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Introduction

The term cancer encompasses a group of neoplastic diseases in which there is a transformation of normal body cells into malignant ones (Adopted from http://medical-dictionary.thefreedictionary.com/Cancer (disease)). There are over 200 different known cancers that afflict the different tissues of humans due to various reasons (Adopted from: http://en.wikipedia.org/wiki/Cancer). In this process, the link between inflammation and cancer has been variously hypothesized. This was supported by the epidemiological evidences which proposed that 25% of all cancers are due to chronic inflammation or infection associated with the inflammatory state (Schetter et al, 2010).

All cancers commence in cells, the body’s fundamental unit of life (Adopted from http://training.seer.cancer.gov/disease/cancer/biology/). Therefore, to know cancer, it’s helpful to know what happens when normal cells become cancerous (Adopted from http://www.cancer.gov/cancertopics/cancerlibrary/what-is-cancer). The body is made up of several cell types, which grow and divide in an organized manner to generate more cells as they are required to maintain the healthy body and when they become old or damaged, they die and are replaced with the new cells (http://en.wikipedia.org/wiki/Cancer_cell) (Figure 1.1). Sometimes this systematic
procedure becomes erroneous and genetic materials become damaged or changed, resulting in
mutations that affect normal cell growth and division (Adopted from http://www.cancer.gov/cancertopics/cancerlibrary/what-is cancer). During this process, the cells do not die in fact when they should (Adopted from http://www.cancer.gov/cancertopics/cancerlibrary/what-is cancer) and there is the formation of new cells against the body’s requirement and the extra mass of tissue formed by the cells is called a tumor (Adopted from http://www.cancer.gov/cancertopics/cancerlibrary/what-is cancer).

Cancer can occur in almost any part of the human body. However, not all tumors are cancerous. Tumor can be benign or malignant (Adopted from https://en.wikipedia.org/wiki/Cancer (Figure 1.2). The term "benign" refers to a tumor condition, or growth that is non-cancerous (Adopted from http://en.wikipedia.org/wiki/Benign_tumor) (Figure 1.2). It is localized and has not spread (or metastasize) to other parts of the body or invaded and destroyed the nearby tissues (Adopted from http://en.wikipedia.org/wiki/Benign_tumor). In general, a benign tumor or such condition is usually not harmful and grows slowly (Adopted from http://www.mesothelioma-treatments.org/benign.html). They can usually be removed and never come back in most of the cases. However, if a benign tumor is large enough, then its size and weight can push the nearby organs, blood vessels, and nerves and thus can cause serious health related problems.

In contrast, malignant tumors are cancer, where the cancer cells not only destroy the host tissues but can also invade and harm the organs and tissues near to it (Adopted from http://www.mesothelioma-treatments.org/benign.html). Also, the cancer cells can break
away from a malignant tumor and enter the lymphatic system or the bloodstream. This is the manner by which cancer spreads from the original tumor to form new tumors in other parts of the body (or metastasize) (Adopted from http://www.mesotheliomatreatments.org/benign.html).

**Colon cancer** is the cancer that forms in the tissue of colon (large intestine) ending in the terminal portion called rectum (Figure 1.3). Most colon or colorectal cancers are adenocarcinomas (cancers that begin in these cells can make and release mucous and other fluids) (Adopted from http://www.cancer.gov/cancertopics/types/colon-and-rectal).

Colon cancer is the second major cause of cancer death worldwide and imparts a substantial amount of morbidity and mortality every year (Jemel et al, 2004). In spite of recent advancements in chemotherapeutic and other mode of treatment, 56,000 deaths occur each year due to this disease (Jemel et al, 2004). Various epidemiological and laboratory animal model studies indicated that etiology of colon cancer is multifactorial and complex (Guruswamy and Rao, 2008).

Colon cancer begins from the colon epithelium as a outcome of the accumulation of genetic variations in defined oncogenes and tumour suppressor genes (Feinberg et al, 2006). Several genetic defects in colon cancer have been found and these play important roles in the carcinogenesis of colon cancer (Adopted from http://www.cancer.gov/cancertopics/pdq/genetics/colorectal /HealthProfessional/page1). It has been found that several signal

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**Figure 1.3:** Various stages of colon cancer. Adopted from http://lancastria.net/blog/colon-cancer-dont-ignore-the-symptoms.html
transduction pathways, including K-ras, Src/PI3-K/Akt, β-catenin, TGFβ and p53 may play critical roles in its pathogenesis. Environmental factors including obesity, diabetes and diet may also play important roles in colon cancer progression (Huang and Chen, 2009). It has been recognized that the colon cancer development proceeds in a multi sequential stage from polyps to adenocarcinoma and it may take a long time for colon cancer to develop, which is estimated to be between 10–17 years (Adopted from http://www.cancer.gov/cancertopics/pdq/genetics/colorectal/HealthProfessional/page1). This time interval provides an opportunity to prevent the disease. However, currently available therapeutic approaches (such as cytotoxic chemotherapy, radiation therapy and biologic response modifiers) for advanced colorectal cancer are only palliative. Therefore, efforts to treat colon cancer at an early stage would consequently be a logical approach for prevention (Krishnan et al, 2000).

**Cyclooxygenase:** Cyclooxygenase (COX) is a bifunctional enzyme catalyzing the first two steps in the biosynthesis of prostaglandins (PGs) from the substrate, arachidonic acid (C20:4) (Smith et al, 1996). Studies carried so far suggest that there is a link between cancer and prostaglandins and it has been observed that tumor tissues contain higher levels of PGs (Cummings and Robertson, 1977). It is now known that at least two forms of COX enzyme exist (Xie et al, 1991). One of these, COX-1 is considered to be a constitutive form and is responsible for maintaining normal physiological functions and the PGs produced by this enzyme may play a protective role in homeostasis in gastrointestinal mucosa (Fournier and Gordon, 2000). The other known form of the enzyme, COX-2, is an inducible protein and its expression is affected by various stimuli such as mitogens, oncogenes, tumor promoters and growth factors (Fournier and Gordon, 2000). COX-2 is the principle isoform that participates in inflammation, and induction of COX-2 is responsible for the production of PGs at the site of inflammation (Dubois et al, 1998). A causative association between increased COX-2 expression and carcinogenesis is supplied by evidence from animal models where chemically and genetically induced colonic tumor growth is inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) and in particular, the selective COX-2 inhibitors (Thun et al, 2002; Barnes and Lee, 1998).

Accumulating evidences strongly suggest 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 et al, 1994). The susceptibility to apoptosis-inducing effects of the chemotherapeutic drugs may depend upon the intrinsic ability of the tumor cells
or not to respond to apoptosis (Tsang et al, 2002). It has been reported that sulindac sulfide can reduce apoptosis in promyelocytic leukemia cell line HL-60, which suggests that the NSAIDs may have antileukemic effect (Shiff et al, 1995). In addition to their general and classical use as reliever of pain, fever and inflammation, the NSAIDs have thus an emerging potential for the chemoprevention of cancer in colon and other tissues (Hinz and Brune, 2002). It has also been shown that the prototype NSAIDs such as aspirin and salicylates, may induce apoptosis in B-CLL cells (Bellesillo et al, 1998).

**COX-2 inhibitors and Cancer:** Evidences strongly suggest a protective effect of NSAIDs in colon cancer (Chung-Faye and Kerr, 2007). Several cohort and case control studies have consistently shown dose related reduction of colorectal cancer in regular users of these drugs. Persons who regularly used NSAIDs had a strikingly lower risk of colon cancer compared to those who did not use NSAIDs (Dubois et al, 1996). In this context, piroxicam which has analgesic, anti-inflammatory and antipyretic properties (Jacoby et al, 1996) and used in rheumatic disorders and rheumatoid arthritis, had also recently been reported to possess anti-tumor properties, via presumably inhibiting the COX activity (Jacoby et al, 1996, 2000a).

Recent randomized clinical trials have shown that the traditional non-steroidal anti-inflammatory drugs (NSAIDs) such as the relatively non-selective COX inhibitors like piroxicam and sulindac as well as the relatively selective COX-2 inhibitors, like celecoxib, both are able to cause regression of adenomas in patients with familial adenomatous polyposis, by up to 100% (Steinbach et al, 2000). The traditional NSAIDs are non-selective inhibitors of both the isoforms of COX, COX-1 and COX-2 while COX-2 selective inhibitors block it in pathological tissues such as in different malignancies where it is overexpressed (Grosch et al, 2006). Studies have however, shown that although the selective COX-2 inhibitors are very effective anti-neoplastic agents, they also suffer from cardiovascular, renal and other side effects (Komers et al, 2001a). Therefore the search is on for more amenable COX inhibitory compounds or a combination thereof.

Piroxicam is a NSAID used to relieve the symptoms of rheumatoid and osteoarthritis, primary dysmenorrhea, postoperative pain and act as an analgesic, especially where there is an inflammatory component (Ding et al, 2003). Piroxicam exerts its growth
inhibitory effects toward premalignant and malignant epithelial cell lines via COX/PGE₂-independent mechanisms targeting signaling pathways regulating cell cycle progression. Recently, anti-tumor attribution of piroxicam has been reported via inhibiting COX activity (Jacoby et al, 2000b).

In cancer research, the use of synthetic drugs as well as the natural bioactive substances to chemoprevent carcinogenesis is an important and rapidly evolving subject (Signorelli and Ghidoni, 2005). In this context, there has recently been a sign of interest in marine bio-reserves, particularly the sea weeds as the source of bioactive substances. Several components of sea weeds such as the polysaccharides, peptides and phycobiliproteins were shown to affect the multiplication of tumor cells (Schwartz et al, 1988; Noda et al, 1989; Riou et al, 1996). Aqueous extracts of green, brown and red algae were also shown to possess the bioactivity against murine immunocytes (Sadnori et al, 1993). In this regard c-phycocyanin from the *Spirulina platensis* was shown to reduce the viability of mouse myeloma cells (Morcos et al, 1988).

C-phycocyanin is one of the water-soluble biliproteins present in *S. platensis*, the blue green algae (cyanobacterium) with antioxidant and free radical scavenger properties (Bhat and Madhyastha, 2000). This water soluble pigment is gaining a lot of importance these days because of its various biological and pharmacological properties. It is shown to be hepatoprotective (Vadiraja et al, 1998), anti-arthritis (Vadiraja et al, 1998) and most importantly anti-inflammatory in nature (Romay et al, 1998a,b; 2000a,b). Very little is known about the mechanism of the anti-inflammatory action although it is shown that c-phycocyanin selectively inhibits COX-2, the inducible isoform of the enzyme responsible for the prostanoid biosynthesis from arachidonic acid (C₂₀:₄) as stated earlier. Morcos et al (1988) have shown its photodynamic properties and use in cancer treatment. They have shown that it specifically binds to cancer cells and thus can be used for anatomical imaging of tumors in vivo. It induces apoptosis in mouse macrophage cell line RAW2647 stimulated with LPS (Reddy et al, 2003) and also in rat histiocytoma cell line, AK5 (Pardhasaradthi and Khar, 2003). C-phycocyanin is effective in causing apoptosis of chronic myeloid leukemia cells (K562) (Subhashini et al, 2004). A dose dependent decrease in cell proliferation was observed and the reduction in the growth of K562 cells in presence of c-phycocyanin could be due to the cellular death by apoptosis.
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The present work therefore focuses on a combination therapy of piroxicam, a tNSAID and c-phycocyanin, a specific COX-2 inhibitor in the chemoprevention of colon cancer and the underlying molecular mechanisms involved in such process.

**Phosphatidyl inositol 3-Kinase (PI3-K):** One of the main focus of the study is the oncogene product, PI3-K which has become a major target of cancer studies because of its involvement in cell survival, proliferation and growth (Yamaguchi et al, 2004). It catalyzes the formation of phosphatidyl inositol 3, 4, 5-triphosphate, which constitutes the second messenger for activating downstream targets such as Akt (protein kinase B) (Vivanco and Sawyers, 2002). There is a growing body of evidence to support the notion that the activation of PI3-K is associated with colon cancer and convert differentiated human gastric or colonic carcinoma cell to a less differentiated and more malignant phenotype (Semba et al, 2002).

**Akt:** Akt is a serine/threonine protein kinase and also known as protein kinase B or Aurora kinase (Bellacosa et al, 1991). It is an inactive cytosolic protein recruited to the plasma membrane, activated by phosphorylation at threonine 308 and serine 473 residues in response to growth factors or cytokines, via the product of PI3-K (Stephens et al, 1998). Upon phosphorylation, Akt has been shown to phosphorylate and to block the action of several pro-apoptotic proteins such as Bad. It also blocks the cytochrome c release from mitochondria through the regulation of Bcl-2 (Hayakawa et al, 2000) while, the effects of PI3-K on tumor growth and progression are thought to be mediated by this protein (FresnoVara et al, 2004). Akt is overexpressed in a number of cancers, including colon, pancreatic, ovarian, and some steroid hormone-insensitive breast cancers (Roy et al, 2002). Moreover, it has been reported that Akt phosphorylation in human colon carcinoma correlates with cell proliferation and apoptotic inhibition, as well as with different clinicopathologic parameters such as invasion grade, vessel infiltration and metastasis to lymph nodes and tumor stage (Itoh et al, 2002).

**Glycogen synthase kinase-3β (GSK-3β):** GSK-3β, a proliferation associated kinase, is one of the primary target for Akt which inactivates GSK-3β by phosphorylation (Frame and Cohen 2001). Apart from inhibition in glycogen synthesis, GSK-3β is known to regulate a diverse array of cell functions such as apoptosis and cell proliferation and also over
expression of it is seen to induce apoptosis in prostate cancer cells (Doble and Woodgett 2003). It inhibits anti-apoptotic molecules including heat shock proteins which in turn may stimulate apoptosis (Xavier et al, 2000). In contrast, GSK-3β mediates cell survival in other cell types as well (Hoeflich et al, 2000). Since, it has been suggested that PI3-K signaling promotes tumorigenesis in human colorectal cancer cells (Khalegpour et al, 2004), it is of great interest to determine whether GSK-3β counteracts the anti-apoptotic effect of the PI3-K pathway and promotes apoptosis in the present experimental colorectal cancer studies.

Rationale of the present study

One of the lessons learned from basic cancer research in recent years is that the combinatorial strategies in cancer therapy can provide dramatic improvement in safety and efficacy over monotherapy regimens, especially if the drugs differ in their mode of action. In this regard, several combinations of non-steroidal anti-inflammatory drugs with other chemopreventive drugs have been investigated (Agarwal et al, 1999; Torrance et al, 2000). For instance, it was found that green tea enhances the effect of sulindac, a classical NSAID that inhibits the activities of both COX-1 and COX-2 isoenzymes (Suganuma, 1999). Similarly, c-phycocyanin has shown to kill tumor cells in vitro which involves the process of apoptosis (Reddy et al, 2003). However its use as an anti-cancer chemopreventive agent could not be demonstrated so far in a suitable animal model. Further, in such mechanism the effect of COX-2 inhibition on PI3-K signaling and the downstream events such as the Akt/Protein Kinase B and GSK-3β are unknown. Therefore, it is important to discover the correlation of PI3-K, Akt, GSK-3β and other target proteins of signal transduction pathways for tumor promotion/suppression. It is also essential to observe such as in the present studies whether c-phycocyanin potentiates the growth inhibitory effects of piroxicam, a non-specific COX-2 inhibitor in a combination regimen, in 1, 2-dimethylhydrazine dihydrochloride (DMH) induced colon cancer in a rat model.

A combination regimen of piroxicam as a traditional NSAID and c-phycocyanin a natural COX-2 inhibitor could be a more potent chemopreventive agent at a much lower dose and expectedly with minimum or no side effects in colon cancer. It is important to keep a traditional NSAID in the preventive armory, as it does not completely shut down the prostaglandin synthetic pathways, which in that case lead to channelize the substrate...
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Arachidonic acid to another related pathway, the lipoxygenase pathway. The product in this pathway, called the leukotrienes are themselves known to promote the carcinogenic pathways. It is proposed to examine the putative signaling mechanism of such effect such as the PI3-K/Akt/GSK-3β and other downstream target proteins in mediating the carcinogenic growth inhibiting effects. Further, the molecular mechanism of apoptosis such as the intrinsic pathway (mitochondrial), the pro and apoptotic proteins such as the Bcl-2, Bax, cytochrome-c, Caspase 3 and 9 and inflammatory marker iNOS will also be elucidated. Activations of different cell cycle regulatory proteins e.g. CDK-2, CDK-4 Cyclin D1, Cyclin E and tumor suppressor proteins e.g. p53, pRb were also investigated, as such a data will help to identify the mechanism of tumor suppression by apoptotic end effect and intracellular signaling proteins for a better designing of chemopreventives in colon cancer.

Objectives

The present work is designed in keeping the following objectives in mind:

Establishment of a credible animal model for colorectal cancer

- Development of colon carcinogenesis by subcutaneous administration of 1, 2-dimethyl hydrazine dihydrochloride (DMH), which is a colon-specific pro-carcinogen in Sprague-Dawley (SD) male rats and its chemoprevention with piroxicam, a traditional NSAID and c-phycocyanin, a cyanobacterium derived COX-2 inhibitor.
- Establishment of anti-inflammatory dose of both piroxicam and c-phycocyanin by carrageenan-induced hind paw edema test in rat model.
- Establishing histopathologically the various prognostic biomarkers of colon cancer such as the aberrant crypt foci (ACF), adenoma and carcinoma in different treatment groups in relation to cancer progression/regression.

Isolation and assessment of viability of colonic epithelial cells

- Colonocytes will be isolated with a metal chelation method in the colonic sac.
- Cell survival of the isolated cell will be assessed by the Trypan blue dye exclusion test.
- Viability of the cells will also be measured with MTT assay.
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Membrane dynamics studies

- Intracellular Ca\(^{2+}\) using Fura-2.
- Membrane phase state determination by using fluorescent membrane probe, Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene).
- Lateral phase separation of the membrane by NBD-PE fluorescence quenching studies.
- Membrane microviscosity study using pyrene excimer formation as a parameter of lateral diffusion in the membrane.
- Fluidity of the membrane by using the fluorescent probe 1, 6-diphenyl-1, 3, 5-hexatriene (DPH).

To study the mechanism of apoptosis (programmed cell death) as an end effect in cancer cell killing

- To study apoptosis by fluorescent staining, TUNEL assay, Comet assay and DNA fragmentation analysis.
- Intracellular reactive oxygen species (ROS) via flow cytometry using DCFH-DA.
- Mitochondrial membrane potential via flow cytometry using JC-1, TMRE and Rhodamine 123.
- Multi-parametric apoptotic assay via flow cytometry.
- mRNA expression by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and further validation by Quantitative Real Time-PCR (qRT-PCR) of Bcl-2, Bax, Bad, Cytochrome c.
- Protein expression and localization studies of Bcl-2, Bax, Bad, cytochrome c, Apaf-1, Caspase-3 and Caspase-9, by Western blot and immunofluorescence.

To study angiogenesis and its inhibition

- Angiogenesis study by Gelatin Zymography of the matrix metalloproteinases.
- HIF-1α DNA binding activity by enzyme linked immunosorbant assay or ELISA.
- Protein expression and localization studies of Wnt, β-catenin, VEGF-A, MMP-2 and MMP-9.
- mRNA expression by RT-PCR and qRT-PCR for various angiogenic factors i.e. VEGF-A, MMP-2 and MMP-9.
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Study of signal transduction pathways involved in inflammation, cell survival and cell cycle regulation by gene and protein expression

- Housekeeping proteins: COX-1 and β-actin.
- Inflammatory enzyme: COX-2 (Enzyme activity and PGE₂ quantification by ELISA).
- Inflammatory cytokines and chemokine: IL-1β, IL-2, IL-4, IFNγ, TNFa, MCP-1, MIP-1β, iNOS.
- Cell survival proteins: PI3-K, IKKβ and IκBβ.
- Proinflammatory transcription factors: Jak3, Stat3, NFkB (DNA binding activity of p56 subunit by ELISA)
- Ligand-dependent transcription factor: PPARα, δ and γ (DNA binding activity by ELISA)
- Tumor suppressor proteins: p53, PTEN, Rb
- Cell cycle regulatory proteins: p21/Waf1, Cyclin D1, Cyclin E, CDK4 and CDK2
- Nuclear marker for cell proliferation and apoptosis: PCNA
- Pro-apoptotic protein: GSK-3β
- DNA mis-match repair proteins: MLH1 and MSH2

Cell proliferation analysis

- By PCNA incorporation in paraffin sections
- Estimation of Nitric oxide synthase and L-citrulline as markers of proliferation.
- To study the gene expression of iNOS by RNA isolation and RT-PCR analysis in the epithelial cells.

In silico molecular docking studies of the piroxicam with various target proteins

- Molecular docking interaction between different proteins in downloaded crystal structure with the ligands piroxicam and phycocyanobilin (a chromophore of c-phycocyanin) using GLIDE software from Schrödinger Suite 2011, USA programme.