Chapter V
CHAPTER V

COPPER COMPLEXES OF CYCLOXYGENASE INHIBITOR: SYNTHESIS, SPECTROSCOPY, MAGNETISM, EPR, CYCLIC VOLTAMETRY AND ANTIPROLIFERATIVE ACTIVITY OF KETOPROFEN HYDRAZONATES AGAINST COLON CANCER CELL LINES

5.1 Introduction:

Colorectal cancer (CRC), which includes cancers of both colon and the rectum, is the second-leading cause of cancer-related deaths in the Western countries other than the lung cancer. The yearly incidence of colorectal cancers is estimated to be one million, whereas approximately 59,000 people die as a result of CRC worldwide. Each year, approximately 155,000 Americans are diagnosed with colorectal cancer. In the year 2000, the reported cases of CRC in Europe were 225,000 accounting for 8% of all malignancies in adults [1,2]

Figure 5.1: The difference between (a) Colon polyp and (b) colon cancer
The colon cancer begins as small, non-cancerous (benign) clumps of cells called adenomatous polyps, which later on become cancerous. These polyps are either mushroom-shaped or flat and may be large or small. The large or flat polyps are more likely to become cancerous than mushroom-shaped or small ones. (Figure 5.1) To improve survival and reduce mortality, strategies are focused on prevention of the disease by early detection in stool or peripheral blood samples using DNA or mRNA identification methods, proteomic techniques, endoscopic screening of pre-cancerous lesions of colon and improvements in oncological surgery [3-5]. Colon and rectal cancers develop in the large intestine, which is the lower part of the intestinal tract (Figure 5.2).

5.2 Nature of disease:

Many people with the colorectal cancers have no symptoms in the early stages of the disease. When the symptoms appear, they vary depending upon the cancer’s size and location in the large intestine. In some cases, symptoms may result from a condition other than cancer, such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), and sometimes diverticulitis respectively.

5.3 Risk Factors:

Age: This is one of the greatest risk factors for colorectal cancer. About 90 percent of people with the disease are older than age 50 and the average age at diagnosis is 62.
Figure 5.2: X-ray photograph showing Colorectal cancer in large intestine.
Nearly 6 percent of the people between the ages of 75 to 80 have had colorectal cancer at some point in life.

**Sex and race:**

In the United States men are at higher risk of colon cancer than are women, and blacks have a greater risk than other racial groups do. Between 1973 and 1992, colon cancer increased by 40 percent among black men and 16 percent among black women. Since the incidence of colon cancer is far less in Africa than the rest of the world, the risk appears to be associated with the life style in an industrialized nation, rather than with race per se. In India, colon cancer incidence has also increased in recent years due perhaps also to change in diet and life style changes.

**Diet:**

This is the most important exogenous factor identified up to now in the aetiology of colon cancer. A diet high in fats, especially in the saturated fats found in red meat, butter, dairy foods, coconut and palm oils, seems to increase the risk of colon cancer as well as the risk of heart disease. Earlier belief that a low-fiber diet is good for avoiding greatly the risk of colon cancer was shattered by results of a study released in June 2001, known as the European Prospective Investigation of Cancer and Nutrition (EPIC), which is the largest study done so far to look at the relationship between diet and cancer. After examining 400,000 case histories the study found that a high-fiber diet can decrease the risk of colorectal cancers by as much as 40 percent. Evidence shows that diet rich in vegetables protects against risk of developing colon cancer, since vegetables contains a large array of substances, both micronutrients (such as carotenoids, folate and ascorbate)
as well as the bioactive compounds (such as phenols, flavones, isothiocyanates and indoles) with anticarcinogenic properties.

**Non-dietary factors:**

High alcohol consumption and excessive drinking has been found to increase risk of developing colon cancers. Physical activity has been associated with decreased risk of colon cancer. An inactive person is likely to develop colon cancer than a person getting regular physical activity although risk of rectal cancer is low as waste stays in the colon longer.

**Genetic factors:**

Genetic vulnerability to colon cancer has been attributed to either polyposis or non-polyposis syndromes. In 1987, the discovery of the first colorectal cancer gene for the autonomous dominant syndrome of familiar adenomatous polyposis (FAP)[6,7] which is associated with the mutations or loss of FAP gene (also called as the adenomatous polyposis coli APC gene), heralded a new and exciting period for further research in this field. In this disease, hundreds of polyps develop in the colon or rectum and if left untreated they progress into cancer. Prophylactic surgical excision of the large bowel is highly essential in such cases. The extracolonic features of FAP include epidermoid cysts of the skin, retinal pigmentation, benign cranofacial and long bone tumors. The malignancy of the GI tract can be one of the major causes of premature death in the FAP patients, which have undergone prophylactic surgery. The hereditary non-polyposis colorectal cancer (HNPCC) also known as Lynch syndrome is associated with germline mutation in six DNA mismatch repair genes [8,9]. The proportion of FAP
related cases are small as compared to HNPCC ones in the overall incidence of the disease [10].

5.4 **Treatment of Colon cancer:**

**a. Surgery:**

Colectomy is the primary treatment for colorectal cancers. Depending upon how far the cancer has penetrated into the wall of the bowel the affected part of colon is removed surgically. The laproscopic techniques in the treatment of colon cancer have also been under evaluation recently.

**b. Radiation therapy:**

Radiation therapy uses X-rays to kill any cancer cells that might remain after surgery or to shrink large tumors before an operation so they can be removed more easily. Radiation is a palliative treatment for metastatic cancers, usually reserved for treatment of rectal cancers, to provide either relief of symptoms or arrest of cell growth to delay these symptoms. No standard radiotherapy regimen exists in these cases and treatment decisions must consider the patient’s general condition, life expectancy, toxicity of the therapy, severity of symptoms and presence of alternative therapies. This may help prevent cancers from reappearing in the same place. Side effects of radiation therapy may include diarrhea, rectal bleeding and occasionally a risk of bowel obstruction.

**c. Chemotherapy:**

Until the last decade, fluoropyrimidines such as 5-FU were the cornerstone of CR cancer chemotherapy both in the adjuvant and in the metastatic settings [11]. The 5-FU is the
most commonly used drug in the colon cancers combined with leucovorin, which modulates the inhibition of thymidylate synthase. New drugs such as oxaliplatin, irinotecan have been used in metastatic colon cancer with significant results in terms of response rate, time to progression and survival [12]. However, all these regimens have the disadvantage of requiring frequent hospital visits, central venous access and possible negative impact of prolonged infusions on quality of life. Several oral pyrimidine analogues and pro-drugs have been developed for colon cancer including doxofluidine, capecitabine and carmofur respectively.

When 5-fluorodeoxyuridine (5′-DFUR) is administered with leucovrin in metastatic colorectal cancer patients, it shows 32% response rates. The compound is also used as a radiosensitizer in advanced CRC and has demonstrated therapeutic efficacy as well as low toxicity [13,14]. Capecitabine (n-4-pentoxy carbonyl-5′-deoxyfluoro-cytidine) is a new fluropyrimidine, a non-toxic pro-drug designed for oral administration. Hepatic and tumoral enzymes such as cytidine deaminase converts capecitabine to 5′-DFUR and then to 5-FU by the action of tumoral thymidine phosphorylase. Van Cutsem et al concluded a randomized, phase II trial using capecitabine as a single agent [15] and phase III using capecitabine along with leucovorin in combination [16]. The overall response rate was 21% for capecitabine alone and 24% with the addition of leucovorin. Carmofur is another 5-FU derivative developed for oral use. In a randomized study, patients receiving this compound for one year have 5-year disease free survival rate [17].

Several other agents have been designed which work on certain targets within colon tumor cells. Almost 50% Colon tumors express RAS genes constitutively. Recently, a phase I clinical trial was conducted using Limonene, which inhibits the
signalling of p21 ras with fairly good results [18]. Use of pro-drugs, such as CB 1954, a non-toxic chemical, which is converted by the enzyme DT-diaphorase into highly bifunctional alkylating agent, yields 10,000 times greater tumor response in cells that express this enzyme [19]. Although preliminary clinical trials concluded that human tumors expressing appropriate enzyme are sensitive to pro-drug therapy, such tumors are rare and unpredictable. In 1980, Bagshawe, et al. suggested use of tumor associated antibodies by linking these to non-mammalian enzymes that act on metastatic cancer cells [20]. This procedure has been termed as ADEPT approach (antibody-directed enzyme prodrug therapy).

A relatively new concept of treating metastatic colon cancers is the development of polymer drug conjugates where an antitumor agent is conjugated to a polymeric carrier that promotes preferential and prolonged intratumoral drug release. The drug conjugates containing N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer and anthracycline antibodies [21] or alkylating agents [22] bound to polymer through peptide linkers for cleavage by thiol dependent enzymes yields [23] products with decreased toxicity and increased efficacy in vivo. Currently, HPMA copolymer bound to Doxorubicin (MW=25,000; Dox. content-10% at total weight) containing a Gly-Phe-Leu-Gly spacer is in a phase I clinical trials and has shown impressive antitumor activity in human colorectal xenograft LS 174T model [24]. It was more effective than either free 5-FU or polymeric matrices containing 5-FU in same model [25].
5.5 Role of Cyclooxygenase enzymes in Colon cancer:

Currently, combination chemotherapy 5-flurouracil and leucovorin with either oxaliplatin or irinotecan with bevacizumab represents the standard of care treatment for metastatic colorectal cancers.[26] Despite recent improvement with the addition of biological agents, novel approaches of treatment are needed to further benefit patients.

Cyclooxygenase (COX-2) inhibition represents one such approach. COX-2 is the enzyme primarily responsible for prostaglandin synthesis triggered by inflammation.[27] Tumor related growth factors induce COX-2 and its presence in variety of human cancers correlates with the tumor stage and patient survival.[28-29] Cyclooxygenase thus represents the best-defined molecular target for treating colon cancers. In the following discussion we have summarized the role of COX in inflammation and its relation to control of COX expression in colorectal neoplasms.

a. Arachidonic acid metabolism by COX isoenzyme:

Phospholipase-A$_2$ (PLA$_2$) converts the cell membrane phospholipids into arachidonoc acid, which is subsequently acted upon and converted to prostaglandins by cyclooxygenase enzyme. (also known as prostaglandin endoperoxide synthase) by acting both as dioxygenase and as a peroxidase. Arachidonic acid is initially converted into prostaglandin G$_2$ (PGG$_2$) (through COX activity) and then into PGH$_2$ (through peroxidase activity) (Figure 5.3). Prostaglandins have been found to play a key role in important physiological processes such as pain, fever, mucosal homeostasis, immunity, haemostasis, reproduction and renal function.
Inactive carcinogens serving as electron acceptors can also become activated by the COX activity. Similarly the COX isoenzymes are also involved in the formation of peroxyl radicals that can activate pro-carcinogens.
b. Distribution in the biological system:

Long before the discovery of the second isoform of cycloxygenase (COX-2), it was widely believed that Cycloxygenase-1 (COX-1) enzyme was expressed constitutively with constant levels in individual tissues. However, it was observed that during inflammation the cycloxygenase activity increases which can be prevented by corticosteroids. In 1990, Needlemann, et al. identified a new inducible isoform of COX in the monocyte cells stimulated by interleukin-1 [30]. The crystal structure of COX-1 was solved by Picot, et al. in 1994 [31], while that of COX-2 in 1996 by Luong, et al. [32] which contributed substantially to the knowledge of genetic make up and regulation of both isoenzymes. In recent years there have been some literature reports on the third isoform of COX-3 enzyme which is believed to be is a splice variant of the COX-1 gene [33].

Of the two COX isoenzymes, COX-1 is constitutively expressed in most tissues and functions as a housekeeping gene by production of prostacyclins, prostaglandins and thromboxane whereas COX-2 is an inducible enzyme, which is activated by different stimuli mediating inflammatory reaction [34]. COX-2 expression is largely undetectable in most tissues under normal conditions and is only constitutively expressed in brain, testis, and renal parenchyma. In the normal non-inflammed gut, little or no COX-2 is present. Intestinal cells express COX-2 expression in response to pro-inflammatory agents, such as liposaccharide[35]. Upregulation of COX-2 expression is also seen in colonic mucosa associated with the inflammatory bowel disease. In vivo studies have shown that increased COX-2 expression is associated with chronic gastritis and ulceration most notably in the leukocytes of the mucosa.
Induced expression of COX-2 leads to a marked increase in the production of prostanoids which leads to enhanced leukocyte infiltration and is thought to be responsible for the pain and inflammation in an arthritic joint. The COX-2 enzyme is important in inflammation, wound healing, regulation of immune response and angiogenesis[36,37]. The COX-2 enzyme is largely expressed by the cells such as synoviocytes, macrophages, and endothelial cells in response to stimuli like cytokines, interleukins (IL-1β), interferons (IFN-γ), growth factors and hormones. The binding of TNF-α, and endotoxins to their receptors has been found to lead to the activation of IKB kinases (IKKs) which in turn activates the transcription factor NF-kB which then enters nucleus and activates transcription of COX-2 and other pro-inflammatory genes that contribute to carcinogenesis in the colon [38-41].

c. Structure of the COX isoenzymes:

COX-1 and COX-2 are membrane associated enzymes that show very similar homology consisting of long narrow channel with hairpin bend at the end. The two enzymes are homo dimers, each monomer consisting of three sites, viz. an epidermal growth factor like domain, a membrane binding domain and a catalytic domain that contains both the cycloxygenase and peroxidase active sites. The membrane binding domain of COX-1 and COX-2 is incorporated in the inner layer of the plasma membrane bilayer which allows liberated arachidonic acid access to the cycloxygenase active site. Both the enzymes show a channel extending from the center of the catalytic domain to the outer surface of the membrane-binding site. Eight amino acid residues play an important role for the substrate and inhibitor binding in the cycloxygenase channel. When cell membranes are damaged, arachidonic acid is released and is pulled inside the
hydrophobic pocket of the enzyme twisted around the hairpin bend, where it interacts with the residues present at the active pocket. Both the isoforms possess polar arginine at the position 120. There is a single amino acid difference between both isoforms where isoleucine molecule is at the position 523 in COX-1 and a valine residue at the same position in COX-2. This leaves a gap in the channel wall of the COX-2 creating a side pocket where many selective drugs bind whereas the bulkier isoleucine at 523 in COX-1 block the access to the side pocket (Figure 5.4).

Figure 5.4: Structural differences between COX-1 and COX-2 enzymes
d. Role of Cox in Colon cancer:

It has been established that COX-1 is constitutively expressed and is involved in the maintenance of normal tissue homeostasis. On the other hand COX-2 is an inducible enzyme that is upregulated at sites of inflammation and is over expressed in approximately 80% of human colorectal carcinoma (CRC). Various types of cancers in humans such as squamous cell carcinoma of head and neck, colorectal adenocarcinoma and cancers of pancreas, lung and breasts show upregulation of COX-2 expression. Cancer specific pathways such as the insulin growth factor (IGF) path [42] via P13/AKT/PKB cascade [43], the proto-oncogene ras and the important APC/β catenin pathway [44] are found to regulate COX-2 expression. Oncogenic ras, as present in majority of CRCs, induces COX-2 expression post transcriptionally via stabilization of COX-2 mRNA by activation of Raf/MEK/ERK pathway [45,46]. Colorectal cancer proceeds through accumulation of series of genetic mutations involving several tumor suppressor genes (APC, p53) and oncogenes (k-ras) respectively. COX-2 is expressed early during these sequences suggesting its important role in carcinogenesis. Raised levels of PGE-2 (the predominant prostaglandin produced by COX-2) are detected in CRC tissue and in macrophages derived from CRC. COX-2 expression is markedly increased in 85-90% of human colorectal adenocarcinomas, whereas COX-1 expression is not expressed in either normal intestinal mucosa or premalignant or malignant lesions.

The first indication that COX might be involved in colorectal cancers came from the results of NSAID treatment to inflammatory disorders in animal models [47,48]. This
was followed by the observation that a patient with a Gardner’s syndrome, a familial form of colorectal cancer, showed profound reduction in the number of rectal polyps following treatment with a NSAID. The protective effect of NSAIDs suggested that an abnormality in eicosanoid metabolism may contribute to tumor growth. Prostaglandin production could be influenced by variations in the levels of COX-2 which is elevated in 85% of adenocarcinomas.

The COX-2 was also found to be increased in the tumors from two animal models for colorectal cancers [49,50]. One such model is the Min (multiple intestinal neoplasia) mouse, which is a mouse model for FAP (familial adenomatous polyposis) where mutations occur in the APC gene. The second animal model for colorectal tumorigenesis involves treatment of rodents with carcinogen azoxymethane (Aom) that results into colon cancers in 52 weeks. The tumors in both the Min and Aom mouse models contain elevated levels of COX-2. In 1966, Oshima, et al. provided compelling evidence that COX-2 contributes positively to the tumor biology in the Min mouse model [51]. The study showed that the effect of NSAIDs on the tumor size and multiplicity could be achieved through COX dependent or COX independent mechanism. The authors concluded that COX-2 acts as a tumor promoter and may not be an absolute requirement for polyp formation in the Min mouse model.

5.6 Role of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) in colon cancer:

NSAIDs are the best-studied chemopreventive agents. Interests in their chemopreventive potential for colon cancers is drawn from epidemiological studies that have shown 40-50% reduction in the incidence of these cancers among users of NSAIDs.
In one of the largest trials evaluated over a period of 7 years, a significant association between low doses of NSAIDs and decreased risk of CRC-related mortality has been observed. Similarly, the Health Professional follow-on study in men reported 32% reduced risk of CRC incidence among regular NSAID users. These drugs are thought to exert their antitumor effects by inhibiting production of the prostaglandins, which are derived from arachidonic acid by the action of COX enzymes. NSAIDs interfere with the uptake of arachidonic acid and its insertion into the membrane as well as uncoupling the oxidative phosphorylation in mitochondria.

b) Classification of NSAIDs:

The NSAID compounds are classified into following subtypes depending upon their preferential and selective actions on the cyclooxygenase isoforms (1) Selective COX-1 inhibitors, (2) Non-selective COX inhibitors, (3) Preferential COX-2 inhibitors, and (4) Highly selective COX-2 inhibitors.

Selective COX-1 inhibitor: Aspirin (1), which is an acetyl derivative of salicylic acid differs from the rest of the NSAIDs due of its action wherein it acetylates the serine group in the active pocket of the cyclooxygenase enzyme in the loss of the enzyme loses the activity of converting arachidonic acid into the respective prostaglandins.
Several epidemiological studies have shown that a substantial decrease of 30-50% in risk of death from colon cancers is associated with the use of Aspirin and other NSAIDs [52,53]

**Non-selective COX inhibitors:**

Some of the non-selective COX inhibitors are summarized in Figure 5.5. The first evidence NSAIDs causing apoptotic DNA fragmentation was provided by Kusuhara [54] who showed that indomethacin (10) and sodium diclofenac (11) cause apoptosis in rat gastric mucosal cells. The studies indicated the involvement of caspases rather than inhibition of prostaglandin synthesis. In 2003, Paraskeva et al. showed that Nimesulide (12) which is a preferential COX-2 inhibitor (Figure 5.6), increases NK-B DNA binding, caspase activation and DNA fragmentation during apoptosis against hypopharyngeal carcinoma cells [55]. However, the compound could not induce apoptosis by itself in these cancer cells but potently augmented chemotherapeutic drug induced apoptosis. Badwai, et al. [56] have studied the effects of acetaminophen, aspirin, naproxen (7) and ibuprofen (8) on cell survival, cell cycle and induction of apoptosis in LnCaP human prostate cells. Ibuprofen was significantly more effective against the prostate cancer cells in vitro. Cell cycle analysis indicated that ibuprofen caused the LnCaP cells to shift from the S and G₂/M phase to G₀/G₁ phase.
Figure 5.5: Non-selective COX-2 inhibitors
Naproxen has almost similar effect as that of ibuprofen but somewhat of lower order.

c) Chemoprevention and NSAIDs-mediated Apoptosis:

The chemopreventive action of optimal dose of NSAIDs in humans is negatively balanced by their gastric and renal toxicity in the biological system [57,58]. In case of some NSAIDs like aspirin, inhibition of platelet function is found to lead to coagulatory problems and bleeding. It has been observed that COX-2 isoform plays a key role in the early stages of intestinal polyp formation.

In 1983, through a double blind, placebo-controlled studies Waddell and Loughry showed that the NSAID sulindac (9) reduces both the number and the size of colorectal adenomas in the patients [59]. Sulindac has two primary metabolites – sulindac sulfide (the active form of sulindac) and sulindac sulfone. Sulindac is thought to be activated to
sulindac sulfide by the intestinal flora, which becomes concentrated in the enterohepatic circulation. Thus, colon is exposed to higher concentrations of the drug than it is present in the systemic circulation. Treated patients experienced a reduction of nearly 60% in the number of tumors developed, compared to patients taking placebo. Sulindac sulfide inhibits proliferation, causes cell cycle quiescence and induces apoptosis in HT-29 colon adenocarcinoma cells [60]. Treatment with another COX inhibitor Piroxicam in some patients has shown complete regression of adenomas. However, gastric toxicity problems have prevented long term use with Piroxicam [61].

Several NSAIDs bring about apoptosis in colon-cancer cells grown in culture which include agents like Sulindac sulfide, Sulindac sulfone, Sulindac, Indomethacin, Naproxen, Pirxicam, Aspirin, Salicylic acid, and COX-2-specific inhibitors like Celecoxib (13), Valdocoxib (14) and Paracoxib (15) respectively. (Figure 5.7)

![Figure 5.7: Highly Selective COX-2 Inhibitors](image)
After treatment with these compounds, colon-cancer cells contract, form micronuclei and develop membrane blebs, all of which are markers of apoptotic activation. These effects can be blocked by drugs that inhibit corresponding gene expression, suggesting that the cell death induced by NSAID treatment is a bona fide programmed cell death and not necrotic cell death caused by general toxic effects of the drugs. The observation that NSAIDs cause apoptosis in colorectal cancer cells \textit{in vitro} suggests that the chemopreventive effects of NSAIDs are cell autonomous, at least in part.

Studies with animals have provided valuable insights into the chemopreventive properties of NSAIDs. The \textit{Min} mouse has a mutation in the \textit{APC} gene and develops intestinal adenomas similar to those in patients with FAP (familial adenomatous polyposis. \textit{Min} mice have been used widely as an experimental model for FAP. Administration of sulindac to \textit{Min} mice causes a dramatic reduction in tumor burden. Other NSAIDs, including Aspirin, Piroxicam, Rofecoxib, Flurbiprofen and Indomethacin, are also effective in reducing, and in some cases nearly abrogating, the tumor burden in these mice. Furthermore, in rats with chemically induced colon cancer, various NSAIDs can prevent tumorigenesis or dramatically decrease tumor load. Recent studies have shown that combinations of chemopreventive drugs may also hold promise for preventing the development of tumors. When \textit{Min} mice are treated with sulindac or EGF-receptor inhibitors, such an EKI-785, they develop about 50–70% fewer tumors [62]. Moreover, when combined with a dose of sulindac that would be too low to prevent disease progression on its own, the frequency of polyp formation was reduced by more than 95%.
Similarly in a recent double blind, placebo-controlled study, Steinbach and colleagues have noted that patients with FAP who received Celecoxib, which is a COX-2 specific NSAID, developed 30% fewer polyps [63].

d) Mechanisms of NSAIDs-mediated Apoptosis:

COX dependent mechanisms:

Analysis of COX expression shows that COX-2 is increased in up to 90% of sporadic carcinomas and 40% of adenomas, but not in normal colonic mucosa. In the adenomas of patients with FAP, and in rats with experimentally induced colon tumors, higher than normal concentrations of COX-2, but not COX-1, prostaglandins, or both COX-2 and prostaglandins together, are seen. Furthermore, the concentration of prostaglandin E₂ (PGE₂) is particularly high in human colon cancers. These findings support the idea that COX-2 over expression is important during colorectal carcinogenesis. The molecular mechanism of colorectal-cancer progression is known to involve several key events and the mutation of a number of crucial genes. Although an increase in the size of colorectal tumors is associated with an increase in concentration of COX-2, it is not yet clear where COX-2 deregulation occurs in the multistep progression of colon cancers. However, the observations that young, small adenomas overexpress COX-2, and that NSAIDs can prevent adenoma formation, imply that COX-2 deregulation happens early in tumor formation.

Several studies have now provided strong evidence for the theory that NSAIDs cause apoptosis in colon-cancer cells by inhibition of COX-2. DuBois [64] found that rat intestinal epithelial cells, modified to increase expression of COX-2, were resistant to
apoptosis. Apoptosis in the colonic epithelium appears to be progressively inhibited during colonic carcinogenesis. One of the ways in which NSAIDs prevent cancer and exert their chemopreventive effects in the colon may be through induction of apoptosis in the colonic mucosa. Relatively high (micromolar rather than nanomolar) concentrations of NSAIDs are required to evoke apoptosis in both _in-vitro_ and _in-vivo_ systems.

Arachidonic acid has been shown to be a critical signal for apoptosis, with NSAIDs triggering apoptosis by inhibiting the metabolism of arachidonic acid and allowing its accumulation. Researchers have, however, questioned, whether the chemopreventive and tumor regression effects of NSAIDs in colorectal patients, are the same biological phenomenons.

Overexpression of COX 2 has also been found to lead to increased production of PGE$_2$, increased adhesion to the extracellular matrix, increased concentrations of Bcl2, reduced TGFb$_2$ receptor expression and the absence of E-cadherin protein respectively. All these changes, suggest increased tumorigenic potential and support the notion that COX-2 overexpression alters the biology of intestinal cells which affects the transformation process. Treatment of such cells with sulindac sulfide has been found block COX-2 activity and restores the apoptotic response, which adds further support to the idea that the antineoplastic activity of NSAIDs involves inhibition of COX-2. Direct genetic evidence for the role of COX-2 in colon cancer has been provided by Oshima and colleagues using _APC_ knock-out mice, which develop polyps in their intestinal tracts because of a truncation mutation in the _APC_ gene.
The question of how COX-2 inhibition leads to apoptosis has been debated. Several studies have suggested that decreased COX-2 activity leads to a reduction of eicosanoids, such as the prostaglandins, and this a lack of prostaglandins, in turn, affects cell proliferation and apoptosis. So far, there has been no definitive evidence to support the existence of a signalling pathway through which prostaglandins can directly affect apoptosis. Arachidonic acid may also provide a mechanism for COX-2 dependent induction of apoptosis. Treatment of colorectal carcinoma cells with various NSAIDs results in inhibition of COX-2 and a dramatic increase in the concentration of arachidonic acid which stimulates the enzyme sphingomyelinase to convert sphingomyelin to ceramide, a potent inducer of apoptosis. Several NSAIDs cause such dramatic increase in ceramide and subsequent activation of apoptosis in colon cancer cells. It is possible that the pro-apoptotic effects of NSAIDs are the result of such induction of the ceramide-induced apoptosis. Other studies have shown that intracellular increases in arachidonic acid can signal apoptosis and that the cellular concentration of unesterified arachidonic acid is a general mechanism by which apoptosis is regulated. It seems that arachidonic acid can alter mitochondrial permeability and cause cytochrome C release, leading to apoptosis.

**COX independent mechanisms:**

Several recent observations cast doubt on the idea that COX is the sole target of NSAID action in the colon. For example, NSAID derivatives such as sulindac sulfone, which lack the ability to inhibit COX, can inhibit colon tumor growth. Additionally, it appears that some NSAIDs can inhibit proliferation and induce cell death in cells that do not express COX. These findings suggest that other targets of NSAIDs that are common
to some neoplastic cells may play a part in NSAID-mediated apoptosis. One potential mechanism involves the transcription factor NF-kB, which promotes cell survival and enhanced proliferation. Several investigators have suggested that NSAIDs could promote apoptosis by inhibiting NF-kB. This may occur by blocking the release of the IkB from NF-kB, leading to a failure in NF-kB activation. Subsequently, genes required for cancer cell growth and survival may not be transcribed.

Another potential COX-independent mechanism of NSAID-mediated apoptosis involves the peroxisome-proliferator-activated receptor d (PPARd), which is a growth-promoting protein. It has recently been reported that NSAIDs such as sulindac can bind to and inhibit PPARd. Normally, when colon-cancer cells are treated with sulindac, they undergo apoptosis. When PPARd is overexpressed in colon-cancer cells, the cells are partially protected from NSAID-induced apoptosis. Furthermore, PPARd is overexpressed in colon cancers. He and colleagues also observed that sulindac can cause the PPARd gene product (a transcription factor) to dissociate from DNA. As a result, the cell is left unable to transcribe the genes necessary for its survival. It is particularly interesting that PPARd is suppressed by APC. When APC is mutated in colorectal cancers, one would therefore expect that PPARd would be higher than in normal cells, promoting unchecked cell growth.

Other mechanisms proposed for the anti-cancer effects of NSAIDs is the hypothesis that COX-2 is required for the angiogenesis process during growth of a tumor. NSAIDs work, in part, by blocking this neo-vascularisation. Using an *in vitro* co-culture system, researchers have shown that COX-2 can regulate the production of angiogenic factors
produced by colon-cancer cells. Inhibition of COX-2 by NSAIDs blocks the production of these factors and thus inhibits angiogenesis.

Angiogenesis is essential for tumor growth beyond 1-2 mm in size. Dubois et al. have shown that PGE2 synthesized by overexpressed COX-2 in CaCo-2 colon carcinoma cells can induce secretion of the angiogenic factors like VEGF, TGF and bFGF leading to stimulation of vascular tube formation. Sulindac and COX-2 selective inhibitors can inhibit angiogenesis by inhibition of these angiogenic factors by tumor cells.

The mechanisms by which NSAIDs exert their chemopreventive effects is currently an area of debate. The COX-dependent and a COX-independent mechanisms are not mutually exclusive, and it is likely that they act in both ways summarizes the chemopreventive mechanisms of action of NSAIDs. Understanding the underlying mechanism of these compounds can lead to the rational development of superior cytotoxic agents.

5.7 Nature and scope of present investigation:

Ketoprofen (16), (3-benzoyl-α-methylbenzeneacetic acid), is a non-selective COX inhibitor which has a close resemblance to the non-steroidal antiestrogen, viz. Trioxefene (17) and Keoxifene (18). Compounds (17) and (18) have been shown to compete for ER binding sites and thereby prevent the growth of malignancies in breast cancers.

In the present work we have kept the structural features of (16) unchanged which are responsible for its COX inhibitory activities with a view to make the compound multi-targeting. We have modified the central ketonic function of (16) with
several hydrazonic pharmacophores such as isonicotinoyl (21), nicotinoyl (20) and salicyloyl (19) respectively and have conjugated these derivatives with copper ions to make them more selective and efficient in their antiproliferative activities.

All synthesized compounds were evaluated against a panel of COX +ve (HT-29 and BXPC3) and COX-ve (MiaP, HCT-116, SW-480, BT-20 and PC3) colon cell lines.
5.8 Experimental:

A. Materials

All chemicals used in the synthesis of ligand and metal conjugates were of AR grade while solvents were distilled prior to their use. Ketoprofen, Salicylhydrazide, Nicotyl hydrazide, Benzoyl hydrazide and copper (II) chloride were products of Aldrich and were used as supplied.

B. Synthesis

i) Synthesis of ketoprofen hydrazine ligand

The ligand was prepared by reacting ketoprofen and the respective hydrazide (salicyl (19), nicotyl (20) and isonicotyl (21) in 1:1 stiochiometric amounts and both dissolved in methanol (5ml) with a few drops of acetic acid. The reaction mixture heated to reflux on the water bath for 6-8 hours. A light yellow colored product separated out when the mixture was allowed to cool. It was washed with ether and dried in vacuum.

ii) Synthesis of copper conjugates of 19-21:

Copper (II) complexes were prepared by a general method described by Sorenson with few modifications. A typical protocol involved mixing of the methanolic solutions of the ligand and of copper-acetate in 1:1 stiochiometric ratio and refluxing the reaction mixture for 3 hrs. The precipitated products were filtered and washed with water and cold acetone and dried under vacumm.

The synthetic strategy followed in the present work is summarized in (Scheme 5.1)
5.9 Results and Discussion:

A. Compositional studies

The elemental analyses of the ligand and its copper conjugates are summarized in Table 5.1, which reveal an adherence to the proposed stiochiometries. The molar conductivity data on metal complexes (3-10 Ω cm² mol⁻¹) in DMSO solvent indicate the non electrolytic nature of the complexes supporting thereby the inclusion of the chloride ion in the metal coordination sphere.

B. Infrared Spectroscopy

The infrared spectra of the hydrazone ligands and their metal complexes (as nujol mulls) are included in the Figure 5.8-5.10 alongwith their assignments. The IR spectrum of the ligand exhibits two strong bands one at 1734 cm⁻¹ attributed to the carboxylate stretch and the other at 1654 cm⁻¹ assigned to the stretching frequency of 3-benzoyl carbonyl group respectively [65]. Condensation with the hydrazone moiety results in the loss of the latter band confirming success of the reaction. Two additional bands seen at 3417 and 3340 cm⁻¹ is due to asymmetric and symmetric stretches of the terminal amino group in the hydrazine side chain [66]. The imine (ν C=Ν) observed at 1668 cm⁻¹ and the amide carbonyl 1676 cm⁻¹ are shifted in the respective ligands to the lower energy side upon metal conjugation indicating its involvement in metal coordination [67,68].
Table 5.1: Analytical data on KF hydrazones (19-21) and its copper complexes (6-8)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Elemental Analysis</th>
<th>Mol weight</th>
<th>Color</th>
<th>Electronic Spectra</th>
<th>E 1/2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C%</td>
<td>N%</td>
<td>H%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KF(16)</td>
<td>60.6</td>
<td>-</td>
<td>9.4</td>
<td>25429</td>
<td>White</td>
</tr>
<tr>
<td>KFSH(19)</td>
<td>70.52</td>
<td>7.03</td>
<td>4.97</td>
<td>388.29</td>
<td>Pale yellow</td>
</tr>
<tr>
<td></td>
<td>(71.08)</td>
<td>(7.21)</td>
<td>(5.15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KFNH(20)</td>
<td>70.31</td>
<td>11.01</td>
<td>4.87</td>
<td>373.29</td>
<td>Pale yellow</td>
</tr>
<tr>
<td></td>
<td>(70.72)</td>
<td>(11.25)</td>
<td>(5.08)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KFINH(21)</td>
<td>70.43</td>
<td>11.04</td>
<td>4.93</td>
<td>373.29</td>
<td>Pale yellow</td>
</tr>
<tr>
<td></td>
<td>(70.72)</td>
<td>(11.25)</td>
<td>(5.08)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><a href="6">Cu(KFINH)Cl2</a></td>
<td>51.39</td>
<td>8.05</td>
<td>3.27</td>
<td>507.83</td>
<td>Green</td>
</tr>
<tr>
<td></td>
<td>(51.98)</td>
<td>(8.27)</td>
<td>(3.74)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><a href="7">Cu(KFNHC12</a></td>
<td>51.43</td>
<td>7.94</td>
<td>3.48</td>
<td>507.83</td>
<td>Green</td>
</tr>
<tr>
<td></td>
<td>(51.98)</td>
<td>(8.27)</td>
<td>(3.74)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><a href="8">Cu(KFSSH)Cl2</a></td>
<td>52.29</td>
<td>5.09</td>
<td>3.47</td>
<td>522.83</td>
<td>Green</td>
</tr>
<tr>
<td></td>
<td>(52.78)</td>
<td>(5.35)</td>
<td>(3.82)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.8: Infrared spectra of Ketoprofen salicyl hydrazone (19) and its Copper conjugate (6)
Figure 5.9: Infrared spectra of Ketoprofen nicotyl hydrazone (20) and its Copper conjugate (7)
Figure 5.10: Infrared spectra of Ketoprofen Isonicotyl hydrazone (21) and its Copper conjugate (8)
C. Electronic Spectroscopy

The electronic spectra of all synthesized copper conjugates are shown in Figure 5.11 and are summarized in Table 5.1. All compounds show a broad peak in the region 686-780 (12870-14577 cm) which is attributed to a combination of the transitions $^2B_{1g} \rightarrow ^2E_g \rightarrow ^2B_{1g} \rightarrow ^2A_{1g}$ respectively in the square planar configuration assigned to these compounds. [69]

D. Magnetic and EPR studies

The room temperature magnetic moments of the complexes are found to be in the range of 1.80-2.0 which is characteristic of the monomeric compound having square planar geometry [70]. These values indicate absence of intermolecular interactions and orbital mixing in these compounds.

The X-band EPR spectra of the copper complexes in DMSO solvent at 77 K (Figure 5.12a-c) show $g_{ll}$ and $g_{\perp}$ signals at 2.07 and 2.06 with All hyperfine values of 85-125 which is consistent with the square planar geometries [71,72].

E. Cyclic Voltametry

The cyclic voltammograms for the two copper conjugates in DMSO solvent are shown in (Figure 5.13a-b) which shows a reduction peak around -1.45 V which is irreversible and probably corresponds to the ligand-based reduction of the imino group. The ill-defined oxidation peaks seen on the reverse scan probably represents the oxidation of the carboxylic groups of the NSAID moiety. All copper complexes of these ligands exhibit a reversible Cu$^{2+}$/Cu$^+$ redox couple in the range +0.40 to -0.47 making it
Figure 5.11: Electronic Spectra of Ketoprofen Hydrazone conjugates (6-8)
Figure 5.12a: X-band EPR spectra of copper complex of Ketoprofen salicyl hydrazone (6)
Figure 5.12b: X-band EPR spectra of copper complex of Ketoprofen nicotyl hydrazone (7)
Figure 5.12c: X-band EPR spectra of copper complex of Ketoprofen isonicotyl hydrazone (8)
Figure 5.13a: Cyclic Voltammogram of copper complex of Ketoprofen Salicyl-hydrazone
Figure 5.13b: Cyclic Voltammogram of copper complex of Ketoprofen Nicotinyl-hydrazone
a very facile redox process which may be contributing to their intracellular biological activity [73].
5.10 Antiproliferative activity

The compounds have been tested on a panel of colon cancer cell lines with and without COX expression. (Figure 5.14 and 5.15). The metal conjugates are found to exhibit more pronounced antiproliferative activities where the facile metal-based redox couple seems to contribute. Among the three copper conjugates, compound of salicylhydrazide Schiff base 19 is more potent with an IC$_{50}$ value of 0.36 μM against the BX PC3 cells.

Comparison of the IC$_{50}$ values of the conjugate 19 against the COX +ve as well as COX –ve cell lines (Table 5.2) reveals much lower values in case of the former which is suggestive that at least large part of the antiproliferative effects of these compounds are mediated through COX expression.

The flow cytometry analysis of the cytotoxicity of 19 on the PC3 cells indicates (Figure 5.16) cell arrest in the G$_1$/S phase characteristic of apoptosis induction through redox mechanism. Such behavior has been noted by Ferrari et al. [74].

5.11 Conclusions

From the present work, it can be observed that use of NSAID compounds as carrier vectors to target colon cancer cells is attractive due to their strong affinity for cyclooxygenase receptors expressed in high concentrations in colon cancer cells. Some of the NSAIDs can be advantageously used to deliver cytotoxic agents to the cancer selectively. The copper conjugation is helpful in this regard for enhancing the liposolubility, adding redox cycling and modulating intracellular oxidative stress for
Figure 5.14: Cytotoxicity assay using HT-29 (COX +ve) colon cancer cell line treated with copper conjugate of Ketoprofen hydrazones (6-8)
Figure 5.15: Cytotoxicity assay using SW-480 (COX –ve) colon cancer cell line treated with copper conjugate of Ketoprofen hydrazones (6-8)
Table 5.2: Comparison of IC<sub>50</sub> values of copper complex of 19

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>COX Positive</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; IN μM</th>
<th>COX Negative</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; IN μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-29</td>
<td>&gt;10</td>
<td>MiaP</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>BXPC3</td>
<td>0.36</td>
<td>HCT-1116</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BT-20</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PC3</td>
<td>187</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.16: Flow cytometry cell cycle analysis of COX dependent PC-3 colon cancer cell line treated with copper conjugate of Ketoprofen salicyl hydrazone (8)

Inhibition in the S-Phase
intracellular oxidative stress for apoptosis induction. Further investigations are required to understand the mechanism of action of these metal conjugates.
References:


34. M.Beinz, H.Clevers, Cell, 103:(2000); 311-320.


57. Hidenubo Kusuhara., Prostaglandins; 54(5)-(1994)


