPS for 4 h

Lane 2: RAW 264.7 cells treated with LPS and 20 μM phycocyanin for 4 h

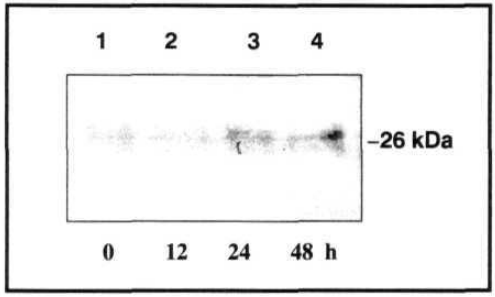
Lane 3: RAW 264.7 cells treated with LPS for 24 h

Lane 4: RAW 264.7 cells treated with LPS and 20 μM phycocyanin for 24 h

Lane 5: RAW 264.7 cells treated with LPS for 48 h

Lane 6: RAW 264.7 cells treated with LPS and 20 μM phycocyanin for 48 h

Fig. 32: Immunoblot analysis of Bcl-2 expression in nuclear extracts of C-Phycocyanin treated RAW 264.7 cells



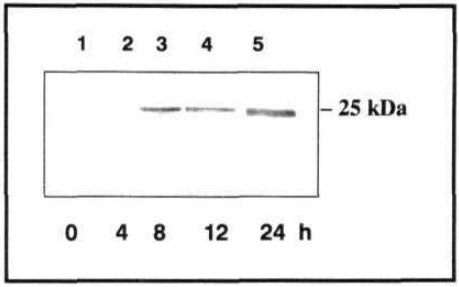
RAW Cells stimulated with LPS were treated with 20 μM C-phycocyanin for the indicated time and lysed, and the whole cell lysates (100 μg) were separated on a 15% SDS-PAGE and the proteins on the gel were transferred to nitrocellulose membrane and probed with affinity purified goat polyclonal anti-Bcl-2.

phycocyanin might be independent of Bcl-2 expression in LPS stimulated RAW 264.7 cells.

3.2.8.5 Cytochrome *c* Release from the Mitochondria as an Early Event

Cytochrome c is a well characterized mobile electron transport protein essential for energy conversion in all aerobic organisms. In mammalian cells, this highly conserved protein is normally localized to the mitochondrial intermembrane space. The release of cytochrome *c* into the cytosol is the hallmark of apoptosis of cells treated with certain apoptosis inducers (Liu ***et al***, 1996; Martinou *et* ***al***, 2000). Hence in the present study the cytosolic cytochrome c was measured by Western blot analysis in RAW 264.7 cells after 4,8,12 and 24 h after the treatment with 20 μM phycocyanin. As shown in fig. 33, the levels of cytochrome *c* in the cytosol were elevated within 4 h of treatment with phycocyanin, and the levels were further increased by 24 h. In contrast to the DNA fragmentation, PARP cleavage, FACS analysis and TUNEL assays, the release of cytochrome *c* from the mitochondria was very rapid and is an early event in phycocyanin induced apoptosis in RAW 264.7 cells.

Fig. 33: Western blot analysis showing C-Phycocyanin-induced release of cytochrome *c* into the cytosol in LPS stimulated RAW 264.7 cells



RAW 264.7 cells stimulated with LPS were treated with 20 μM C-phycocyanin for 0-24 h. Both floating and adherent cells were collected for extraction of cytosolic protein and the proteins (100 μg) were separated on a 15% SDS-PAGE. After electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with cytochrome c antibodies.

Discussion

4.1 Effect of C-Phycocyanin on LOX Pathway

Lipoxygenases (LOXs) are the key enzymes involved in the oxygenation of polyunsaturated fatty acids, to generate bioactive lipids such as hydroperoxy fatty acids, leukotrienes and lipoxins, which play an important role in allergy, asthma and other hypersensitive reactions. Based on the position of incorporation of molecular oxygen into the fatty acid, AA, LOXs are broadly classified into 5-, 12- and 15-lipoxygenases. Among these, 5-LOX plays a key role in view of its involvement in the formation of leukotrienes (Engels and Nijkamp, 1998). 12-LOX, mainly expressed in platelets, leukocytes and many solid tumor cells, plays an important role in hemostasis and other immunological activities (Shanon *et al,* 1993). 15-LOX, mainly expressed in reticulocytes, is known to play an important role in atherogenesis by promoting the oxidation of LDL (Kuhn and Chan, 1997; Feinmark and Cornicelli, 1997). In view of their role in many pathological manifestations, several attempts are being made to develop selective inhibitors of lipoxygenases.

C-phycocyanin is a biliprotein extracted from the blue green algae, *Spirulina platensis.* It is composed of two different subunits: a- and β-subunits, and functioning as a light-harvesting protein in cyanobacteria (Katjusa *et al,* 1995; Zhang *et al,* 1996). A number of studies have demonstrated the potential of C-phycocyanin in the treatment/ prevention of many disorders like inflammation, arthritis and hepatotoxicity. Many of these therapeutic effects were attributed to its potent antioxidant (Romay *et al,* 1998 a; Romay and Gonzalez, 2000) and free radical scavenging properties (Vadiraja and Madyastha, 2000). Since eicosanoids, the

oxygenated metabolites of arachidonic acid *via* lipoxygenase and cyclooxygenase pathway, are known to mediate many of the above degenerative disorders, an attempt is made in the present study to test the effect of C-phycocyanin on LOX and COX pathways.

The present study on lipoxygenase pathway showed no inhibition on recombinant human 5-lipoxygenase and rabbit reticulocyte 15-lipoxygenase activity levels, even upto 100 μM concentration of C-phycocyanin. These studies indicate that the therapeutic effects of C-phycocyanin may not be mediated through inhibition of lipoxygenase pathway.

4.2 Effect of C-Phycocyanin on COX Pathway

Cyclooxygenase, the key enzyme involved in the biosynthesis of prostaglandins (Smith *et al,* 1996), plays an important role in inflammation and variety of other disorders (Cummings and Robertson, 1977; Vane *et al*, 1998; Basu, 1999). With the discovery of inducible form of cyclooxygenase, COX-2 (Xie *et al,* 1991), it has been postulated that PGs that contribute to inflammatory process are derived exclusively from COX-2, while many of the "house keeping" effects of COX appear to be mediated by COX-1. Selective inhibitors of COX-2 would exhibit anti-inflammatory and analgesic effects without the deleterious side effects of conventional NSAIDs. Many pharma companies have, therefore, started searching for compounds that would selectively inhibit COX-2 without affecting COX-1 activity. These efforts have led to the introduction of selective COX-2 inhibitors such as celecoxib by Searle and rofecoxib by Merck, as new class of NSAIDs into the market.

The *in vitro* potency of a drug is reflected by its IC_{50} value. This is the concentration at which the drug achieves 50% of its maximal inhibition of COX. The lower the IC_{50} value, the more potent is the drug. In the present study, employing recombinant human COX-2 and ram seminal vesicles COX-1 it has been shown that phycocyanin is a selective inhibitor of COX-2, with an IC_{50} value of 180 nM. It is more potent inhibitor than celecoxib (IC_{50} 255 nM) and rofecoxib (IC_{50} 401 nM). The human whole blood COX-2 assay provides an additional and a more relevant measure of COX-2 inhibition selectivity under pathophysiological environment rich in plasma protein and cells (Patrignani ***et al***, 1994; Brideau ***et al***, 1996). In the present study it was observed that C-phycocyanin very efficiently inhibited COX-2 activity with an IC_{50} value of 80 nM in the human whole blood assay, wherein the fresh heparinized human whole blood was incubated with LPS and the PGE_2 formed was measured. In fact the IC50 value obtained in the whole blood assay (80 nM) is much lower than the value obtained with the partially purified enzyme (180 nM). It will be interesting to probe further into this enhanced efficiency of C-phycocyanin in inhibiting COX-2 under cellular environment. Inhibition of COX-2 activity is a favorable condition for treating inflammation, arthritis and preventing cancer (Kawamori *et al,* 1998). Earlier studies have demonstrated the anti-inflammatory property of C-phycocyanin (Remirez *et al,* 1999) and this property of phycocyanin can be explained, in part, by the specific inhibition of COX-2. The mechanism of inhibition of COX activity by C-phycocyanin appears to be similar to those reported for COX-2 selective inhibitors, occurring through a time dependent mechanism leading to a possible

formation of a tightly bound inhibitor complex (Copeland *et al,* 1994; Marnett, and Kalgutkar, 1999). Additionally, C-phycocyanin has been shown earlier to possess radical scavenging and antioxidant properties (Romay *et al,* 1998 a; Romay, and Gonzalez, 2000; Vadiraja, and Madyastha, 2000), which could also contribute substantially towards its anti-inflammatory and anti-arthritic effects. It is increasingly recognized that reactive oxygen species are involved in rheumatoid arthritis (Miesel and Zuber, 1993; Flugge *et al,* 1999). When compared to the toxicities associated with the currently available COX-2 selective anti-inflammatory drugs, C-phycocyanin would likely provide safer therapeutic alternative since it is as efficacious as currently used drugs (NSAIDs), if not more. But most importantly, this water-soluble biliprotein is from a natural source with least toxic effects. Several other natural products also were shown to inhibit either COX-2 activity or gene expression. Resveratrol, a phytoalexin found in grapes and other foods, was shown to inhibit COX-2 activity and suppress the activation of COX-2 gene expression (Subbaramaiah *et al,* 1998). It is also known that compounds such as radicicol, genistein, curcumin and retinoids inhibit COX-2 gene expression (Subbaramaiah *et al,* 1997).

The ratios of IC_{50} COX-2 / IC_{50} COX-1 provide a useful comparison of relative values for a series of NSAIDs tested in the same system. NSAIDs with values <1 would be said to have selectivity for COX-2, while those with ratios equal to 1 are nonselective. However, this ratio for a particular NSAID will vary according to whether it is measured using intact cells, cell homogenates, variety assays, purified enzymes or recombinant

proteins expressed in bacterial, insect or animal cells (Kurumbali *et al*, 1996; Pairet, 1998). It also varies when measured in different types of cells derived from various species (Vane, and Botting, 1998). Earlier studies indicate that a high degree of *in vitro* biochemical selectivity for COX-2 will be required in order to achieve effective functional selectivity *in vivo.* In the present study C0X-2/C0X-1 ratio of the IC_{50} value calculated for C-phycocyanin *in vitro* is very low and comparable to those calculated for celecoxib and rofecoxib. These findings suggest that C-phycocyanin is a selective inhibitor of COX-2.

It is known that the active site of COX-2 is larger than that of COX-1 so that it can accommodate bigger structures (Luong *et al,* 1996). C-phycocyanin is significantly much bigger in size (~39 kDa) than NSAIDs. Its three dimensional structure probably would facilitate the proper binding with the active site of COX-2. In fact reduced phycocyanin, where the C-10 methine bridge in the chromophore is reduced (Terry *et al,* 1993), is significantly less active COX-2 inhibitor and also lost COX-2 selectivity. It is quite possible that the reduced phycocyanin with an altered native conformation may not favor proper binding at the active site. This appears to be true with phycocyanobilin, the chromophore of phycocyanin, which also significantly lost COX-2 selectivity, suggesting that the apoprotein plays a prominent role in the inhibition of COX-2. However, at present it is not known whether the whole protein or part of it is involved in the inhibition of COX-2 and the site of interaction i.e. active site or allosteric site. It is quite unlikely that the whole protein is involved in binding to the active/ allosteric site of the enzyme. Also the mechanism of entry of C-

phycocyanin into the cells is not clear. It would be interesting to probe further into the kinetics of inhibition and the mechanism of interaction between the enzyme and the whole phycocyanin or its fragment.

In summary, it is demonstrated that C-phycocyanin is a potent selective inhibitor of COX-2 activity with potency comparable to celecoxib and rofecoxib, the known selective COX-2 inhibitors. Reduced phycocyanin and phycocyanobilin are poor inhibitors of COX-2 activity without COX-2 specificity. Also phycocyanin has no effect on 5- and 15- lipoxygenases *in vitro.*

4.3 Effect of C-Phycocyanin on Mouse Macrophage Cell Line, RAW 264.7

Macrophages play an important role in the regulation of inflammation and immune response. When activated, macrophages release growth factors, cytokines and lipid mediators such as prostaglandins and leukotrienes, which promote inflammation by directing cellular migration into the site of inflammation through the production and release of proinflammatory cytokines such as IL-6. Induced PG synthesis is associated with the onset of symptoms resulting from acute immune system activation. For example, COX-2 knock out mice do not develop fever in response to normally pyrogenic doses of bacterial endotoxin (Li *et al*, 1999). Elevated PGs levels are also associated with conditions of both chronic inflammation and cancer (DeWitt, 1991; Smith and Marnett, 1991). Macrophages secrete PGs upon activation by the bacterial endotoxin, lipopolysaccharide, primarily due to the induction of the COX-2 gene transcription and subsequent production of the COX-2 protein (Reddy and

Herschman, 1994; Lee *et al*, 1992). Along with COX-2, another immediate early gene, the inducible isoform of nitric oxide synthase, is expressed in LPS stimulated macrophages, RAW 264.7. Numerous reports suggest a complex "cross talk" between the COX-2 and iNOS pathways. Although not all published data are consistent, most findings indicate that nitric oxide down regulates COX-2 expression. Effective inhibition of NO synthesis by inhibitors of NOS activity such as N-monomethyl-L-arginine (L-NMMA) have been shown to increase COX-2 protein expression in rodent macrophages (Stadler *et al,* 1993; Swierkosz *et al*, 1995; Habib *et al*, 1997; Patel *et al,* 1999). In addition, Habib *et al* (1997) found increased COX-2 mRNA levels after treatment of LPS activated rat peritoneal macrophages with L-NMMA and aminoguanidine.

In the present study the effect of phycocyanin on COX-2 expression was monitored in LPS stimulated mouse macrophage cell line, RAW 264.7. In order to prevent the interference of NO in LPS stimulated mouse macrophages, inhibitor of NOS activity-NMMA was employed throughout the present study. These studies have shown no effects of C-phycocyanin on COX-1, COX-2 and iNOS protein levels in mouse macrophages. C-phycocyanin, on the other hand, decreased the levels of PGE_2 significantly in LPS stimulated macrophages. However, the concentration of C-phycocyanin required for 50% inhibition of PGE_2 levels in the macrophages is much higher (~ 20μM) compared to the IC_{50} value obtained for *in vitro* inhibition of COX-2 enzyme (180 nM) and to that obtained in whole blood assay (80nM). The reasons for this unusually high concentration of C-phycocyanin required for inhibition of PGE_2 levels in mouse macrophage

cell line are not quite clear. The problems associated with the entry of C-phycocyanin and the reduced bioavailability of C-phycocyanin with in the macrophages may be one of the possibilities. Further studies in this direction, however, are required to elucidate the mechanism and explain the discrepancy. The foregoing studies, thus indicate that the decreased levels of PGE_2 observed in C-phycocyanin treated mouse macrophage cells, could be due to selective inhibition of COX-2 activity, without any effect on the expression of COX enzymes.

Epidemiological and experimental evidences demonstrate the use of COX-2 inhibitors in reducing the risk of colorectai cancers (Giardiello *et al,* 1993; Giovannucci *et al,* 1994; 1995; Hanif *et al,* 1996; Barnes and Lee, 1998; Kawamori ef *al,* 1998; Reddy *et al,* 1993; 2000). Clinical trails also have shown the use of NSAIDs in reducing the number and size of colorectai polyps in patients with Familial Adenomatous Polyposis (FAP) (Giardiello *et al,* 1993; Steinbach *et al,* 2000). However the signaling mechanisms responsible for the chemopreventive action of these drugs have not been clearly established (Gupta and DuBois, 1998). Recent studies have demonstrated the induction of apoptosis in tumor cells by NSAIDs (Shiff *et al,* 1995; Hanif *et al,* 1996; Piazza *et al,* 1997, Sheng *et al,* 1997; Elder *et al,* 1997; Liu *et al*, 1998; Hsu *et al,* 2000; Li *et al,* 2000). This NSAID induced apoptosis in tumor cells was shown to be due to the inhibition of COX-2 induced PGE_2 synthesis. In addition NSAIDs were shown to inhibit COX-2 induced angiogenesis. Similar to other NSAIDs, phycocyanin is known to exhibit anticancer properties (Liu ef *al,* 2000). In the present study also C-phycocyanin reduced the growth and

multiplication of mouse macrophage cell line. Although the mechanism behind this effect is not fully understood, based on the present studies, it is proposed that C-phycocyanin inhibited PGE_2 levels may partially be responsible for this effect. This C-phycocyanin mediated decrease in the growth of macrophage cell line may be due to either necrosis or apoptosis. In order to test this, further studies were undertaken on the characteristic markers of apoptosis.

C-phycocyanin induced inhibition in the growth of RAW 264.7 cells was examined by confocal and phase contrast microscopy. When cells were exposed to 20 μM phycocyanin for 48 h several morphological changes, such as cell shrinkage and nuclear condensation that are ascribed to apoptosis, were observed. Phase contrast microscopic studies also revealed the presence of cells with web like activated membrane structure and detached cells from the culture flask. Similar inhibition in the growth of human leukemia-blast K562 cells by C-phycocyanin was reported (Liu *et al*, 2000).

There has recently been a surge of interest in marine bioresources, particularly seaweeds, as sources of bioactive substances. Several preparations of seaweed such as polysaccharide, peptide and phycobiliproteins were shown to affect the multiplication of tumor cells (Schwartz *et al,* 1988; Noda *et al,* 1989; Riou *et al,* 1996). Many aqueous extracts of green, brown and red algae were shown to possess bioactivity against murine immunocytes (Sadanori *et al,* 1993). C-Phycocyanin was shown to reduce the viability of mouse myeloma cells after irradiation by 300 J cm-2 at 514 nm for 3 days (Morcos ef *al,* 1988).

Overexpression of Bcl-2 is correlated with the progression of prostate carcinoma (McDonnell *et al,* 1992). Ectopic expression of Bcl-2 was shown to impair apoptotic signaling by inactivating c-Jun NH2-terminal kinase, leading to apoptosis (Herrmann ***et al***, 1997). To determine whether the cell death induced by C-phycocyanin in the present study has any relation to the expression of Bcl-2, phycocyanin treated RAW 264.7 cells were analyzed for changes in the levels of Bcl-2 protein. Western blot analysis of the proteins from cells exposed to C-phycocyanin, in the present study, showed no significant changes in the levels of Bcl-2. These studies suggest that C-phycocyanin induced apoptosis is independent of Bcl-2 expression. Similar operation of Bcl-2 independent pathways was reported in celecoxib induced apoptosis in prostate cancer cells (Hsu *et al,* 2000) and human primitive neuroectodermal tumors (Ratnakar *et al,* 2001) and NS-398 induced apoptosis of colon cancer cells (Li *et al,* 2001). C-phycocyanin also was shown to have no effect on the expression of Bcl-2 protein in human leukemia K562 cells (Liu *et al,* 2000). Bcl-2 family of proteins belong to two distinct classes-death repressors (Bcl-2, Bcl-XL) and death promoters (Bcl-Xs). The ratio between death repressors and death promoters in the Bcl-2 family will determine a cells destiny upon diverse stimuli (Ray ef *al,* 1996; Luo *et al,* 1999). The C-phycocyanin induced apoptosis observed in the present study may be mediated through other members of the Bcl-2 family.

Several apoptosis inducing agents are known to trigger mitochondrial uncoupling leading to the rupture of outer membrane. This inturn causes the release of pro-apoptotic factors such as apoptosis

inducing factor (AIF), cytochrome *c* and the apoptosis protease activating factor (Apaf-1) into the cytosol. Cytochrome *c* and apaf-1, after leaking out of the mitochondria, act as cohorts in the activation of caspase-9. Hence, cytochrome *c* release from the mitochondria is considered as a key signal that initiates the irreversible events in cell death. Functional studies indicate that drug induced opening or closing of the mitochondrial megachannel (permeability transition pore) can induce or prevent apoptosis. The anti-apoptotic protein Bcl-2 acts on mitochondria to stabilize membrane integrity and prevent the opening of the megachannel (Yang *et al,* 1997; Susin ef *al,* 1998; Tsujimoto and shimizu, 2000). In the present study, it is examined whether or not cytochrome *c* is released into the cytosol in response to C-phycocyanin by employing Western blot analysis. These studies have shown the release of cytochrome c as early as 4 h after treatment of RAW 264.7 cells with C-phycocyanin, with later increase upto 24 h. This observation suggests that cytochrome c release is one of the early events of C-phycocyanin-induced apoptosis. This is quite against the **PARP** cleavage in RAW 264.7 cells, which is induced 24 h after exposure to C-phycocyanin. NS-398, the selective inhibitor of COX-2, was also shown to induce apoptosis in colon cancer cells by cytochrome c dependent pathway (Li *et al,* 2001). The present studies on mouse macrophage cell line, **RAW 264.7,** exposed to C-phycocyanin reveal the induction of apoptosis through cytochrome c release, followed by PARP cleavage, and finally leading to the degradation of DNA into oligonucleosomal fragments.

Several studies have revealed the overexpression of the COX-2 enzyme in human cancers, including colorectal cancer. Furthermore, an

elevated level of COX-2 expression is associated with poor prognosis of cancer patients (Taketo 1998 a&b; Fujita *et al,* 1998). The overexpression of COX-2 in intestinal epithelial cells causes phenotypic changes that may enhance the tumorigenic or metastatic potential of colorectal cancer cells (Tsujii and DuBois, 1995; Tsujii ef *al,* 1997). Oshima *et al.* (1996) demonstrated that COX-2 null mutation dramatically reduces the number and size of the intestinal polyps in mice and that a novel COX-2 inhibitor was more effective than sulindac in reducing the number of polyps in APCd716 mice. These results provide evidence that COX-2 is important in tumorigenesis and indicate that COX-2 selective inhibitors may be a novel class of therapeutic agents for colorectal polyposis and cancer (Oshima *et al,* 1996). Based on these conclusive evidences, COX-2 specific inhibitors have been approved recently by FDA for treatment and prevention of cancers in FAP patients. Further clinical trails are in progress for the use of COX-2 specific inhibitors either alone or in combination for the treatment and prevention of several other cancers. In this connection, natural compounds such as C-phycocyanin assume much greater prominence for the treatment/ prevention of cancers. Further detailed studies involving animal/ human experiments, however, are required to test the efficacy of C-phycocyanin in the treatment /prevention of cancers, where in COX-2 has been implicated. In this respect studies of Romay *et al* (2000) on the effects of C-phycocyanin extracts on decreasing the levels of PGE_2 in mouse ear inflammation model appear quite promising.

In summary, motivated by several claims on the therapeutic role of C-phycocyanin, particularly its anti-inflammatory, antiarthritic and anti-

cancer properties, it is planned to address the question as to whether this natural product C-phycocyanin might interfere with arachidonic acid cascade. The preliminary studies on activity and mechanism of action revealed the effects of C-phycocyanin resembling that of NSAIDs, which are thought to display a chemopreventive effect by their common ability to inhibit prostaglandin synthesis. C-phycocyanin induced apoptosis in LPS stimulated RAW 264.7 cells appears to be mediated through cytochrome *c* pathway but independent of Bcl-2 expression.

Conclusions

Recombinant human 5-Lipoxygenase and rabbit reticulocyte 15-lipoxygenase are not inhibited by C-phycocyanin, even at higher concentration (100μM).

C-phycocyanin selectively inhibited COX-2 with an IC_{50} value of 180 nM, which is much lower compared to those of celecoxib and rofecoxib, the selective inhibitors of COX-2.

C-phycocyanin also inhibited the production of PGE_2 in heparinized human whole blood incubated with lipopolysaccharide for 24 h with an IC_{50} value of 80 nM.

Inhibition of COX-2 by C-phycocyanin is dependent on the period of pre-incubation with the enzyme.

The COX-2/COX-1 ratio (0.04) obtained for phycocyanin is indicative of the COX-2 selectivity of C-phycocyanin.

Reduced phycocyanin and phycocyanobilin are poor inhibitors of COX-2, without COX-2 specificity.

C-phycocyanin inhibited the growth and multiplication of LPS stimulated mouse macrophage cell line, RAW 264.7.

C-phycocyanin inhibited the production of PGE_2 in the lipopolysaccharide stimulated RAW 264.7 macrophages, in a dose dependent manner.

Studies involving specific apoptotic hallmarks, including PARP cleavage, confocal microscopy, TUNEL assay, FACS analysis and DNA fragmentation have revealed the induction of apoptosis in LPS stimulated mouse macrophages.

Induction of apoptosis by C-phycocyanin is independent of Bcl-2 expression.

C-phycocyanin induced apoptosis in RAW 264.7 cells, appears to be mediated through cytochrome c dependent pathway.

References

Amin AR, Attur M, Patel RN, Thakker GD, Marshall PJ, Rediske J, Stuchin SA, Patel IR and Abramson SB. Superinduction of cyclooxygenase-2 activity in human osteoarthritis-affected cartilage. Influence of nitric oxide. **J Clin Invest** 99: 1231-1237, 1997.

Anderson GD, Hauser SD, McGarity KL, Bremer ME, Isakson PC and Gregory SA. Selective inhibition of cyclooxygenase-2 (COX-2) reverses inflammation and expression of COX-2 and interleukin 6 in rat adjuvant arthritis. **J Clin Invest** 97: 2672-2679, 1996.

Anderson ME, Allison DRD and Meister A. Interconversion of leukotrienes catalyzed by purified γ-glutamyltranspeptidase: concomitant formation of leukotriene D4 and γ-glutamyl amino acids. **Proc Natl Acad Sci USA** 79: 1088-1091, 1982.

Andre E, and Hou K. **Comptes Rendus** 194: 645-647, 1932.

Balazy M, Metabolism of 5,6-epoxyeicosatrienoic acid by the human platelet. Formation of novel thromboxane analogs. **J Biol Chem** 266: 23561-23567, 1991.

Balcarek JM, Theisin TW, Cook MN, Varrichio A, Hwang SM, Strohsacker MW and Crooke ST. Isolation and characterization of cDNA clone encoding rat 5-lipoxygenase. **J Biol Chem** 263: 13937-13941, 1988.

Barnes CJ and Lee M. Chemoprevention of spontaneous intestinal adenomas in the adenomatous polyposis coli Min Mouse model with aspirin. **Gastroenterology** 114: 873-877, 1998.

Basu S. Oxidative injury induced cyclooxygenase activation in experimental hepatotoxicity. **Biochem Biophys Res Commun** 254: 764-767, 1999.

Berkely HO and Galliard T. Measurement of lipoxygenase activity in crude and partially purified potato extracts. **Phytochemistry** 15: 1475-1479,1976.

Bisgaard H. Leukotrienes and prostaglandins in asthma. **Allergy** 39: 413-420, 1984.

Blum H, Beier H and Gross HJ. Improved silver staining of plant proteins, RNA, DNA in polyacrylamide gels. **Electrophoresis** 8: 93-97, 1987.

Borgeat P and Samuelsson B. Arachidonic acid metabolism in polymorphonuclear leukocytes: effects of ionophore A23187. **Proc Natl Acad Sci USA** 76:2148-2152,1979.

Boussiba S and Richmond AE. Isolation and Characterization of Phycocyanins from the Blue-green Alga *Spirulina platensis.* **Arch Microbiol** 120: 155-159,1979.

Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principal of protein dye binding. **Anal Biochem** 72: 248-254, 1976.

Brash AR, Hughes MA, Hawkins DJ, Boeglin WE, Song WC and Meijer L. Allene oxide and aldehyde biosynthesis in starfish oocytes. **J Biol Chem** 266:22926-22931, 1991.

Brash AR, Boeglin WE, Chang MS and Sheih BH. Purification and molecular cloning of an 8R-lipoxygenase from the coral Plexaura homomalla reveal the related primary structures of R- and S-lipoxygenases. **J Biol Chem 271**: 20949-20957, 1996.

Brash AR, Boeglin WE and Chang MS. Discovery of a second 15s-lipoxygenase in humans. **Proc Natl Acad Sci USA** 94: 6148-6152, 1997.

Breder CD and Saper CB. Expression of inducible cyclooxygenase mRNA in the mouse brain after systemic administration of bacterial lipopolysaccharide. **Brain Res** 713:64-69, 1996.

Brideau C, Kargman S, Liu S, Dallob AL, Ehich EW, Rodger IW and Chan CC. A human whole blood assay for clinical evaluation of biochemical efficacy of cyclooxygenase inhibitors. **Inflamm Res** 45: 68-74, 1996.

Brooks PM. COX-2 inhibitors. **Aust Prescriber** 23: 30-32, 2000.

Brown DM, Warner GL, Ale-Martinez JE, Scott DW and Phipps RP. Prostaglandin E2 induces apoptosis in immature normal and malignant B lymphocytes. **Clin Immunol Immunopathol** 63: 221-229, 1992.

Brown PH and Lippman SM. Chemoprevention of breast cancer. **Breast Cancer Res Treat** 62: 1-17, 2000.

Buttar NS and Wang KK. The "Aspirin" of the new mellenium: Cyclooxygenase-2 inhibitors. **Mayo Clin Proc** 75: 1027-1038, 2000.

Campbell WB, Gebremedhin D, Pratt PF and Harder DR. Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. **Circ Res** 78: 415-423,1996.

Campling BG, Pym J, Galbraith PR and Cole SP. Use of MTT assay for rapid determination of chemosensitivity of human leukemic blast cells. **Leukemia Research** 12: 823-831, 1988.

Capdevila J, Chacos N, Werringloer J, Prough RA and Estabrook RW. Liver microsomal cytochrome P-450 and the oxidative metabolism of arachidonic acid. **Proc Natl Acad Sci USA** 78: 5362-5366,1981.

Carroll MA, Balazy M, Margiotta P, Falck JR and McGiff JC. Renal vasodilator activity of 5,6-epoxyeicosatrienoic acid depends upon

conversion by cyclooxygenase and release of prostaglandins. **J Biol Chem** 268: 12260-12266, 1993.

Cashman JR, Hanks D and Weiner RI. Epoxy derivatives of arachidonic acid are potent stimulators of prolactin secretion. **Neuroendocrinology** 46:246-251, 1987.

Chan CC, Dubois L and Young V. Effects of two novel inhibitors of 15-lipoxygenase, L-651, 392 and L-651, 896, in a guinea pig model of epidermal hyperproliferation. **Eur J Pharmacol** 139: 11-18, 1987.

Chen JK, Falck JR, Reddy KM, Capdevila J and Harris RC. Epoxyeicosatrienoic acids and their sulfonamide derivatives stimulate tyrosine phosphorylation and induce mitogenesis in renal epithelial cells. **J Biol Chem** 273:29254-29261,1998.

Chen JK, Capdevila J and Harris RC. Overexpression of C-terminal Src kinase blocks 14, 15-epoxyeicosatrienoic acid-induced tyrosine phosphorylation and mitogenesis. **J Biol Chem** 275: 13789-13792, 2000.

Chen X, Kurre U, Jenkins NA, Copeland NG and Funk CD. cDNA cloning, expression, mutagenesis c-terminal iso-leucine, genomic structure, and chromosomal localizations of murine 12-lipoxygenases. **J Biol Chem** 269: 13979-13987,1994.

Cipollone F, Ganci A, Panara MR.Greco A, Cuccurullo F, Patrono C and Patrignani P. Effects of nabumetone on prostanoid biosynthesis in humans. **Clin Pharmacol Ther** 58: 335-341, 1995.

Claesson HE and Haeggstrom J. Human endothelial cells stimulate leukotriene synthesis and convert granulocyte released leukotriene A_4 into leukotriene B_4, C_4, D_4 and E_4. **Eur J Biochem** 173: 93-100,1988.

Copeland RA, Williams JM, Giannaras J, Nurnberg S, Covington M, Pinto D, Pick S and Trzaskos JM. Mechanism of selective inhibition of the inducible isoform or prostaglandin G/H synthase. **Proc Natl Acad Sci USA** 91: 11202-11206, 1994.

Crofford LJ, Wilder RL, Ristimaki AP, Sano H, Remmers EF, Epps HR and Hla T. Cyclooxygenase-1 and-2 expression in rheumatoid synovial tissue. **J Clin Invest** 93: 1095-1101, 1994.

Cummings KB and Robertson RP. Prostaglandin: increased production by renal cell carcinoma. **J Urol** 118: 720-723, 1977.

Davis R and Brogden RN. Nimesulide: an update of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy. **Drugs** 48: 431-454, 1994.

Dempke W, Rie C, Grothy A and Schmoll HJ. Cyclooxygenase-2: a novel target for cancer chemotherapy? **J Cancer Res Clin Oncol** 127: 411-417, 2001.

DeWitt DL. Prostaglandin endoperoxide synthase: regulation of enzyme expression. **Biochim Biophys Acta** 1083: 121-134, 1991.

DeWitt DL, Meade EA and Smith WL. PGH synthase isoenzyme selectivity: the potential for safer nonsteroidal anti-inflammatory drugs. **Am** J **Med** 95: 40 S -44 S, 1993.

Dinchuk JE, Car BD, Focht RJ, Johnston JJ, Jaffe BD, Covington MB, Contel NR, Eng VM, Collins RJ, Czerniak PM, Corry SA and Trzaskos JM. Renal abnormalities and an altered inflammatory responses in mice lacking cyclooxygenase II. **Nature (Lond.)** 378: 406-409, 1995.

Dix CJ, Habberfield AD, Sullivan MH and Cooke BA. Inhibition of steroid production in leydig cells by non-steroidal anti-inflammatory and released compounds: evidence for the involvement of lipoxygenase products in steroidogenesis. **Biochem** J 219: 529-537, 1984.

Dixon RA, Jones RE, Diehl RE, Bennett CD, Kargman S and Rouzer CA. Cloning of the cDNA for human 5-lipoxygenase. **Proc Natl Acad Sci USA** 85:416-420, 1988.

DuBois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, van de Putte LB and Lipsky PE. Cyclooxygenase in biology and disease. **FASEB J** 12: 1063-1073, 1998.

Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S and DuBois RN. Up-regulation of cyclooxygenase-2 gene expression in human colorectal adenomas and adenocarcinomas. **Gastroenterology** 107: 1183-1188, 1994.

Elder DJ, Halton DE, Hague A and Paraskeva C. Induction of apoptotic cell death in human colorectal carcinoma cell lines by a cyclooxygenase-2 (COX-2)-selective nonsteroidal anti-inflammatory drug: independence from COX-2 protein expression. **Clin Cancer Res** 3: 1679-1683, 1997.

Engelhardt G, Bogel R, Schnitzler C and Utzmann R. Meloxicam: influence on arachidonic acid metabolism. Part II. *In vivo* findings. **Biochem Pharmacol** 51: 29-38,1996.

Engels F and Nijkamp FP. Pharmacological inhibition of leukotriene actions. **Pharm World Sci** 20: 60-65,1998.

Erickson BA, Longo WE, Panesar N, Mazuski JE and Kaminski DL. The effect of selective cyclooxygenase inhibitors on intestinal epithelial cell mitogenesis. **J Surg Res** 81:101-107. 1999.

Everts B, Wahrborg P and Hedner T. COX-2 specific inhibitors- the emergence of a new class of analgesic and anti-inflammatory drugs. **Clin Rheumatol** 19: 331-343,2000.

Falck JR, Manna S, Moltz J, Chacos N and Capdevila J. Epoxyeicosatrienoic acids stimulate glucagon and insulin release from isolated rat pancreatic islets. **Biochem Biophys Res Commun** 114:743-749,1983.

Feinmark SJ and Cornicelli JA, Is there a role for 15-lipoxygenase in atherogenesis? **Biochem Pharmacol** 54: 953-959, 1997.

Fels AOS, Pawlowski NA, Cramer EB, King TKC, Cohn ZA and Scott WA. Human alveolar macrophages produce leukotriene B_4. **Proc Natl Acad Sci USA** 79: 7866-7870, 1982.

Fitzpatrick FA, Ligget W, McGee J, Bunting S, Morton D and Samuelsson B. Metabolism of leukotriene A_4 by human erythrocytes **J Biol Chem** 259: 11403-11407, 1984.

Flugge LA, Miller-Deist LA and Petillo PA. Towards a molecular understanding of arthritis. **Chem Biol** 6: R157-R166, 1999.

Fosslien E. Molecular pathology of cyclooxygenase-2 in neoplasia. **Ann Clin Lab Sci** 30: 3-21, 2000.

Fournier DB and Gordon GB, COX-2 and colon cancer: Potential targets for chemoprevetion. **J Cell Biochem** 34: 97-102, 2000.

Fujita T, Matsui M, Takaku K, Uetake H, Ichikawa W, Taketo MM and Sugihara K. Size-and invasion-dependent increase in cyclooxygenase-2 levels in human colorectal carcinomas. **Cancer Res** 58:4823-4826, 1998.

Fukino H et al (In Eisei Kagaku, Japan) Effect of *Spirulina* on the renal toxicity induced by inorganic chemistry and cisplatin. 36: 5-11, 1990.

Funk CD, Hoshiko S, Matsumoto T, Radmark O and Samuelsson B. Characterization of the human 5-lipoxygenase gene. **Proc Natl Acad Sci USA** 86: 2587-2591, 1989.

Funk CD, Furci L and Fitzgerald GA. Molecular cloning, primary structure and expression of the human platelet/erythroleukemia cell 12-lipoxygenase. **Proc Natl Acad Sci USA** 87: 5638-5642,1990.

Funk CD, Funk LB, Fitzgerald GA and Samuelsson B. Characterization of human 12-lipoxygenase genes. **Proc Natl Acad Sci USA** 89:3962-3966, 1992.

Giardiello FM, Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P, Booker SV, Robinson CR and Offerhaus GJ. Treatment of colonic and

rectal adenomas with sulindac in familial adenomatous polyposis. **N Engl J Med** 328: 1313-1316, 1993.

Gillmor SA, Villasenor A, Fletterick R, Sigal E and Browner MF. The structure of mammalian 15-lipoxygenase reveals similarity to the lipases and the determinants of substrate specificity. **Nature Struct Biol** 4: 1003-1009, 1997.

Giovannucci E, Rimm EB, Stampfer MJ, Colditz GA, Ascherio A and Willett WC. Aspirin use and the risk for colorectal cancer and adenoma in male health professionals. **Ann Intern Med** 121: 241-246, 1994.

Giovannucci E, Egan KM, Hunter DJ, Stampfer MJ, Colditz GA, Willett WC and Speizer FE. Aspirin and the risk of colorectal cancer in women. **N Engl J Med** 333:609-614, 1995.

Glaser K, Sung ML, O'Neill K. Belfast M, Hartman D, Carlson R, Kreft A, Kubrak D, Hsiao CL and Weichman B. Etodolac selectively inhibits human prostaglandin G/H synthase 2 (PGHS-2) versus human PGHS-1. **Eur J Pharmacol** 281: 107-111, 1995.

Glazer AN. Light guides, directional energy transfer in a photosynthetic antenna. **J Biol Chem** 264: 1-4,1989.

Gonzalez R, Rodriguez S, Romay C, Ancheta O, Gonzalez A, Armesto J, Remirez D and Merino N. Anti-inflammatory activity of phycocyanin extract in actetic acid induced colitis in rats. **Pharmacol Res** 39: 55-59, 1999.

Goppelt-Struebe M and Beiche F. Cyclooxygenae-2 in the spinal cord: localization and regulation after a peripheral inflammatory stimulus. **Adv Exper Med Biol** 433: 213-216, 1997.

Gu JL, Natarajan R, Ben-Ezra J, Valente G, Scott S, Yoshimoto T, Yamamoto S, Rossi JJ, L J and Nadler L. Evidence that a leukocyte-type of 12-lipoxygenase is expressed and regulated by angiotensin II in human adrenal glomerulosa cell. **Endocrinology** 134: 70-77,1994.

Gupta RA and DuBois RN. Aspirin, NSAIDs and colon cancer prevention: mechanisms? **Gastroenterology** 114: 1095-1098,1998.

Habib A, Bernard C, Lebret M, Creminon C, Esposito B, Tedgui A and Maclouf J. Regulation of the expression of cyclooxygenase-2 by nitric oxide in rat peritoneal macrophages. **J Immunol** 158: 3845-3851, 1997.

Hada T, Ueda N, Takahashi Y and Yamamoto S. Catalytic properties of human platelet 12-lipoxygenase as compared with the enzymes of other origins. **Biochim Biophys Acta** 1083: 89-93, 1991.

Hada T, Swift LL and Brash AR. Discovery of 5R-lipoxygenase activity in oocytes of the surf clam, Spisula solidissima. **Biochim Biophys Acta** 1346: 109-119, 1997.

Hamberg M and Samuelsson B. Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets. **Proc Natl Acad Sci USA** 71: 3400-3404,1974.

Hammarstrom S, Hamberg M, Samuelsson B, Duell EA, Stawiski M and Voorhees JJ. Increased concentrations of nonesterified arachidonic acid, 12L-hydroxy-5, 8,10,14-eicosatetraenoic acid, prostaglandin E2, and prostaglandin F2alpha in epidermis of psoriasis. **Proc Natl Acad Sci USA** 72:5130-5134,1975.

Hanif R, Pittas A, Feng Y, Koutsos MI, Qiao L, Staiano-Coico L, **Shift** SI and Rigas B. Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin independent pathway. **Biochem Pharmacol** 52: 237-245, 1996.

Haque MS, Arora JK, Dikdan G, Lysz TW and Zelenka PS. The Rabbit Lens Epithelial Cell Line N/N1003A Requires 12-Lipoxygenase Activity for DNA Synthesis in Response to EGF. **Mol Vis** 5: 8, 1999.

Hara A, Yoshimi N, Niwa M, Ino N and Mori H. Apoptosis induced by NS-398, a selective cyclooxygenase-2 inhibitor, in human colorectal cancer cell lines. **Jpn J Cancer Res** 88:600-604, 1997.

Harris RC, Homma T, Jacobson HR and Capdevila J. Epoxyeicosatrienoic acids activate Na+/H+ exchange and are mitogenic in cultured rat glomerular mesangial cells. **J Cell Physiol** 144: 429-437,1990.

Harris RC, Mckanna JA, Akai Y, Jacobson HR, DuBois RN and Breyer MD. Cyclooxygenase-2 is associated with the macula densa of rat kidney and increases with salt restriction. **J Clin Invest** 94: 2504-2510, 1994.

Hawkins DJ and Brash AR. Eggs of the sea urchin, Strongylocentrotus purpuratus, contain a prominent (11R) and (12R) lipoxygenase activity. **J Biol Chem** 262: 7629-7634, 1987.

Herrmann JL, Menter DG, Beham A, von Eschenbach A and McDonnell TJ. Regulation of lipid signaling pathways for cell survival and apoptosis by Bcl-2 in prostate carcinoma cells. **Exp Cell Res** 234:442-451, 1997.

Hogaboom GK, Cook M, Newton JF, Varrichio A, Shorr RG, Sarau HM and Crooke ST. Purification, characterization, and structural properties of a single protein from rat basophilic leukemia (RBL-1) cells possessing 5-lipoxygenase and leukotriene A4 synthetase activities. **Mol Pharmacol** 30: 510-519,1986.

Honn KV, Grossi IM, Timar J, Chopra H and Taylor JD. Platelets and Cancer metastasis. In: Microcirculation and cancer metastasis. Eds: Weiss L, Buchanan M, Orr FW, **CRC Press,** London-Oxford, 1991.

Honn KV, Tang DG, Gao X, Butovich IA, Liu B, Timar J and Hagmann W. 12-Lipoxygenases and 12(S)-HETE: role in cancer metastasis. **Cancer and Metastasis Reviews** 13:365-396, 1994.

Hsu AL, Ching TT, Wang DS, Song X, Rangnekar VM and Chen CS. The cyclooxygenase-2 inhibitor celecoxib induces apoptosis by blocking Akt activation in human prostate cancer cells independently of Bcl-2. **J Biol Chem.** 275: 11397-11403,2000.

Hulting AL, Lindgren JA, Hokfelt T, Eneroth P, Werner S, Patrono C and Samuelsson B. Leukotriene C_4 as a mediator of luteinizing hormone release from rat anterior pituitary cells. **Proc Natl Acad Sci USA** 82: 3834-3838, 1985.

Hunter JA, Finkbeiner WE, Nadel JA, Goetzl EJ and Holtzman MJ. Predominant generation of 15-lipoxygenase metabolites of arachidonic acid by epithelial cells from human trachea. **Proc Natl Acad Sci USA** 82: 4633-4637, 1985.

Ikawa H, Kamitani H, Calvo BF, Foley JF and Eling TE. Expression of 15-lipoxygenase-1 in human colorectal cancer. Cancer **Res** 59: 360-366, 1999.

Izumi T, Hoshiko S, Radmark O and Samuelsson S. Cloning of the cDNA for human 12-lipoxygenase. **Proc Natl Acad Sci USA** 87: 7477-7481,1990.

Jakobsson PJ, Steinhilber D, Odlander B, Radmark O, Claesson HE and Samuelsson B. On the expression and regulation of 5-lipoxygenase in human lymphocytes. **Proc Natl Acad Sci USA** 89:3521-3525, 1992.

Jakschik B, Harper T and Murphy RC. Leukotriene C_4 and D_4 formation by paniculate enzymes. **J Biol Chem** 257: 5346-5349, 1982.

Jung TM and Dailey MO. A novel and inexpensive source of allophycocyanin for multicolor flow cytometry. **J Immunol Methods** 121: 9-18,1989.

Kang RY, Freire-Moar J, Sigal E and Chu CQ. Expression of cyclooxygenase-2 in human and an animal model of rheumatoid arthritis. **Br J Rheum** 35: 711-718, 1996.

Kargman S, Charleson S, Cartwright M, Frank J, Riendeau D, Mancini J, Evans J and O'Neill G. Characterization of Prostaglandin G/H Synthase 1 and 2 in rat, dog, monkey, and human gastrointestinal tracts. **Gastroenterology** 111: 445-454, 1996.

Karim A, Tolbert D, Burton E. SC-58635 (celecoxib): a highly selective inhibitor of cyclooxygenase-2 disposition kinetics in man and identification of its major CYP450 isozyme in its biotransformation. **Pharm Res** 14: S-617 Abstract 3469, 1997.

Kawamori T, Rao CV, Seibert K and Reddy BS. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. **Cancer Res** 58: 409-412, 1998.

Keppler D, Hagmann W and Rapp S. Role of leukotrienes in endotoxin action in vivo. **Rev** Infect **Dis** 9: S580-S584, 1987.

Kim JA, Gu JL, Natarajan R, Esteban J, Berliner JA and Nadler JL A leukocyte-type of 12-lipoxygenase is expressed in human vascular and mononuclear cells: evidence for upregulation by angiotensin II. Arterioscler **Throm Vasc Biol** 15: 942-948,1995.

Kroemer G, Dallaporta B and Resche-Rigon M. The mitochondrial cell death/life regulator in apoptosis and necrosis. **Annu Rev Physiol** 60:619-642, 1998.

Kroemer G and Reed JC. Mitochondrial control of cell death. **Nat** Med 6:513-519,2000.

Kuhn H and Chan L. The role 15-lipoxygenase in atherogenesis: Pro- and anti-atherogeneic actions. **Curr Opin Lipidol** 8: 111-117, 1997.

Kuhn H, Heydeck D, Hugou I and Gniwotta C. *In vivo* action of 15-lipoxygenase in early stages of human atherogenesis. **J Clin Invest** 99:888-893,1997.

Kujubu DA, Fletcher BS, Barnum BC, Lim RW and Herschman HR. TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/ cyclooxygenase homologue. **J Biol Chem** 266: 12866-12872, 1991.

Kurumbail RG, Stevens AM, Gierse JK, McDonald JJ, Stegeman RA, Pak JY, Gildehaus D, Miyashiro JM, Penning TD, Seibert K, Isakson PC and Stallings WC. Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. **Nature** 384: 644-648, 1996.

Kurumbail RG, Stevens AM, Gierse JK, McDonald JJ, Stegeman RA, Pak JY, Gildehaus D, Miyashiro JM, Penning TD, Seibert K, Isakson PC and Stallings WC. Kurumbail RG, Stevens AM, Gierse JK, McDonald JJ, Stegeman Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. **Nature** 384: 644-648, 1996.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **Nature** 227: 680-685, 1970.

Langenbach R, Morham SG, Tiano HF, Loftin CD, Ghanayem BI, Chulada PC, Mahler JF, Lee CA, Goulding EH and Kluckman KD. Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin induced gastric ulceration. **Cell** 83:483-492, 1995.

Lazer ES, Miao CK, Cywin CL, Sorcek R, Wong HC, Meng Z, Potocki I, Hoermann M, Snow RJ, Tschantz MA, Kelly TA, McNeil DW, Coutts SJ, Churchill L, Graham AG, David E, Grob PM, Engel W, Meier H and Trummlitz G. Effect of structural modification of enol-carboxamide-type nonsteroidal anti-inflammatory drugs on COX-2/COX-1 selectivity. **J Med Chem** 40: 980-989, 1997.

Lee SH, Soyoola E, Chanmugam P, Hart S, Sun W, Zhong H, Liou S, Simmons D and Hwang D. Selective expression of mitogen-inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide. J **Biol Chem** 267:25934-25938, 1992.

Li M, Lotan R, Levin B, Tahara E, Lippman SM and Xu XC. Aspirin induction of apoptosis in esophageai cancer: a potential for chemoprevention. **Cancer Epidemiol Biomark Prev** 9: 545-549, 2000.

Li M, Wu X and Xu XC. Induction of apoptosis by cyclo-oxygenase-2 inhibitor NS398 through a cytochrome C-dependent pathway in esophageai cancer cells. **Int J Cancer** 93:218-22, 2001.

Li M, Wu X and Xu XC. Induction of apoptosis in colon cancer cells by cyclooxygenase-2 inhibitor NS398 through a cytochrome c-dependent pathway. **Clin** Cancer **Res.** 7: 1010-1016, 2001.

Li S, Wang Y, Matsumura K, Ballou LR, Morham SG and Blatteis CM. The febrile response to lipopolysaccharide is blocked in cyclooxygenase-2(-/-), but not in cyclooxygenase-1 (-/-) mice. **Brain Res** 825: 86-94, 1999.

Lim HY, Joo HJ, Choi JH, Yi JW, Yang MS, Cho DY, Kim HS, Nam DK, Lee KB and Kim HC. Increased expression of cyclooxygenase-2 protein in human gastric carcinoma. **Clin Cancer Res** 6:519-525, 2000.

Lipsky LP, Abramson SB, Crofford L, DuBois RN, Simon LS and van de Putte LB. The classification of cyclooxygenase inhibitors. **J Rheumatol** 25:2298-2303,1998.

Liu B, Marnett LJ, Chaudhary A, Ji C, Blair LA, Johnson CR, Diglio CA and Honn KV. Biosynthesis of 12(S)-hydroxyeicosatetraenoic acid by B16 amelanotic melanoma cells is a determinant of their metastatic potential. **Lab Invest** 70: 314-323, 1994.

Liu X, Kim CN, Yang J, Jemmerson R and Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. **Cell** 86: 147-157, 1996.

Liu XH, Yao S, Kirschenbaum A and Levine AC. NS398, a selective cyclooxygenase-2 inhibitor, induces apoptosis and down-regulates bcl-2 expression in LNCaP cells. **Cancer Res.** 58: 4245-4249,1998.

Liu Y, Xu L, Cheng N, Lin L and Zhang C. Inhibitory effect of phycocyanin from *Spirulina platensis* on the growth of human leukemia K562 cells. **J Appl Phycology** 12: 125-130, 2000.

Luo D, Cheng SCS and Xie Y. Expression of Bcl-2 family proteins during chemotherapeutic agents-induced apoptosis in the hepatoblastoma HepG2 cell line. **Br J biomed Sci** 56: 114-122, 1999.

Loeffler M and Kroemer G. The mitochondrion in cell death control: certainties and incognita. **Exp Cell Res** 256: 19-26, 2000.

Luong C, Miller A, Barnett J, Chow J, Ramesha C and Browner MF. Flexibility of the NSAID binding site in the structure of human cyclooxygenase-2. **Nat Struct Biol** 3: 927-933, 1996.

Maccarrone M, Ranalli M, Bellincampi L, Salucci ML, Sabatini S, Melino G and Finazzi-Agro A. Activation of different lipoxygenase isozymes induce apoptosis in human erythroleukemia and neuroblastoma cells. **Biochem Biophys Res Commun** 272: 345-350, 2000.

Maloney CG, Kutchera WA, Albertine KH, Mclntyre TM, Prescott SM and Zimmerman GA. Inflammatory agonists induce cyclooxygenase type 2 expression by human neutrophils. J **Immunol** 160: 1402-1410, 1998.

Marnett LJ and Kalgutkar AS. Cyclooxygenase 2 inhibitors: discovery, selectivity and the future. **Trends Pharmacol Sci** 20:465-469,1999.

Marshall LA and Roshak A. Coexistence of two biochemically distinct phopholipase A_2 activates in human platelet, monocyte and neutrophil. **Biochem Cell Biol** 71: 331-339,1993.

Marshall LA, Bolognese B, Winkler JD and Roshak A. Depletion of human monocyte 85 kDa phospholipase A_2 does not alter leukotriene formation. **J Biol Chem** 272: 759-765, 1997.

Martinou JC, Desagher S and Antonsson B. Cytochrome c release from mitochondria: all or nothing. **Nat Cell Biol** 2: E41-E43, 2000.

Mastino A, Piacentini M, Grelli S, Favalli C, Autuori F, Tentori L, Oliverio S and Garaci E. Induction of apoptosis in thymocytes by prostaglandin E2 in vivo. **Dev immunol** 2: 263-271,1992.

Matsumoto T, Funk CD, Radmark O, Hoog JO, Jornvall H and Samuelsson B. Molecular cloning and amino acid sequence of human 5-lipoxygenase. **Proc Natl Acad Sci USA** 85: 26-30, 1988.

McDonnell TJ, Transco P, Brisbay SM, Logothetis C, Chung LW, Hsieh JT, Tu SM and Campbell ML. Expression of the protooncogene Bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. **Cancer Res** 52:6940-6944, 1992.

Mead JF, Alfin-Slater RB, Howton DR and Popjak G. Prostaglandins, thromboxanes and prostacyclin. *In* Lipids: Chemistry, Biochemistry and Nutrition. Eds. Mead JF. **Plenum Press.** Pp.149-216, 1986.

Miesel R and Zuber M. Elevated levels of xanthine oxidase in serum of patients with inflammatory and autoimmune rheumatic diseases. **Inflammation** 17: 551-561, 1993.

Mitchell JA, Belvisi MG, Akaresereenont P, Robbins RA, Kwon OJ, Croxtall J, Barnes PJ and Vane JR. Induction of cyclooxygenase-2 by cytokines in human pulmonary epithelial cells: Regulation of dexamethasone. Br J **Pharmacol** 113: 1008-1014, 1994.

Mizuno H, Sakamoto C, Matsuda A, Wada K, Uchida T, Noguchi H, Akamatsu T and Kasuga M. Induction of cyclooxygenase-2 in gastric mucosal lesions and its inhibition by the specific antagonist delays healing in mice. **Gastroenterology** 112: 387-397, 1997.

Morcos NC and Henry WL. Medical treatments of tumors with phycocyanin. United States patent # 5,163,898. Nov 17th, 1992.

Morcos NC, Berns M and Henry WL. Phycocyanin: laser activation, cytotoxic effect, and uptake in human atherosclerotic plaque. **Laser Surg Med** 8: 10-17,1988.

Morham SG, Langenbach R, Loftin CD, Tiano HF, Vouloumanos N, Jennette JC, Mahler JF, Kluckman KD, Ledford A, Lee CA and Smithies O. Prostaglandin synthase-2 gene disruption causes severe renal pathology in the mouse. **Cell** 83:473-482, 1995.

Murphy RC, Hammarstrom S and Samuelsson B. Leukotriene C: A slow reacting substance from murine mastocytoma cells. **Proc Natl Acad Sci USA** 76: 4275-4279, 1979.

Nadel JA, Conrad DJ, Ueki IF, Schuster A and Sigal E. Immunocytochemical localization of arachidonate 15-lipoxygenase in erythrocytes, leukocytes, and airway cells. **J Clin Invest** 87: 1139-1145, 1991.

Natarajan R, Gonzales N, Hornsby PJ and Nadler JL Mechanism of angiotensin II-induced proliferation in bovine adrenal cortical cells. **Endocrinology** 131: 1174-1180, 1992.

Natarajan R, Gu JL, Rossi J, Gonzales N, Lanting L, Xu L and Nadler JL Elevated glucose and angiotensin II increase 12-lipoxygenase activity and expression in porcine aortic smooth muscle cells. **Proc Natl Acad Sci USA** 90: 4947-4951,1993.

Natarajan R, Gonzales N, Lanting L and Nadler JL. Role of the lipoxygenase pathway in angiotensin ll-induced vascular smooth muscle cell hypertrophy. **Hypertension** 23[suppl I]: I-142-I-147, 1994.

Natarajan R, Bai W, Gu JL, Rangarajan V and Nadler JL. Platelet-derived growth factor BB mediated regulation of 12-lipoxygenase in porcine aortic smooth muscle cells. **J Cell Physiol** 169: 391-400, 1996.

Natarajan R, Rosdahl J, Gonzales N and Bai, W. Regulation of 12-lipoxygenase by cytokines in vascular smooth muscle cells. **Hypertension** 30:873-879,1997.

Needleman P and Isakson PC. The discovery and function of COX-2. **J Rheumatol** 24 (Suppl 49): 6-8, 1997.

Nicoletti I, Migliorati G, Pagliacci MC, Grignani F and Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. **J Immunol Metb** 139: 271-279, 1991.

Noble S and Balfour JA. Meloxicam. **Drugs** 51: 424-432, 1996.

Noda H, Amano H, Arashima K, Hashimoto S and Nishizawa K. Studies on the antitumor activity of marine algae. **Bull Jap Soc Sci Fish** 55: 1254-1264, 1989.

Node K, Hou Y, Ruan X, Yang B, Spiecker M, Ley K, Zeldin DC and Liao JK. Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. **Science** 285: 1276-1279, 1999.

Node K, Ruan XL, Dai J, Yang SX, Graham L, Zeldin DC and Liao JK. Activation of Galpha s mediates induction of tissue-type plasminogen activator gene transcription by epoxyeicosatrienoic acids. **J Biol Chem** 276: 15983-15989,2001.

Nugteren DH, Arachidonate lipoxygenase in blood platelets. **Biochim Biophys Acta** 380: 299-307, 1975.

Oliw EH. Metabolism of 5(6) oxidoeicosatrienoic acid by ram seminal vesicles. Formation of two sterioisomers of 5-hydroxyprostaglandin I1. **J Biol Chem** 259: 2716-2721, 1984.

Orning L, Hammarstrom S and Samuelsson B. Leukotriene D: a slow reacting substance from rat leukemia cells. **Proc Natl Acad Sci USA** 77: 2014-2017, 1980.

Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, Trzaskos JM, Evans JF and Taketo MM. Suppression of intestinal polyposis in Apc 6716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). **Cell** 87: 803-809, 1996.

Pairet M. Inhibition of cyclooxygenase-1 and cyclooxygenase-2. Analysis of *in vitro* test systems and their clinical relevance. **J Clin Rheum** 4:S17-25, 1998.

Pascual JM, McKenzie A, Yankaskas JR, Falck JR and Zeldin DC. Epoxygenase metabolites of arachidonic acid affect electrophysiologic properties of rat tracheal epithelial cells. **J Pharmacol Exp Ther** 286: 772-779,1998.

Patel R, Attur MG, Dave M, Abramson SB and Amin AR. Regulation of cytosolic COX-2 and prostaglandin E_2 production by nitric oxide in activated murine macrophages. J **Immunol** 162: 4191-4197, 1999.

Patrignani P, Panara MR, Greco A, Fusco O, Natoli C, Iacobelli S, Cipollone F, Ganci A, Creminon C, Maclouf J and Patrono C. Biochemical and pharmacological characterization of the cyclooxygenase activity of human blood prostaglandin endoperoxide synthases. **J Pharmacol Exp Ther** 271:1705-1712, 1994.

Patrono C. Aspirin as an antiplatelet drug. **N Engl J Med** 330: 1287-1294, 1994.

Pek SB and Walsh MF. Leukotrienes stimulate insulin release from the rat pancreas. **Proc Natl Acad Sci USA** 81: 2199-2202, 1984.

Penning TD, Talley JJ, Bertenshaw SR, Carter JS, Collins PW, Docter S, Graneto MJ, Lee LF, Malecha JW, Miyashiro JM, Rogers RS, Rogier DJ, Yu SS, Anderson GD, Burton EG, Cogburn JN, Gregory SA, Koboldt CM, Perkins WE, Seibert K, Veenhuizen AW, Zhang YY and Isakson PC. Synthesis and biological evaluation of the 1,5-diarylpyrazole class of cyclooxygenase-2 inhibitors: identification of 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]-benzenesulfonamide (SC-58635, celecoxib). **J Med Chem** 40: 1347-1365, 1997.

Piazza GA, Rahm AK, Finn TS, Fryer BH, Ll H, Stoumen AL, Pamukcu R and Ahnen DJ. Apoptosis primarily accounts for the growth-inhibitory properties of sulindac metabolites and involves a mechanism that is independent of cyclooxygenase inhibition, cell cycle arrest and p53 induction. **Cancer Res** 57: 2452-2459, 1997.

Pilbeam CC, Fall PM, Alander CB and Raisz LG. Differential effects of nonsteroidal anti-inflammatory drugs on constitutive and inducible prostaglandin G/H synthase in cultured bone cells. **J Bone Miner Res 12**: 1198-1203, 1997.

Piper PJ. Leukotrienes, potent mediators of airway constriction. **Int Arch Allergy Appl Immunol** 76:43-48, 1985.

Proctor KG, Falck JR and Capdevila J. Intestinal vasodilation by epoxyeicosatrienoic acids: arachidonic acid metabolites produced by a cytochrome P450 monooxygenase. **Circ Res** 60: 50-59,1987.

Rao CV, Tokumo K, Rigotty J, Zang E, Kelloff G and Reddy BS. Chemoprevention of colon carcinogenesis by dietary administration of piroxicam, alpha-difluoromethylornithine, 16 alpha-fluoro-5-androsten-17-one, and ellagic acid individually and in combination. **Cancer Res** 51: 4528-4534, 1991.

Rapoport SM, Schewe T, Wiesner R, Halangk W, Ludwig P, Janicke-Hohne M, Tannert C, Hiebsch C and Klatt D. The lipoxygenase of reticulocytes. Purification, characterization and biological dynamics of the lipoxygenase: its identity with the respiratory inhibitor of the reticulocyte. **Eur J Biochem** 96: 545-561, 1979.

Rapoport SM, Schewe T and Thiele BJ. Maturational breakdown of mitochondria and other organelles in reticulocytes. **Blood Cell Biochem** 1: 151-194, 1990.

Ratnakar P, Kiranmai G, Reddanna P, Sutton LN, Phillips PC and Reddy CD. Overexpression of Cyclooxygenase-2 (COX-2) in human primitive Neuroectodermal Tumors: Effects of Celecoxib and Rofecoxib **Cancer Lett** (In Press).

Ray S, Bullock G, Nunez G, Tang C, Ibrado AM, Huang Y and Bhalla K. Enforced expression of Bcl-Xs induces differentiation and sensitizes chronic myelogenous leukemia blast crisis K562 cells to 1-beta-D-arabinofuranosylcytosine-mediated differentiation and apoptosis. **Cell Growth and Differentiation** 7: 1617-1623, 1996.

Raz A, Wyche A and Needleman P. Temporal and pharmacological division of fibroblast cyclooxygenase expression into transcriptional and translational phases. **Proc Natl Acad Sci USA** 86: 1657-1661, 1989.

Reddanna P, Whelan J and Reddy CC. A new pathway for the biosynthesis of leukotriene F_4. **Annal N Y Acad Sci** 524: 12339-12345,1988.

Reddy BS, Rao CV, Rivenson A and Kelloff G. Inhibitory effect of aspirin on azoxymethane induced colon carcinogenesis in F344 rats. **Carcinogenesis (Lond.)** 14: 1493-1497, 1993.

Reddy BS, Hirose Y, Lubet R, Steele V, Kelloff G, Paulson S, Seibert K and Rao CV. Chemoprevention of colon cancer by specific cyclooxygenase-2 inhibitor, celecoxib, administered during different stages of carcinogenesis. **Cancer Res** 60: 293-297,2000.

Reddy N, Everhart A, Eling T and Glasgow W. Characterization of a 15-lipoxygenase in human breast carcinoma BT-20 cells: Stimulation of 13-HODE formation by TGF /EGF. **Biochem Biophys Res Commun** 231: 111-116, 1997.

Reddy ST and Herschman HR. Ligand-induced prostaglandin synthesis requires expression of the TIS10/PGS-2 prostaglandin synthase gene in murine fibroblasts and macrophages. **J Biol Chem** 269:15473-15480, 1994.

Reed DW, Bradshaw WS, Xie W and Simmons DL In vivo and in vitro expression of a non-mammalian cyclooxygenase-1. **Prostaglandins** 52: 269-284,1996.

Reich R, Kohen F, Slager R and Tsafriri A. Ovarian lipoxygenase activity and its regulation by gonadotropin in the rat. **Prostaglandins** 30: 581-590, 1985.

Remirez D, Gonzalez A, Merino N, Gonzalez R, Ancheta O, Romay C and Rodriguez S. Effect of phycocyanin in zymosan-induced arthritis in mice-phycocyanin as an antiarthritic compound. **Drug Dev Res** 48: 70-75, 1999.

Rimbau V, Camins A, Romay C, Gonzalez R and Pallas M. Protective effect of C-phycocyanin against kainic acid-induced neuronal damage in rat hippocampus. **Neurosci Lett** 276: 75-78,1999.

Rimbau V, Camins A, Pubill D, Sureda FX, Romay C, Gonzalez R, Jimenez A, Escubedo E, Camarasa J and Pallas M. C-Phycocyanin protects cerebellar granule cells from low potassium/serum deprivation-induced apoptosis. **Naunyn Schmiedebergs Arch Pharmacol** 364:96-104, 2001.

Riou D, Colliec-Jouaults S, Pinczon du Sel D, Bosch S, Siavoshian S, Le Bert V, Tomasoni C, Sinquin C, Durand P and Roussakis C. Antitumor and antiproliferative effects of a fucan extracted from ascophyllum nodosum against a non-small-cell bronchopulmonary carcinoma line. **Anticancer Res** 16: 1213-1218, 1996.

Ristimaki A, Narko K and Hla T. Down-regulation of cytokine-induced cyclo-oxygenase-2 transcript isoforms by dexamethasone: evidence for post-transcriptional regulation. **Biochem** J 318: 325-331, 1996.

Ristimaki A, Honkanen N, Jankala H, Sipponen P and Harkonen M. Expression of cyclooxygenase-2 in human gastric carcinoma. **Cancer Res** 57:1276-1280, 1997.

Rokach J and Fitzsimmons B. The lipoxins. **Int J Biochem** 20: 753-758, 1988.

Roman RJ and Alonso-Galicia M. P-450 eicosanoids: A novel signaling pathway regulating renal function. **News Physiol Sci** 14: 238-242, 1999.

Romay C, Armesto J, Remirez D, Gonzalez R, Ledon N and Garcia I. Antioxidant and anti-inflammatory properties of C-phycocyanin from blue green algae. **Inflamm Res** 47: 36–41, 1998 a.

Romay C, Ledon N and Gonzalez R. Further studies on anti-inflammatory activity phycocyanin in some animal models of inflammation. **Inflamm Res** 47: 334-338,1998 b.

Romay C and Gonzalez R. Phycocyanin is an antioxidant protector of human erythrocytes against lysis by peroxyl radicals. **J Pharm Pharmacol** 52: 367-368, 2000.

Romay C, Ledon N and Gonzalez R. Effects of phycocyanin extract on prostaglandin E2 levels in mouse ear inflammation test. **Arzneimittelforschung** 50: 1106-1109, 2000.

Romero MF, Madhun ZT, Hopfer U and Douglas JG. An epoxygenase metabolite of arachidonic acid 5,6 epoxy-eicosatrienoic acid mediates angiotensin-induced natriuresis in proximal tubular epithelium. **Adv Prostaglandin Thromboxane Leukotriene Res** 21 A: 205-208,1991.

Roth SH. NSAID gastropathy. A new understanding. **Arch Intern** Med 156: 1623-1628, 1996.

Rouzer CA, Matsumoto T and Samuelsson B. Single protein from human leukocytes possesses 5-lipoxygenase and leukotriene A4 synthase activities. **Proc Natl Acad Sci USA** 83: 857-861, 1986.

Sadanori M, Hiroyoshi M, Kyoko N, Fuminori K. Effect of seaweed preparations on murine immunocytes. **J Appl Phycol** 5: 629-637, 1993.

Samuelsson B, Dahlen SE, Lindgren JA, Rouzer CA and Serhan CN. Leukotrienes and lipoxins: Structure, biosynthesis and biological effects. **Science** (Washington DC) 237: 1171-1176, 1987.

Sarith M, Souvannavong V and Adam A. Nitric oxide synthase induces macrophages death by apoptosis. **Biochem Biophys Res Commun** 191: 503-508, 1993.

Sawaoka H, Kawano S, Tsuji S, Tsujii M, Gunawan ES, Takei Y, Nagano K and Hori M. Cyclooxygenase-2 inhibitors suppress the growth of gastric cancer xenografts via induction of apoptosis in nude mice. **Am J Physiol** 274:G1061-G1067.1998.

Schewe T, Halangk W, Hiebsch C and Rapoport SM. A lipoxygenase in rabbit reticulocytes which attacks phospholipids and intact mitochondria. **FEBS Lett** 60: 149-152, 1975.

Schewe T, Rapoport SM and Kuhn H. Enzymology and physiology of reticulocyte lipoxygenase: comparison with other lipoxygenases. **Adv Enzymol Relat Areas Mol Biol** 58:191-272, 1986.

Schewe T and Kuhn H. Do 15-lipoxygenases have a common biological role? **Trends Biochem Sci** 16: 369-373, 1991.

Schmassmann A, Peskar BM, Stettler C, Netzer P, Stroff T, Flogerzi P and Halter F. Effect of inhibition of prostaglandin endoperoxide synthase-2 in chronic gastro-intestinal ulcer models in rats. **Br J Pharmacol** 123: 795-804, 1998.

Schreinemachers DM and Everson RB. Aspirin use and lung, colon, and breast cancer incidence in a prospective study. **Epidemiology** 5:138-146, 1994.

Schwartz JL, Sklar G, Reid S and Trickier D. Prevention of experimental oral cancer by extracts of Spirulina dunaliella algae. **Nutr Cancer** 11: 127-134, 1988.

Scott LJ. and Lamb HM. Rofecoxib. **Drugs** 58: 499-505, 1999.

Seibert K, Zhang Y, Leahy K, Hauser S, Masferrer J and Isakson P. Distribution of COX-1 and COX-2 in normal and inflamed tissues. **Adv Exp Med Biol** 400A: 167-170, 1997.

Setty BNY, Graeber JE and Stuart MJ. The mitogenic effects of 15- and 12-hydroxyeicosatetraenoic acids on endothelial cells may be mediated via diacylglycerol kinase inhibition. **J Biol Chem** 262: 17613-17622, 1987.

Shah GM, Shah RG and Poirier GG. Different cleavage pattern of poly (ADP-Ribose) polymerase during necrosis and apoptosis in HL 60 cells. **Biochem Biophys Res Commun** 229: 838-844, 1996.

Shannon VR, Stenson WF and Holtzman. Induction of epithelial arachidonate 12-lipoxygenase at active sites of inflammatory bowel disease. **Am** J **Physiol** 264:G104-111, 1993.

Sheng H, Shao J, Kirkland SC, Isakson P, Coffey RJ, Morrow J, Beauchamp RD and DuBois RN. Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. **J Clin Investig** 99: 2254-2259, 1997.

Sheng H, Shao J, Morrow JD, Beauchamp RD and DuBois RN. Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. **Cancer Res** 58: 362-366, 1998.

Sheu HL, Omata K, Utsumi Y, Tsutsumi E, Sato T, Shimizu T and Abe K. Epoxyeicosatrienoic acids stimulate the growth of vascular smooth muscle cells. **Adv** Prostaglandin **Thromboxane Leukotriene Res** 23: 211-213,1995.

Shift SJ, Qiao L, Tsai LL and Rigas B. Sulindac sulfide, an aspirin-like compound, inhibits proliferation, causes cell cycle quiescence and induce apoptosis in HT-29 colon adenocarcinoma cells. **J Clin Investig** 96: 491-503, 1995.

Shimokawa T and Smith WL Prostaglandin endoperoxide synthase: The aspirin acetylation region. **J Biol Chem** 267: 12387-12392, 1992.

Siegle I, Klein T, Backman JT, Saal JG, Nusing RM and Fritz P. Expression of cyclooxygenase 1 and cyclooxygenase 2 in human synovial tissue: differential elevation of cyclooxygenase 2 in inflammatory joint diseases. **Arthritis Rheum** 41: 122-129,1998.

Sigal E, Craik CS, Highland E, Grunberger D, Costello LL, Dixon RAF and Nadel JA. Molecular cloning and primary structure of human 15-lipoxygenase. **Biochem Biophys Res Commun** 157: 457-464,1988.

Sigal E, Dicharry S, Highland E and Finkbeiner WE. Cloning of human airway 15-lipoxygenase: identity to the reticulocyte enzyme and expression in epithelium. **Am J Physiol** 262: L392-L398, 1992.

Simon LS and Smith TJ. NSAID mechanisms of action, efficacy, and relative safety. **Postgrad** Med **Special Rep** 12-16, 1998.

Smalley WE and DuBois RN. Colorectal cancer and nonsteroidal anti-inflammatory drugs. **Adv Pharmacol** 39: 1-20, 1997.

Smith WL and Marnett LJ. Prostaglandin endoperoxide synthase: structure and catalysis. **Biochim Biophys Acta** 1083: 1-17, 1991.

Smith WL, Garavito RM and DeWitt DL. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1and-2. **J Biol Chem** 271: 33157-33160,1996.

Sok DE, Pai JK, Atrache V, Kang YC and Sih CJ. Enzymatic inactivation of SRS-Cys-Gly (leukotriene D). **Biochem Biophys Res Commun** 101: 222-229, 1981.

Stadler J, Harbrecht BG, Di Silvio M, Curran RD, Jordan ML, Simmons RL and Billiar TR. Endogenous nitric oxide inhibits the synthesis of cyclooxygenase products and interleukin-6 by rat kupffer cells. **J Leukoc Biol** 53: 165-172, 1993.

Steinbach G, Lynch PM, Phillips RK, Wallace MH, Hawk E, Gordon GB, Wakabayashi N, Saunders B, Shen Y, Fujimura T, Su LK and Levin B. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. **N Engl J Med** 342: 1946-1952, 2000.

Stewart WF, Kawas C, Corrada M and Metter EJ. Risk of Alzheimer's disease and duration of NSAID use. **Neurology** 48: 626-632, 1997.

Subbaramaiah K, Zakim D, Weksler BB and Dannenberg AJ. Inhibition of cyclooxygenase: a novel approach to cancer prevention. **Proc Soc Exp Biol Med** 216: 201-210, 1997.

Subbaramaiah K, Chung WJ, Michaluart P, Telang N, Tanabe T, Inoue H, Jang M, Pezzuto JM and Dannenberg AJ. Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. **J Biol Chem** 273: 21875-21882, 1998.

Susin SA, Zamzami N and Kroemer G. Mitochondria as regulators of apoptosis: doubt no more. **Biochim Biophys Acta** 1366: 151-165, 1998.

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM and Kroemer G. Molecular characterization of mitochondrial apoptosis-inducing factor. **Nature (Lond.)** 397:441-446, 1999.

Swierkosz TA, Mitchell JA, Warner TD, Botting RA and Vane JR. Co-induction of nitric oxide synthase and cyclooxygenase: interactions between nitric oxide and prostaglandins. **Br J Pharmacol** 114:1335-1342, 1995.

Synder GD, Yadagiri P and Falck JR. Effect of epoxyeicosatrienoic acids on growth hormone release from somatotrophs. **Am** J **Physiol** 256: E221-E226, 1989.

Taketo MM. Cyclooxygenase-2 inhibitors in tumorigenesis (part I). **J Natl Cancer Inst** (Bethesda) 90: 1529-1536, 1998 a.

Taketo MM. Cyclooxygenase-2 inhibitors in tumorigenesis (part II). **J Natl Cancer Inst** (Bethesda) 90: 1609-1620, 1998 b.

Talley JJ. Bertenshaw SR, Brown DL, Carter JS, Graneto MJ, Koboldt CM, Masferrer JL, Norman BH, Rogier DJ Jr, Zwwifel BS and Seibert K. 4,5 - Diaryloxazole inhibitors of cyclooxygenase-2 (COX-2). **Med Res Rev** 19: 199-208, 1999.

Tappel AL. In The enzymes (eds. Boyer PD, Lardy H, Myrback K) **Academic Press, New York.** 8: 275-283, 1963.

Terry MJ, Maines MD and Lagarias JC. Inactivation of phytochrome- and phycobiliprotein-chromophore precursors by rat liver biliverdin reductase. **J Biol Chem** 268: 26099-26106, 1993.

Thun MJ, Namboodiri MM and Heath CW Jr. Aspirin use and reduced risk of fatal colon cancer. N **Engl** J **Med** 325:1593-1596. 1991.

Thun MJ, Namboodiri MM, Calle EE, Flanders WD and Heath CW Jr. Aspirin use and risk of fatal cancer. **Cancer Res** 53:1322-1327,1993.

Tocco G, Freire-Moar J, Schreiber S, Sakhi S, Aisen P and Pasinetti G. Maturational regulation and regional induction of cyclooxygenase-2 in rat brain: implications for Alzheimer's disease. **Exp Neurol** 44: 339-349, 1997.

Towbin H, Staehelin T and Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. **Proc Natl Acad Sci USA** 76: 4350-4354, 1979.

Tsujii M and DuBois RN. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. **Cell** 83:493-501, 1995.

Tsujii M, Kawano S and DuBois RN. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. **Proc Natl Acad Sci USA** 94:3336-3340, 1997.

Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M and DuBois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. **Cell** 93: 705-716, 1998.

Tsujimoto Y and Shimizu S. Bcl-2 family: life-or-death switch. **FEBS Lett** 466:6-10,2000.

Tucker ON, Dannenberg AJ, Yang EK, Zhang F, Teng L, Daly JM, Soslow RA, Masferrer JL, Woerner BM, Koki AT and Fahey TJ. Cyclooxygenase-2 expression is up-regulated in human pancreatic cancer. **Cancer Res** 59: 987-990, 1999.

Turk J, Maas RL, Brash AR, Roberts LJ, and Oates JA. Arachidonic acid 15-lipoxygenase products from human eosinophils. **J Biol Chem** 257:7068-7076, 1982.

Ueda N, Hiroshima A, Natsui K, Shinjo F, Yoshimoto T, Yamamoto S, Ii K, Gerozissis K and Dray F. Localization of arachidonate 12-lipoxygenase in parenchymal cells of porcine anterior pituitary. **J Biol Chem.** 265: 2311-2316, 1990.

Vadiraja BB, Gaikwad NW and Madyastha KM. Hepatoprotective effect of C-phycocyanin: protection for carbon tetrachloride and R- (+)-pulegone-

mediated hepatotoxicity in rats. **Biochem Biophys Res Commun** 249:428–431, 1998.

Vadiraja BB and Madyastha KM. C-phycocyanin: a potent peroxyl radical scavenger in vivo and in vitro. **Biochem Biophys Res Commun** 275: 20-25, 2000.

van Leyen K, Duvoisin RM, Engelhardt H and Wiedmann M. A function for lipoxygenase in programmed organelle degradation **Nature** 395: 392-395, 1998.

Vanderhoek JY. Role of the 15-lipoxygenase in the immune system. **Ann NY Acad Sci** 524: 240-251,1988.

Vane J. Pharmacology: Towards a better aspirin. **Nature (Lond.)** 367: 215-216, 1994.

Vane JR and Botting RM. ***in*** Selective COX-2 inhibitors (Vane JR and Botting JH. Ed), **Kluwer Academic Publishers and William Harvey Press,** UK, pp. 1-18, 1998.

Vane JR, Bakhle YS and Botting RM. Cyclooxygenases 1 and 2. **Annu Rev Pharmacol Toxicol** 38: 97–120, 1998.

Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. **Nature (Lond.) New Biol** 231: 232-235, 1971.

Vane L. Differential inhibition of cyclooxygenase isoforms: An explanation of the action of NSAIDs. **J Clin Rheumatol** 4 (Suppl): S 3-S10, 1998.

Veera Reddy K, Hammarberg T and Radmark O. Mg^{2+} activates 5-lipoxygenase in vitro: dependency on concentrations of phosphotidylcholine and arachidonic acid. **Biochemistry** 39: 1840-1848, 2000.

Warburg C, Christian W. **Biochem J** 310: 384-402, 1941.

Watson AJ. Chemopreventive effects of NSAIDs against colorectal cancer: regulation of apoptosis and mitosis by COX-1 and COX-2. **Histol Histopathol** 13: 591-597, 1998.

Wolff H, Saukkonen K, Anttila S, Karjlainen A, Vainio H and Ristimaki A. Expression of cyclooxygenase-2 in human lung carcinoma. **Cancer Res** 58: 4997-5001, 1998.

Wu SH, Breshahan BA and Lianos EA. Hemodynamic role of arachidonate 12- and 15-lipoxygenases in nephrotoxic serum nephritis. **Kidney Int** 43: 1280-1285, 1993.

Wyllie AH, Kerr JF and Currie AR. Cell death: the significance of apoptosis. **Int Rev Cytol** 68: 251-306, 1980.

Xie W, Chipman JG, Robertson DL, Erikson RL and Simmons DL. Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. **Proc Natl Acad Sci USA** 88: 2692-2696, 1991.

Xie W, Robertson DL and Simmons DL. Mitogen-inducible prostaglandin G/H synthase: a new target for nonsteroidal anti-inflammatory drugs. **Drug Dev Res** 25: 249-265, 1992.

Yamagata K, Andreasson KI, Kaufman WE, Barnes CA and Worley PF. Expression of a mitogen-inducible cyclooxygenase in brain neurons; regulation of by synaptic activity and glucocorticoids. **Neuron** 11: 371-386, 1993.

Yamamoto S. Mammalian lipoxygenases: molecular structures and functions. **Biochim Biophys Acta Lipids Lipid Metab** 1128:117-131, 1992.

Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP and Wang X. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. **Science (Washington DC)** 275: 1129-1132, 1997.

Yoshimoto T, Suzuki H, Yamamoto S, Takai T, Yokoyama C and Tanabe T. Cloning and sequence analysis of the cDNA for arachidonate 12-lipoxygenase of porcine leukocytes. **Proc Natl Acad Sci USA** 87:2142-2146, 1990.

Zhang CW, Tseng CT and Zhang YZ. Purification and physiochemical properties of phycobiliprotein of *Spirulina platensis* var Nanjingensis. **Natural Product Research and Development** 8: 29-34, 1996.

Zimmermann KC, Sarbia M, Weber AA, Borchard F, Gabbert HE and Schror K. Cyclooxygenase-2 expression in human esophageal carcinoma. **Cancer Res** 59: 198-204, 1999.

Zipser RD, Nast CD, Lee M, Kao HW and Duke R. In vivo production of leukotriene B4 and leukotriene C4 in rabbit colitis: relationship to inflammation. **Gastroenterology** 92: 33-39, 1987.

Zou AP, Fleming JT, Flack JR, Jacobs ER, Gebremedhin D, Harder DR and Roman RJ. Stereospecific effects of epoxyeicosatrienoic acids on renal vascular tone and K (+)-channel activity. **Am J Physiol** 270:F822-F832, 1996.