C- Phycocyanin and Celecoxib, selective COX- 2 inhibitors, induce apoptosis in chronic myeioid leukemia cell line - K562

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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Dedicated
to
Almighty
&
Beloved Parents

University of Hyderabad

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HYDERABAD - 500 046, INDIA

DECLARATION

I hereby declare that the work embodied in this thesis entitled " ***C-Phycocyanin and Celecoxib, selective COX-2 inhibitors, induce apoptosis in chronic myeloid leukemia cell*** *line-K562 "* has been carried out by me under the supervision of Prof. P. Reddanna and this has not been submitted for any degree or diploma of any other university earlier.

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CERTIFICATE

This is to certify that **Miss. J. Subhashini** 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 her thesis " ***C-Phycocyanin and Celecoxib, selective COX-2 inhibitors, induce apoptosis in chronic myeloid leukemia cell line-K562*** " for submission for the degree of Doctor of Philosophy of this University.

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J. Subhashini

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ABBREVIATIONS

µCi	micro curie
µM	micro molar
^{o}C	degree centigrade/ degree celsius
AA	arachidonic acid
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair
COX	cyclooxygenase
cpm	counts per minute
C-terminal	carboxy terminal
DAPI	diamino phenyl indole
DNA	deoxy ribonucleic acid
EDTA	ethylene diamine tetra acetic acid
FACS	fluorescence activated cell sorter
FBS	fetal bovine serum
g	gram
h	hour(s)
kb	kilobase pair
kDa	kilodalton
l	litre
mg	milligram
min	minutes
ml	milliliter
mM	millimolar
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NBT	nitroblue tetrazolium
nm	nanometers
NSAIDs	non-steroidal anti-inflammatory drugs
N-terminal	amino terminal
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKC	protein kinase C
pmole	picomole
rpm	revolutions per minute
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylene diamine
Tris	tris-(Hydroxymethyl) aminoethane
UV	ultraviolet

Introduction

1.1 Cancers

Cancer may be a malady of genes, arising from genetic damage of diverse sorts recessive and dominant mutations, large rearrangements of DNA and point mutations, all leading to distortions of either the expression or biochemical function of genes (Armitage and Doll, 1954). Each alteration, whether an initiating or a progression association event, may be mediated through a gross chromosomal change and therefore has the potential to be cytogenetically visible. Three main cytogenetic changes have been detected in cancer cells: chromosomal deletions, inversions and translocations, with translocations being by far the most frequent (Rabbits, 1994; Rowley, 1999). Recurring chromosomal abnormalities have been identified in a variety of cancers more frequently with leukemias, lymphomas and sarcomas. At present, more than 500 recurring cytogenetic abnormalities have been reported in hematological malignancies, a frequency several times higher than that reported in mesenchymal and epithelial cancers, according to the Cancer Genome Anatomy Project/ Cancer Chromosome Aberration Project of the National Cancer Institute. In lymphoid leukemias and lymphomas, chromosomal translocations frequently lead to the transcriptional activation of proto-oncogenes by bringing their coding regions in the vicinity of immunoglobulin or T-cell receptor gene-regulating elements, thus leading to their inappropriate expression (Kuppers and Dalla-favena, 2001). In addition to proto-oncogene transcriptional activation, chromosomal translocations might cause gene fusions, as frequently observed in human leukemias.

Cancer occurs when cell division gets out of control. Usually, the timing of cell division is under strict constraint, involving a network of signals that work together to say when a cell can divide, how often it should happen and how errors can be fixed. Mutations in one or more of the nodes in this network can trigger cancer, be it through exposure to some environmental factor (e.g. tobacco smoke) or because of a genetic predisposition, or both. The predominant mechanisms for the cancers featured here are (i) impairment of a DNA repair pathway (ii) the transformation of a normal gene into an oncogene and (iii) the malfunction of a tumor supressor gene.

1.1.1 Different Kinds of Cancer

Cancer can originate almost anywhere in the body.

Carcinomas the most common types of cancer arise from the cells that cover external and internal body surfaces. Lung, breast, and colon are the most frequent cancers of this type in the United States of America.

Sarcomas are cancers arising from cells found in the supporting tissues of the body such as bone, cartilage, fat, connective tissue, and muscle.

Lymphomas are cancers that arise in the lymph nodes and tissues of the body's immune system.

Leukemias are cancers of the immature blood cells that grow in the bone marrow and tend to accumulate in large numbers in the bloodstream.

1.1.2 What Causes Cancer?

Cancer is often perceived as a disease that strikes for no apparent reason. This is because scientists don't know all the reasons. But many of the causes of cancer have already been identified. Besides heredity, scientific studies point to the existence of three main categories of factors that contribute to the development of cancer: chemicals (e.g., from smoking or diet), radiation, and viruses or bacteria.

1.1.3 *Genes* and Cancer

Chemicals (e.g., from smoking), radiation, viruses, and heredity all contribute to the development of cancer by triggering changes in a cell's genes. Chemicals and radiation act by damaging genes, viruses introduce their own genes into cells, and heredity passes on alterations in genes from one generation to the next. These altered or mutated genes make a person more susceptible to cancer.

1.1.4 Cancer Prevention

Since exposure to carcinogens (cancer-causing agents) is responsible for triggering most human cancers, people can reduce their cancer risk by taking steps to avoid such agents. Hence the first step in cancer prevention is to identify the behaviors or exposures to particular kinds of carcinogens and viruses that represent the greatest cancer hazard.

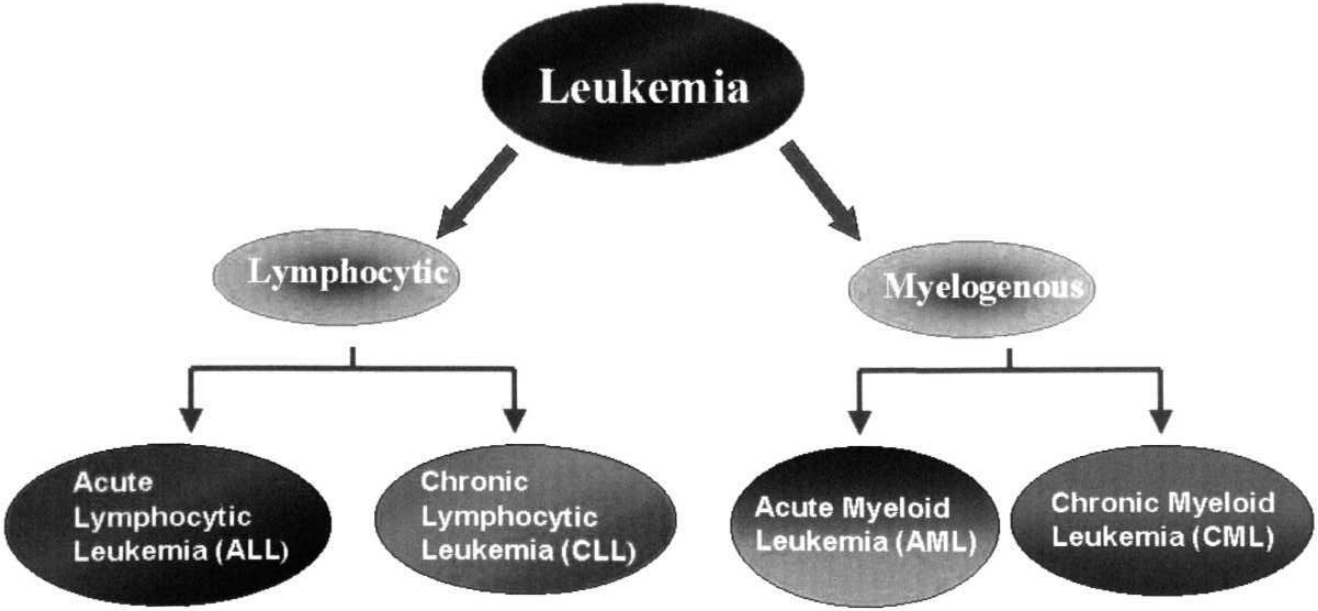
1.2 Leukemia

1.2.1 Types of leukemia

Leukemia, the term coined by Virchow is a cancer of white blood cells. Leukemia occurs when a white blood cell, whose development is frozen, continues to duplicate itself. The resulting progeny of cells are all in the same stage of development and bear the distinctive hallmarks of the type of ancestral white blood cell that gave rise to them. Based on this understanding, by 1900 leukemia was no longer seen as a single disease. Instead it was imagined akin to a tree with two main limbs that inturn have two primary branches, all of which reflect from what type of cell the leukemia originates (Fig.1). One limb, myelogenous leukemia, has its hallmark in the blood and bone marrow either a predominance of immature myeloblasts (acute myeloid leukemia-AML) or mature myeloid cells (chronic myeloid leukemia-CML). With the other limb, lymphocytic leukemia, the blood and bone marrow is over populated by either precursor B or T cells (acute lymphocytic leukemia-ALL) or mature B or T cells (chronic lymphocytic leukemia-CLL).

Despite their divergent origins, all types of leukemias share the same set of symptoms, just as fever and congestion can be the symptoms for a number of different microbial infections. When people develop leukemia, their abnormal white blood cells crowd out or hamper the functioning of their red blood cells, fostering the tiredness and paleness that are the hallmarks of anemia. These white blood cells also do not effectively fight infections, which can be fatal. A lack of functioning cells that clot blood platelets makes people with leukemia prone to

Fig. 1: Classification of leukemia



life-threatening bleeding episodes. Leukemia cells that congregate at various spots in the body can also spur a variety of other symptoms, including bone or joint pain, or enlarged organs. Untreated acute leukemia progresses rapidly to death, whereas untreated chronic leukemia can be longer lasting, although just as deadly in the long run. Although leukemia afflicts both adults and children, by the 1920's it was recognized that the disease mostly attacks children. In 1930 it was considered a relatively rare disease. But by 1960, statistics collected in Great Britain revealed that leukemia had become the second leading cause of death in children.

1.2.2 Chronic myeloid leukemia

Chronic myeloid leukemia (CIVIL) is a cancer of granulocytes (one of the main types of white blood cells). These leukemia cells fill up the bone marrow and thus lead to an increased risk of infection. The disease usually develops very slowly, which is why it is called 'chronic' myeloid leukemia. Chronic myeloid leukemia can occur at any age, but affects more commonly middle-aged and older people. It is rare in children.

It is mainly divided into two phases.

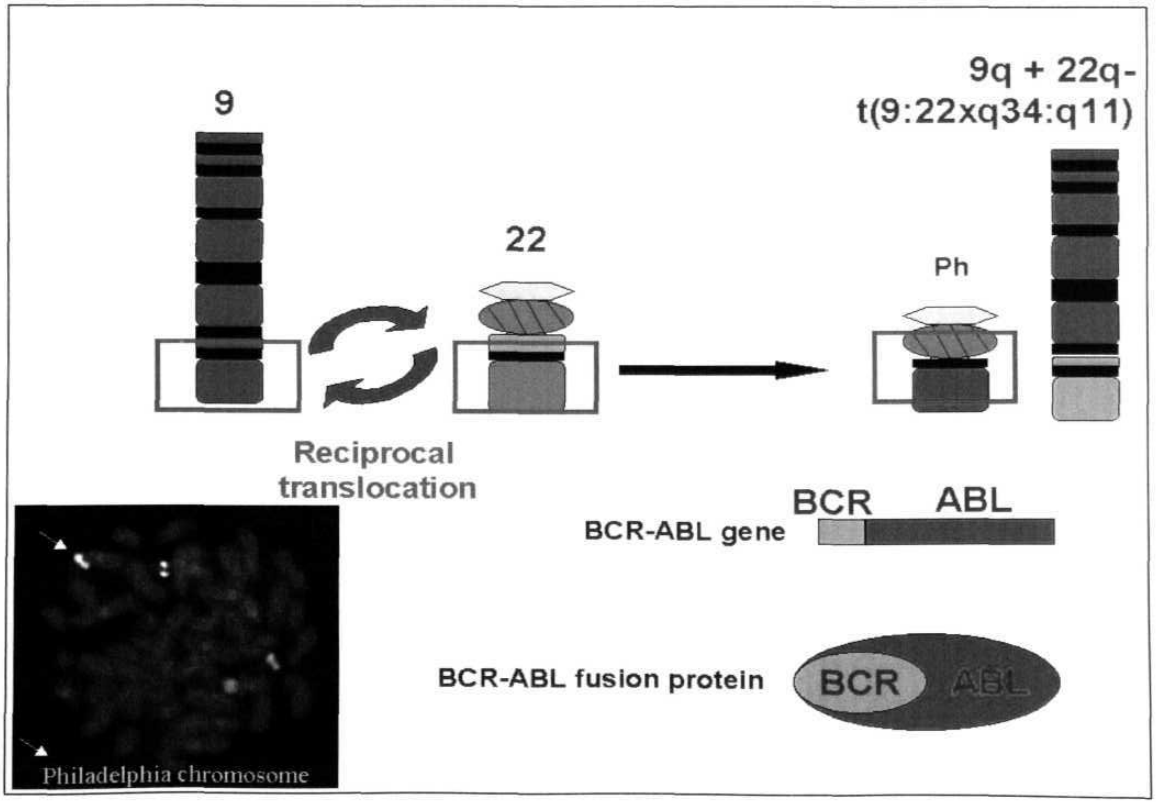
i. *The chronic phase:* The phase when most people are diagnosed. At this phase CML progresses very slowly and is often stable for long periods. It is also called as "stable phase" as it lasts on an average of about 4-5 years.

ii. *The accelerated and blast phase (advanced phase):* Leukemia gradually develops into the accelerated phase, during which the disease develops more quickly. During this phase immature cells (blast cells) fill up the

bone marrow. After some months leukemia transforms into blast phase, which is more like an acute leukemia.

CML is a disorder characterized by a massive expansion of progenitor cells in all stages of maturation (Daley *et ai,* 1990). CML is associated with the Philadelphia chromosome (Ph), a cytogenic abnormality generated by a reciprocal translocation between the bcr gene (break point cluster region) on chromosome 22q and the c-abl (ableson leukemia virus) protooncogene on chromosome 9q (Fialkow *et ai,* 1997; Caspersson *et ai,* 1970; Rowley, 1973; Heisterkamp *et ai,* 1983; Groffen *et ai,* 1984). The fusion gene produces a chimeric 8.5 kb transcript that codes for the p210 $^{bcr\text{-}abl}$ protein (Shlivelman *et a/.*, 1985) (Fig. 2). Bcr-abl signaling causes transformation through several mechanisms (Coretz *et ai,* 1997; Salgia *et ai,* 1997). It has been postulated that the altered tyrosine kinase activity of p210 $^{bcr\text{-}abl}$ stimulates uncontrolled cell proliferation, leading to the massive clonal expansion of hematopoietic progenitors detected in CML (Stryekmans *et ai,* 1976; Eaves *et ai,* 1986). Recent studies revealed that CML progenitors have similar proliferation rates to their normal counterpart and that p210 $^{bcr\text{-}abl}$ increases cell survival by inhibiting apoptosis (Bedi *et ai,* 1994). Thus, p210 $^{bcr\text{-}abl}$ may act through an anti-apoptotic mechanism (McGahon ef *ai,* 1994). Furthermore, inhibition of bcr-abl kinase activity by the tyrosine kinase inhibitor, genestein, induced inhibition of cell growth associated with apoptosis (Carlo-Stella ef *ai,* 1996). However, a normal reaction of CML cells to death-inducing stimuli has also been observed (Amos ef *ai,* 1995).

Fig. 2: Molecular Genetics of CML



1.2.3 Diagnosis

In the analysis of bone aspirate, if more than 5 % of cells in the preparation are myeloblasts, this indicates CML. The type of blast, lymphoblast vs. myeloblast, is usually determined primarily by morphology, with cytochemistry and immunophenotyping used as supportive evidence. CML can be diagnosed from a peripheral blood smear, but the bone marrow is usually examined for confirmation. The blood smear will show a raised white blood cell count (30,000-400,000/uL), granulocytes at all stages of development, increased eosinophils and basophils, blast cells, and an elevated platelet count (300,000-600,000). Bone marrow will show increased cellularity, a relatively low red blood cell count, and small megakaryocytes. The patient's lactate dehydrogenase levels in serum will also be high. A cytogenic study will show the presence of the Ph chromosome.

1.2.4 Treatment

The treatment of CML is primarily based on the phase of the disease. The chronic phase will be treated in a slightly different way than the accelerated or blastic phases. The treatment of chronic myeloid leukemia will depend on the stage or phase of the illness. In the chronic phase the aim of treatment is usually to control the condition, often for several years. Treatment can be a drug called interferon alpha, which is given as a small injection under the skin, a drug called imatinib (Glivec) or chemotherapy tablets. Sometimes a combination of interferon and chemotherapy may be used. A bone marrow or stem cell transplant may be a suitable treatment for some patients and can cure leukemia in some people.

This is more likely to be possible in younger patients who have a brother or sister whose bone marrow is a close match to their own.

Over the last 10 years it has been proposed that understanding signal transduction pathways and how they go awry, would change thinking of medical community about disease therapy in general and cancer therapy in particular (Levitzki, 1990, 1992, 1994, 1995; Levitzki and Gazit, 1995). Researches suggested that signal transduction therapy would become an important therapeutic modality. Today, there are a number of new agents (signal transduction inhibitors) in clinical development. Imatinib (STI571, Glivec) is a small molecule drug selected for its ability to inhibit the Bcr-Abl kinase, the pathogenic molecular abnormality in chronic myelogenous leukemia (CML). It also is an efficient inhibitor of the Kit and platelet-derived growth factor receptor tyrosine kinases. *In vitro* studies have demonstrated that this drug potently inhibits proliferation and induces apoptosis of cells that depend on activation of these kinases. Phase I clinical studies have demonstrated remarkable activity against CML. However, the emergence of resistance, particularly in patients with acute leukemias, has propmpted immense research, and many are concerned about the future prospects of imatinib.

The continuous CML cell line K562, used in the present study, was established by Lozzio & Lozzio (1975) from the pleural effusion of a 53- year old female with chronic myelogenous leukemia in terminal blast crisis. The cell population has been characterized as highly undifferentiated and of the granulocytic series. K562 blasts are multipotential, hematopoietic malignant cells

that spontaneously differentiate into recognizable progenitors of erythrocytic, granulocytic and monocytic series.

1.3 Nonsteroidal anti-inflammatory drugs as chemopreventives

1.3.1 Epidemiological evidences

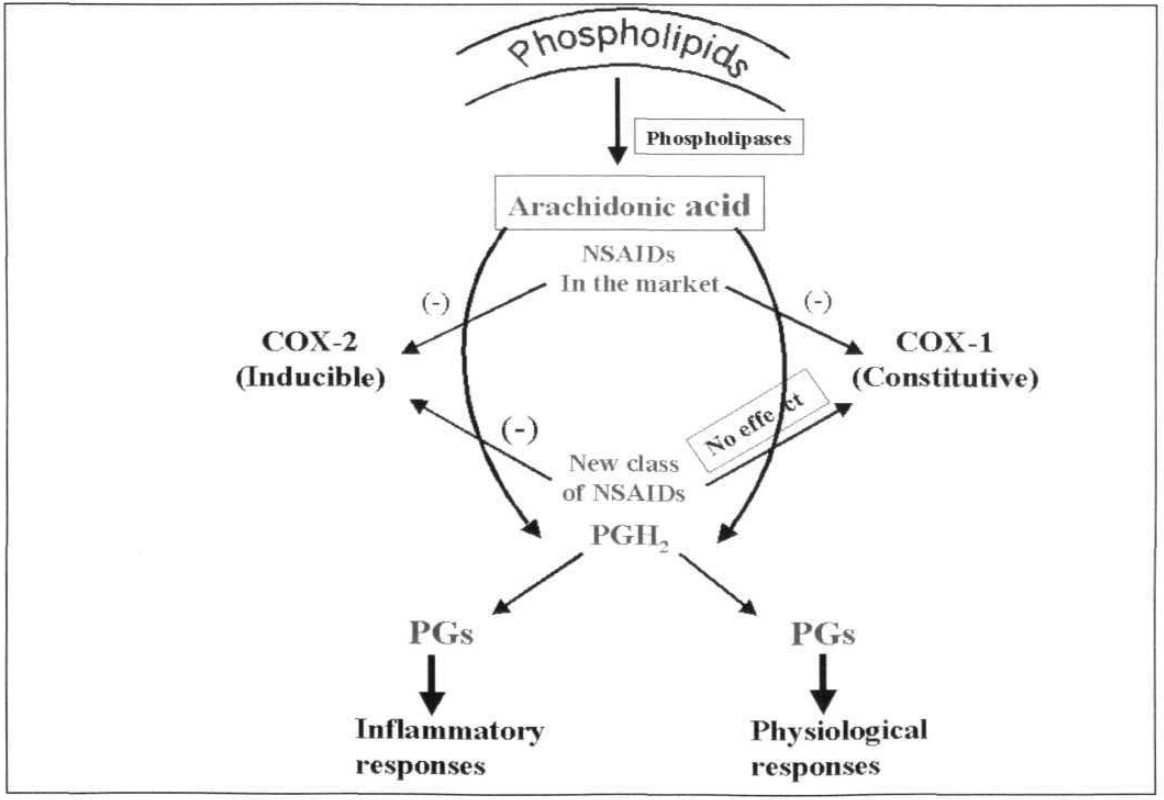
Epidemiological studies have shown that chronic intake of aspirin is associated with a reduction in the incidence of colorectal cancer (Thun *et* a/., 1991; Giovannucci *et* a/., 1995). In a clinical trial, Celecoxib (Steinbach ef a/., 2000) was found to reduce the number and size of polyps in patients with familial adenomatous polyposis (FAP). The effects of several non-steroidal anti-infflammatory drugs (NSAIDs) on tumor growth have also been demonstrated in animal models of FAP (Oshima *et* a/., 2001) and chemical colon carcinogenesis (Takahashi *et* a/., 1996; Kawamori *et al.*, 1998; Yoshimi *et* a/., 1999; Kishimoto *et* a/., 2000). These observations suggest that NSAIDs have a potent chemopreventive effect.

1.3.2 How do NSAIDs mediate their antineoplastic activity?

NSAIDs inhibit the cyclooxygenase (COX) enzymes, COX-1 and COX-2, which catalyse the conversion of arachidonic acid to prostaglandins. COX-1 is expressed constitutively and is required for physiological processes such as maintenance of gastrointestinal mucosa and platelet aggregation, whereas COX-2 is induced by cytokines, growth factors, and mitogens. NSAIDs vary in their abilities to inhibit COX-1 and COX-2 (Thun ef a/., 2002). Classic NSAIDs not only inhibit COX-2, but also inhibit COX-1, resulting in the common side effect of gastric mucosal damage. To reduce the gastrointestinal side effects of NSAIDs,

selective COX-2 inhibitors were developed (Jackson and Hawkey, 2000) (Fig. 3). Selective inhibitors of COX-2 have been demonstrated to induce apoptosis in a variety of cancer cells, including those of colon (Hara *et* a/., 1997), stomach (Sawaoka *et* a/., 1998), prostate and breast (Liu *et* a/., 1998). These observations are consistent with the cancer chemopreventive effects of NSAIDs. The biochemical mechanism underlying COX-2 inhibitor induced apoptosis, however, remains elusive. Tumour inhibition by NSAIDs may be mediated by distinct cellular processes. These processes involve the ability of NSAIDs to restore apoptosis, induce cell-cycle arrest, and inhibit angiogenesis (Chan, 2002; Thun ef a/., 2002). One of the main ways by which NSAIDs exert their effects is modulation of apoptosis, although there is considerable debate about how these effects are mediated. Because COX-2 expression is increased in up to 90% of sporadic colon carcinomas and 40% of adenomas, but not in normal colonic mucosa, NSAIDs were presumed to mediate apoptosis via COX-2 inhibition. The relevance of COX-2 for adenoma formation was genetically demonstrated by a reduced number of adenomas in APCmin/+ mice, the mouse model for FAP, with an additional targeted deletion of COX-2 (Oshima *et* a/., 1996). However, compounds that do not inhibit COX-2, such as sulindac sulphone, also induce apoptosis *in vitro* and inhibit colorectal carcinogenesis in animal models. In addition, low dose aspirin, which has virtually no COX-2 inhibitory effects, had a chemopreventive effect (81 mg) in individuals at increased risk for developing colorectal cancer (Baron, 2003). COX-independent mechanisms are also suggested by the finding that some NSAIDs inhibit proliferation and induce cell death in cells that do not express COX-1 and COX-2 (Hanif *et* a/., 1996).

Fig. 3: Cyclooxygenase-2: the target for new class of NSAIDs



Compounds that are structurally similar to NSAIDs, but do not inhibit COX, also have chemopreventive and proapoptotic properties. COX-dependent and COX-independent mechanisms of apoptosis induction are not mutually exclusive, and it is likely that both have a role in the biological activity of NSAIDs (Fig. 4).

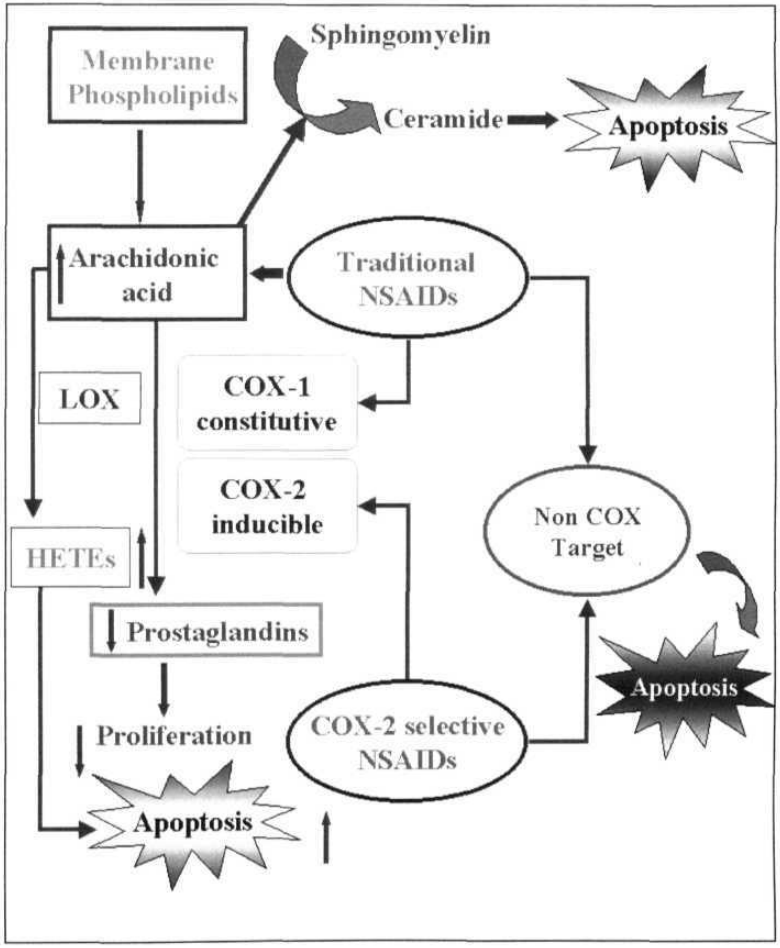
However, the issue of whether COX-2 expression is exclusively responsible or even rate limiting for chronic inflammation-related cancer promotion is not resolved. The precise mechanisms by which chronic inflammation stimulates cancer development are not fully understood and are likely to be complex and multifactorial (Coussens and Werbz, 2002). Indeed, NSAIDs have been shown to inhibit other pathways that contribute to inflammation. For example, NSAIDs inhibit activation of NF-kB, which controls the transcription of a variety of proinflammatory cytokines, independently of COX inhibition (Tegeder *et a*/., 2001). It is therefore quite possible that NSAIDs inhibit inflammation stimulated carcinogenesis by affecting the function of these different pathways in addition to those of COX-1 or COX-2. However, because these agents are potent inhibitors of COX catalytic activity, it might be difficult to judge the relative contribution of these COX and non-COX mechanisms.

1.4 Cyclooxygenases

1.4.1 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 *et a*/., 1996), and also in their

Fig. 4: POSSIBLE MECHANISMS OF NSAIDs INDUCED APOPTOSIS



intracellular localization. COX-2 accepts a wider range of fatty acids as substrates when compared to 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 of a single methyl group. This substitution allows COX-2 inhibitors to access the secondary internal side pocket of the molecule that is obstructed by isoleucine in the COX-1 isoform (Kurumbail *et* a/., 1996; Vane, 1998). The COX-2 active site is about 20 % larger and has a slightly different shape than that of COX-1 (Luong ef a/., 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 *et* a/., 1994; Anderson *et* a/., 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" 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 72 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). In certain cell lines a 90 kDa band is seen and is due to post-translational modifications (DeWitt and Smith, 1988). 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 22 kb 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 a continuosly 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 1).

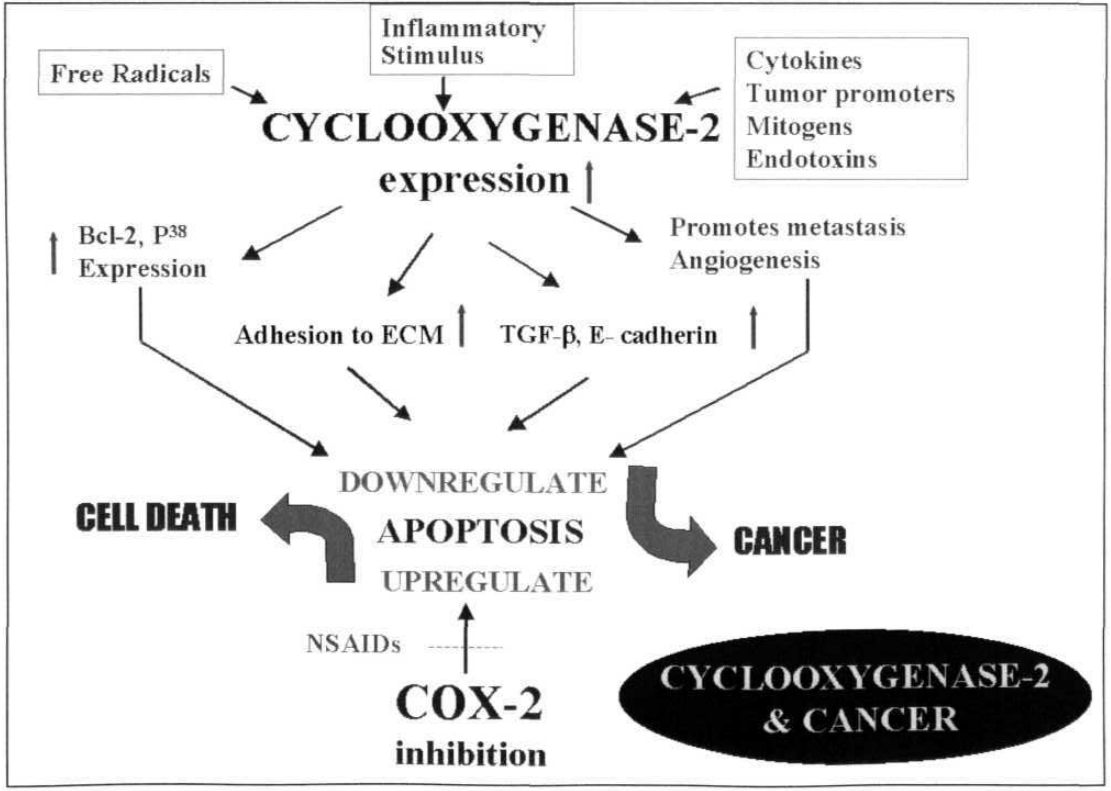
1.4.2 *COX-* 2 inhibitors *& Cancer* treatment

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 *et* a/., 1993; Simon and Smith, 1998). 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* a/., 1994; Ristimaki *et* a/., 1997; Zimmermann *et* a/., 1999; Wolff *et* a/., 1998; Tucker *et* a/., 1999) (Fig. 5). 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* a/., 1998). Human gastric and breast (Brown and Lipmann, 2000) tumors express higher levels of COX-2 than surrounding normal tissue. The importance of PGs in tumorigenesis is evidenced by the data demonstrating the ability of the NSAIDs to inhibit growth and metastasis of tumors *in vivo* (Snyderman *et* a/., 1995) and most notably, for colon cancer (Rao *et* a/., 1991; Sheng *et* a/., 1997). Several population based studies have detected a 40-50 % decrease in relative risk for colorectal cancer in persons who regularly use NSAIDs (Thun *et* a/., 1993: Smalley and DuBois, 1997). Two selective COX-2 inhibitors, Celecoxib and Rofecoxib, have received approval from the Food and Drug Administration (FDA) in the United States. The cancer preventive actions of COX-2 inhibitors may

Table 1: Comparison of cyclooxygenase enzymes

Property	COX-1	COX-2
Cellular expression	Constitutive in most tissues in particular, stomach, kidney, monocytes/macrophage and 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)
Relative size of active site	< COX-2	>COX-1
Effect of glucocorticoids	Little or none	Inhibits expression
Endogenous inducers	~	Growth factors, Oncogene expression
Endogenous inhibitors	-	Glucocorticosteroids IL-1
Patho/physiological activity	Cytoprotective	Inflammatory

Fig. 5: Role of Cyclooxygenase-2 in Cancer



involve inhibition of angiogenesis (Tsujii *et a*/,, 1998) or modulation of mitosis and apoptosis (Watson, 1998).

1.4.3 C-Phycocyanin

Chemoprevention may be an effective way to reduce cancer risk. Natural products have been the mainstay of cancer chemotherapy for the past 30 years. Blue-green algae are the most primitive life forms on earth with nutrient-dense, edible forms like Nostoc, Spirulina, Aphanizomenon species etc. Spirulina is non-nitrogen fixing blue-green algae with over 30 years long history of safe human consumption. Spirulina is gaining attention as a nutraceutical and source of potential Pharmaceuticals. Spirulina with 62% protein content is the world's richest source of Vitamin B-12. It is also rich in beta-carotene and other carotenoids, Vitamin E, minerals (e.g. manganese, copper, iron, zinc, selenium), trace minerals (e.g. selenium) and essential fatty acid (e.g. gamma-linolenic acid). Recent studies have demonstrated antioxidant (Miranda ef a/., 1998), antimutagenic (Chamorro ef a/., 1996), antiviral (Ayehunie ef a/., 1998; Hayashi ef a/., 1996), anticancer (Mishima ef a/., 1998; Chen and Zhang, 1995; Schwartz et a/, 1998, Schwartz and Shklar, 1987), anti-allergic (Kim *et a*/., 1998;Yang ef a/., 1997), immune enhancing (Hayashi ef a/., 1994; 1998), hepato-protective (Torres-duran et a/., 1998; Gonzalez de rivera ef a/., 1993), blood vessel relaxing (Parades-carbajal ef a/., 1997) and blood lipid-lowering effects (Iwata ef a/., 1990 ; Kato ands Takemoto, 1984) of Spirulina extracts. The biological and pharmacological properties of Spirulina were attributed mainly to calcium-spirulan and C-Phycocyanin (C-PC) (Hayashi ef a/., 1996). However, C-PC

gained more attention because of its anti-cancer activity and is believed that it might enhance the body's immunity (Zhang *et a*/., 1994) to fight against diseases.

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). Phycocyanin, allophycocyanin, phycoerythrin and phycoerythrocyanin are the principal phycobiliproteins. C-PC consists of a- and β-subunit polypeptides to which one or more linear tetrapyrrole chromophores are covalently attached (Fig.6). Phycocyanobilin is the chromophore of allophycocyanin and phycocyanin. In both proteins the chromophores are attached by cysteinyl thioether linkages at α-84 and (3-84 positions; in phycocyanin a second Phycocyanobilin is found attached to cysteine β-155 in a loop not found in allophycocyanin. Phycobiliproteins are stable at 2-5°C as ammonium sulfate precipitates. Purified biliproteins may dissociate into subunits under acidic or basic conditions, but are relatively stable at room temperature and 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 convenient markers in such applications as gel electrophoresis, isoelectric focusing and gel exclusion chromatography.

Spirulina platensis also called as *Arthrospira platensis* is unicellular filamentous blue green algae. C-PC, the water-soluble non-toxic biliprotein pigment isolated from *Spirulina platensis* has significant antioxidant (Romay *et* a/., 1998 a; Romay and Gonzalez, 2000) and radical scavenging properties (Vadiraja and Madyastha, 2000). Phycocyanin was shown to inhibit inflammation

in mouse ears (Romay ef *al.*, 1998 a, b: Gonzalez *et al.,* 1999) and prevent acetic acid induced colitis in rats (Gonzalez *et al.,* 1999). C-PC is used for the treatment of diseases such as Alzheimer's and Parkinson's (Rimbau *et al.,* 1999; 2001) and prevents experimental oral and skin cancers (Morcos and Henry, 1992). Of major interest to ongoing research in inflammation as well as cancer is the finding that C-PC selectively inhibits Cyclooxygenase-2 (Reddy *et al.,* 2000). Recently we have reported that C-PC induces apoptosis in mouse macrophage cell line RAW 264.7(Reddy ef a/., 2003) and rat histiocytoma cell line AK5 (Pardhasaradhi *et al.*, 2003).

1.4.4 Celecoxib

Celecoxib (Celebrex also known as SC-58635) (4-[5-(4-methylphenyl)-3-trifluoromethyl] 1 H-pyrazol-1-yl) benzene sulfonamide) is a 1,5-diaryl substituted pyrazole (Fig.7) (Penning ef *al.,* 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 chemopreventive properties against colon cancer. Peak plasma concentrations occur approximately three hours after an oral dose and, when taken with a high-fat meal, peak concentrations 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 ef *al.,* 1997), which is larger than expected V/F when compared to other NSAIDs, and probably relates to the lipophilic nature of

Fig. 6: **Structure of C-Phycocyanin**

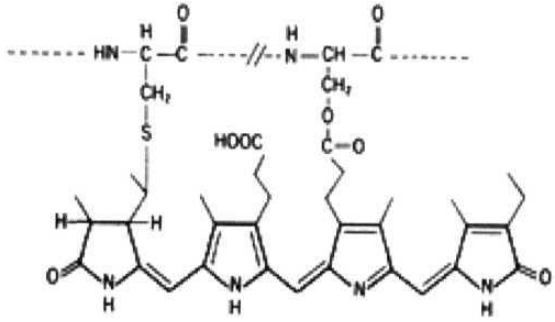
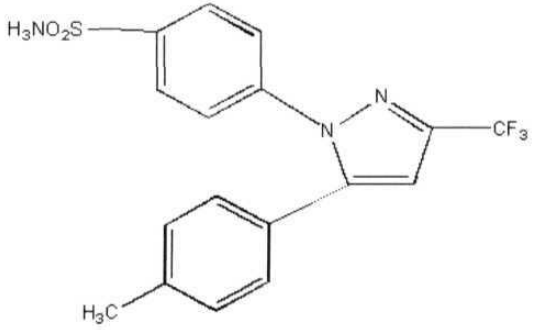


Fig. 7: **Chemical structure of Celecoxib**



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 of drugs metabolized by this isoenzyme, such as some (3-blockers, antidepressants and antipsychotics. Celecoxib is well tolerated, with an adverse effect profile similar to placebo. The most commonly reported adverse effects include headache, diarrhoea, rhinitis, nausea, sinusitis, dyspepsia and abdominal pain.

1.5 Apoptosis

1.5.1 Definition

As much as the definition of life may be controversial, the definition of death also may prove problematic. The death of a living cell may result from an external physical injury or it may be an outcome of activating an internal pathway for cell suicide i.e. programmed cell death (PCD). PCD was coined initially to explain the cell death occurring during development, whereas apoptosis was used to describe cell death that exhibits a set of morphological features. The process of cell death and apoptosis are distinct, however, apoptosis often has been used interchangeably with programmed cell death.

Cell death occurs either by necrosis or apoptosis. Necrosis results from physical injury, metabolic block, mutations, toxic substances etc., and not genetically controlled. It is characterized by cell swelling, mitochondrial dilation, dissolution of other organelles, non-caspase proteolytic cascades depending on

serine proteases, calpains or cathepsins, plasma membrane rupture and spillage of the cytoplasmic contents ultimately leading to the inflammatory response (Denecker *et* a/., 2001). By contrast, apoptosis is a genetically controlled process exhibiting constellation of structural and functional changes including calcium flux, cytochrome c redistribution, caspase activation, loss of plasma membrane asymmetry, reduction in cell volume, selective proteolysis of a subset of cellular proteins, chromatin condensation, nucleosomal DNA fragmentation and ultimately breakdown into apoptotic bodies that are rapidly phagocytosed (Desagher and Martinou, 2000) (Table. 2).

1.5.2 Regulation of Apoptotic pathways

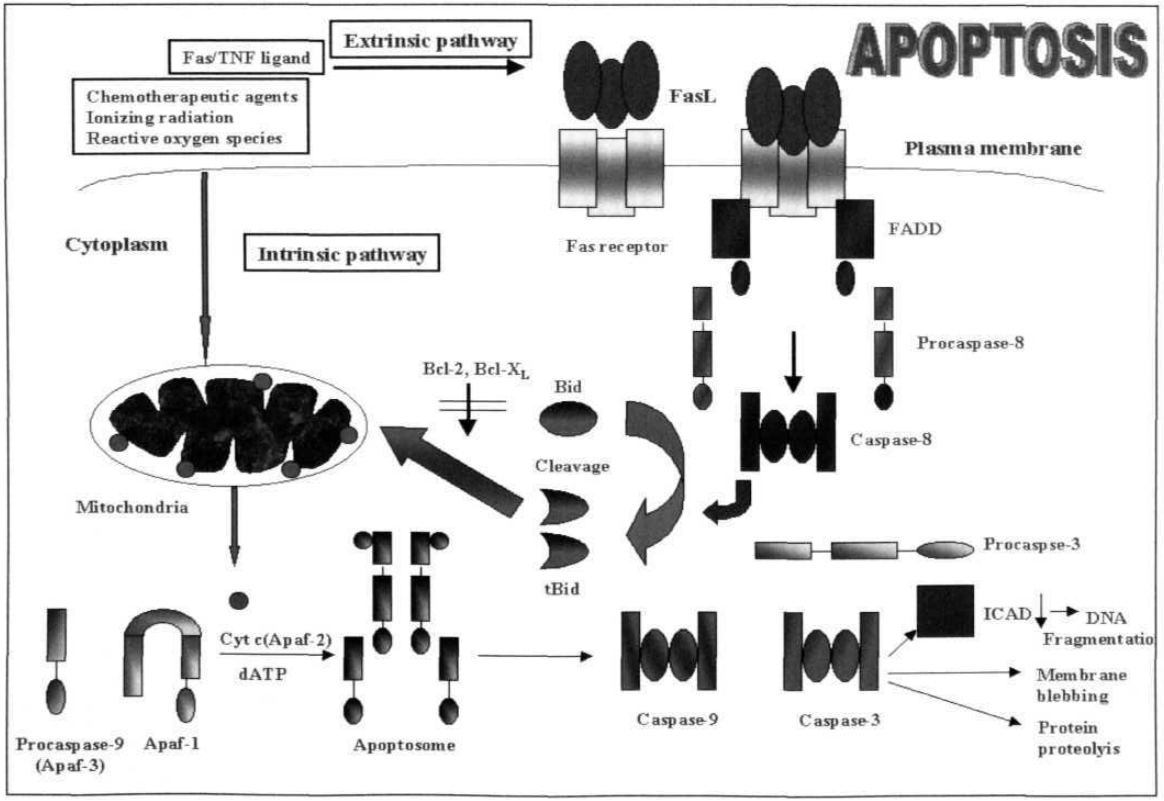
The genetically conserved intrinsic cell suicide program can be divided into three phases. i. Initial phase ii. Effector phase and iii. Execution phase. The initial phase comprises the exposure of healthy cells to various extrinsic/ intrinsic death signals including physiological, genetic and environmental factors that trigger apoptosis (Olie *et* a/., 1998). During the effector phase, release of cytochrome c from the mitochondrial compartment into cytosol occurs, which is regulated by the Bcl-2 family proteins (Thornberry and Lazebnik, 1998). Execution phase is characterized by the activation of constitutive caspases in the presence of cytochrome c and the activated caspases play crucial role in the proteolysis of specific proteins resulting in the apoptotic phenotype (Kidd *et* a/., 2000).

There are three distinct apoptotic pathways that exist in the cell. Of these, the two major pathways i.e. the extrinsic one activated through ligand - dependent death receptor oligomerization (Fig. 8), the intrinsic pathway acting

Table 2: Differences between apoptosis and necrosis

Apoptosis	Necrosis
Morphological features	
Membrane blebbing, but no loss of integrity Aggregation 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 small 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	No energy requirement
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 neighbouring cells or macrophages	Phagocytosis by macrophages
No inflammatory response	Significant inflammatory response

Fig. 8: An Overview of Apoptotic Signaling Pathways



through mitochondrial involvement and the third minor one induced through stress-mediated events involving the endoplasmic reticulum have been identified. (Thornberry, 1998). These pathways may interact and amplify weak apoptotic signals and shorten cellular execution time.

The intrinsic apoptotic cascade involves formation of "apoptosome" consisting of Apaf-1, Apaf-2 (cytochrome c) and Apaf-3 (procaspase-9) (Chinnaiyan, 1999). The binding of dATP to Apaf-1 induces the formation of a multimeric Apaf-1 that interacts with cytochrome c and procaspase-9. Apoptosome formation leads to the activation of caspase-9, which inturn activates the downstream caspases including caspase-3 that orchestrate the biochemical execution of cells. The extrinsic apoptotic pathway involves the ligand-receptor interactions triggering the activation of proteases whose actions culminate in the destruction of cell structure. A complex containing several components forms at the receptor. Fas ligand activates Fas receptor on the cell membrane followed by FADD (Fas associated death domain) recruitment; TNF receptor (TNF R1) activation leads to TRADD (Tumor necrosis factor receptor associated death domain) binding which in turn interacts with FADD and in either case, FAD binds caspase-8 with a death domain as well as protease activity and may trigger a common pathway (Chang and Yang, 2000).

The death signals mediated by Fas/TNF-R1 receptors usually activate caspases directly, bypassing the need for mitochondria. However, Bid (a pro-apoptotic Bcl-2 family protein) is cleaved by caspase-8 in response to Fas/TNF-R1 death receptor signals (Yin, 2000) and translocated to miochondria and induces cytochrome c release, which inturn activates the downstream caspases.

Such a connection between the two-apoptotic pathways could be important for induction of apoptosis in certain types of cells and is responsible for the pathogenesis of a number of human diseases.

1.6 Molecular markers of apoptosis

1.6.1 Cytochrome c

The complex role of mitochondria in mammalian cell apoptosis came into focus when biochemical studies identified several mitochondrial proteins that are able to activate cellular apoptotic programs directly. Normally, these proteins reside in the intermembrane space of mitochondria. In response to a variety of apoptotic stimuli, they are released into the cytosol and (or) the nucleus. They promote apoptosis either by activating caspases and (or) by neutralizing cytosolic inhibitors of this process.

Cytochrome c is encoded in the nucleus as a procytochrome c, and undergoes transport across the outer membrane of the mitochondria, where it combines with heme to become the mature protein. How cytochrome c crosses the outer membrane of mitochondria is enigmatic, thus providing no clues to how holo-cytochrome c might escape. Atleast two competing theories have been proposed. First, a strong correlation has been established between the phenomenon of mitochondrial permeability transition (PT) and apoptosis (Petit *et al.,* 1996). The PT pore remains poorly characterized but appears to consist of several proteins located in both the inner and outer mitochondrial membranes that collaborate with each other at the contact sites where these two membranes come into close apposition. The swelling that follows PT pore opening causes

rupture of the outer membrane, because the surface area of inner membrane with its cristae is considerably larger than the surrounding outer membrane. Thus, one model of how cytochrome c escapes from mitochondria envisions outer membrane rupture as the culprit, occurring as a secondary consequence of PT pore opening. A second model predicts a specific channel located in the outer membrane that allows release of cytochrome c. Moreover, the antiapoptotic proteins Bcl-2 and Bcl-x_L, which reside in the outer mitochondrial and some other intracellular membranes, prevent release of cytochrome c and activation of caspase-3, while protecting cells from death.

1.6.2 PARP (Poly (ADP-ribose) Polymerase)

Apoptosis is a mechanism by which a cell commits suicide in response to differential stimuli like DNA damage, signals from neighboring cells, or extracellular chemical signals. The DNA repair enzyme poly (ADP-ribose) polymerase (PARP), also known as poly (ADP-ribose) synthetase or poly (ADP-ribose) transferase (PADRT), has emerged as a major player along the continuum of cell death. Among the first responses of a cell to DNA damage is the synthesis of poly (ADP-ribose) by the ploy (ADP-ribose) polymerase (PARP, PARP-1 EC 2.4.2.30).

PARP plays a multifunctional role in many cellular processes, including DNA repair, recombination, cell proliferation and apoptosis, as well as genomic stability (Menissier-de *et al*., 1994; Jeggo, 1998; Szabo and Dawson, 1998; Pieper *et al.,* 1999; Herceg and Wang, 2001). PARP-1 has an estimated molecular weight of 116 kDa and is organised in three major functional domains: a 42 kDa DNA binding domain (DBD) located at the N-terminus, a 16 kDa central

fragment, bearing 15 potential automodification sites, and a 55 kDa catalytic and nucleotide binding domain in the c-terminal fragment. DNA binding domain contains two zinc finger motifs and a nuclear localization signal. This region recognises both the double and single stranded DNA breaks in a nonsequence dependent manner (Menissier-de *et ai,* 1989) through the first and second zinc fingers respectively (Gradwohl *et ai,* 1990).

PARP is efficiently cleaved and inactivated in programmed cell death into a 24 kDa fragment containing N-terminal DNA binding domain and an 89 kDa peptide comprising the central automodification domain and the C-terminal NAD^+ binding and catalytic domains. The site of PARP cleavage (DEVD213G in human PARP, $DEVE_{216}$ in bovine PARP) is located within the nuclear localization signal and is highly conserved. Activation of cytosolic proteases and a fairly specific degradation of proteins, including PARP, are important for apoptosis (Pettmann and Henderson, 1998). PARP cleavage may interfere with its key homeostatic function as a DNA double-strand break repair enzyme (Casciola-Rosen *et ai,* 1996), which might facilitate or allow the DNA degradation characteristic of apoptosis (Cohen, 1997).

1.6.3 Internucleosomal DNA fragmentation

The biochemical hallmark of apoptosis is degradation of DNA by endogenous DNases, which cut the internucleosomal regions into double-stranded DNA fragments of 180-200 base pairs (bp) (Wyllie ,1980). The DNA fragments contain blunt ends (Alnemri and Litwack, 1990) as well as single 3^1 overhangs (Didenko and Hornsby, 1996). Internucleosomal fragmentation has been demonstrated in a wide variety of situations and cell types. A variety of

caspase substrates are involved in the regulation of DNA structure, repair and replication (Nicholson and Thornberry, 1997). The DNase enzyme responsible for the fragmentation during apoptosis include DNA fragmentation factor (DFF 40) (Liu *et al.*, 1998) caspase activated DNase (CAD) (Enari *et al.* , 1998; Sakahira *et al.,* 1998) and in hematopoietic cells NUC70 (Urbano *et al.,* 1998). DFF40 and CAD are present in normal cells as inactive heterodimers with the inhibitor proteins DFF45 (Liu *et al.,* 1997) and ICAD (inhibitor of CAD) (Sakahira *et al.,* 1998). These enzymes are selectively activated upon cleavage by caspase 3 (Sakahira *et al.,* 1998; Liu *et al.,* 1997) or by other members of the caspase family (Tang and Kidd, 1998). Exposure of nuclei to activated CAD or DFF40 is sufficient to induce the nuclear morphologic changes typical of apoptosis (Liu *et al.,* 1998; Enari *et al.,* 1998). DNA degradation during apoptosis is a complex, nuclease-mediated process that proceeds through an ordered series of steps, not all of which need be completed (Oberhammer *et al.* 1993; Zakeri *et al.* 1993; Rusnak *et al.,* 1996). DNA degradation begins with the generation of high-molecular-weight fragments of ~300 kb, which are converted to ~ 50-kb fragments which, in turn, give rise to 10-40-kb fragments. The latter give rise to the oligosomal DNA fragments, detectable as the characteristic " DNA ladders" on conventional agarose gels.

1.6.4 Bcl-2 family proteins

The Bcl-2 family of proteins are central regulators of apoptosis because they integrate diverse suvival and detah signals that are generated outside and inside the cell (Adams & Cory 2001, Strasser ef *al.,* 2000). The family is subdivided into two classes: anti-apoptotic members such as Bcl-2 and Bcl-x_L

(the Bcl-2 like survival factors) which protects cells from apoptosis, and pro-apoptotic members such as Bax and Bak (the Bax like death factors) and the large group of only BH3 only death proteins (Antonsson, 2001; Huang *et al.,* 2000) which trigger or sensitize for apoptosis. They reside predominantly in the outer mitochondrial membrane, endoplasmic reticulum, and the outer nuclear envelope. Mutual interactions between pro- and anti-apoptotic members set the threshold that determines whether a cell should die or not. Thus, Bcl-2 family members act like check points through which survival and death signals must pass before they determine the cell's fate.

Bcl-2 family members interact with each other to regulate apoptosis

One of the unique features of Bcl-2 family proteins is hetero-dimerization between anti-apoptotic and pro-apoptotic proteins, which is considered to inhibit the biological activity of their partners (Oltvai *et al.,* 1993; Yang *et al*., 1995). This heterodimerization is mediated by the insertion of a BH3 region of a pro-apoptotic protein into a hydrophobic cleft composed of BH1, BH2 and BH3 from an anti-apoptotic protein.

How Bcl-2 family members act to regulate apoptosis is of central importance, and one approach taken by many in the field has been to identify cellular proteins that interact with members of the Bcl-2 family. The first Bcl-2 associated protein to be identified was Bax (Oltvai *et al.* 1993). Bax is homologous to Bcl-2 in sequence and coimmunoprecipitates with Bcl2 in cell extracts and *in vitro* (Oltvai *et al.,* 1993). In functional assays Bax suppresses the ability of Bcl-2 to block apoptosis. Thus, not all Bcl-2 family members are inhibitors of apoptosis, but instead some may regulate apoptosis by functional

antagonism through the formation of heterodimers. The overall theme that emerges is that protectors and promoters of cell death interact with each other and the outcome that prevails depends on the ratio of the death promoter to the death suppressor. A model whereby Bcl-2 family members regulate apoptosis by forming homodimers and heterodimers has been proposed (Oltvai *et al.* 1993), although it is still not clear which Bcl-2 family member is the antagonist and which is the effector. One hint that Bax may be a death effector, that is functionally sequestered by Bcl-2 comes from the observation that BH1 mutants of Bcl-2 fail to associate with Bax and suppress apoptosis but are still capable of forming Bcl-2 homodimers (Yin *et al.,* 1994). The observation that Bax and Bak can act as both inducers and inhibitors of apoptosis suggests that the cellular context of expression may be a determining factor (Kiefer *et al.,* 1995, Knudson and Korsmeyer *et al.,* 1997).

1.6.5 c-Myc

c-Myc is a member of the Myc- family of transcription factors, which also include N-Myc, L-Myc, and the viral v-Myc proteins (Nau *et al.,* 1985; Schwab *et al,.* 1985; Sheiness and Bishop 1979; Sheiness *et al.,* 1978). The *c-myc* gene is evolutionarily conserved but absent from the genomes of yeast and *C.elegans*. The human *c-myc* can encode three polypeptides of molecular weights 45-50 [c-MycS (Spotts *et al.,* 1997), 64, and 67 kDa], the 64-kDa protein being the major product of the gene (Hann *et al.,* 1988). The 64-kDa c-Myc has several structural and functional domains. The nuclear localization signal directs c-Myc to the nucleus (Dang and Lee, 1988), the C-terminal basic-helix-loop-helix-leucine zipper (bHLHZip)-domain is required for protein-protein interactions and binding

to DNA (Landschulz ef a/., 1988; Murre *et* a/., 1989), and the N-terminal transactivation domain (TAD) mediates the activation of the basal transcription machinery (Kato *et* a/., 1990).

The *c-myc* protooncogene is an important regulator of growth promotion, differentiation and apoptosis. A deregulated expression of this gene is often observed in tumour cells (Nesbit *et* a/., 1999). *c-myc* mainly acts as a transcription factor and its biological activity in proliferation, malignant transformation and apoptosis is dependent on its binding to DNA in a sequence-specific manner in association with Max protein (Blackwood and Eisenman, 1991; Facchini and Penn, 1998). *c-myc* is an immediate early gene whose transcription correlates directly with mitogenic stimulation. In quiescent cells, c-*myc* mRNA and the resulting protein are undetectable but *c-myc* expression is rapidly induced by mitogen or serum stimulation to reach a transient maximum which is followed by a gradual decline to a detectable steady-state level in proliferating cells (Thompson *et* a/., 1985).

Apoptosis induced by c-Myc: In addition of being a regulator of cell growth and differentiation, c-Myc can also cause cells to undergo apoptosis. Interestingly, both under- and overexpression of c-Myc can be apoptotic depending on the circumstances. For example, the ability of some lymphocytic cells to undergo apoptosis induced by various treatments is dependent on reduction in c-Myc expression (Fischer *et* a/., 1994; Wang ef a/., 1999; Wu *et* a/., 1996). If the level of c-Myc is manipulated to remain high, apoptosis can be prevented. On the other hand, also an increased expression of c-Myc can lead apoptotic cells into the apoptotic route. This has been shown in several cell lines including CHO

(Wurm *et al.* , 1986), fibroblats (Evan *et al.* ,1992; Wyllie *et al.,* 1987), and myeloid 32D cells (Askew *et al.* ,1991). Also, apoptosis in T-cells can be prevented by the administration of c-Myc antisense oligonucleotides (Shi *et al.* ,1992).

Currently, the mechanism(s) by which c-Myc induces apoptosis is elusive. Being a transcription factor, one obvious way for c-Myc to influence the survival of cells would be via transcriptional regulation of target genes. Such potential target genes mediating apoptosis induced by c-Myc include odc, idh A, and cdd25A, as the expression of these three genes has been shown in certain circumstances to lead into apoptosis (Galaktionov *et al.* 1996; Packham and Cleveland 1994; Shim *et al.,* 1998). However, also other mechanisms need to be investigated, as it has been shown that c-Myc can induce apoptosis without transcnptional activation (Evan *et al.,* 1992). Further, c-MycS, a shorter translational product of c-Myc which lacks an intact N-terminal TAD, is able to induce apoptosis (Xiao *et al.,* 1998).

1.6.6 Protein kinase *C*

Protein phosphorylation and dephosphorylation are important processes in the regulation of protein function. Phosphorylation occurs on serine, threonine, and tyrosine residues and is catalysed by protein kinases thereby mediating various cellular signaling events that can be associated with human diseases. Protein kinase C (PKC) is a family of serine-threonine kinases with important roles in cellular functions such as growth, differentiation, tumor promotion and apoptosis. There are atleast 11 isozymes that are classified on the basis of their cofactor requirements: conventional (a, βI, βII,γ), novel (8, ε, η, θ, μ), and typical

(ξ,ι). Members of PKC family consists of a single polypeptide, comprised of an N-terminal regulatory region (approximately 20-40 kDa) and a C-terminal catalytic region (approximately 45 kDa).

PKC is a calcium-dependent and phospholipids dependent protein kinase. It is an integral part in cell signaling machinery. Receptor mediated hydrolysis of phophatidyl inositol is a common mechanism involved in transmembrane signaling through which inositol 1,4,5 triphosphate (IP3) and diacylglycerol are generated in the cell (Berridge and Irvine, 1984; Nishizuka, 1984). Diacylglycerol in the presence of calcium, released from intracellular stores by IP_3, and thus initiating the reactions that change the biochemical state in the cell.

1.6.7 Heat shock proteins

Heat shock proteins (Hsps) are the set of conserved proteins induced in prokaryotes and eukaryotes by elevated temperatures and a number of other types of cellular stress conditions. (Lindquist, 1986). Hsps are classified according to the apparent molecular weight, intracellular location, and main inducer (Craig *et a*/., 1989, Pelham, 1986). In general, five classes of chaperone proteins have been distinguished: Hsp60, Hsp70, Hsp90, Hsp100 and small Hsp. Members of the Hsp70, Hsp60 and Hsp100 families have been identified in mitochondria. Hsps act as molecular chaperones and are involved in protein folding, refolding, transport, and translocation (Beckmann *et a*/., 1992; Gething and Sambrrok, 1992; Itoh *et a*/., 1995).

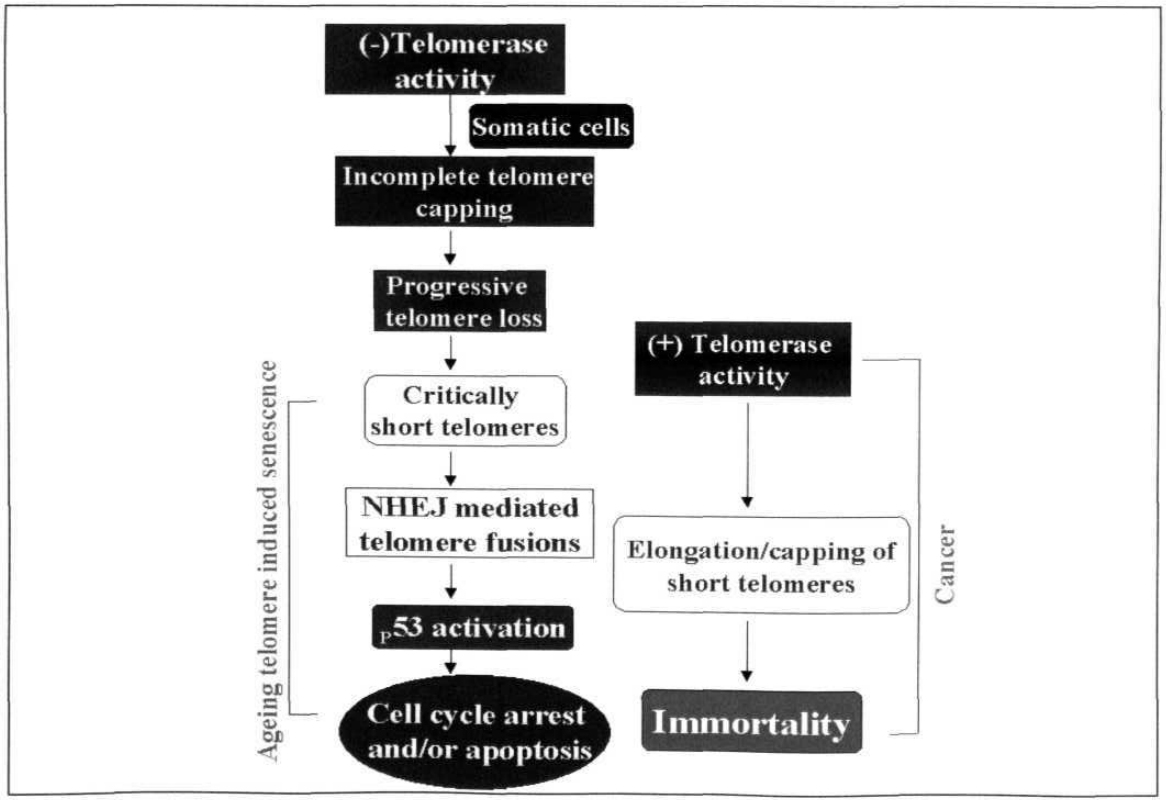
Hsp60 is the eukaryotic homolog of the bacterial chaperonin GroES. Hsp60 contains 14 identical subunits of relative molecular mass 58 kDa that are

assembled as two heptameric rings stacked back to back. These are mitochondrial matrix proteins induced by stress and form, within the mitochondria, the chaperonin complex that is important for mitochondrial protein folding and function. Preferential substrates for Hsp60 are folding intermediates that have not acquired their native structure. The important role of Hsp60 in mitochondrial biogenesis was initially identified by the analysis of temperature sensitive mutants. Null mutations of Hsp60 in *S.cerevisiae* are inviable due to the severe defects in folding of mitochondrial proteins. Conditional mutants accumulate unfolded proteins in the matrix that are not able to assemble into active enzyme complexes. So, the mitochondrial chaperonins, Hsp60 are likely to be important in the process of apoptosis.

1.7 Telomerase

Telomeres are tandem repeats of hexanucleotide sequences (TTAGGG), associated with specific proteins, located at the end of eukaryotic chromosomes (Moyizis *et* a/., 1988; Blackburn, 1991). This is required to maintain chromosomal physiology, and loss of these telomeric sequences or mutations of telomere-binding proteins trigger a series of events including chromosomal fusions and genomic instability that ultimately compromise cell proliferative capacity and/ or viability (Depinho, 2000; Blackburn, 2000) (Fig .9). In normal somatic cells, telomere shortening occurs *in vitro,* with each cell division and *in vivo* with age, reflecting the cumulative effect of cell divisions (Harley *et* a/., 1990, Allsupp *et al.,* 1992, Vaziri *et* a/., 1994). This telomere shortening has been attributed to the end-replication defect of DNA polymerase and single DNA strands incompletely replicated, would lead to a loss of terminal telomeric repeats after each cell

Fig. 9: Telomerase and Cancer



division. Telomerase is a ribonucleoprotein complex responsible for de novo telomere synthesis and addition of telomeric repeats to existing telomeres (Greider, 1998).

Telomerase is active in 70-90 % of malignant tissues and many immortal cell lines. Most somatic cells have no detectable telomerase activity, with the exception of certain stem cells, lymphocytes and germline cells (Liu *et al.*, 1999). Telomere activation doesnot seem to cause carcinogenesis but it allows a cell to continue division and attain immortality, a necessary achievement for a cancerous cell to be successful. The fundamental components of telomerase are RNA subunit (hTR), telomerase RNA component (TERC) that provides the template for the telomere synthesis reaction and a rate-limiting catalytic protein subunit (telomerase reverse transcriptase, TERT), and the telomerase associated protein (TEP1) (Meyerson et al., 1 997; Kirkpatrick and Mokbel, 2001; Liu *et al.,* 1999). Of these TERC and TEP1 are expressed ubiquitously in both normal and cancerous tisuue (Meyerson *et al.,* 1997), whereas hTERT is detectable in tumor cells but not in normal somatic cells (Kirkpatrick and Mokbel, 2001; Liu *et al.,* 1999).

As telomerase is overexpressed in a large number of tumors and is not expressed in most somatic cells, gives a rationale for evaluation of telomerase as a target for new anticancer drugs. Telomerase activity can be measured *in vitro* by using telomere repeat amplification protocol (TRAP) assay that uses a polymerase amplification step after telomerase extension of a primer. (Kim *et al.,* 1994).

Purpose of the study

Many natural products as well as synthetic compounds have been shown to be potent cancer protective or preventive agents against chemical-induced carcinogenesis in animal models. Many of these chemopreventive agents which interfere with the process of cancer development or carcinogenesis are promising since they are generally non-toxic substances. Although substantial progress has been made in the basic understanding of carcinogenesis, the precise molecular and cellular targets for effective chemoprevention are largely unknown. Furthermore, the cellular signal transduction events related to these molecular targets elicited by many of these agents are not well characterized and are poorly understood, more specifically COX-2 inhibitors.

A number of studies have shown that NSAIDs induce apoptosis in cancer cell lines. In an effort to gain insight into the biochemical mechanism underlying COX-2 inhibitor induced apoptosis, in the present study we have evaluated the effects of C-PC (a natural COX-2 inhibitor) and Celecoxib (a synthetic COX-2 inhibitor) on growth and multiplication of human chronic myeloid leukemia K562 cell line. Also the molecular mechanisms involved in C-PC induced cellular effects were investigated.

The specific objectives of the present study are:

- To study the comparative effect of C-PC, a natural COX-2 inhibitor and Celecoxib, synthetic COX-2 inhibitor on growth and multiplication of chronic myeloid leukemia cells.
- To study the external and internal structural deformities in C-PC induced cell death.
- To understand the molecular mechanism of action of C-PC.
- To elucidate the upstream and downstream signaling pathways involved in C-PC - induced cellular effects in K562 cells.

Materials & Methods

2.1 Materials

The human chronic myeloid leukemia cell line, K562, was provided by National Centre for Cell Science (NCCS), Pune, India.

Phosphate buffered saline (PBS), RPMI 1640, Fetal Bovine Serum (FBS), Penicillin, Gentamycin and Streptomycin were purchased from GIBCO, Ltd. (BRL Life Technologies, Inc., Grand Island, NY). DEAE-cellulose, Poly-L-lysine, glutaraldehyde, MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide], DAPI (4, 6-diamidino-2-phenylindole), Proteinase K, RNase A, Propidium iodide, Phenylmethylsulfonyl fluoride (PMSF), Leupeptin, Aprotinin, Pepstatin A, Trypsin, Tween-20, Triton X-100, Ponceau S, Igepal CA-630, Sodium orthovanadate, Sodium Bicarbonate, EDTA and Calcium chloride were purchased from Sigma Chemical Company (St.Louis, USA). Low fat milk powder was purchased from E-Merck. Nitrocellulose membranes and the enhanced chemiluminescence (ECL) kit were purchased from Amersham Life Science (Amersham, Bucks, UK). X-ray film and development solutions were from Kodak. Mouse monoclonal antibodies against cytochrome c and Bax were purchased from Santa Cruz, CA, USA. Polyclonal antibodies of Bcl-2 and PARP were purchased from R&D systems Inc, USA. FITC conjugated anti rabbit IgG was purchased from Molecular Probes Europe (Leiden, The Netherlands).

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

C-PC was kindly provided by Green India Natural Products Ltd., Madurai, India, and Celecoxib was a generous gift from Unichem Laboratories Ltd., Mumbai, India.

2.2 Cell culture and treatment

The human chronic myeloid leukemia K562 cells were grown in suspension in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2mM L-glutamine. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C. The cultured cells were passed twice each week, seeding at a density of about $2X10^5$ cells/ml. For treatment exponentially growing K562 cells were collected and resuspended in fresh culture medium. Stock solutions of C-PC in PBS and Celecoxib in DMSO were freshly prepared for each experiment. The final concentration of DMSO in all the cultures was 0.1%. Cell viability was determined by the trypan blue dye exclusion method.

2.3 Cell proliferation assay

Cell proliferation was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) staining as described by Mosmann (1983). Since the MTT colorimetric technique was developed, it has been applied to the quantitation of antitumor activity mediated by macrophages, LAK cells and NK cells against tumor cell targets as well as cell proliferation and drug sensitivity to tumor cells. The advantages which it offers include sensitivity, simplicity, rapidity and the avoidance of radioactivity. The MTT assay is based on the reduction of the tetrazolium salt, MTT, by viable cells. The dehydrogenases using NADH or

NADPH as coenzyme can convert the yellow form of the MTT salt to insoluble, purple formazan crystals (Liu *et* a/., 1997). Formazan solution is read spectrophotometrically after the crystals are dissolved by organic solvent (DMSO). K562 cells ($5X10^3$ cells/well) were incubated in 96-well plates in the presence or absence of C-PC and Celecoxib (10, 25, 50, 100 μM) for 24, 48, 72, 96 h in a final volume of 100μl. At the end of the treatment, 20 μl of MTT (5 mg/ml in PBS) was added to each well and incubated for an additional 4 hours at 37°C. The purple-blue MTT formazan precipitate was dissolved in 100 μl of DMSO. The activity of the mitochondria, reflecting cellular growth and viability, was evaluated by measuring the optical density at 570 nm on μ Quant Bio-tek Instruments, Inc microtiter plate reader. Each concentration was tested in three different experiments run in four replicates. Means and standard deviations were calculated and reported as the percentage of growth vs control. The viable cells were counted by the trypan blue exclusion assay with a hemocytometer.

2.4 Morphological differentiation

K562 cells were incubated with C-PC and Celecoxib (50μM). The appearance of morphological differentiation was assessed after 48 h. The cells were viewed on a phase contrast inverted microscope and photographed with Nikon F-601 AF Camera.

2.5 Analysis of nuclear morphology

After treatment of cells with C-PC and Celecoxib (25 &50 μM) for 48 hours, cells were harvested and washed with ice cold PBS and fixed in a solution of methanol: acetic acid (3:1) for 30 min after which staining was carried with

DAPI (1 mg/ml). Stained cells were placed on slides and assessed for morphological signs of apoptosis under a fluorescence microscope (Olympus BH2RFC). Apoptotic cells were defined on the basis of nuclear morphology changes such as chromatin condensation and fragmentation (bead-like formation). Apoptotic cells were photographed under high magnification and counted on atleast four independent slides. Each experiment was repeated atleast three times.

2.6 Scanning Electron Microscopy

After treatment cells were collected, washed with PBS and concentrated to $1X10^5$ cells/ ml, one drop of such suspension was placed onto a plastic cover slip previously coated with 1% poly-L-lysine (Sigma, St.Louis, MO, USA) in water. The cover slip was then allowed to stand in a small Petri dish at room temperature for 15-30 min to facilitate the cells to adhere to the cover slip. Fixative (15% glutaraldehyde in 0.01 mol/L phosphate buffer, pH 7.4) was added to the Petri dish to cover the slip and the cells were fixed for 1 h at 40°C. The cover slip was then taken through graded alcohols for dehydration and dried by the critical-point technique (EMS850 critical point drier Electron Microscopy Sciences). After trimming, mounting, and coating with gold-platinum (JFC 1600 JEOL Co auto fine sputter coater), the specimens were observed with SEM (JSM-5600, JEOL Co).

2.7 Transmission Electron microscopy

Electron microscopy was performed to confirm that the ultrastructural features of apoptosis were present in cells exposed to C-PC. In the present

study, K562 cells treated with C-PC at 50 μM for 24 hours were fixed in 2.5% glutaraldehyde (pH 7.3) buffered with 0.1 mol/L sodium cacodylate overnight at 4°C and then washed with 0.1 mol/L sodium cacodylate buffer for 15 minutes before post- fixation with 1% osmium tetroxides buffered with 0.1 mol/L sodium cacodylate for 1 hour on ice. After another wash with 0.1 mol/L sodium cacodylate buffer for 15 minutes, cells were dehydrated with increasing concentrations of alcohol (30%, 50%, 70%, 80%, 90%, and 100%; three times at each concentration) for 10 minutes each. Next, cells were infiltrated with propylene oxide for 15 minutes, followed by 1:1 propylene oxide: epoxy resin for 2 hours, and finally 100% epoxy resin for 2 hours. Cells were embedded with fresh epoxy resin into molds and placed in a 60° C oven for 2 hours. Semithin sections were examined to confirm proper cross-sectional orientation before ultrathin sectioning. Ultrathin sections (Leica ultracut CUT) were stained with uranyl acetate and lead citrate and were examined with TEM (H7500 Hitachi Co).

2.8 DNA fragmentation assay

Cells were treated with vehicle alone or C-PC or Celecoxib (10, 25 & 50 μM)for 48 h. DNA laddering was detected by isolating fragmented DNA using the SDS/ Proteinase K/ RNase A extraction method, which allows the isolation of only fragmented DNA without contaminating genomic DNA (Hermann *et a*/., 1994). Five million cells were pelleted, washed in cold PBS and lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.2 % Triton X-100 for 20 min at 4°C. After centrifugation at 14,000 g for 15 minutes, the supernatant was treated with Proteinase K (0.5 mg/ml) and 1% SDS for 1 hour at 50° C. DNA was

extracted twice with buffered phenol and precipitated with 140mM NaCl and 2 volumes of ethanol at -20° C overnight. DNA precipitates were washed in 70% ethanol, dissolved in TE, and treated for 1 hour at 37° C with RNase A. Fifteen microliters of DNA was mixed with 3ul DNA sample buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol and 30 % glycerol), and DNA was resolved in 1 % agarose gel in TBE (44.6 mM Tris, 44.5 mM boric acid and 1 mM EDTA) using 100 bp ladder as DNA standard. DNA fragmentation was visualized upon staining gel with ethidium bromide (0.5 mg/ml) and exposed to UV light. The presence of apoptosis was indicated by the appearance of a ladder of oligonucleosomal DNA fragments that are approximately 180–200 bp multiples.

2.9 Quantification of apoptosis by flow cytometry

One of the major characteristics, which is used to assess apoptosis, is the state and content of nuclear DNA. The later is readily assessed by flow cytometric quantitation of red fluorescence from fixed propidium iodide-stained, RNase-treated cells. Apoptotic activity is heralded by sub-G1 events on DNA histograms, as described by Nicoletti *et at.* (1991). The advantage of flow cytometry is that it permits measurements on a large number of cells within a short time period. Flow cytometry has been used to analyze numerous features of the apoptotic process (Gorczyca, 1999; Vermes *et at.,* 2000; Lecoeur *et* a/., 2002). Apoptosis starts with cell shrinkage expressed by changes in light - scatter signals. The FS-SSC pattern obtained by flow cytometry was used to detect the changes in size and granularity induced by the apoptotic process. The FS-SSC histogram was used to divide the cells into separate populations, differing in size and granulation. Gates were set on the populations and the

staining of the cells in the gates and of all cells, respectively, was measured as mean fluorescence intensity (MFI), peak position of fluorescence intensity or relative number of positive cells (%), depending on the agent.

To quantitate apoptosis, a flow cytometric analysis using propidium iodide was performed as described previously. Cells which were less intensively stained than G1 cells (sub- G1 cells) in flow cytometric histograms were considered apoptotic cells. DNA analysis of the aforesaid samples by flow cytometry was performed to evaluate the percentage of apoptotic cells. As is generally accepted, apoptotic cells can be recognized by their diminished stainability with DNA specific fluorochromes, which could be attributed to DNA degradation and its subsequent leakage from the cell. The method for DNA labeling was as described previously (Reddy *et al.*, 2003) with minor modifications. Briefly, K562 cells were treated with C-PC and Celecoxib (25 & 50 μM) for 48 h. After treatment, cells were prepared as single cell suspension in 200 μl PBS, fixed with 2 ml of ice-cold 70 % ethanol, and maintained at 4 ° C overnight. The cells were harvested by centrifugation at 500X g for 10 min, resuspended in 500 ul PBS supplemented with 0.1 % Triton X-100 and RNase A (100 ug/ml), incubated at 37 ° C for 30 min, and stained with 50 ug/ml propidium iodide (PI) in the dark at 4° C for 30 min. The red fluorescence of individual cells was measured with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). A minimum of 10,000 events was collected per sample. The relative DNA content per cell was obtained by measuring the fluorescence of PI that bound stoichiometrically to DNA.

2.10 Preparation of whole cell extracts and immunoblot analysis

The protocol was based on Sambrook *et al*. (1989). To prepare the whole cell extract, cells were washed with PBS and suspended in a lysis buffer (20 mM Tris, 1 mM EDTA, 150 mM NaCl, 1%NP 40, 0.5% deoxy cholic acid, 1mM p-glycerophosphate, 1mM sodium orthovanadate, 1mM PMSF, 10 μg/ml leupeptin, 20 μg/ml aprotinin). After 30 min of shaking at 4°C, the mixtures were centrifuged (10,000X g) for 10 min, and the supernatants were collected as the whole-cell extracts. The protein content was determined according to the Bradford method (Bradford, 1976). An equal amount of total cell lysate was resolved on 8-12 % SDS-PAGE gels along with protein molecular weight standards, and then transferred onto Nitrocellulose membranes. The membranes were blocked with 5% w/v nonfat dry milk and then incubated with the primary antibodies (COX-1, COX-2, Cytochrome c, PARP, Bcl-2, Bax, c-Myc, Hsp60) in 10 ml of antibody-diluted buffer (1X Tris-buffered Saline and 0.05% Tween with 5% milk) with gentle shaking at 4°C for 8-12 hours and then incubated with peroxidase conjugated secondary antibodies. Signals were detected using an ECL Western blotting detection kit.

2.11 Detection of cytochrome c release

Release of cytochrome *c* from mitochondria to cytosol was measured by Western blot as previously described (Chandra *et al*., 1998) with some modifications. Cells were washed twice with ice cold PBS and sonicated (3 X 5 sec on ice) in buffer containing 20 mM HEPES (pH 7.2), 10 mM KCl, 1.5 mM EDTA, 1 mM EGTA, 250 mM sucrose and protease inhibitors. The homogenates

were centrifuged at 750X g for 5 min, and the supernatant was then centrifuged at 10,000X g for another 5 min. The supernatant was subjected to further ultracentrifugation at 100,000X g for 60 min. The resulting supernatant represented the cytosolic fraction. Following the quantification, proteins were separated on polyacrylamide gel, transferred onto a nitrocellulose membrane and probed with antibody against cytochrome c followed by incubation with a secondary antibody conjugated with horseradish peroxidase. Detection was performed using the ECL kit.

2.12 Measurements of PKC activity

K562 cells were treated with C-PC and Celecoxib (25 uM & 50 uM) for 15 and 30 min. After treatment cells were harvested washed twice with ice cold PBS and pelleted by centrifugation at 100 x g for 5 min at 4^0C. Cells were resuspended in 50 mM Tris-HCl (pH 7.5) containing 0.3 % (w/v) p-mercaptoethanol, 1.0 mM EDTA, 2.5 mM EGTA, 50 ug/ml of phenylmethylsulfonyl fluoride and 10 mM benzamidine. The cells were disrupted by sonication and the homogenates were cleared by centrifugation at 135,000 x g for 45 min. Total cellular PKC activity in sonicates was examined by measuring the rate of phosphate incorporation into a peptide corresponding to the phosphorylation site domain of myelin basic protein [QKRPSQRSKYL, MBP4-14] substrate. PKC isozyme activities were measured using an assay (75 µl) consisting of 50 mM Tris/HCl (pH 7.40), 10 µg/ml phosphatidylserine vesicles and $CaCl_2$ (0.1 mM), MBP4-14 (50 AM), DAG. After thermal equilibration to 30°C, assays were initiated by the simultaneous addition of the sample along with 5 mM Mg^{2+}, 100 uM ATP, 0.3 uCi [γ^{32}P] ATP and terminated after 30 min

with 100 ul of 175 mM phosphoric acid. Following this, 100 ul was transferred to P81 filter papers, which were washed three times in 75 mM phosphoric acid. Phosphorylated peptide was quantitated by scintillation counting.

2.13 Extraction, Purification and characterization of C-PC

2.13.1 Preparation of crude extract

Approximately 1g of algal extract was dissolved in 20 ml of 10 mM potassium phosphate buffer pH 7.0 and centrifuged at 10000 g for 30 min, and a C-PC containing clear blue supernatant was collected.

2.13.2 Separation and purification of C-PC

The blue supernatant was fractionated with ammonium sulfate at 0-30 % and 30-50%. The precipitate from 0-30% (w/v) ammonium sulfate was discarded. The precipitate from the 30-50% (W/V) ammonium sulfate was dissolved in a known volume of sodium phosphate buffer pH-7.0 and dialyzed against the same buffer. The dialyzed sample of C-PC was chromatographed on a DE-52 anion exchange column. The C-PC containing sample was eluted with NaCl solution of a linear increasing ionic concentration (0-0.5 M, 100 ml) at 1 ml min^{-1}. The fractions were collected using 1 ml glass tube.

2.13.3 Spectroscopic measurements

All visible and UV spectra of C-PC samples were measured on a Shimadzu 2001 spectrophotometer. The maximum absorption wavelength for C-PC is 620 nm. The purity was evaluated according to the absorbance ratio A_{620}/A_{280} (Boussiba and Richmond 1979).

2.13.4 SDS-PAGE analysis

SDS-PAGE analysis of protein fractions was carried out on vertical slab gels according to the method of Laemmli (1970). Samples containing 5ug of protein were dissolved in sample buffer containing 1% SDS (W/V), 5% (3-mercaptoethanol (V/V), 0.003% bromophenol blue (W/V) and 12% glycerol (V/V), in 0.063 M Tris-HCI, pH-6.8. The samples were boiled at 100° C for 5 minutes and subjected to electrophoresis. The stacking gel contained 4.5% polyacrylamide in 0.125 M Tris-HCI, pH-6.8 and the resolving gel contained 10% polyacrylamide in 0.375 M Tris-HCI pH-8.8. The ratio of acrylamide to N, N, N', N' - methylene bis acrylamide was 29.2: 0.8. Proteins were electrophoresed at constant potential difference (80 volts) with an electrode buffer containing 0.025 M Tris-HCI, 0.192 M glycine and 0.1 % SDS with pH-8.5. Molecular weight markers were run simultaneously with the samples.

2.13.5 Native -*PAGE* analysis

To check the molecular weight and intactness of the protein polyacrylamide gel under non-denaturing conditions was done. The separating gel was cast on vertical slabs. The acrylamide to bis-acrylamide ratio was 29.2: 0.8 and they were polymerized in Tris - HCl, pH-8.8. The protein samples were dissolved in sample buffer containing 10% sucrose and 0.003% bromophenol blue in Tris-HCI buffer, pH-6.8 and loaded onto the stacking gel. The proteins were separated using 60 V at 4^0 C. Resolved proteins were transferred to nitrocellulose membranes. After 1 hour of incubation with the blocking solution containing 5% low-fat milk in TBS (20 mM Tris-HCI, pH-7.5 and 0.9% NaCl), the

membranes were washed twice with TBST (TBS containing 0.05% Tween 20). After being washed with in TBS-T, the blots were incubated with the primary antibody (anti -C-PC) at a dilution of 1:1000 in blocking solution for 1 hour at room temperature. After being washed in TBS-T, the blots were incubated with horseradish peroxidase- conjugated anti-rabbit IgG (1:2000) for 1 hour, washed for three times, and the proteins were detected using enhanced chemiluminiscence system (Amersham).

2.13.6 Silver staining

The silver staining procedure was carried according to the procedure of Blum *et* a/ (1987). After completion of electrophoresis, the gel was incubated in the fixative (50 % methanol, 12% acetic acid and 0.05% formaldehyde) for one hour followed by three washings in 50% ethanol for twenty minutes each. Gels were next treated in Sodium thiosulphate (2mg/100 ml) for one minute and then washed in double distilled water thrice. Gels were then stained in 0.2% silver nitrate containing 0.05% formaldehyde for 30 minutes. The gels after being washed with double distilled water thrice, 20 seconds each were developed with 6% sodium carbonate containing 0.075% formaldehyde. Reaction was stopped with 1% acetic acid solution and stored in 50% methanol after thorough washing.

2.13.7 Raising of polyclonal antibodies

To study the mode of action of C-PC polyclonal antibodies were raised against C-PC. The antibodies were raised by injecting C-PC into rabbits (New Zealand variety) subcutaneously after mixing with Freunds complete adjuvant and emulsification. Prior to immunization, the lateral ear vein was bled to collect

pre-immune serum. After two weeks a booster injection of protein emulsified with Freund's incomplete adjuvant was given. The second booster injection was given after a week of first booster injection and finally the blood was collected after 10 days of the second booster injection The collected blood was left overnight at 4°C for clotting and the serum was collected by centrifuging at 7000 rpm for 20 minutes. The serum was aliquoted and stored at -20° C after adding 0.01% azide.

2.13.8 Immunolocalization of *C-PC* by Confocal microscopy

After treatment cells were washed with PBS and fixed in 4% paraformaldehyde pH-7.4 for 20 min at 4°C. After fixation cells were washed twice with PBS. The cells were permeabilized for 5 min at RT in 0.2% saponin, PBS, 0.03M sucrose, 1 % BSA. After permeabilization cells were washed with PBS and blocked for 60 minutes in blocking solution comprising 0.5% NP-40, 5% normal goat antiserum in PBS. The cells were washed with PBS and incubated with primary antibody at a dilution of 1:200 (in blocking solution) for 60 min at RT .The cells were washed with PBS thrice and incubated with secondary antibody, FITC-conjugated anti-rabbit IgG (1:1000 dilution in blocking solution) for 30 min at RT. The cells were then washed twice with PBS and once with distilled water, and cells were observed on Meridian ULPIMA confocal microscope with the use of a mounting medium for fluorescence.

2.14 Assay for telomerase activity

Telo TAGGG telomerase PCR ELIS ^{PLUS} kit is an extension of the original method described by Kim *et a/.* (1994). It allows highly specific amplification of

telomerase-mediated elongation products combined with non-radioactive detection following an ELISA protocol. Furthermore, because telomerase has an essential RNA component, aliquots of samples were treated with RNase to assess the specificity of the reaction. In brief, K562 cells were treated with C-PC (1, 3, 5, 10 pM) for 5 days. . After treatment cells were harvested, washed with PBS and lysed with 200 ul of ice-cold lysis buffer (10 mM Tris-HCl [pH 7.5], 1 mM EGTA, 0.5 % CHAPS, 10 % [v/v] glycerol, 5 mM ft-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 30 minutes. Cell lysate was centrifuged at 13,000X g for 20 minutes at 4^0C. Supernatant was carefully removed and transferred to a fresh tube and the protein concentration was determined using the Bradford method. TRAP assay was performed using the Telo TAGGG telomerase PCR ELIS ^{PLUS} kit (Roche Diagnostic GmbH Mannheim, Germany) and the procedures were followed from the manufacturer's protocol. In brief, supernatant of the cell extracts was split into two aliquots before performing the assay: one was used to prepare a negative control by heat inactivation of telomerase for 10 min at 80 ° C, the other one was used to evaluate the telomerase mediated adding of telomeric sequence. For PCR amplification, 2 ul of supernatant and 25 µl of reaction mixture were transferred into a suitable tube, then sterile water was added to a final volume of 50 µl. An amplification reaction was carried out by the following protocol: mixture was kept at 25°C for 30 min, 94^0C—30 s, 50^0C—30 s, 72 ^{0}C—90 s (30 cycles), and at 72°C for 10 min. The 2.5 µl amplification product was mixed with 10 µl of denaturation reagent and incubated at room temperature for 10 min.

An aliquot of 100 ul of hybridization buffer was added and mixed thoroughly, then 100 ul of mixture per well was transferred into the precoated MTP modules and incubated at 3 °C on a shaker for 2 h. Anti-DIG-HRP working solution (100 ul) was added and incubated at room temperature for 30 min with shaking. The solution was removed completely and the precipitate was rinsed five times with 250 µl of washing buffer per well for a minimum of 30 s. After removing the washing buffer, 100 ul of TMB substrate solution was added and incubated for color development at room temperature for 20 min with gentle shaking. Finally, 100 ul of stop reagent was added to each well to stop color development. The amount of TRAP products was determined by the measurement of the absorbance at 450 and 690 nm (Fig. 10).

The relative telomerase activity (RTA) was obtained using the following formula:

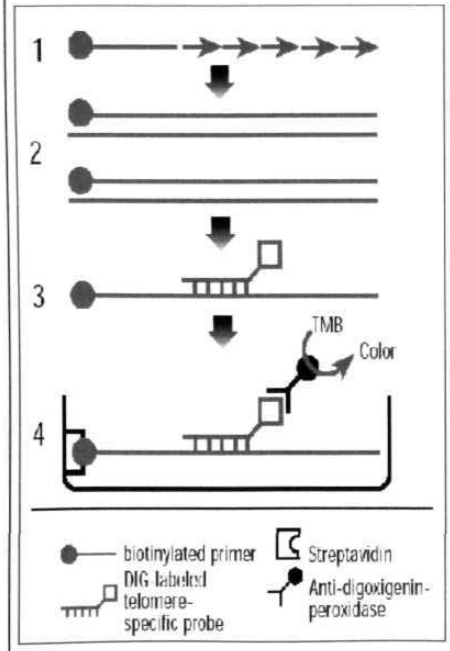
$$RTA = [(A_S - A_{S0}) / A_{S,\ IS}] / [(A_{TS8} - A_{TS8,\ 0}) / A_{TS8,\ IS}] \times 100$$

Where A_S is the absorbance of sample; $A_{S0,}$ absorbance of heat inactivated sample; $A_{TS8,}$ absorbance of control template; $A_{TS8,\ 0,}$ absorbance of lysis buffer; and $A_{TS8,}$ IS. is the absorbance of internal standard of the control template.

2.15 Statistical analysis

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

Fig. 10: Telomerase Activity Assay – TRAP ELISA Method



Step1: Telomerase adds telomeric repeats to the 3' end of the biotin labeled synthetic primer.

Step 2: Amplification by PCR using biotinylated primer.

Step 3: PCR products are denatured and hybridized to digoxigenin labeled detection probes.

Step 4: The DNA hybrid is bound to the streptavidin coated microtitre plate and anti digoxigenin peroxidase and TMB substrate generate a colored product measurable with a microplate reader.

Results

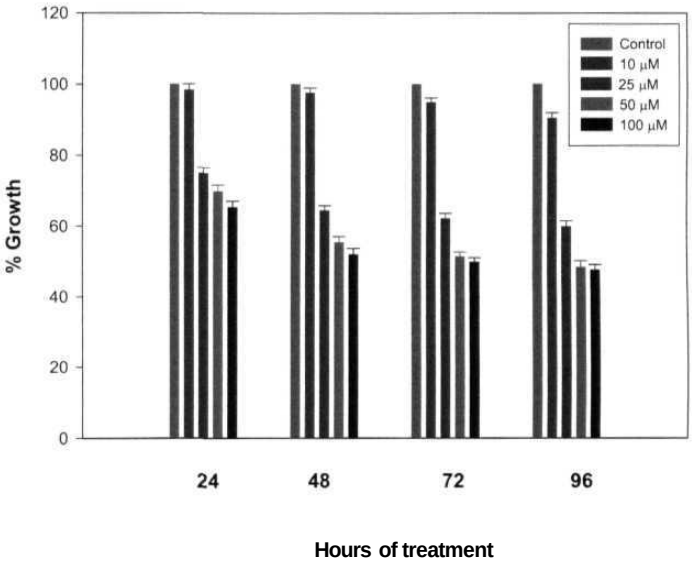
3.1 Effects of C-PC and Celecoxib on gowth of K562 cells

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 a*/., 2001). Growth inhibition and apoptosis have been observed in several other cancer cell lines by COX-2 inhibitors (Everts *et a*/., 2000; Buttar and Wang, 2000; Fournier and Gordon 2000; Fosslien, 2000; Dempke *et a*/., 2001). To test the effects of C-PC (a natural COX-2 inhibitor) and Celecoxib (a synthetic COX-2 inhibitor), human chronic myeloid leukemia cell line, K562, cultured in RPMI 1640 medium was incubated with different concentrations of C-PC and Celecoxib and the viability was examined by MTT assay. Cells were cultured in 10% FBS containing medium with or without C-PC or Celecoxib (10-100 μM) for 24, 48, 72, 96 h and cell proliferation was evaluated by the MTT assay. Under these experimental conditions a dose dependent decrease in K562 cell proliferation was observed until 48 hours after C-PC (Fig.11) and Celecoxib (Fig. 12) treatment with maximum decrease in cell proliferation being at 50 μM where the percent inhibition was 49% and 53% respectively. Since the maximum inhibition was observed in cells exposed to 50 μM C-PC / Celecoxib for 48 h, further experiments were carried under these conditions.

3.2 Effect of C-PC and Celecoxib on COX-1 and COX-2 protein expression levels

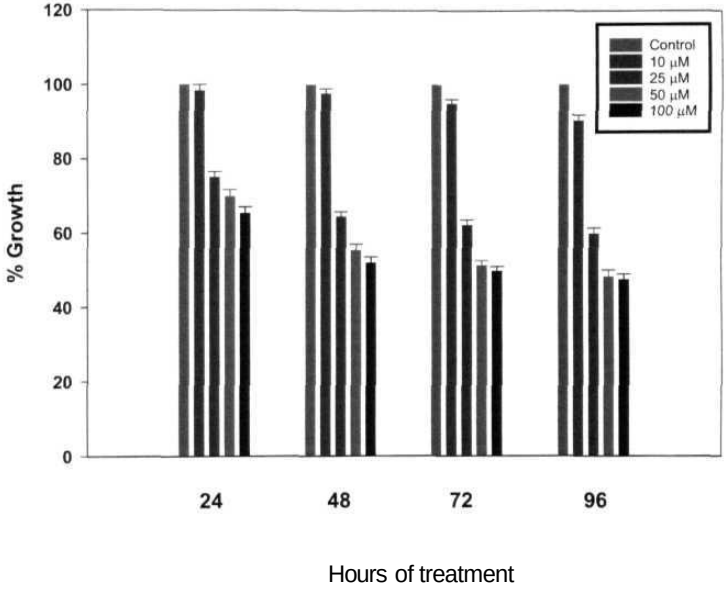
K562 cell line, cultured in RPMI 1640 medium was incubated with C-PC (25 & 50 uM) and Celecoxib (25 & 50 uM) for 48 h and the expression of COX-1 and COX-2 was monitored by Western blot analysis, with the whole cell lysate made from C-PC and Celecoxib treated cells. The results presented in Fig. 13

Fig. 11: Effect of C-PC on cell proliferation in K562 cells (MTT assay)



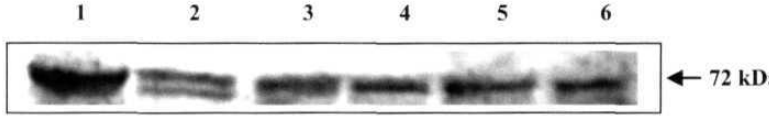
K562 cells were treated with 10, 25, 50, 100 uM of C-PC and the cell survival was determined after 24, 48, 72 and 96 h by MTT assay. **The** % viable cells were calculated in comparison to untreated cells. **The** number of cells in the control were taken as 100 %. Each data point represent the mean ± SD of four replicates.

Fig. 12: **Effect of Celecoxib on cell proliferation in K562 cells (MTT assay)**



K562 cells were treated with increasing concentrations (10, 25, 50, 100 uM) of Celecoxib and the cell survival was determined after 24, 48, 72 and 96 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 %. Values are the means ± SD of four replicates.

Fig. 13: **Western blot analysis showing the effects of C-PC and Celecoxib on COX-1 protein expression in K562 cells**



Whole cell lysates (50 ug) 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 antibodies.

Lane 1: COX-1 protein positive control (from ram seminal vesicles)

Lane 2: K562 control cells

Lane 3: K562 cells treated with C-PC 25 uM

Lane 4: K562 cells treated with C-PC 50 uM

Lane 5: K562 cells treated with Celecoxib 25 uM

Lane 6: K562 cells treated with Celecoxib 50 µM

showed no significant changes in COX-1 (lanes 3-6) protein levels in C-PC and Celecoxib treated cells compared to control cells (lane 2). These results indicate that C-PC and Celecoxib have no effect on the protein levels of COX-1 in K562 cells. The results in western blot analysis of COX-2 in cells treated with C-PC/Celecoxib were presented in figure 14. As shown in figure, COX-2 in K562 cells showed a molecular weight of 90 kDa (lanes 1-5) as against the COX-2 positive control (lane 6). Also the results showed increase in COX-2 protein levels in C-PC (lane 2 & 3) and Celecoxib (lane 4 & 5) treated cells compared to that of untreated cells (lane 1).

3.3 Morphological and ultrastructural changes

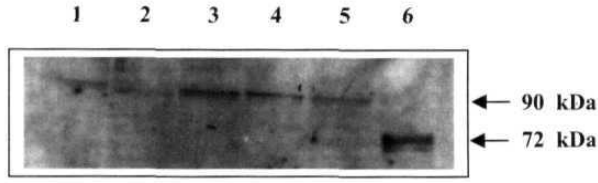
3.3.1 Phase contrast microscopy

Phase contrast microscopy pictures of K562 cells treated with C-PC and Celecoxib (50 uM) for 48 h were taken to observe the altered morphological features. Cells grown in complete medium in the absence of C-PC or Celecoxib were round in shape with characteristic features of lymphoid cells (Fig. 15A). However, after 48 h of incubation with C-PC (Fig.15B) Celecoxib (Fig. 15C) showed decrease in cell number with cytoplasmic shrinkage and marked convolution of cellular surfaces. Many cells displayed protuberances of the plasmamembrane that would eventually separate into membrane-bound apoptotic bodies.

3.3.2 Fluorescence microscopic studies

A distinguishing feature of apoptosis is the condensation and fragmentation of nuclear chromatin, which can be monitored by fluorescence

Fig. 14: Western blot analysis showing the effects of C-PC and Celecoxib on COX-2 protein expression in K562 cells



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Whole cell lysates (50 ug) 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 antibodies.

Lane 1: K562 control cells

Lane 2: K562 cells treated with C-PC 25 uM

Lane 3: K562 cells treated with C-PC 50 uM

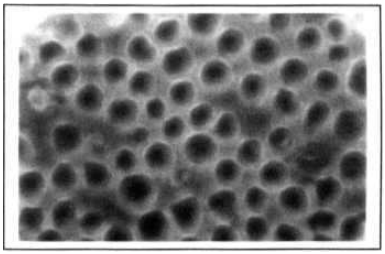
Lane 4: K562 cells treated with Celecoxib 25 uM

Lane 5: K562 cells treated with Celecoxib 50 uM

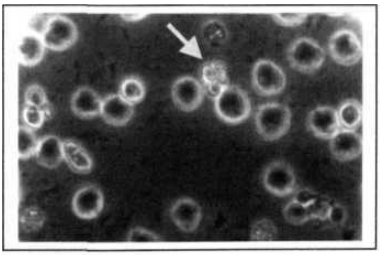
Lane 6: COX-2 positive control (rh COX-2)

Fig. 15: **Phase contrast photomicrographs showing the effect of C-PC and Celecoxib in K562 cells**

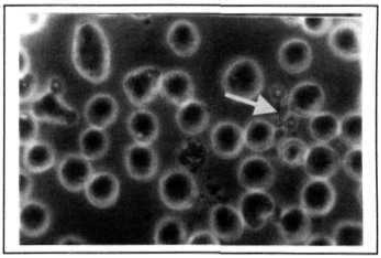
A. Control



B. C-PC 50



C. Celecoxib 50 µM



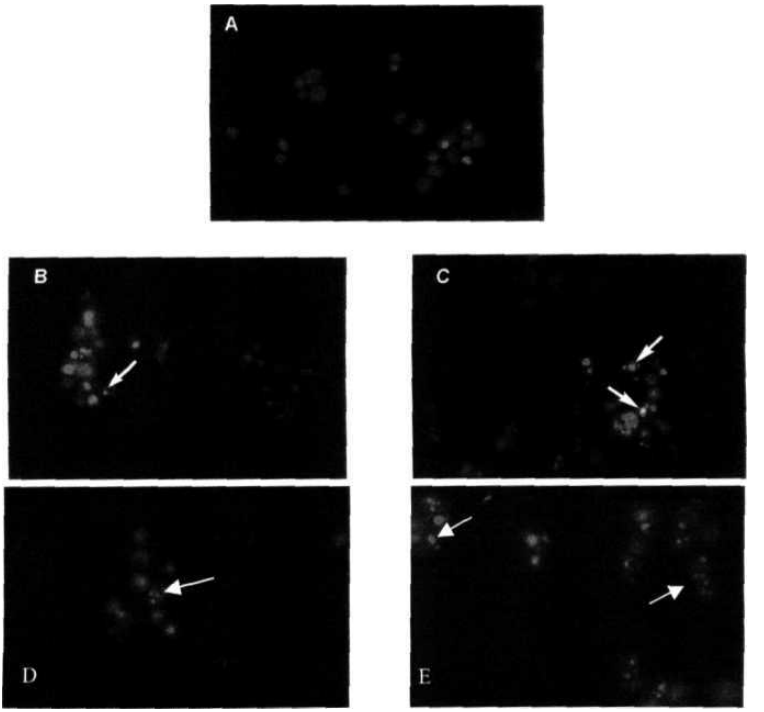
K562 cells were treated with C-PC (50 uM) and Celecoxib (50 µM) for 48 h and cells were photographed under phase contrast microscopy (Magnification 400 X). Arrows indicate a typical apoptotic cell with apoptotic bodies.

microscope. K562 cells were exposed to various concentrations of C-PC and Celecoxib (25 & 50 uM) for 48 h, and then assessed for morphological signs of apoptosis by staining with DAPI. Nuclear condensation and apoptotic bodies, a hallmark of apoptosis, were observed in cells treated with C-PC (Fig 16 B & C) and Celecoxib (Fig. 16 D &E). Also the number of apoptotic cells increased with the increasing concentration of C-PC/ Celecoxib. Chromatin of apoptotic cells was segregated and compacted into sharply delineated masses, very close to the nuclear envelope, as indicated by the arrows.

3.3.3 Ultrastructural changes - SEM & TEM

In the light of changes observed under phase contrast and fluorescence microscopes, further studies were undertaken for detailed analysis of morphological and ultrastructural changes on SEM and TEM. To determine whether the antiproliferative effects of C-PC were associated with apoptosis, we examined the ultrastructural changes of K562 cells treated with 50 uM C-PC / Celecoxib for 48h. Apoptotic cell death was confirmed by scanning and transmission electron microscopy, which revealed characteristic ultrastructural features of apoptosis. SEM studies of C-PC and Celecoxib treated cells revealed the presence of membrane blebbing, which might be due to a deep cytoskeleton rearrangement, causing progressive changes in cell shape, organelle distribution, cell shrinkage and severing junctions with its neighbours and loss of microvilli (Fig. 17B). TEM gives the qualitative bidimensional image of the sectioned samples. The cell volume of the C-PC/Celecoxib treated cells was reduced, which indicated shrinkage of cytoplasm, while the plasma membrane remained well defined. The cells showed typical nuclear fragmentation and condensed

Fig. 16: **Fluorescence microscopic studies showing C-PC and Ceiecoxib induced nuclear DNA fragmentation in K562 cells stained with DAPI**

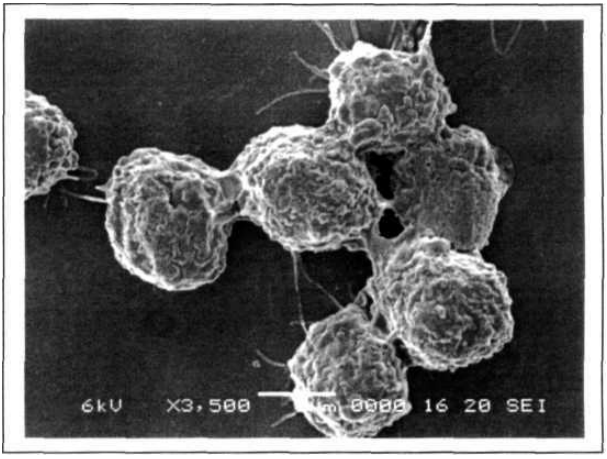


Nuclear morphology of K562 cells was observed under a fluorescence microscope (Olympus BH2RFC) after treatment with C-PC & Ceiecoxib for 48 h. The arrows are pointed to the apoptotic cells. (Magnification 400X).

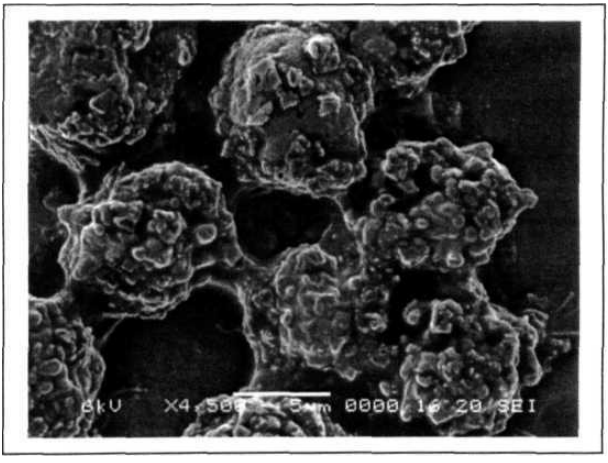
A: K562 control cells
B: K562 cells treated with C-PC 25 uM
C: K562 cells treated with C-PC 50 uM
D: K562 cells treated with Ceiecoxib 25 uM
E: K562 cells treated with Ceiecoxib 50 uM

Fig. 17: **Scanning electron micrographs showing C-PC treated K562 cells**

A. Control



B. C-PC 50 µM



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

chromatin with the formation of apoptotic bodies (Fig. 18 B & C). However in the control cells, the nuclei are intact with high nucleus/cytoplasm ratio (Fig. 18A).

3.3.4 *C-PC* and Celecoxib induced DNA fragmentation in K562 cells

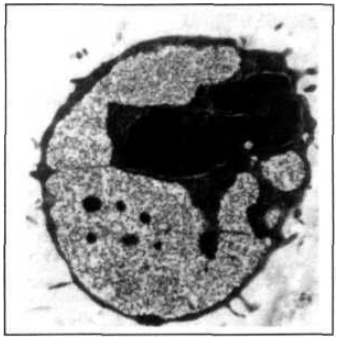
In addition to morphological evaluation, apoptosis induction by C-PC and Celecoxib was ascertained by using an assay developed to measure DNA fragmentation, a biochemical hallmark of apoptosis. During later satges of apoptosis internucleosomal cleavage of cellular DNA by endonucleases to 180 bp or oligomers of 180 bp fragments could be detected by extraction of nuclear DNA and agarose gel electrophoresis. As illustrated in (Fig. 19), agarose gel electrophoresis of DNA extracted from K562 cells treated with C-PC and Celecoxib at concentrations of 10, 25 and 50 uM for 48 h revealed a progressive increase in the non-random fragmentation into a ladder of 180–200 bp (lanes 2-7). The degree of nuclear DNA fragmentation was directly proportional to the concentration of C-PC/Celecoxib. Such a pattern corresponds to internucleosomal cleavage, reflecting the endonuclease activity characteristic of apoptosis. Control cells did not show any internucleosomal DNA fragmentation (lane 1).

3.4 DNA content assay by fluorescence activated cell sorter (FACS)

The induction of apoptosis in C-PC and Celecoxib treated cells was further verified by flow cytometric analysis of DNA content. Loss of DNA is a typical feature of apoptotic cells. Propidium iodide (PI) staining of DNA, which is taken up into the nucleus of apoptotic and necrotic cells, was used to measure the relative numbers of dead cells (Pullen *et* a/., 1981). Furthermore, since

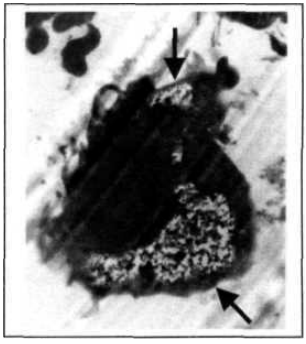
Fig. 18: Transmission electron micrographs of C-PC and Celecoxib treated K562 cells

A. Control (x 8240)



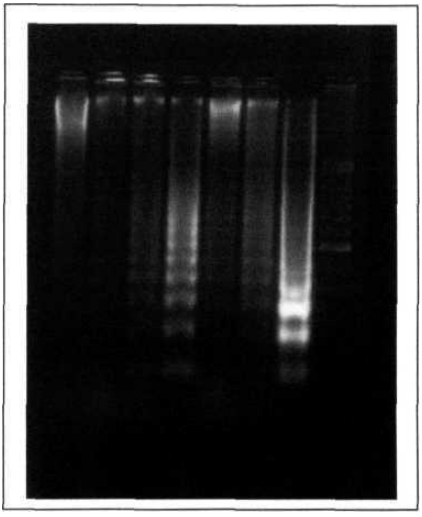
B. C-PC 50 µM (X 4740)

C. Celecoxib 50 µM (x 9450)



Ultrastructural changes induced by C-PC & Celecoixb (50 uM) in K562 cells. Cells were harvested, fixed in 2.5 % glutaraldehyde and analyzed by transmission electron microscopy. Chromatin condensation and nuclear fragmentation is clearly seen in C-PC and Celecoxib treated cells. Control cells showed distinguishable diffused interchromatin.

Fig. 19: **Agarose gel electrophoresis showing *internucleosomal* DNA fragmentation induced by C-PC and Celecoxib in K562 cells**



Agarose gel electrophoresis of DNA extracted from K562 cells treated with C-PC and Celecoxib for 48 h. After treatment cells were lysed and total cellular DNA was extracted and electrophoresed on a 1% agarose gel containing 0.05 mg/ml ethidium bromide at 5 V/cm. The gels were then photographed under UV illumination.

Lane 1: K562 control cells

Lane 2: K562 cells treated with C-PC 10 uM

Lane 3: K562 cells treated with C-PC 25 uM

Lane 4: K562 cells treated with C-PC 50 uM

Lane 5: K562 cells treated with Celecoxib 10 µM

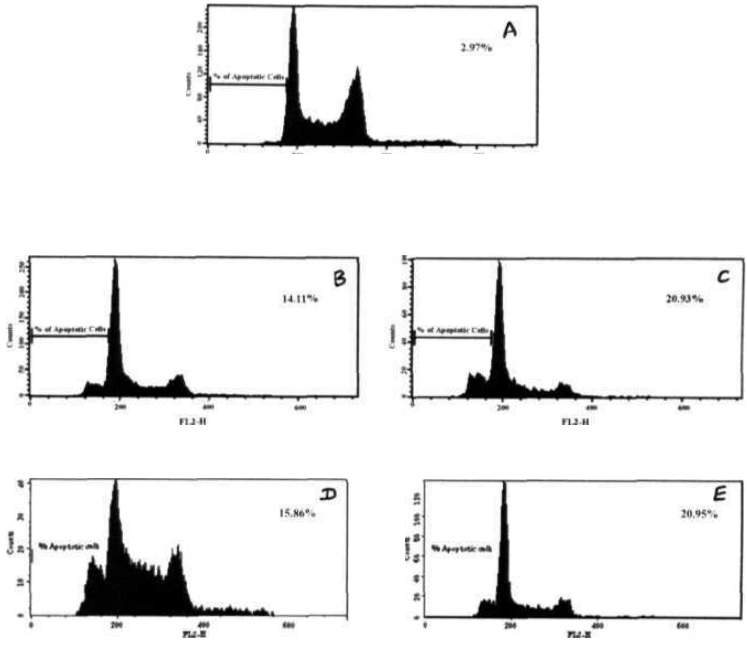
Lane 6: K562 cells treated with Celecoxib 25 uM

Lane 7: K562 cells treated with Celecoxib 50 uM

Lane 8: 100 bp ladder

apoptosis, but not necrosis, involves degradation of DNA, the staining pattern obtained with PI was used to establish whether cell death was due to apoptosis or necrosis. Two different ways of staining with PI are generally observed: PI in the presence of a permeabilising and fixing agent results in the staining of DNA in living, apoptotic and necrotic cells; and PI in a physiological buffer, stain cells that are dead due to apoptosis or necrosis. In the present study K562 cells treated with C-PC/ Celecoxib (25 & 50 μM) for 48 h were taken for FACS analysis. Fig. 20 illustrates the DNA content histograms obtained after PI staining of permeabilized cells that had been treated with C-PC and Celecoxib (25 & 50 uM) for 48 h. In agreement with DNA fragmentation results, a typical sub-diploid apoptotic peaks were observed in K562 cells treated with 25 μM and 50 μM C-PC (Fig. 20 B & C) and Celecoxib (Fig 20 D & E) for 48 h. The FACS analysis of control cells, on the other hand, showed prominent G1, followed by S and G2/M phases (Fig. 20 A). Only 2.97 % of these cells showed hypodiploid DNA (sub G0/G1 peaks). This value of 2.97 % hypodiploid DNA in control cells increased to 14.11 % and 20.93 % cells in C-PC (25 & 50 uM) treated cells (Fig.20 B& C) and 15.86% and 20.95 % in Celecoxib (25 & 50 uM) treated cells (Fig 20 D &E). These studies thus reveal increase of hypodiploid apoptotic cells in response to C-PC and Celecoxib treatment in a concentration-dependent manner and the decrease of the cells at S and G2 phase of cell cycle. This result suggested a possibility that C-PC induced apoptosis occurs at S and G2 phase of the cell cycle.

Fig. 20: Quantification of apoptosis by flow cytometric analysis (FACS)



Celecoxib for 48 h were determined using propidium iodide staining by flow cytometry.

A. Control

B. K562 cells treated with C-PC (25 uM)

C. K-562 cells treated with C-PC (50 uM)

D. K-562 cells treated with Celecoxib (25 uM)

E. K-562 cells treated with Celecoxib (50 uM)

3.5 Signal transduction pathways

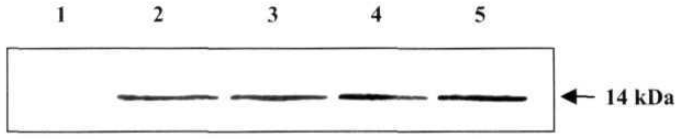
3.5.1 *C-PC* and Celecoxib treatment evokes cytochrome c release

One of the major apoptotic pathways is activated by the release of apoptogenic protein, cytochrome c, from mitochondria into the cytosol. The release of cytochrome c, one of the most important respiratory-chain proteins, from the mitochondria into the cytosol is the hallmark of cells undergoing apoptosis (Liu *et a*/., 1996; Martinou *et a*/., 2000). To specify the molecular basis of apoptosis the release of cytochrome c into the cytosol was measured in K562 cells treated with C-PC and Celecoxib, by Western blot analysis employing mouse monoclonal cytochrome c antibodies. As shown in Fig. 21 untreated cells cytochrome c (lane 1) was not detectable in the cytoplasm, whereas the levels of cytosolic cytochrome c significantly increased after C-PC (lanes 2 & 3) and Celecoxib treatment (lanes 4 & 5). As shown in Fig.22 the levels of cytochrome c in the cytosol were elevated within 6 hours after treatment with C-PC (lanes 2 & 3) and the levels were further increased by 12 hours (lanes 4 & 5) with later stabilization (lanes 6 & 7).

3.5.2 PARP cleavage in response to *C-PC* and Celecoxib treatment

PARP, poly (ADP-ribose) polymerase, has been implicated in many cellular processes including apoptosis and DNA repair. PARP is primarily found in the nucleus and is activated by DNA strand breaks. PARP is a 116-kDa protein, which converts nicotinamide adenine dinucleotide (NAD) to nicotinamide and protein-linked ADP-ribose polymers. The DNA repair enzyme, PARP has been recognized as a representative death substrate that is cleaved and

Fig. 21: Western blot analysis showing C-PC and Celecoxib induced release of cytochrome c into the cytosol in K562 cells



K562 cells were treated with C-PC and Celecoxib for 48 h, cytosolic proteins (50 ug) were separated on a 15 % SDS-PAGE, and after electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with mouse monoclonal cytochrome-c antibodies.

Lane 1: K562 control cells

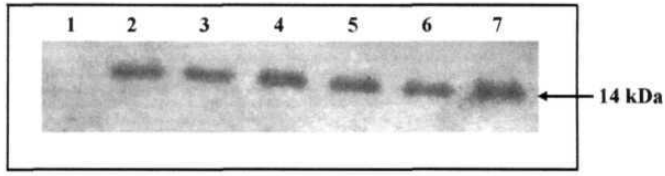
Lane 2: K562 cells treated with C-PC 25 uM

Lane 3: K562 cells treated with C-PC 50 uM

Lane 4: K562 cells treated with Cele 25 uM

Lane 5: K562 cells treated with Cele 50 uM

Fig. 22: **Western blot analysis showing release of cytochrome c into the cytosol in K562 cells treated with C-PC for different time periods**



K562 cells were treated with C-PC (0-24 h), cytosolic proteins (50 ug) were separated on a 15 % SDS-PAGE, and after electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with mouse monoclonal cytochrome-c antibodies.

Lane 1: K562 control cells (0 h)

Lane 2: K562 cells treated with C-PC 25 uM (6 h)

Lane 3: K562 cells treated with C-PC 50 uM (6 h)

Lane 4: K562 cells treated with C-PC 25 uM (12 h)

Lane 5: K562 cells treated with C-PC 50 uM (12 h)

Lane 6: K562 cells treated with C-PC 25 uM (24 h)

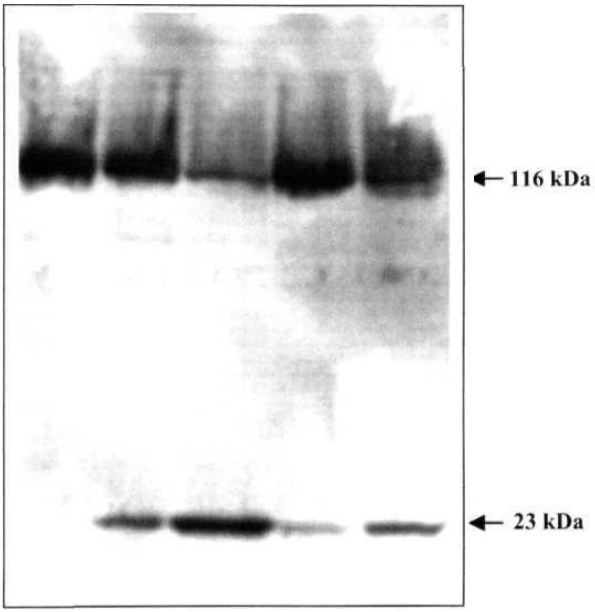
Lane 7: K562 cells treated with C-PC 50 uM (24 h)

inactivated by down-stream caspases. In response to growth factor withdrawal or on exposure to a variety of chemotherapeutic compounds (Shah *et* a/., 1996), PARP is cleaved to generate 85 and 23 kDa fragments. To determine whether PARP is cleaved in C-PC and Celecoxib induced cell death, we treated K562 cells with 25 & 50 μM C-PC and Celecoxib for 48 h and PARP cleavage was monitored with PARP antibodies, which specifically recognizes the 23 kDa fragment of the cleaved PARP and uncleaved 116 kDa PARP. Fig. 23 illustrates the gradual increase in the proportion of the M_r 23,000 cleavage product and decrease in the proportion of 116 kDa uncleaved PARP with increasing concentrations of C-PC (lanes 2 & 3) and Celecoxib (lanes 4 & 5). In the control cells, however, no fragment of PARP was observed, except the uncleaved 116 kDa protein (lane 1). The extent of PARP Cleavage in C-PC treated cells (lanes 3 & 4), however, was much higher than the same in Celecoxib treated cells (lanes 4&5).

3.5.3 Bcl-2/ Bax ratio modulation

Different proteins of the Bcl-2 family have been implicated in triggering or preventing apoptosis. Bax and Bcl-2 are the proteins associated with the mitochondrial membrane and their ratio is crucial for cell survival. In light of the recent reports that attributed COX-2 inhibitor-induced apoptosis to bcl-2 downregulation (Liu *et* a/., 1998; Sheng *et* a/., 1998), studies were undertaken to test whether Bcl-2 expression is affected after C-PC/Celecoxib treatment in K562 cells. Changes in the expression of cellular anti-apoptotic proteins, Bcl-2 and of the pro-apoptotic protein Bax, following C-PC and Celecoxib treatment (25 & 50 uM) for 48 h were examined by Western blotting. As shown in Fig. 24 untreated

Fig. 23: **Western blot analysis showing the cleavage of PARP in cell extracts of C-PC and Celecoxib treated cells**



Whole cell lysates from K562 cells treated with C-PC and Celecoxib for 48 h, were fractionated on a 12 % 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 (116 kDa) and the cleaved fragment (23 kDa).

Lane 1: K562 control cells

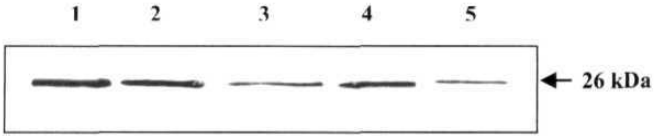
Lane 2: K562 cells treated with C-PC 25 uM

Lane 3: K562 cells treated with C-PC 50 uM

Lane 4: K562 cells treated with Celecoxib 25 uM

Lane 5: K562 cells treated with Celecoxib 50 uM

Fig. 24: **Immunoblot analysis of Bcl-2 expression in K562 cells treated with C-PC and Celecoxib**



K562 cells were treated with C-PC and Celecoxib (25 & 50 uM) for 48 h, and the whole cell lysates (50 ug) 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 antibodies.

Lane 1: K562 control cells

Lane 2: K562 cells treated with C-PC 25 uM

Lane 3: K562 cells treated with C-PC 50 uM

Lane 4: K562 cells treated with Celecoxib 25 uM

Lane 5: K562 cells treated with Celecoxib 50 uM

cells expressed high levels of Bcl-2 protein (lane 1) where as in cells treated with C-PC (lane 2 & 3) and Celecoxib (lane 4 & 5) the expression of Bcl-2 protein is down regulated in a dose dependent manner. Bax protein levels, however, were not altered on C-PC and Celecoxib treatment (Fig. 25). As a result of decreased Bcl-2 with no change in Bax, the ratio of **Bcl-2/Bax** reduced significantly during C-PC and Celecoxib treatment.

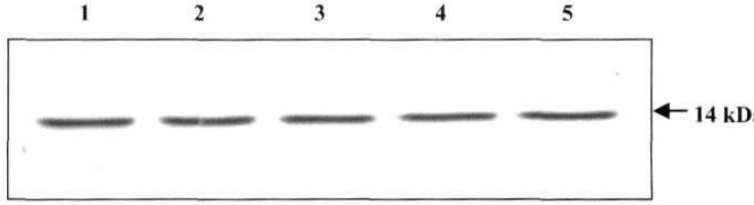
3.5.4 c-Myc expression and modulation

Myc proteins are known to be critical regulators of apoptotic mechanisms. To examine the possible contribution of c-Myc to the C-PC and Celecoxib induced cell death, Western blot analysis was performed in K562 cells with specific antibodies. Under conditions of exponential growth, continuous exposure to C-PC (lanes 2 & 3) and Celecoxib (lanes 4 & 5) produced a marked increase in c-Myc expression in a dose dependent manner (Fig. 26).

3.5.5 Effect of C-PC and Celecoxib on PKC activity

Protein kinase C (PKC) is a Ca^{2+} and phospholipid-dependent serine/threonine protein kinase with fundamental importance in cellular growth control. Alterations in PKC have been linked to the increased cell proliferation in response to tumor promotion. Therefore measurements were carried out as function of time of exposure inorder to assess the effect of C-PC and Celecoxib on PKC activity. After treatment with C-PC and Celecoxib PKC activity decreased in a dose dependent manner (Fig.27), indicating desensitization of PKC upon treatment. This study suggests that antiproliferative effects of C-PC and Celecoxib might be mediated through PKC pathway.

Fig. 25: Immunoblot analysis of Bax expression in C-PC and Celecoxib treated K562 cells



K562 cells were treated with C-PC and Celecoxib for 48 h, and the whole cell lysates (50 ug) were separated on a 12 % SDS-PAGE and the proteins on the gel were transferred to nitrocellulose membrane and probed with mouse monoclonal anti-Bax.

Lane 1: K562 control cells

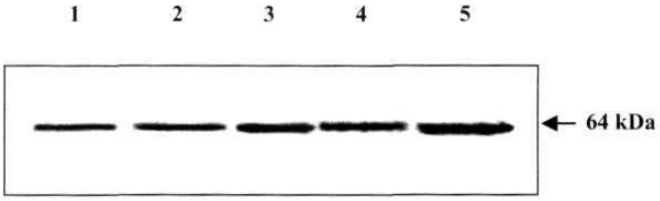
Lane 2: K562 cells treated with C-PC 25 uM

Lane 3: K562 cells treated with C-PC 50 uM

Lane 4: K562 cells treated with Celecoxib 25 uM

Lane 5: K562 cells treated with Celecoxib 50 uM

Fig. 26: Immunoblot analysis of c-Myc expression in C-PC and Celecoixb treated K562 cells



K562 cells were treated with C-PC and Celecoxib for 48 h, and the whole cell lysates (50 ug) were separated on a 10 % SDS-PAGE and the proteins on the gel were transferred to nitrocellulose membrane and probed with affinity purified rabbit polyclonal anti c-Myc.

Lane 1: K562 control cells

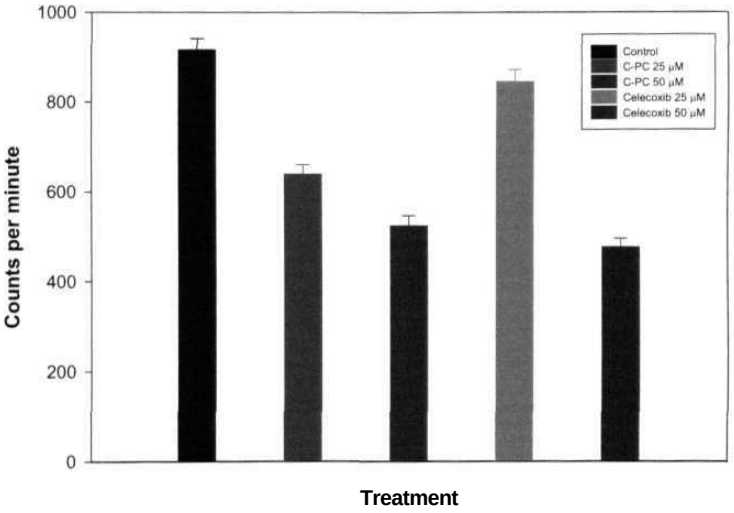
Lane 2: K562 cells treated with C-PC 25 uM

Lane 3: K562 cells treated with C-PC 50 uM

Lane 4: K562 cells treated with Celecoxib 25 uM

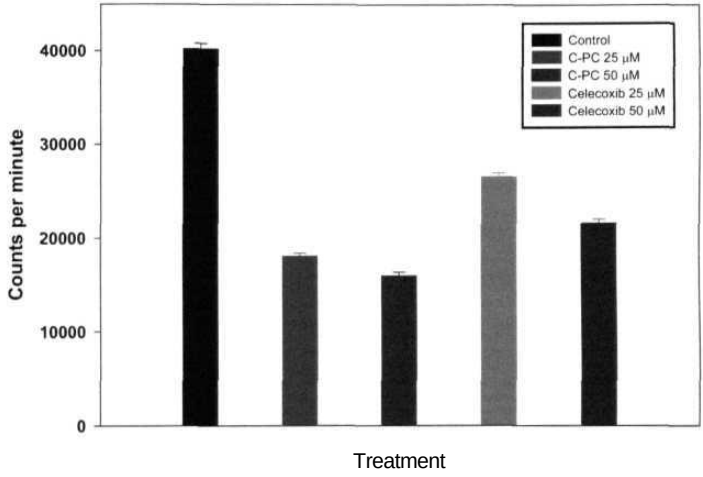
Lane 5: K562 cells treated with Celecoxib 50 uM

Fig. 27 A: **Effect of C-PC and Celecoxib on PKC activity (at 15 min)**



K562 cells were treated with C-PC and Celecoxib (25 uM & 50 uM) for 15 min. PKC activity was determined using MBP (4-14) as described in materials and methods and percent of total PKC activity (bars) from three experiments performed in duplicate were calculated. Results are the mean ± SD of three experiments performed in duplicate.

Fig.27 B: Effect of C-PC and Celecoxib on PKC activity (at 30 min)



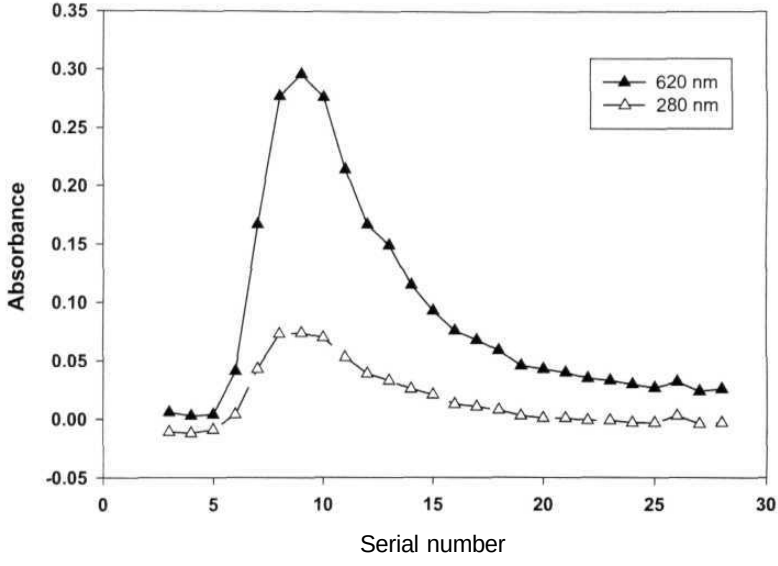
K562 cells were treated with C-PC and Celecoxib (25 uM & 50 uM) for 30 min. PKC activity was determined using MBP(4-14) as described in materials and methods and percent of total PKC activity (bars) from three experiments performed in duplicate were calculated. Results are the mean ± SD of three experiments performed in duplicate.

3.6. Purification of *C-PC* from *Spirulina platensis*

Purification of C-PC from *Spirulina platensis* was achieved by a combination of salt precipitation and ion-exchange chromatography. C-PC was precipitated from Spirulina extract by ammonium sulfate (0-30% initially and 30-50 % later). The 30-50 % ammonium sulfate precipitate was used for further purification on DE-52 column. The dialyzed ammonium sulfate extract was loaded on to DE-52 column. The bound C-PC was eluted with a linear increasing salt concentration (0-0.5 M NaCl, 100 ml) at 1 ml/min (Fig.28). The sample is considered pure, if the ratio of A620/A280 is above 4.0(Boussiba & Richmond, 1979). In the ion-exchange column chromatography only fractions having A620/A280 ratio above 4.0 were pooled and scanning spectrum of the pooled samples were taken. Fig.29 presents the typical visible scanning spectrum of highly purified C-PC, showing absorbance maximum at 620 nm. Details of the purification steps are given in Table 3. As shown in Table 3, the absorbance ratios for the C-PC increased at each step of purification. From one gram of dry Spirulina cells, 60-70 mg pure C-PC was isolated. The yield was sacrificed for the sake of purity.

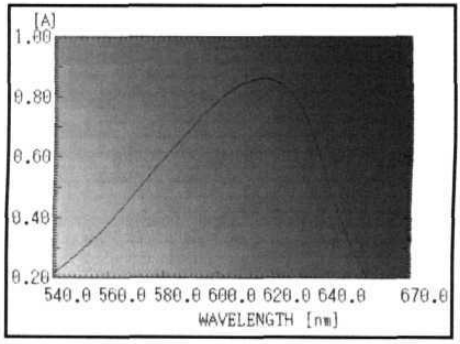
The homogeneity of the purified protein was checked by electrophoresis under non-denaturing (Native-PAGE) and denaturing (SDS-PAGE) conditions. Native PAGE revealed the presence of a single band at 39 kDa (Fig.30 A) and SDS-PAGE performed under denaturing conditions showed the presence of two bands corresponding to a (19 kDa) and p subunits (20 kDa) (Fig.30 B). In order to study the mode of action of C-PC, polyclonal antibodies were raised against

Fig. 28: Elution profile of C-PC



Anion exchange chromatography of C-PC on DEAE-Cellulose (DE-52) column: 30-50 % ammonium sulfate fractionated proteins were loaded on to pre equilibrated DE-52 column and after washing the column bound proteins were eluted with linear salt gradient (0-0.5 M NaCl). The eluted fractions were checked for absorbance at 280 and 620 nm.

Fig.29: **Visible scanning spectrum of C-PC purified from *Spirulina platensis***

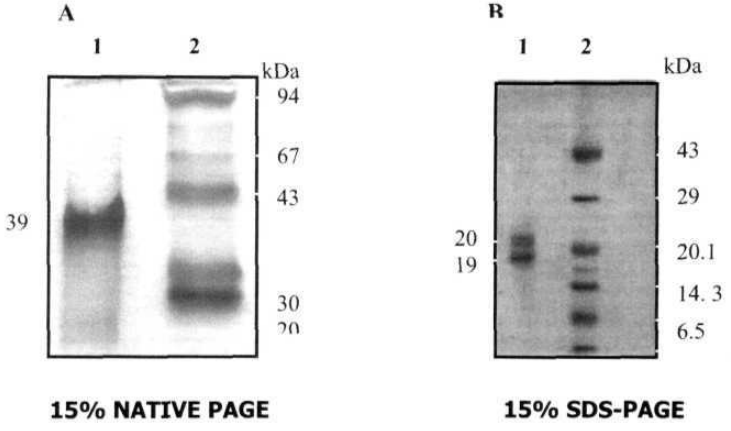


SCANNING SPECTRUM OF C-PC

Table. 3: **Purification profile of C-PC from *Spirulina platensis***

Step of purification	**Absorbance ratios (A_{620}/A_{280})**
Crude extract	**1.08**
Precipitation with $(NH_4)_2SO_4$ (0-15%)	0.45
Precipitation with $(NH_4)_2SO_4$ (30-50%)	2.98
DE-52	**4.8**

Fig. 30: **Native PAGE and SDS-PAGE of C-PC**



15% NATIVE PAGE **15% SDS-PAGE**

purified C-PC. Western blotting with increasing concentration of C-PC confirmed that the antibody is specific to protein of interest (Fig.31).

3.7 Effects of *C-PC* -mechanistic studies

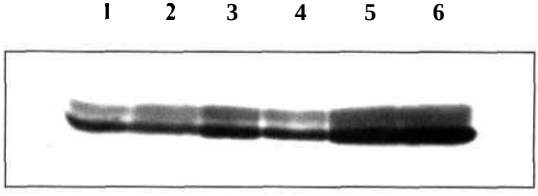
3.7.1 Immunolocalization

The subcellular distribution of C-PC was determined in K562 cells using laser scanning confocal microscopy. Polyclonal antibodies of C-PC were used to determine its *in situ* localization in C-PC treated cells. This was done by using fluorescence labeled anti-rabbit antisera and monitoring the cells exposed to anti-Phycocyanin antiserum on confocal microscopy. These studies showed a strong immunofluorescence signal in the cytosol with no signal in the nuclear compartment (Fig.32) of C-PC treated cells. Native-PAGE analysis confirmed the intactness of the protein in C-PC treated cells (Fig.33). As shown in figure, there is time dependent increase in the uptake of C-PC into the cells, reaching maximum by 24 h exposure. Since the size of the protein is equal to that of the positive control (lane 6), it is assumed that the whole C-PC is entering the cells. These studies thus demonstrate the entry of intact C-PC into the cells and then probably showing effects on K562 cells.

3.7.2 Expression of stress proteins

Hsp60, the eukaryotic homolog of the bacterial chaperonin, is the mitochondrial matrix protein induced by stress and form within the mitochondria. This chaperonin complex that is important for mitochondrial protein folding and function. Apoptosis is closely linked to mitochondrial dysfunction such as reduction in mitochondrial transmembrane potential or release of cytochrome c

Fig. 31: **Western blot analysis of C-PC**



Various fractions collected during C-PC purification were separated on 15 % SDS-PAGE and after electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with anti-C-PC antibodies raised against pure C-PC in rabbit.

Lane 1: Crude

Lane 2: Pellet (8000 rpm)

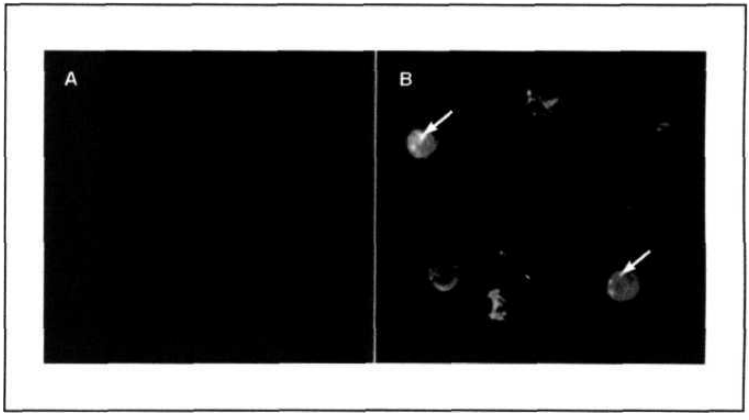
Lane 3: Supernatant (8000 rpm)

Lane 4: 0-30 % $(NH_4)_2SO_4$ supernatant

Lane 5: 30-50 % $(NH_4)_2SO_4$ pellet

Lane 6: DE-52 fraction

Fig.32: Immunolocalization of C-PC by confocal microscopy

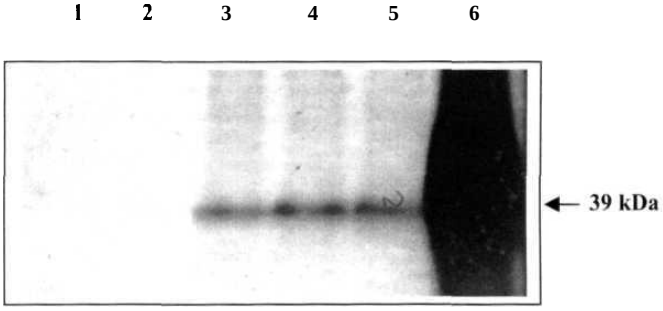


Confocal image showing the localization of C-PC in K562 cells fixed in paraformaldehyde using C-PC polyclonal antibodies. After treatment cells were permeabilized, washed with PBS, incubated with rabbit polyclonal anti C-PC antibodies and then with incubated with anti rabbit FITC conjugated secondary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

A: Control K-562 cells

B: K-562 cells treated with C-PC (50 uM)

Fig. 33: Immuno-localization of C-PC in K562 cells (Native-PAGE)



Whole cell lysates of K562 cells treated with 50 uM C-PC for different time intervals were separated on 15 % native - PAGE, transferred to nitrocellulose membrane and probed with rabbit polyclonal anti-C-PC.

Lane 1: K562 control cells

Lane 2: K562 cells treated with C-PC 50 uM (0 h)

Lane 3: K562 cells treated with C-PC 50 uM (12 h)

Lane 4: K562 cells treated with C-PC 50 uM (24 h)

Lane 5: K562 cells treated with C-PC 50 uM (48 h)

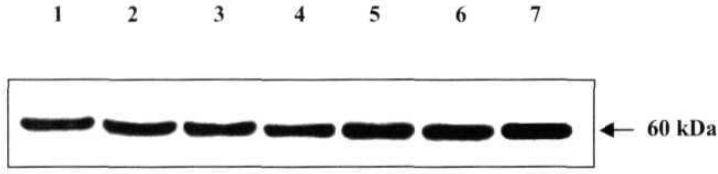
Lane 6: + ve control C-PC

into the cytosol. Therefore in the present study it is evaluated whether mitochondrial Hsp60 is involved in the apoptosis induced by C-PC. As shown in the Fig 34 a dose-dependent and time dependent increase in the levels of mitochondrial stress protein, Hsp60, was observed in the cytosolic extracts of C-PC (lanes 2-7) treated cells, when compared to the untreated control samples (Lane 1).

3.7.3 *C-PC* suppressing telomerase activity of K562 cells

The disruption of the telomeric structure and/or function by drugs interacting with the telomerase enzyme has evolved into a promising way of anticancer drug development. To assess the effects of C-PC on telomerase activity of K562 cells, TRAP (Telomerase repeat amplification protocol) assay was employed. After 5-day incubation of cells with different concentrations of C-PC (1-10 uM), telomerase activity of K562 cells was measured. As shown in Fig.35, the telomerase activity of K562 cells decreased in a concentration-dependent manner.

Fig. 34: Effect of C-PC on Hsp60 protein expression by immunoblot analysis



K562 cells were treated with C-PC and cytosolic extracts were separated on 10 % SDS-PAGE. After electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with mouse monoclonal anti-hsp60 antibodies.

Lane 1: K562 control cells

Lane 2: K562 cells treated with C-PC 25 uM (6 h)

Lane 3: K562 cells treated with C-PC 50 uM (6 h)

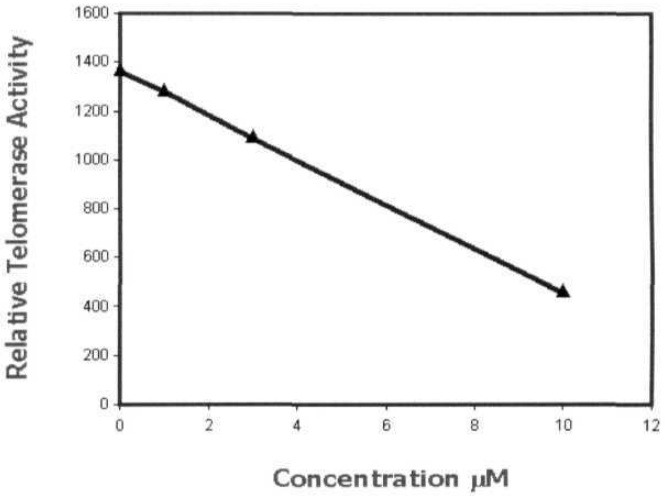
Lane 4: K562 cells treated with C-PC 25 uM (12 h)

Lane 5: K562 cells treated with C-PC 50 uM (12 h)

Lane 6: K562 cells treated with C-PC 25 uM (24 h)

Lane 7: K562 cells treated with C-PC 50 uM (24 h)

Fig. 35: Effect of C-PC on Telomerase Activity (TRAP-ELISA Method)



Detection of telomerase activity in K562 cells treated with C-PC for 5 days. Telomerase activity was determined using the TRAP - ELISA method as described in materials and methods. Values are the mean + SD of data from at least three independent experiments.

Discussion

Chemoprevention, the use of drugs or natural substances to inhibit carcinogenesis, is an important and rapidly evolving subject of cancer research. There has recently been a surge of interest in marine bioresources, particularly seaweeds, as sources of bioactive substances. Several preparations of seaweeds such as polysaccaharide, peptide and phycobiliproteins were shown to affect the multiplication of tumor cells (Schwartz *et a*l. 1988; Noda ef *al.* 1989; Riou *et al.,* 1996). Aqueous extracts of green, brown and red algae were shown to possess bioactivity against murine immunocytes (Sadnori *et al.,* 1993). C-Phycocyanin (C-PC) from *Spirulina platensis* 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).

C-PC is one of the major water-soluble biliprotein present in *Spirulina platensis.* This water-soluble protein pigment is gaining a lot of importance these days because of its various biological and pharmacological properties. C-PC is extensively used as a food colorant and in cosmetics because of its blue colour and its strong fluorescence in the visible region. It is also non-carcinogenic. However, most of its pharmacological properties are not known except a few. Morcos ef *al.* (1988 & 1992) have shown its photodynamic properties and its use in cancer treatment. They have shown that, C-PC specifically binds to cancer cells, and thus can be used for anatomical imaging of tumors *in vivo* (Morcos ef *al.,* 1988). Recently its hepatoprotective (Vadiraja ef *al.,* 1998), anti-oxidant (Romay ef *al.,* 1998 a), radical scavenging (Vadiraja and Madyastha, 2000) and anti- inflammatory properties have been demonstrated (Romay ef *al.,* 1998 b). Earlier studies from the laboratory revealed that C-PC is a selective inhibitor of

COX-2 (Reddy ef a/., 2000) and induces apoptosis in mouse macrophage cell line, RAW 264.7 induced with LPS (Reddy ef a/., 2003) and in rat histiocytoma cell line, AK5 (Pardhasaradhi ef a/., 2003).

Epidemiological studies showed that use of non-steroidal anti-inflammatory drugs (NSAIDs) was associated with a reduced risk of developing several malignant diseases including colorectal cancer (Giardiello ef a/., 1993; Giovannucci ef a/., 1994; 1995; Hanif ef a/., 1996; Barnes and Lee, 1998; Kawamori ef a/., 1998; Reddy ef a/., 1993; 2000). Selective COX-2 inhibitors cause fewer serious adverse effects than traditional NSAIDs. The improved safety profile of selective COX 2 inhibitors makes it realistic to consider their long-term use in individuals at low to moderate risk of cancer. Earlier studies from the laboratory revealed that C-PC is a selective inhibitor of COX-2 (Reddy ef a/., 2000). Similar to other NSAIDs, C-PC is known to exhibit anti-cancer properties (Liu ef a/., 2000). When compared to the toxicities associated with the currently available COX-2 selective anti-inflammatory drugs, C-PC would likely provide safer therapeutic alternative since it is as efficacious as currently used NSAIDs, if not more. But most importantly, this water-soluble biliprotein is from a natural source with least toxic effects.

In an effort to gain insight into effects of C-PC on other cancer cell lines and tounderstand the biochemical mechanism underlying COX-2 inhibitor induced apoptosis, in the present study we have evaluated the effects of C-PC (a natural COX-2 inhibitor) and Celecoxib (a synthetic COX-2 inhibitor) on the proliferation and apoptosis of chronic myeloid leukemia cells (K562 cells). The effects of C-PC and Celecoxib on the viability of K562 cells were evaluated after

24, 48, 72, 96 h in culture. These studies have clearly shown the inhibition in the growth of K562 cells in a dose and time dependent manner. A dose dependent decrease in K562 cell proliferation was observed until 48 hours after C-PC (Fig. 1) and Celecoxib (Fig.2) treatment with maximum decrease in cell proliferation being at 50 μM where the percent inhibition was 49% and 53% respectively. This reduction in the growth of K562 cells in the presence of C-PC / Celecoxib could be due to either apoptosis or necrosis. In order to test the factors responsible for reduced growth of K562 cells, further studies were undertaken on the characteristic markers of apoptosis.

Although a clinical significance of COX-2 inhibitors is well established, the mechanism of the chemopreventive action of these compounds is largely unknown. As COX-2 inhibitors such as Celecoxib and Nimesulide are already in use in several clinical trials, it will be tremendously important to decipher and understand the pathways that underlie the apoptosis promoting activities of these compounds. Indeed, there is accumulating evidence suggesting that the antineoplastic effect of NSAIDs may not be solely mediated by the inhibition of COX-2 activity and a subsequent decrease of prostaglandin E_2 (PGE_2) synthesis, but by other cellular targets besides COX-2. This assumption is largely based on the observation that significantly higher concentrations of NSAIDs are necessary to inhibit cell growth and to induce apoptosis than those required for the inhibition of prostaglandin production (Tegeder *et a*l., 2001). Furthermore, NSAIDs reduced cell survival not only in COX-2 expressing cells, but also in COX-2 deficient cell lines (Elder *et a*l., 1997; Zhang *et a*l., 1999; Grosch *et a*l., 2001). In the present study the effect of C-PC and Celecoxib was monitored on COX-1 and

COX-2 expression in K562 cell line. Both C-PC and Celecoxib showed significant reduction in growth only at very high concentrations i.e 50 μM, while their COX-2 IC50 values are in 0.18 and 0.26 uM range. It is to be noted at this juncture, that both C-PC and Celecoxib inhibit COX-1 also at high concentrations employed in the present study. COX-1 IC_{50} for C-PC and Celecoxib are 4.5 and 16.3 uM respectively (Reddy *et al.*, 2000). The concentrations of C-PC and Celecoxib required for inducing apoptosis in K562 cells is much higher compared to the IC50 values obtained for *in vitro* inhibition of enzyme. The present data indicate that the effects of C-PC and Celecoxib on K562 cells may not necessarily be related to inhibition of prostaglandin synthesis. However, the effective concentrations of C-PC and Celecoxib available in the cells for inhibition of COX-1 and COX-2 is to be evaluated in the present study.

Recent accumulating evidence suggests that defects in the process of apoptosis may be closely associated with carcinogenesis and that many cancer cells have defective machinery for self-destruction (Yano ef a/., 1994). It is suggested that the susceptibility to apoptosis-inducing effects of chemotherapeutic drugs may depend on the intrinsic ability of tumor cells to respond to apoptosis (Yano *et* a/., 1994; Tseng ef a/., 2002). It has been reported that sulindac sulfide can induce apoptosis in promyelocytic leukemia cell line HL-60, which suggests that nonsteroidal anti-inflammatory drugs (NSAIDs) have anti-leukemic effect (Shiff *et* a/., 1995). Recently, it has been shown that aspirin and salicylate induce apoptosis of B-CLL cells (Bellosillo ef a/., 1998). The present study demonstrates the induction of apoptosis in K562 cells treated with C-PC and Celecoxib, the selective COX-2 inhibitors. Apoptosis is a specific mode

of cell death recognized by a characteristic pattern of morphological, biochemical, and molecular changes. C-PC and Celecoxib treated cells showed pronounced morphological changes like cell shrinkage, formation of membrane blebs, and micronuclei characteristic of apoptosis as evidenced by fluorescence and electron microscopic studies. A ladder-like DNA fragmentation pattern, a biochemical marker of apoptosis (cleavage of DNA into nucleosomal size fragments of 180-200 bp) was observed in K562 cells treated with C-PC and Celecoxib. The flow cytometer has recently become the instrument of choice for analysis of cell kinetics and offers a mean for the rapid and accurate analysis of a large population of individual cells. Flow cytometric analysis of treated cells showed the increase of hypodiploid apoptotic cells in a concentration-dependent manner and the decrease of the cells at S and G2 phase of cell cycle. This result suggested a possibility that C-PC and Celecoxib induced apoptosis occurs at S and G2 phase of the cell cycle. This result is similar to that found by Hanif *et al.* (1996) in the study of colon cancer cells for NSAIDs.

In mitochondria, cytochrome c is required as an electron carrier in oxidative phosphorylation, a process which generates the majority of intracellular ATP (Hatefi, 1985). Cytochrome c resides in the space between the outer and inner membranes of mitochondria, where it snuggles up to the cytochrome c oxidase complex located in the inner membrane. 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. In cytoplasm, cytochrome c is known to

get associated with caspase-9, Apaf-1 and dATP to form the apoptosome complex (Li *et* a/., 1997), which inturn activates caspase-9, 3 and 7. Caspase activation leads to the cleavage of cellular substrates and apoptosis. The molecular mechanism responsible for the translocation of cytochrome c from mitochondria to cytosol is at present unknown. A principal hypothesis for how cytochrome c exits mitochondria during apoptosis is that the permeability transition pore in the mitochondrial IM (inner membrane) opens, causing mitochondrial swelling and rupture of the mitochondrial OM (outer membrane) (Szabo and Zoratti, 1991; 1992; Von Ashen *et al.,* 2000). 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 *et al.*, 1998; Tsujimoto and Shimizu, 2000). In the present study, it is examined whether cytochrome c is released or not into the cytosol in response to C-PC and Celecoxib treatment by employing Western blot analysis. These studies have shown the release of cytochrome c after treatment of C-PC and Celecoxib treatment. Cytochrome c release was observed as early as 6 h after treatment with C-PC, with later increase upto 24 h. In this report, we show that the release of cytochrome c from mitochondria to cytosol is an early event in the apoptotic process, preceding morphological signs of apoptosis. This is in support with the earlier finding that C-PC indced apoptosis in RAW 26.7cells through cytochrome c release into the cytosol (Reddy ef *al.,* 2003). 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).

In the present model a biochemical evidence is provided for cellular damage in the from of activation of potential substrates for an ICE/CED 3 like proteases during apoptosis called, Poly (ADP) ribose polymearse (PARP). Activation of caspases leads to cell demise (Nicholson and Thornberry, 1997) via cleavage of cellular substrates, such as actin (Mashima *et* a/., 1997), fodrin (Martin *et* a/., 1995), PARP (Lazebnik *et* a/., 1994) and gelsolin (Kothakota *et* a/., 1997). By processes that are not altogether clear, poly (ADP) ribosylation of variety of proteins facilitates DNA repair. Activation of PARP by DNA damage depletes energy stores and thus may prevent apoptosis. PARP cleavage, on the other hand, seems to be important to preserve the energetic substrates for apoptotic events. In the present study, C-PC and Celecoxib accelerated the cleavage of PARP leading to the formation of a 23 kDa product. This cleavage of PARP might then preclude the catalytic domains of PARP being recruited to the sites of DNA damage, and presumably disable PARP from coordinating subsequent repair and maintain genome integrity. Also PARP is known to negatively regulate the Ca^{+2} and Mg^{+2} dependent endonucleases (Yoshihara *et al.*, 1975; Yoshihara *et* a/., 1974;Tanaka et a/, 1984). Since C-PC and Celecoxib are promoting the PARP cleavage in K562 cells, it may result in activation of Ca^{+2} and Mg^{+2} dependent endonucleases, which would eventually cleave DNA into oligonucleosomal fragments. Caspase-3 cleavage product was not detected in the present study, although PARP cleavage was observed. It is possible that the amount of caspase-3 cleavage was too low to be detected by Western blot analysis or, alternatively, that other caspases may be participating in C-PC induced apoptosis.

Bcl-2 belongs to a growing family of proteins, which can either inhibit (Bcl-2, Bcl-X_L, Bcl-2w, Mcl-1, Bfl-1, A1 etc.) or favor (Bax, Bcl-X_S, Bad, Bak, Bik etc.) apoptosis, which in cells reside predominantly in the outer mitochondrial membrane, endoplasmic reticulum, and the outer nuclear envelope (Adams and Cory 1998; Zamzami *et* a/., 1996). Bcl-2 is known to protect cells against apoptosis triggered by a wide range of factors. Activated *bcl-2* gene could prevent apoptosis induced by c-Myc (Bissonnette *et al.*, 1992), and Bax, another *bcl-2* family gene was observed to be increased when c-Myc was overexpressed (Sakamuro *et* a/., 1995). Enhanced expression of Bcl-2 or of its apoptosis-inhibitory homologs is involved in the pathogenesis of numerous human cancers. Overexpression of Bcl-2 is correlated with the progression of prostate carcinoma (McDonnell *et* a/., 1992). Ectopic expression of Bcl-2 was shown to impair apoptotic signaling by inactivating c-Jun NH2-terminal kinase, leading to apoptosis (Herrmann *et* a/., 1997). Bcl-2 regulates apoptosis atleast inpart, due to their capacity to act on mitochondria, perhaps as an endogenous inhibitor of the pore forming protein Bax (Antonsson *et* a/., 1997). Bcl-2 proteins regulate the translocation of mitochondrial ions or proteins (cytochrome *c)* into the cytoplasm (Kluck ef a/., 1997). Bax might function as a death effector molecule that is neutralized by Bcl-2. However, the inhibitory effect of Bcl-2 on apoptosis is determined by the interaction with Bax, a 21 kDa protein with a degree of homology to Bcl-2. Bcl-2 can form heterodimers with Bax and lose its protective effect. When Bcl-2 is present in excess, cells are protected from apoptosis. However, when Bax is in excess and the homodimers of Bax dominate, cells are susceptible to programmed cell death. So, it appears to be the relative ratios of

Bcl-2 and Bax that determine the fate of a cell, rather than the absolute concentrations of either (Oltvai ef *al.,* 1993). C-PC induced apoptosis in AK5 cells through the down regulation of Bcl-2 (Pardhasaradhy *et al.,* 2003). To determine whether the cell death induced by C-PC and Celecoxib in K562 cells has any relation to the expression of Bcl-2, C-PC and Celecoxib treated cells were analyzed for changes in the levels of Bcl-2 protein. Bcl-2 and Bax are expressed in cultured K562 cells. However, the changes in these two proteins induced by C-PC and Celecoxib were different. The level of Bcl-2 in K562 cells decreased with increasing C-PC and Celecoxib concentrations. In contrast to the decreased Bcl-2 levels, the expression of Bax showed no apparent changes after treatment with C-PC and Celecoxib at various concentrations. The net effect resulted in a lowered ratio of Bcl-2/Bax, which might be responsible for C-PC and Celecoxib induced apoptosis in K562 cells. Similar operation was reported in Indomethacin induced apoptosis in chronic myeloid leukemia cells (Zhang *et al.,* 2000).

Apoptosis is modulated by the expression of a number of regulatory genes, especially some oncogenes and tumor suppressor genes, such as p53, *bcl-2, bax* and *c-myc* (Staunton and Gaffney, 1998). The *c-myc* proto-oncogene has two coupled functions: proliferation and apoptosis. These opposing roles of *c-myc* require the interaction with other gene products to determine the final outcome of cells. A candidate for such a modifying gene is *bcl-2* (Reed, 1994). The interaction between the proto-oncogene *c-myc* and members of the Bcl-2 family may play an important role in the control of cell apoptosis (Bissonnette *et al.,* 1992). Myc proteins are known to be critical regulators of apoptotic mechanisms (Furhmann ef *al.,* 1999; Thompson, 1998). Among various

proapoptotic factors, Myc oncoprotein has been reported to promote apoptotic responses as well as proliferative activity, although the precise mechanism by which Myc regulates both unlinked pathways remains obscure (Shi ***et*** *al.,* 1992; Sakamuro ***et*** *al.,* 1995; Evan and Littlewood, 1998). Constitutive c-Myc expression in an II-3 dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis (Askew ***et*** *al.,* 1991). The present results have demonstrated an increase in the expression of c-Myc in response to C-PC and Celecoxib treatment. Recent experiments have demonstrated that c-Myc-induced sensitization to apoptotic stimuli is mediated by changes in the mitochondrial membrane resulting in the release of cytochrome *c* into cytoplasm (Juin ef *al.,* 1999). The translocation of mitochondrial cytochrome c into the cytoplasm observed in the present study supports such a possibility. The expression of other regulatory proteins, with which c-Myc complexes, in response to C-PC/ Celecoxib treatment might be responsible for driving the cells towards apoptosis (Morris ef *al.,* 2003). Further studies, however, are required to elucidate the mechanism(s) of c-Myc- induced apoptosis in the present study, and explain how c-Myc can promote both cell growth and cell death and what are the factors influencing that decision.

Overexpression of PKC is associated with carcinogenesis, whereas inactivation of PKC is associated with tumor suppression, cell cycle arrest, decreased proliferation, and apoptosis. Inhibitors of PKC have been studied as potential anticancer agents precisely because they are effective in inducing apoptosis (Caponigro et *al,* 1997). C-PC and Celecoxib inhibited PKC activity in K562 cells, suggesting that the antiproliferative signal from C-PC and Celecoxib

may be partially transmitted through PKC. However, the target of PKC-mediated suppression of proliferation in K562 cells is not known. Also the present data demonstrating the induction of apoptosis is preceded by arrest of cells in the S and G2 phase of the cell cycle suggesting that C-PC and Celecoxib probably interact with components of the cell cycle engine such as cyclin-dependent kinases and phosphorylation events regulated by other kinases.

Mode of action of C-PC

C-PC has been shown to induce apoptosis in mouse macrophage cell line (RAW264.7) stimulated with LPS (Reddy *et* a/., 2003). In addition to inducing apoptosis, this biliprotein is known to have anti-inflammatory (Romay *et* a/., 1998 b), anti-oxidant (Romay ef a/., 1998 a) properties with potent inhibition of COX-2 activity (Reddy *et* a/., 2000). It is not clear whether any of these activities contribute to the ability of C-PC to induce apoptosis.

To understand the biochemical properties and to raise polyclonal antibodies to C-PC it was necessary to have pure C-PC. C-PC from *Spirulina platensis* was isolated and purified to homogeneity and the procedure involved precipitation with ammonium sulfate (30-50 % saturation), and ion exchange column chromatography on a DEAE-cellulose column. As reported earlier, the A620/280 ratio of C-PC can be used to check the purity of C-PC preparation and if the A620/280 ratio is >4, it is considered as pure. So peak fractions having A620/A280 ratio above > 4.0 were pooled. The UV-Visible spectrum exactly matches the reported spectra of C-PC from *Spirulina platensis* (Boussiba, s 1979). The native PAGE and SDS-PAGE data indicate that, the C-PC is pure and contains two subunits, a (17 kDa) and (3 (20 kDa). In order to study the mode

of action of C-PC, polyclonal antibodies were raised against purified C-PC in rabbits. Western blot analysis with increasing concentrations of C-PC confirmed that the antibody is specific to protein of interest.

Heat shock proteins are a family of highly conserved molecules involved in protein folding in both prokaryotic and eukaryotic cells. Hsp60 strictly interacts in a two-step folding mechanism in normal prokaryotic and eukaryotic cells. The role of Hsps during carcinogenesis was postulated and investigated *in vivo* in many sites, as lung (Koomagi *et al.,* 1996), breast (Yano *et al.,* 1996), esophageal (Nakajima *et a*l., 2002) and ovarian (Yamamoto *et al.,* 2001) melanoma (Protti *et al.,* 1994), lymphoblastic leukemia and nephroblastoma (Stammler and Volm, 1996). Mitochondria, that can release pro-apoptotic factors such as cytochrome-c and pro-caspase, are central regulators of the apoptosis. It has been suggested that the activation of caspase-3 and -6, key components of the apoptotic machinery forms a part of a multiprotein complex which contains the mitochondrial Hsp60, and pro-caspase-3 was activated and dissociated from the Hsp60 complex following induction of apoptosis, suggesting that Hsp60 may serve as positive regulator of caspases during apoptosis (Samali *et al.,* 1999; Xanthoudakis *et al.,* 1999). Heat shock proteins are known to modulate apoptotic cell death induced by various stimuli and would modulate the balance between cell death and survival (Ciocca *et al.,* 1992; Jaattela, 1999). Recent results from several laboratories have made it clear that cytoplasmic Hsp70 and Hsp27 prevent apoptosis induced by several anticancer drugs as well as other apoptotic stimuli, suggesting that these heat shock proteins have anti-apoptotic action and thus limit the efficacy of cancer therapy (Dix ef *al.,* 1996; Creagh and

Cotter 1999; Mosser et a/, 1999). In contrast, mitochondrial heat shock proteins such as Hsp60 and mtHsp70 were upregulated by the treatment of anticancer drug in HeLa cells (Kim ef a/., 1999). Also, it has been revealed that Hsp60 accelerates the maturation of pro-caspase-3 by upstream activator proteases during apoptosis in Jurkat cells (Samali *et* a/., 1999; Xanthoudakis *et al.*, 1999). The present study also demonstrated an increase in mitochondrial Hsp60 levels in a dose and time dependent manner during C-PC induced apoptosis.

Telomerase activation and telomerase catalytic subunit gene *(hTERT)* are correlated with the deregulation of apoptosis. Telomerase is active in stabilizing telomeres of certain self-renewing cell populations and most malignant cells and, in humans, telomeres in these cells are maintained at about 15 kilobase pairs (kbp). In contrast, telomerase is not expressed in most normal human somatic cells, and telomere length is significantly shorter (Greider, 1998). The telomere hypothesis postulates stabilization of telomere length and telomerase activation as key events in cellular immortalization and carcinogenesis (Hayakawa *et* a/., 1999). Accordingly, telomerase could be a novel and highly selective target for antitumor drug design, a number of reports have examined factors believed to inhibit telomerase (Boklan ef a/., 2002). The present study indicates a decrease in telomerase activity in K562 cells with C-PC treatment for up to 5 days suggesting that this biliproetin from *Spirulina platensis* could be a candidate for cancer therapy. The positive correlation was also detected between *hTERT* or telomerase activation and Bcl-2 expression (Wang ef a/., 2000). Recently, it is shown that down-regulation of Bcl-2 and telomerase is considered to play an important role in drug-induced apoptosis (Lyu ef a/., 2002; Ji ef a/., 2002). It is

reported that telomerase activity in the colorectal carcinomas with expression of Bcl-2 is higher than in the carcinomas without expression of Bcl-2, suggesting that expression of Bcl-2 is importantly associated with telomerase activity. In this study, C-PC decreased the expression of anti-apoptotic gene, Bcl-2 suggesting that C-PC exerts an inhibitory effect on the cell growth of K562 cells through down regulation of Bcl-2, but not the Bax gene mediated pathway.

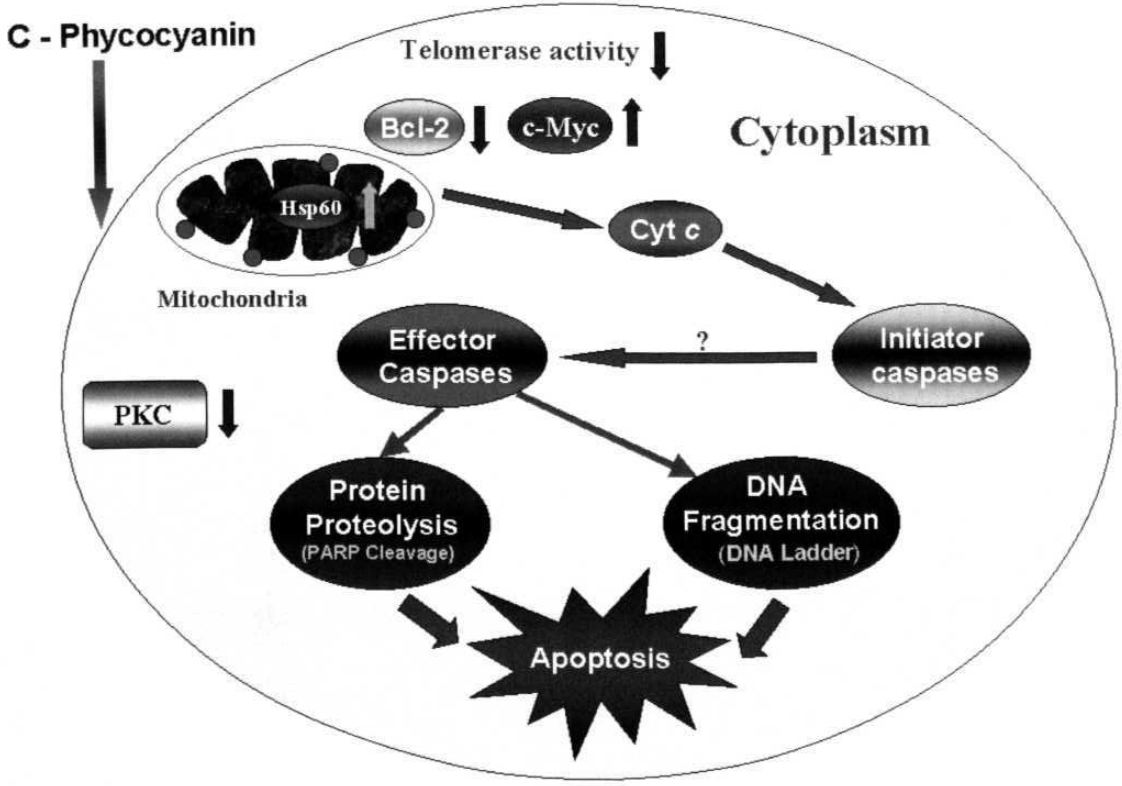
Immunological studies employing C-PC polyclonal antibodies and fluorescent *in situ* hybridisation on confocal microscope suggest that intact C-PC enters into the K562 cells and is concentrated in the cytosol. However, it is not clear whether the entry of C-PC into the cells is mediated by any cell surface receptors or by other mechanisms. It was demonstrated earlier that C-PC is specifically taken up by actively proliferating cancer cells (Morcos *et at.,* 1988), suggesting such a possibility in K562 cells also.

In summary, this work presents evidence that COX-2 inhibitors C-PC and Ceiecoxib, induce apoptosis in human chronic myeloid leukemia cells. Both C-PC and Ceiecoxib induced ultrastructural changes such as cell shrinkage, formation of membrane blebs, and micronuclei characteristic of cells undergoing apoptosis. This induction of apoptosis in K562 cells by C-PC and Ceiecoxib appears to be mediated by cytochrome c release, PARP cleavage, Bcl-2 down regulation, c-Myc upregulation, overexpression of Hsp60 and inhibition of PKC activity., and by inhibiting PKC activity. Since very high concentrations of C-PC and Ceiecoxib (much higher than IC_{50} values for COX-2) are required to induce apoptosis in these cells, it is suggested that COX-2 independent pathways also may be operating in inducing apoptosis. Howevr, it is not clear at this juncture that how

much of C-PC/ Celecoxib supplied to K562 cells is available for action. The present study also demonstrated that C-PC, being a protein of 39 kDa enters into the K562 cells. However, the mechanism of its entry is quite unclear. The overall signal transduction mechanism involved in C-PC induced apoptosis in K562 cells is presented in Fig. 36.

Since C-PC being a natural pigment and a component of edible Spirulina extracts, it forms a good alternative to highly toxic chemotherapeutic products in the market. Also it can form a good candidate for enhancing the sensitivity of cancer cells to conventional anticancer drugs and thus ultimately reducing their toxic side effects. However, further studies are required to test the efficacy of C-PC and Celecoxib on CML and other leukemias in animal models.

Fig. 36: Proposed Mechanism of Action of C-Phycocyanin



Summary

C-Phycocyanin (C-PC) is a non-toxic water soluble phycobiliprotein pigment isolated from *Spirulina platensis.* This water- soluble protein pigment is of greater importance because of its various biological and pharamacological properties. C-PC is reported to have anti-inflammatory, antioxidant, and antiarthritic properties. Recent studies have shown that C-PC selectively inhibits cyclooxygenase-2 (COX-2) and also induces apoptosis in RAW 264.7 cells stimulated with LPS, and AK5 rat histiocytoma cell line. Celecoxib was the first COX-2 inhibitor approved for use in U.S.A. In addition to the analgesic, antipyretic and anti-inflammatory activity, it has chemopreventive properties against colon cancer. Celecoxib significantly reduces the number of colon polyps in patients with familial adenomatous polyposis (FAP) and was recently approved by FDA for the treatment of FAP patients. Celecoxib was recently shown to induce apoptosis in COX-2 expressing androgen responsive (LNCap) and non-responsive (PC3) prostate cancer cells.

Prostaglandins (PGs) are a family of intercellular and intracellular messengers derived from arachidonic acid. These mediators exert a wide range of effects on processes such as smooth muscle tone, vascular permeability, cellular proliferation and inflammatory/immune function. The initial step in the synthesis of PGs from arachidonic acid is mediated by cyclooxygenases (COX also known as prostaglandin H synthase or prostaglandin endoperoxide synthase), of which two isoforms are recognized. COX-1 is expressed constitutively in most cell types, and prostanoids derived from COX-1 are thought to be important in gastric and renal homeostasis. COX-2 is the product of immediate early gene and is rapidly expressed only after exposure of cells to

hormones, mitogenic stimuli, bacterial lipopolysaccharides and inflammatory mediators. The induction of COX-2, with the resultant production of prostanoids can contribute to parturition, inflammation, pain, fever and certain types of cancer.

NSAIDs are used to treat acute and chronic inflammatory disorders. This anti-inflammatory mechanism of NSAIDs is due to a reduction of PG synthesis by the direct inhibition of cyclooxygenase. Since COX-2 is responsible for the production of prostaglandins at the site of inflammation, the selective inhibition of COX-2 results in potent anti-inflammatory effect. Unlike the non-specific NSAIDs, the selective COX-2 inhibitors have lesser gastrointestinal and other side effects. In addition to the role of COX-2 in inflammation, a number of studies have shown its role in cancer. A strong correlation has been established between the use of NSAIDs and the decreased incidence of colorectal, breast and lung cancers.

In view of involvement of cyclooxygenases in the mediation of cancers, particularly COX-2 which is over expressed in many cancers and inhibition of COX-2 leads to a markedly reduced tumor growth & block angiogenesis, the therapeutic role of COX-2 inhibitors towards a variety of cancers appear promising. Hence in the present study it is proposed to test the effect of COX-2 inhibitors on the growth and multiplication of CML cell line in K562. For this study C-PC a natural COX-2 inhibitor, and Celecoxib, a synthetic COX-2 inhibitor in the market, were employed. An attempt also was made to understand the mechanism(s) of C-PC induced cell death and to correlate the same with Celecoxib.

The present study demonstrates that C-PC (natural COX-2 inhibitor) and Celecoxib (synthetic COX-2 inhibitor) induce apoptosis in chronic myelogenous leukemia cells (K562 cells). The results indicate that C-PC and Celecoxib reduced the growth and multiplication of K562 cells. Phase contrast microscopic studies also revealed the presence of cells with web like activated membrane structure and also decrease in cell number. Ultrastructural changes, like membrane blebbing and nuclear condensation, typical of apoptosis, were observed by Scanning and Transmission electron microscopy.

In the present study the effect of C-PC and Celecoxib was monitored on COX-1 and COX-2 expression in K562 cell line. Both C-PC and Celecoxib showed significant reduction in growth only at very high concentrations i.e. 50 uM, while their COX-2 IC_{50} values are in 0.18 and 0.26 uM range. It is to be noted at this juncture, that both C-PC and Celecoxib inhibit COX-1 also at high concentrations employed in the present study. COX-1 IC_{50} for C-PC and Celecoxib are 4.5 and 16.3 uM respectively (Reddy *et a*/., 2000). The concentrations of C-PC and Celecoxib required for inducing apoptosis in K562 cells is much higher compared to the IC50 values obtained for *in vitro* inhibition of enzyme. The present data indicate that the effects of C-PC and Celecoxib on K562 cells may not necessarily be related to inhibition of prostaglandin synthesis. However, the effective concentrations of C-PC and Celecoxib available in the cells for inhibition of COX-1 and COX-2 is to be evaluated in the present study.

The enzymatic cleavage at the DNA linker region renders a "classical laddering" of DNA, regarded as a marker of apoptosis, was clearly detected in cells treated with C-PC and Celecoxib. Flow cytometric analysis of the K562

cells treated with C-PC and Celecoxib showed 14-21 % of cells in sub G0/G1 phase. Flow cytometric analysis of treated cells showed the increase of hypodiploid apoptotic cells in a concentration-dependent manner and the decrease of the cells at S and G2 phase of cell cycle. This result suggested a possibility that C-PC and Celecoxib induced apoptosis occurs at S and G2 phase of the cell cycle.

Cytochrome c is known to be an essential factor in the mitochondrial respiratory chain and is released in response to various stimuli that elicit apoptosis. The precise mechanism for cytochrome c translocation is still unclear. Western blot analysis showed that cytochrome c is released significantly in the treated cells as early as 6 h compared to the control cells. Poly-ADP-Ribose-Polymerase (PARP), a 116-kDa protein that binds specifically at DNA strand breaks, is also a substrate for certain caspases (for example, caspase 3 and 7) activated during early stages of apoptosis. These proteases cleave PARP to fragments of approximately 85 kDa and 23 kDa. Detection of the 23 kDa PARP fragment with anti-PARP antibodies thus serves as an early marker of apoptosis. In the present study, a dose dependent increase in PARP cleavage was observed in cells treated with C-PC as well as Celecoxib.

Bcl-2 prevents the release of apoptosis inducing factor and cytochrome c from mitochondria, which is considered to be a key event during apoptosis. The present studies revealed a down regulation of Bcl-2 in K562 cells treated with C-PC and Celecoxib. Bcl-2 can form heterodimers with Bax protein, a Bcl-2 associated protein, which antagonizes Bcl-2 action and induces apoptosis. In this study no influence was seen on Bax protein levels in K562 cells treated with C-

PC and Celecoxib. The resulting net effect could thus lead to a lowered ratio of Bcl-2/Bax, which might be responsible for the C-PC and Celecoxib induced apoptosis in K562 cells. The interaction between the proto-oncogene *c-myc* and members of the Bcl-2 family may play an important role in the control of cell apoptosis. Among various proapoptotic factors, Myc oncoprotein has been reported to promote apoptotic responses. The present studies have demonstrated an increase in the expression of c-Myc in response to C-PC and Celecoxib treatment. C-PC and Celecoxib inhibited PKC activity in K562 cells, suggesting that the antiproliferative signal from C-PC and Celecoxib may be partially mediated through PKC. Also the present data demonstrating the induction of apoptosis is preceded by arrest of cells in the S and G2 phase of the cell cycle engine such as cyclin-dependent kinases and phosphorylation events regulated by other kinases.

Heat shock proteins are known to modulate apoptotic cell death induced by various stimuli and would modulate the balance between cell death and survival. The present study also demonstrated an increase in mitochondrial Hsp60 levels during C-PC and Celecoxib induced apoptosis. It was reported that telomerase activation and telomerase catalytic subunit gene (*hTERT*) are correlated with the deregulation of apoptosis. The telomere hypothesis postulates stabilization of telomere length and telomerase activation as key events in cellular immortalization and carcinogenesis. Accordingly, telomerase could be a novel and highly selective target for antitumor drug design. The present studies indicate an apparent decrease in telomerase activity in K562 cells with C-PC treatment for upto 5 days and hence this biliprotein from *Spirulina platensis* could

be a candidate for cancer therapy. Immunological studies employing C-PC polyclonal antibodies and fluorescent *in situ* hybridisation on confocal microscope suggest that C-PC enters into the K562 cells and is concentrated in the cytosol. However, it is not clear whether the entry of C-PC into the cells is mediated by any cell surface receptors or by other mechanisms.

In conclusion our studies reveal that C-PC, a naturally occurring biliprotein from *Spirulina platensis* and a known COX-2 inhibitor, induces apoptosis in chronic myeloid leukemia K562 cells and is comparable with Celecoxib, a known COX-2 inhibitor and a chemopreventive agent. This is confirmed by MTT assay, nuclear condensation assay (DAPI staining), electron microscopic studies (SEM & TEM), DNA fragmentation, PARP cleavage and FACS analysis. The induced apoptosis is accompanied by the release of cytochrome c into the cytoplasm and downregulation of Bcl-2 expression, c-Myc overexpression, and downregulation of PKC activity. The overall signal transduction mechanism of C-PC induced apoptosis in K562 cells is presented in Fig.36. Being a natural compound and having characteristic stability and solubility in aqueous solution, C-PC could form a more acceptable chemo preventive and/or chemotherapeutic agent for CML patients. Further in depth studies on CML patients, however, are required to test this possibility.

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