Chapter 2

REVIEW OF LITERATURE

A disorder called colon cancer

The fight against Cancer today in general and Colon Cancer in particular, stands at a turning point in its history. The explosion of information and progress in the understanding of the cellular and molecular biology of cancer in recent years presents tremendous opportunities for the control of this terrible disease. Over the last three decades, a great deal of research has been made regarding the genetics, diagnosis, staging and therapeutic modalities of colon cancer. Even though surgery remains the cornerstone of the treatment of colon cancer, new guidelines have been established for a multimodality treatment resulting in improved survival rate and quality of life. The key challenge, however, remains the translation of the basic knowledge generated in the laboratories into more efficacious, preventative, diagnostic and therapeutic products.

Diverse molecular events are integrated in the development and progression of colorectal cancer which is a leading cause of cancer related death worldwide (Jemal et al, 2002). A complex combination of genetic, epigenetic, and postgenetic (e.g., posttranslational) alterations are involved in the multistage development of colon cancer (Chung, 2000; Jass et al, 2002; Feinberg et al. 2006). Infact, carcinogenesis is a multistep process involving the activation of oncogenes, inactivation of tumor suppressor genes (Compagni et al, 2000) and paralleling these genetic events, cancer cells also induce profound changes in the normal neighboring tissue. This altered tissue which is referred to as tumor stroma, provides an environment for tumor growth, invasion and metastatic spreading (Liotta and Clair, 2000).

Colorectal cancer is the second most common cancer in both incidence and mortality among men and women in the developed countries such as North America and Western Europe (Stewart and Kleinhues 2003). However, many Asian
countries, including China, Japan, South Korea, and Singapore, have also experienced an increase of two to four times in the incidence of colorectal cancer during the past few decades (Sung et al, 2005). The rising trend in incidence and mortality from colon cancer is more striking in the affluent than in poorer sections of the societies and also differs substantially among the different ethnic groups.

Only very little data is available concerning the recent trends of occurrence of colon cancer in India. Nonetheless, malignancy is one of the leading cause of death, and cancer of the large bowel or colon, although not as common as in the West, is one of the most common forms of malignancy encountered in Indian medical practice as well. The incidence rate of colon cancer is comparably low in India, which vary from 3.7 to 0.7/100,000 among men and 3 to 0.4/100,000 among women in eight population regions (Mohandas and Desai, 1999). Rural incidence rate in India is approximately half to that of urban rate. However, significant increase in the incidence of colon cancer has been reported for both men and women over the last two decades.

Dietary habits remained a central theme for most of the studies on the prevention and development of colon cancer in human population. Despite promising data in epidemiologic studies, most dietary changes have not been successful in preventing colorectal cancer and remained sketchy (Wong et al, 2007). Specifically, clinical trials have shown no benefit with fiber, beta carotene, and vitamin A, C, and E interventions (Greenberg et al, 1994; Alberts et al, 2000; Schatzkin et al, 2000; Albanes et al, 2000). Other studies suggest that calcium may prevent colorectal carcinoma by binding bile and fatty acids and inhibiting the proliferation of colonic epithelial cells (Shaukat et al, 2005).

Colon cancer prevention has now focused on novel targeted therapies, such as the nonsteroidal anti-inflammatory drugs (NSAIDs), which critically showed the crucial link of inflammation and development of cancer. However, unresolved questions about the mechanism(s) by which these drugs act, the optimal drug, dose, treatment regimen, and the balance of risks and benefits in specific populations must be answered.

Development of colon cancer

Most colorectal cancers develop via a characteristic series of pathological steps (Figure 2.1 a & b). Colorectal epithelial cells acquire abnormal growth and
morphological characteristics and form an adenoma (adenomatous polyps), a tumor mass often protruding into the lumen of the colon or rectum. In time, these lesions enlarge and a subset of cells can acquire additional abnormal growth behaviors, which allow them to invade into the bowel wall and metastasize.

Figure 2.1: Human colon cancer. (a) The formation of colon polyps and inset showing the structure and its position, (b) Colorectal carcinogenesis. Normal epithelial cells acquire abnormal growth and morphological characteristics and form an aberrant crypt focus, which enlarges to become an adenoma. With time, adenomas increase in size and can become adenocarcinomas, which have the ability to invade and metastasize. Presumably driving this process are somatic mutations that develop in the designated key genes in the colonocytes that make up these lesions. Abnormalities in DNA mismatch repair mechanisms can also contribute. Aspirin and other NSAIDs inhibit this process, perhaps at several distinct points. Figure is adopted from Kinzler and Vogelstein, 1996.
At this point, the tumors are classified as adenocarcinomas and can be lethal. In the course of this typical multistep process, somatic mutations develop in key genes in the cells that comprise these lesions, such as in: the adenomatous polyposis coli (APC) and p53 tumor suppressor genes; the K-Ras oncogene; and various genes that mediate DNA mismatch repair (Kinzler and Vogelstein, 1996). The mutational events presumably drive the evolution of the pathological lesions and their neoplastic and malignant behavior. Various end point biomarkers indicate the likely progression to cancer such as colon polyps, aberrant crypt foci and aggregates of morphologically abnormal crypts, which have been recently recognized to precede the development of adenoma (Srivastava et al, 2001). The multistep nature of carcinogenesis provides opportunities for the intervention with the agents targeted at specific mechanisms involved in the initiation, promotion, and progression stages of cancers.

**Etiology of colon cancer**

As outlined above, colon carcinogenesis is a protracted process that occurs over a period of decades (see Fig. 2.2). During this time, cancer-associated gene mutations successively accumulate, and a benign (but initiated) enterocyte progresses to an invasive cancer (Fearon and Vogelstein, 1990).

![Figure 2.2: Colorectal carcinogenesis: adenoma-carcinoma sequence](Adopted from Kelloff et al, 1996 and Ilyas et al, 1999)
The early changes include mutations in key oncogenes and tumor suppressor genes \[e.g., APC\] and \(K-Ras\) \cite{Vogelstein1988}. Also associated with the carcinogenic process are epigenetic alterations \[e.g., DNA hypermethylation leading to silencing of tumor suppressor genes\] \cite{Grady2002}. A number of these early molecular events and lesions can be assessed in blood or in cells exfoliated into the stool and may serve as markers of cancer risk and/or of response to therapy. Examples include gene mutations and polymorphisms \(e.g.,\) of oncogenes and carcinogen-metabolizing enzymes as well as alterations in gene expression \(e.g.,\) aberrant gene promoter hypermethylation of \(hMLH1\) \cite{Grady2001}, loss of imprinting of the insulin-like growth factor II gene \(C\) \cite{Grady2003}, as well as secreted cancer-associated proteins and antigens \(e.g.,\) carcinoembryonic antigen \(M\) \cite{MacDonald1999}.

Ongoing developments have focused on applying proteomic, genomic, and gene expression microarray profiling approaches to tissue, blood, or stool tests. For example, a stool-based genomic panel targeting 19 alterations associated with colorectal neoplasia \(e.g.,\) mutational hot spots on \(K-Ras, APC,\) and \(p53,\) as well as long fragment DNA \(S\) \cite{Ahlquist2002, Ahlquist2002}.

Development of biomarkers

Among the first histologically detectable changes that may be associated with colon cancer development are subtle alterations in the regular pattern of the intestinal crypts known as aberrant crypt foci \(ACF\). \(ACF\) appear to arise as the result of premalignant genetic alterations; they often show \(APC\) loss \cite{Grady1996}, as well as \(K-Ras\) mutations \cite{Grady1993}. These alterations are associated with the formation of early and intermediate adenomas, respectively. Alteration of the transcription factors Smad2 and Smad4 appear to mediate the transition to late adenoma. Finally, mutations in the tumor suppressor gene \(p53\) pave the way for transformation of adenomas into malignant cancers \(D\) \cite{Figuero2002}.

Additional alterations that occur early in colorectal tumorigenesis include genome-wide DNA hypomethylation and genomic instability, via defects in chromosome segregation or DNA replication fidelity \cite{Wong2007}. The number, size, and dysplastic features of \(ACF\) correlate with the number of adenomatous polyps \(adenomas\) \cite{Takayama1998}, which in turn constitute one of the most well-established colon cancer risk markers \cite{O'Shaughnessy2002}.
Risk and Burden

Stryker et al. found a relative risk of colon cancer development of approximately 1% per year for adenomas >1 cm (Stryker et al., 1987). In addition to size, important determinants of colon cancer risk include adenoma number (O’Brien et al., 1990) and clinical features such as histological architectural type. For example, tubular histology is associated with the lowest lifetime risk (5% overall), while villous lesions are associated with the highest (up to 50%), and tubulovillous lesions with an intermediate lifetime risk [15-20% (Tobi, 1999)], respectively.

Screening studies have indicated that colon cancer is typically diagnosed 10-15 years after adenoma detection (Kozuka et al., 1975). This temporal lag presents a compelling rationale and opportunity, if not responsibility, for screening and intervention as colon cancer represents a significant public health burden worldwide. Adenomas are a very prevalent lesion; nearly half of men and 30% of women develop adenomas by the age of 50 years (Villavicencio and Rex, 2000). In addition to environmental and lifestyle factors (dietary fat and calcium, exercise, NSAID use, and so forth), multiple inherited and acquired genetic factors contribute to colon cancer risk (Potter, 1999).

Heritable colon cancer

At the highest risk for colon cancer are patients with familial adenomatous polyposis (FAP), an autosomally dominant hereditary syndrome caused by mutation or truncation of the \( \text{APC} \) gene. FAP is defined by an autosomal dominant germline mutation in the \( \text{APC} \) gene (Kinzler et al., 1991a). Patients with FAP develop hundreds to thousands of adenomatous polyps in the colorectum by their teenage years and colorectal carcinoma by the fourth decade of life (King et al., 2000). Although FAP is rare, accounting for <1% of colon cancer cases, the lifetime colon cancer risk in FAP patients is nearly 100%. The molecular biology and etiology of colon cancer in FAP is thought to parallel sporadic cancers. Indeed, 80% or more of sporadic colon cancers contain a mutation in either \( \text{APC} \) or an associated oncogene, \( \beta \)-catenin (Kinzler et al., 1991b; Groden et al., 1991; Morin et al., 1997; Morin, 1999).

Other familial colon cancer syndromes include hereditary nonpolyposis colon cancer (HNPCC), which accounts for 5% of all colon cancer cases. In HNPCC,
germ line mutations in two genes are commonly found, hMSH2 and hMLH1 (Kinzler and Vogelstein, 1996). These genes encode for mismatch repair proteins, which when abnormal will lead to genomic microsatellite instability and a two- to three-times higher mutation rate (Shibata et al, 1994; Bhattacharyya et al, 1994; Eshleman et al, 1995; Lynch et al, 1996). HNPCC has an associated lifetime colon cancer risk of up to 90% (Lynch et al, 1996; Lenz and Harpster, 2003). Other high-risk populations include patients who have had a prior colon cancer, as well as patients with inflammatory bowel disease.

**Nonheritable colon cancer**

In nonheritable colon cancer, at least seven independent genetic events are needed over decades and in the correct order to develop colorectal cancers (Vogelstein et al, 1988). This process begins with a normal colonic epithelial cell developing an adenomatous polyposis coli (APC) mutation, migrating to the top of the colonic crypt, expanding, and then forming an early adenoma (Smith et al, 1993; Miyashiro et al, 1995). Accumulation of a K-Ras mutation then promotes intermediate adenoma formation followed by the transition to a late adenoma after mutations on chromosome 18q21 (candidate genes DCC, DPC4, JV18) occur. Mutations in the p53 gene then transform the premalignant lesion to invasive carcinoma, and other additional genetic hits lead to metastasis (Kinzler and Vogelstein, 1996).

**Window of opportunity**

The above data underscore the need for effective strategies for both colon cancer prevention and treatment. The colon is an ideal target organ in which to develop chemopreventive interventions for a number of reasons. First, it is estimated that colorectal cancers develop over a period of 10-20 yr, providing a large window of opportunity for therapeutic intervention. Second, the relative sequence of genetic events required for tumor formation has been investigated most extensively in the colon (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996, Wong et al, 2007). Third, the established histopathological progression of normal tissue to an intermediate adenoma and, ultimately, invasive cancer presents milestones with respect to where a particular lesion is in the carcinogenic sequence, both in the presence and absence of chemopreventive agent exposure (Ilyas et al, 1999). Finally, the adenomatous polyp serves as a preneoplastic marker of colon cancer risk, aiding in the identification of the
subpopulation of individuals who would benefit most from the chemopreventive therapy.

Experimental models of colon carcinogenesis

One of the most widely used animal models for assessing the efficacy of chemopreventive agents against colorectal cancer is the 1,2-dimethylhydrazine (DMH) induced colon cancer. DMH requires metabolic activation in vivo to azoxymethane (AOM), which is then converted to the ultimate carcinogen methylazoxymethanol (MAM). In rats, DMH is injected i.p. once a week for 15-20 wk (Klurfed, 1990). Dysplastic and aberrant crypt foci (ACF), the putative cancer precursors, can be identified from 6 week onwards following DMH injection (Filipe, 1975) and gross colonic tumors (one per animal) are present after the minimum time duration of 20 wk post-DMH (Descner and Long, 1977).

There are several advantages of using the DMH model of colon carcinogenesis for chemoprevention studies. First, experimentation to date indicates that the promotional and protective effects of experimental diets can be discriminated in this model (Steele et al, 1994; Chang et al, 1997). Second, the evolution of colon tumors in the DMH model is similar to that in humans, including the progression of ACF to adenomas (often polyps), and ultimately carcinomas. Third, the histopathological features of DMH-induced colon tumors are similar to those of human tumors. Finally, 30-60% of DMH-induced colon tumors possess K-Ras mutations as seen in human colon tumors. The pitfalls of using the DMH model system to study colorectal carcinogenesis include the fact that both DMH and AOM are carcinogens to which humans are not exposed either environmentally or in their diet. Furthermore, unlike human colon tumors, DMH-induced tumors seldom exhibit mutations in either Apc (approx 8%) or p53 (Chang et al, 1997).

Currently, more than 400 studies have been performed using either the DMH or Min mouse model to assess the chemopreventive activity of synthetic or naturally occurring agents or diets against colorectal cancer (Corpet and Tache, 2002; Corpet and Pierre, 2003). Agents that afford strong protection against intestinal tumorigenesis in the DMH/AOM rat and/or Min mouse models include piroxicam, sulindac, celecoxib, difluoromethylornithine, polyethylene glycol, thiosulfonate, protease inhibitors, sphingomyelin, epidermal growth factor receptor kinase inhibitor, resveratrol, fish oil, curcumin, and calcium (Tsao et al, 2004).
Metabolism of DMH

On the basis of biological effects of DMH and its chemical derivatives, Druckrey (1970) and Druckrey et al (1967) had summarized the scheme for the metabolic activation of this carcinogen (Figure 2.3 a & b). In this pathway, DMH undergoes a series of oxidations via azomethane (AM) and azoxymethane (AOM) to form methylazoxymethanol (MAM). The latter may form a conjugate, e.g., a 6-glucuronide or sulfate which is transported by the bile or the blood to the colon. At this site the conjugate is split to reform MAM. The compound undergoes either a spontaneous or a tissue-specific enzyme-catalyzed breakdown into formaldehyde, hydroxyl ion, and the active methylating species, methyl diazonium (MD).

Pozharisski et al, (1975) established that DMH and/or its metabolites entered the intestinal lumen not only with the bile but also directly through its wall after subcutaneous administration in rats and once it has entered the epithelial cells, DMH methylates the macromolecules. DMH induces tumors of the colon but not of other organs whatever the treatment route (Pozharisski et al, 1975). According to Flala, (1975) the explanation for the organ-specificity of DMH could be that the colonic mucosa contains specific enzymes, not found in non susceptible tissues, which catalyze the hydroxylation of AOM and/or the conversion of MAM to the ultimate carcinogenic species. Also it has been shown by Pozharisski et al, (1975) that the regions of the gut with preferential incidence of neoplasms (colon) are characterized by a higher quantity of stem cells and, particularly by larger indices of their proliferative pool and shorter life cycles compared to the...
small bowel in which tumors appear very rarely. The works of other researchers also suggested that the stem cells entering mitosis are carcinogenic acceptors and the peculiarities of these cell population kinetics may play a significant role in the expression of the carcinogenic effect (Warwick, 1971; Rajewsky, 1972).

Inflammation and cancer

Inflammation may be considered as a secret killer owing to its surprising link between several human diseases (Figure 2.4). There is evidently a strong functional relationship between inflammation and cancer. In 1863, Virchow hypothesized the origin of cancer from the sites of chronic inflammation as inflammatory signals are known to enhance cell proliferation (Balkwill and Mantovani, 2001). Although it is now clear that proliferation of cells alone does not cause cancer, sustained cell proliferation in an environment rich in inflammatory cells, growth factors, activated stroma, and DNA damage-promoting agents, certainly potentiate and/or promote neoplastic risk (Coussens and Werb, 2002).

Chronic inflammatory diseases

The strongest association of chronic inflammation with malignant disease lies in colon carcinogenesis, which arises in individuals with inflammatory bowel diseases, for example, chronic ulcerative colitis and Crohn’s disease (Coussens and Werb, 2002) (Table 2.1). The plausible link behind the association of the inflammatory cells and neoplastic process is that many malignancies arise from
areas of infection and inflammation, simply as a part of the normal host response. There is a growing body of evidence indicating that persistent infections within the host induce chronic inflammation (Scholl et al, 1994; Shacter and Weitzman, 2002).

Table 2.1 Chronic inflammatory conditions associated with neoplasms

<table>
<thead>
<tr>
<th>Pathogenic condition</th>
<th>Associated neoplasm(s)</th>
<th>Aetiologic agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asbestosis, silicosis</td>
<td>Mesothelioma, Lung carcinoma</td>
<td>Asbestos fibres, silica particles</td>
</tr>
<tr>
<td>Bronchitis</td>
<td>Lung carcinoma</td>
<td>Silica, asbestos, smoking (nitrosamines, peroxides)</td>
</tr>
<tr>
<td>Cystitis, bladder inflammation</td>
<td>Bladder carcinoma</td>
<td>Chronic indwelling, urinary catheters</td>
</tr>
<tr>
<td>Gingivitis, lichen planus</td>
<td>Oral squamous cell carcinoma</td>
<td></td>
</tr>
<tr>
<td>Inflammatory bowel disease, Crohn's disease, chronic ulcerative colitis</td>
<td>Colorectal carcinoma</td>
<td></td>
</tr>
<tr>
<td>Lichen sclerosus, hereditary pancreatitis</td>
<td>Vulvar squamous cell carcinoma</td>
<td></td>
</tr>
<tr>
<td>Reflux oesophagitis, Barrett's oesophagus</td>
<td>Pancreatic carcinoma</td>
<td>Alcoholism, mutation in trypsinogen gene on Ch7</td>
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<tr>
<td>Sialadenitis</td>
<td>Salivary gland carcinoma</td>
<td></td>
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<tr>
<td>Sjögren syndrome</td>
<td>MALT lymphoma</td>
<td></td>
</tr>
<tr>
<td>Skin inflammation</td>
<td>Melanoma</td>
<td>Ultraviolet light</td>
</tr>
</tbody>
</table>

Adapted from Coussens and Werb, 2002. MALT, mucosa-associated lymphoid tissue

Leukocytes and other phagocytic cells induce DNA damage in proliferating cells, through their generation of reactive oxygen and nitrogen species that are produced normally by these cells to fight infection (Maeda and Akaike, 1998). These species react to form peroxynitrite, a mutagenic agent (Maeda and Akaike, 1998). Hence, repeated tissue damage and regeneration of tissue, in the presence of highly reactive nitrogen and oxygen species released from the inflammatory cells, interacts with DNA in proliferating epithelium resulting in permanent genomic alterations such as point mutations, deletions, or rearrangements. Indeed, p53 mutations are seen at frequencies similar to those in tumors in chronic inflammatory diseases such as inflammatory bowel disease (Yamanishi et al, 2002).

Immuno-modulation

It is now evident that in colonic physiology, inflammatory cells have powerful effects on the tumor development. Early in the neoplastic process, these
cells are powerful tumor promoters, producing an attractive environment for tumor growth, facilitating genomic instability and promoting angiogenesis. The inflammatory cells, and the chemokines /cytokines that they produce, influence the whole tumor organ, regulating the growth, migration and differentiation of all cell types in the tumor microenvironment, including the neoplastic cells, fibroblasts and endothelial cells. Later in the tumorigenic process, neoplastic cells divert inflammatory mechanisms to favor neoplastic spread and metastasis. This may be the part of an attempt by the tumor to subvert immune cell functions, therefore favoring tumor development (Brigati et al, 2002). The pro-tumor actions of the inflammatory cells include releasing growth and survival factors, promoting angiogenesis, stimulating DNA damage, remodeling the extracellular matrix to facilitate invasion, coating tumor cells to make available receptors for disseminating cells via lymphatics and capillaries, and evading host defense mechanisms.

Although inflammatory responses should also be anti-tumor, cancer patients are often defective in their inflammatory responses. This may arise by two distinct tumor mediated mechanisms: a failure to upregulate the anti-inflammatory cytokines, or subversion of the host response resulting from desensitization of receptors owing to high chemokine /cytokine concentration than blunt systemic responses (Allavena et al, 2000; Coussens and Werb, 2002).

These new insights on relationships of inflammation with cancer thus, provide a strong anti-cancer therapeutic opportunity and rationalize the use of certain drugs, called the non-steroidal anti-inflammatory drugs (NSAIDs) to harness the activities that are anti-tumor while suppressing those that are pro-tumor in the tumor tissues.

Cyclooxygenase and carcinogenesis

Prostaglandin H synthase (also known as Cyclooxygenase or COX), is an enzyme that catalyses the first two steps in the conversion of arachidonic acid (C20:4) to prostaglandins (PG) H2. PGH2 is further processed by the respective synthase to give rise to the primary prostanoids, such as the pro-inflammatory prostaglandin PGE2 (Vane and Botting, 1998). NSAIDs are known as low molecular weight inhibitors for COX. There are two known isoforms of COX: COX-1 and COX-2 (Vane et al, 1998) and one less established variant, COX-3 (Chandrashekharan et al, 2002).
Aside from producing several proinflammatory eicosanoids, COX may promote carcinogenesis by activating carcinogens via its peroxidase activity, which can operate on substrates other than PGG\(_2\); or by producing either malondialdehyde (MDA), a direct-acting mutagen, or peroxyl radicals (see Figure 2.5). The fact that COX-2 gets induced, and aspirin and other NSAIDs taken on regular basis decrease the relative risk of colorectal cancers (DuBois et al, 1996), suggests a possible role for COX-2 and PGs in the induction of colorectal cancers.

**Figure 2.5: Arachidonic acid metabolism by COX isoenzymes.** Phospholipase A\(_2\) (PLA\(_2\)) releases arachidonic acid (AA) from membrane phospholipids, which is in turn converted by either COX-1 or COX-2 to PGG\(_2\) (C, cyclooxygenase catalytic activity of COX) and then to PGH\(_2\) (P, peroxidase catalytic activity of COX). PGH\(_2\) is converted to either PGs (e.g., E\(_2\), F\(_2\)\(_\alpha\), I\(_2\), D\(_2\)), thromboxane A\(_2\) (TxA\(_2\)), or malondialdehyde (MDA). MDA is a direct-acting mutagen and can be produced without COX by direct lipid peroxidation. AA can be converted directly to 15-(R)-HETE by both COX isoenzymes. COX-1 is constitutively expressed in most tissues, whereas COX-2 is induced by cytokines, growth factors, tumor promoters, or other agents after the initiation of specific physiological events. Compounds other than PGG\(_2\), e.g., procarcinogenic hydroperoxides, can serve as substrates for the peroxidase activity of both COX enzymes. Inactive carcinogens serving as electron acceptors can also become activated by this activity. The COX isoenzymes are also involved in the formation of peroxyl radicals that can activate procarcinogens. *Figure is adapted from Shiff and Rigas, 1997.*

**COX-1 and COX-2 proteins: relative role in colonic neoplasia**

COX-1 is constitutively expressed in most tissues and plays an important role in tissue homeostasis, such as maintaining the protective lining of the gastrointestinal mucosa and mediating the platelet aggregation (DuBois et al, 1998). In contrast COX-2 is absent from most normal tissues and its expression is
induced by inflammatory cytokines and cellular transformation (Coyne et al, 1992; Simmons et al, 1999). Transcription of the COX-2 gene is rapidly induced in response to cytokines, growth factors and numerous pharmacological agents, and in pathological conditions (DuBois et al, 1994; Di Popolo et al, 2000). Further to the finding that COX-2 expression is commonly upregulated in various human cancers, including colon carcinomas (Masferer et al, 2000), genetic and pharmacological studies have shown a cause-and-effect connection between the expression of COX-2 and carcinogenesis (Eberhart et al, 1994; Oshima et al 2001; Jacoby et al 2000; Yao et al 2004).

Studies in vitro or in animal models suggest that prostaglandins produced by COX-2 reduce the rate of apoptosis induction and increased proliferation of cancer cells (DuBois et al, 1998). Subsequently, it was shown that COX-2 antagonists (e.g. Celecoxib, Rofecoxib, Nimesulide etc.) inhibited the growth factor-induced angiogenesis, while COX-1 inhibitors were ineffective. Several hypotheses have been proposed to explain how increased COX-2 expression might contribute to the carcinogenesis, including elevated Bcl-2 protein and inhibition of apoptosis (Tsuji and DuBois, 1995), increased angiogenesis (Tsuji et al, 1998) and enhanced metastatic ability (Jiang et al, 2001).

Other studies (Langenbach et al, 1995, Langenbach et al, 1999) suggest that COX-1 activity, perhaps through the induction of COX-2, may also be essential for the development of colorectal neoplasia. In mouse knockout studies deletion of either the COX-1 or COX-2 genes in Apc-deficient mice caused a 70%-80% reduction in intestinal polyposis. It is hypothesized that COX-1 in activated platelets may signal the increased expression of COX-2 in other cells through the release of lipid or protein paracrine mediators (Patrono et al, 2001). A role for COX-1 in the induction of COX-2 might explain why, in epidemiologic studies, aspirin use is associated with reduced risk of colorectal cancer even at doses and also at dosing intervals that could not sustain COX-2 inhibition in nucleated cells (Thun et al, 1991, Giovannucci et al, 1995).

Identification of COX genes

The human genes for COX-1 and COX-2 are located on chromosomes 9 and 1, respectively. The COX-2 gene is 8.3 kilobases (kb) whereas the COX-1 is much larger at 22 kb (Wu, 1995; Seibert et al, 1997; Vane et al, 1998; DuBois et al, 1998; Mitchell and Warner, 1999; Smith et al, 2000; FitzGerald and Patrono,
In general terms, the COX-1 gene exhibits the features of a housekeeping gene whereas the gene for COX-2 is a primary early response gene with many regulatory sites.

In vivo local increases in COX-2 expression have been associated with inflammation, rheumatoid arthritis and other pathological disorders. The intracellular pathways regulating these events appear numerous and complicated (Figure 2.6), varying between cell types and cellular stimulus, with nuclear receptors such as peroxisome proliferator-activated receptor-γ (PPAR-γ) attracting more recent attention (Subbaramaiah et al, 2001; Yang and Frucht, 2001; Marrache et al, 2002). There is also regulation of COX-2 expression at the post-transcriptional level. For example, Ras, which is implicated in many cancers and a key regulator of COX-2 expression, exerts this influence at least partly through a post-transcriptional process via protein kinase B (Sheng et al, 2000 and 2001) leading to the stabilization of COX-2 mRNA.

It has been suggested recently that there is another COX enzyme formed as a splice variant of COX-1 (Chandrasekharan et al, 2002). In the initial report of this enzyme it was named COX-3, although it may more appropriately have been named COX-1b. COX-3 is made from the COX-1 gene but retains intron 1 in its mRNA; it was initially reported to be expressed in canine cerebral cortex and in lesser amounts in other tissues analyzed. In humans, COX-3 mRNA was found to be expressed as an ~5.2 kb transcript that was most abundant in the cerebral cortex and heart. The difference at the protein level between COX-3 and COX-1 is the insertion of 30-34 aa, depending on the mammalian species, into the hydrophobic signal peptide. In COX-3 this signal peptide is not cleaved; the protein is glycosylated and displays COX activity (Warner and Mitchell, 2004).

NSAIDs as anticancer therapeutics

The observation that ‘NSAIDs reduce by about half both the incidence of and mortality from colon cancer’, made in 1988, by Kune et al represents a quantum advance in the field of colon cancer chemoprevention. Subsequent epidemiological studies have shown a significant inverse association between the intake of certain NSAIDs (such as aspirin, piroxicam, and sulindac) and the risk of colon cancer in general in the human population (Thun et al. 1991; Giardiello et al, 1993; Giovannucci et al. 1995; Rao et al, 2002). Further studies have
confirmed that NSAIDs reduce the risk of colon cancer by 40-60% in animal models (Skinner et al, 1991; Charalambous et al 1996; Shiff and Rigas, 1997) and FAP cohort studies (Labayle et al, 1991).

Figure 2.6: NSAIDs, COX inhibition and prostaglandins. The main targets of cyclooxygenase-2 (COX 2)-specific inhibitors (coxibs) and nonsteroidal anti-inflammatory drugs (NSAIDs) are COX 1 and COX 2 enzymes that are central to the conversion of arachidonic acid to prostaglandins. An alternative pathway of arachidonic acid metabolism involves the lipoxygenases. Both the lipoxygenase and COX pathways are regulated by peroxide concentrations, with COX-2 being induced at lower peroxide concentrations than COX 1. Prostaglandins (PGs) influence angiogenesis, apoptosis, cell proliferation and migration. The balance between prothrombotic factors (for example, thromboxane A2) and anti-thrombotic factors (for example, prostacyclin) might be of particular relevance to cardiovascular toxicity. IL-6, interleukin 6; NF-κB, nuclear factor-κB; PDGF, platelet-derived growth factor; PLA2, phospholipase A2, which cleaves fatty acids, such as arachidonic acid, from phospholipids; VEGF, vascular endothelial growth factor.
Cancers of most organs, including colon, bladder, breast, liver, and lung express increased levels of COX-2 as compared to the non-neoplastic adjacent tissue, making the COX-2 gene an important target in the study of carcinogenesis (Umar et al, 2003). Evaluation of NSAIDs, including newer selective COX-2 inhibitors, in carcinogen-induced and genetically manipulated animal models of colorectal cancer demonstrates that these drugs are effective chemopreventive agents (Gwyn and Sinicrope, 2002). Furthermore, in human subjects with FAP treated with specific COX-2 inhibitors, Celecoxib (Steinback et al, 2000) or Rofecoxib (Hallak et al, 2003), regression was shown in colorectal cancer and polyps. Therefore, taken together these findings suggest that the inhibition of COX-2 by the NSAIDs is relevant to the suppression of carcinogenesis. But NSAIDs may act through mechanisms other than inhibition of COX enzyme activity (Figure 2.6), as some NSAIDs lacking COX-inhibitory function show efficacy in inhibiting colon carcinogenesis (Elder et al, 1997).

Apoptosis is the dominant anti proliferating end-effect of the potentially effective chemopreventive drugs, and sensitivity to NSAIDs- induced apoptosis may simply be increased with the malignant potential of the tumor (Elder et al. 1996). The induction of apoptosis by NSAIDs may result from the inhibition of the COX isoforms but other as yet unidentified pathways to NSAIDs-induced apoptosis clearly exist, which may include interference with cell-cycle progression, reduction of carcinogen activation and stimulation of immune surveillance (Brigati et al, 2002).

NSAIDs with different selectivity towards COX

Many studies since the early 1990s have shown that the broad range of classical NSAIDs inhibit both COX-1 and COX-2 although with a general tendency toward COX-1 selectivity (Wu, 1995; Seibert et al, 1997; Vane et al, 1998; DuBois et al, 1998; Mitchell and Warner, 1999; Smith et al, 2000; FitzGerald and Patrono, 2001). This appears to be associated with gastrointestinal toxicity: the more COX-1-selective drugs appear to have the tendency to cause more gastrointestinal damage. This has provided the rationale for the development of selective inhibitors of COX-2. The first two compounds to enter the market place...
following deliberate development as COX-2-selective agents were rofecoxib (Vioxx™) and celecoxib (Celebrex™); these were joined by some existing NSAIDs, most notably etodolac (Lodine™), meloxicam (Mobic™, Mobicox™), and nimesulide (Aulin™, Mesulid™, Nimed™, and others), that display some level of COX-2 selectivity. Recently the number of therapeutically available COX-2-selective agents has been increased by the addition of valdecoxib (Bextra™; Talley et al, 2000), etoricoxib (Arcoxia™; Riendeau et al, 2001) and lumiracoxib (Prexige™).

This range of agents illustrates the important point that there are a number of chemically different structural classes of COX-2-selective inhibitors, which are still evolving (van Ryn et al, 2000) (Table 2.2). These COX-2-selective drugs together with the NSAIDs cover a wide range of selectivities toward COX-1 and COX-2.

<table>
<thead>
<tr>
<th>Structural Class</th>
<th>Members</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>COX-1-non-selective</td>
</tr>
<tr>
<td>alkanones</td>
<td>nabumetron</td>
</tr>
<tr>
<td>antranilic acids</td>
<td>meclofenamic acid, mafenamic acid</td>
</tr>
<tr>
<td>arylpropionic acids</td>
<td>ibuprofen, flurbiprofen, ketoprofen, naproxen</td>
</tr>
<tr>
<td>diaryheterocycles</td>
<td>SC560</td>
</tr>
<tr>
<td>di-tert-butyl phenols</td>
<td>darbufelone</td>
</tr>
<tr>
<td>enolic acids</td>
<td>piroxicam, tenoxicam, phenylbutazone</td>
</tr>
<tr>
<td>heteroaryl acetic acids</td>
<td>diclofenac, ketorolac, tolmetin</td>
</tr>
<tr>
<td>indole and indene acetic acids</td>
<td>indomethacin, sulindac</td>
</tr>
<tr>
<td>para-aminophenol derivatives</td>
<td>acetaminophen</td>
</tr>
<tr>
<td>salicylic acid derivatives</td>
<td>aspirin, diflunisal, sulfasalazine</td>
</tr>
<tr>
<td>sulfanilides</td>
<td>nimesulide, flosulide</td>
</tr>
</tbody>
</table>

Mechanism of COX- inhibition by NSAIDs

Aspirin

Historically, the 1st discovered drug by Hoffman Felix in 1897, aspirin still occupies the most important and bench mark NSAID. Aspirin transfers its acetyl
moiety to a single serine hydroxyl group of PGH synthase (Ser$^{530}$), which blocks the approach of arachidonic acid to the substrate binding site and inhibits cyclooxygenase activity (Figure 2.7) (Van Der-Ouderaa et al, 1980; DeWitt et al, 1990).

![Figure 2.7: Chemical Equation depicting that Aspirin transfers its acetyl moiety to a single serine hydroxyl group of PGH synthase (Ser$^{530}$)](image)

The acetylated enzyme however, exhibits normal level of peroxidase activity (Van Der-Ouderaa et al, 1980, Higgs et al, 1987). Site-directed mutagenesis of the hydroxyl group to a hydrogen atom (Ser$^{530-\rightarrow}$Ala$^{530}$) produces an enzyme with undiminished cyclooxygenase activity but no sensitivity to irreversible aspirin inhibition (DeWitt et al, 1990). This suggests that Ser$^{530}$ is not essential for cyclooxygenase catalysis and indicates an element of serendipity in the pharmacological activities of aspirin.

There are two major determinants of the preferential reactivity of Ser$^{530}$ to aspirin. The first is the heme prosthetic group (Chen and Marnett, 1989; Kulmacz, 1989). Although Ser$^{530}$ is well removed in linear sequence from the putative heme-binding residues of PGH synthase, acetylation of its hydroxyl group by aspirin is accelerated at least 100-fold by binding heme to the apoprotein. The other major determinant of acetylation selectivity is the salicylate moiety of aspirin. Reaction of PGH synthase with other acetyl transferring agents does not selectively acetylate Ser$^{530}$. This suggests that a specific binding site for the salicylate portion of the molecule exists at or near the substrate binding site of the protein.

These selectivity determinants account for the fact that PGH synthase is the principal target of aspirin acetylation in intact cells (Roth and Majerus, 1975). Considering the short half-life of aspirin in humans, this means that it is essentially a titrant for active holoenzyme (Chen and Marnett, 1989; Higgs et al, 1987).
The Coxibs: Celecoxib and Etoricoxib

Selective COX-2 inhibitors (coxibs) are approved for the relief of acute pain and symptoms of chronic inflammatory conditions such as osteoarthritis and rheumatoid arthritis. They have similar pharmacological properties but a slightly improved gastrointestinal (GI) safety profile if compared to the traditional nonsteroidal anti-inflammatory drugs (tNSAIDs) like aspirin. All coxibs have comparable efficacy to tNSAIDs but a slightly better GI safety profile.

Celecoxib belongs to the first generation of coxibs launched in 1999; while etoricoxib belongs to the second-generation drugs (Shi and Klotz, 2008). The different chemical structures of the coxibs are responsible for their distinct pharmacokinetic properties. Celecoxib possess a sulfonamide group while etoricoxib have a methylsulfone moiety (Brune and Hinz, 2004; Pratico and Dogne, 2005; Guzman et al, 2007).

Celecoxib (Figure 2.8)

Due to the poor aqueous solubility of celecoxib, its oral bioavailability is low (20-40%) (Paulson et al, 2000; Babu et al, 2002). Celecoxib can be given with or without food (Paulson et al, 2001) and extensively metabolized, primarily by CYP2C9 (80%); CYP3A4 has a minor role (Tang et al, 2000; Kirchheiner et al, 2003). The metabolism involves hydroxylation at the methyl moiety followed by further oxidation of the hydroxyl group to form a carboxylic acid, which is the major metabolite of celecoxib. In healthy adults, the elimination half-life (t1/2) is approximately 12 h and the apparent plasma clearance averages 450 ml/min (Paulson et al, 2000, Itthipanichpong et al, 2005).
Etoricoxib (Figure 2.9)

The pharmacokinetics of etoricoxib is linear over the clinically relevant dose range (Agrawal et al, 2001 and 2003). Etoricoxib is rapidly absorbed and the oral bioavailability approaches 100%. Steady-state conditions are achieved within 7 days of once-daily dosing with an accumulation ratio of approximately 2.0. Since t1/2 is approximately 25 h, once-daily dosing can be accomplished in experimental conditions. Etoricoxib is metabolized extensively, and the metabolites are largely excreted into the urine (70%) (Rodrigues et al, 2003).

Etoricoxib is metabolized via 6'-methyl-hydroxylation (major pathway) and 1'-N-oxidation, which is catalyzed by multiple P450 forms. CYP3A4 accounts for the majority of the activity (approximately 60%); the remaining part is divided over several CYPs such as CYP2C9, CYP2D6, CYP1A2, and possibly CYP2C19 (Kassahun et al, 2001). So, the metabolic pattern of etoricoxib differs from that of the other coxibs (celecoxib) that are primarily (>80%) metabolized by a single P450 form (CYP2C9) in human liver microsomes (Rodrigues et al, 2003).

COX-independent mechanism of the NSAIDs

Whether COX or eicosanoids are essential or even necessary for colorectal carcinogenesis has not been fully assessed. Clearly, some colorectal cancers develop without overexpressing COX or producing high levels of PGs. However this still does not rule out a central role for COX enzymes, at least for most colorectal cancers. A potentially important means by which NSAIDs prevent colorectal neoplasia is to affect cell turnover in the colorectal epithelium (Shiff and Rigas, 1997). Cell death and renewal are critical for the regulation of the structural
integrity of all tissues. NSAIDs may inhibit cell proliferation by inducing cell cycle quiescence in colonocytes, in part by reducing the levels of several key molecules that catalyze transitions through the various phases of the cell cycle (Shiff et al, 1995; 1996). However, the detailed molecular pathways that induce quiescence have yet to be fully elucidated.

It had been shown that several NSAIDs, including acetyl salicylic acid (ASA), sulindac, indomethacin, piroxicam, naproxen, as well as selective COX-2 inhibitors, simultaneously retard the proliferation and induce apoptosis in colon cancer cells (Shiff and Rigas, 1997; Elder et al, 1997). However, several groups contend that they have identified mechanisms by which NSAIDs induce PG- or COX-independent apoptosis (Zhang et al, 1999; Grosch et al, 2001; Maier et al, 2004; Rigas and Kashfi, 2005; Jana, 2008), which make the strong case that COX inhibition is not required for certain presumed chemopreventive effects of NSAIDs. Others have shown that NSAID treatment of colon cancer cells generates the proapoptotic lipid, ceramide (Chan et al, 1998).

This apparent inconsistency is not merely of theoretical interest, but has important implications for the rational design of strategies for colon chemoprevention and for assessing the relative significance of each mechanism in carcinogenesis. In all likelihood this is not a contradiction at all; rather, NSAIDs bring about their chemopreventive effects in the colon through both COX-dependent and -independent mechanisms (Shiff and Rigas, 1999).

Evidence of restoration of apoptosis and inhibition of angiogenesis

The molecular basis for the activity of NSAIDs in the prevention and treatment of cancer is thought to be pleiotropic. Despite continuing uncertainty about the molecular pathways, there is mounting evidence that tumor inhibition may be mediated by at least two distinct cellular processes. These involve the ability of NSAIDs to restore apoptosis in APC-deficient cells (Tsujii and DuBois, 1995; He et al, 1999) and their capacity, particularly in the case of coxibs, to inhibit angiogenesis.
Apoptosis

Apoptosis, or programmed cell death, is needed to maintain homeostasis in continuously replicating tissues such as the intestine (Afford and Randhawa, 2000). Partial suppression of apoptosis occurs early in tumorigenesis in approximately 85% of human colorectal cancers due to the inactivation of both alleles of the APC gene (Morin et al, 1996; Kinzler and Vogelstein, 1998). The suppression of apoptosis allows APC-deficient cells to accumulate in adenomatous polyps. Further suppression of apoptosis occurs as these cells may develop additional genetic mutations and phenotypic changes (Bedi et al, 1995).

In vitro, both nonselective NSAIDs and selective COX-2 inhibitors stimulate apoptosis in APC-deficient cells that have not yet undergone malignant transformation. This is also seen clinically in FAP patients treated with sulindac (Pasricha et al, 1995) and in experimental studies of ApcMin mice (Boolbol et al, 1996; Mahmoud et al; 1998, Jacobasch et al, 1998) and rats exposed to chemical carcinogens (Samaha et al, 1997). Nonselective NSAIDs lose their ability to inhibit chemically induced tumors when polyps undergo malignant transformation. In contrast, selective COX-2 inhibitors stimulate apoptosis and suppress growth in many carcinomas, including cultured human cancers of the stomach (Uefuji et al, 2000), esophagus (Li et al, 2000a, Souza et al, 2000), tongue (Sumitani et al, 2001), brain (Joki et al, 2000), lung (Tsubouchi et al, 2000), and pancreas (Molina et al, 1999).

Despite these observations, results from other studies challenge the conventional wisdom that COX inhibition is the only shared function of NSAIDs (Vane, 1971) or that the products rather than the substrate of COX activity mediate its biologic effects. For example, in some experimental models, the concentration of free arachidonic acid itself regulates apoptosis in colorectal epithelial cells (Chan et al, 1998; Cao et al, 2000). Other experimental models suggest that NSAIDs may affect apoptosis through a mixture of prostaglandin-dependent and prostaglandin-independent pathways (Marx, 2001). NSAIDs have also been reported to induce apoptosis through 15-lipoxygenase-1, independent of COX-2 (Shureiqi et al, 2000). However, many of these effects have been
demonstrated only with high concentrations of NSAIDs in vitro and are of uncertain clinical relevance.

Angiogenesis

A second cellular process by which COX-2 inhibitors may inhibit tumor growth is believed to be through the inhibition of angiogenesis and neovascularization (Tsujii et al., 1998; Jones et al., 1999). Solid tumors must stimulate the formation of new capillary blood vessels to grow larger than approximately 2 mm in diameter which is essential for tumor survival (Masferrer et al., 1996; Jones et al., 1999; Holash et al., 1999). COX-2 expression is widely induced in the angiogenic vasculature of colorectal adenomatous polyps and in carcinomas of the colon, lung, breast, esophagus, and prostate (Holash et al., 1999, Masferrer et al., 2000). Selective COX-2 inhibitors suppress the growth of corneal capillary blood vessels in rats exposed to basic fibroblast growth factor (Masferrer et al., 2000) and inhibit the growth of several human tumors transplanted into mice (Williams et al., 2000).

Therapeutic (low micromolar) concentrations of coxibs also suppress the release of angiogenic growth factors by human or rodent colorectal cancer cells that are co-cultured with vascular endothelial cells (Tsujii et al, 1998; Jones et al, 1999) and block migration and tube formation by the endothelial cells. In contrast, toxic concentrations of aspirin or indomethacin are required to block vascular endothelial tube formation.

These experiments suggest that COX-2 may be essential for tumor vascularization and growth. However, the relevance of the experimental models to human colorectal cancer remains uncertain. Such therapeutic dilemmas surrounding the use of COX-2-selective inhibitors highlight several of the challenges in chemoprevention research. Potential agents must have both chemopreventive effects and acceptable risk-benefit profiles for the target population.

Coxibs and cancer chemoprevention: Promise and reality

Coxibs are assumed to be administered in the long-term treatment of patients suffering from arthritis due to lower incidence of gastrointestinal side
effects. Several recent studies however, pointed to an increased incidence of thromboembolic events associated with the use of coxibs (Howard and Delafontaine, 2004; Kearney et al, 2006). Increased incidence of vascular events was reported for ibuprofen and diclofenac, too. Rofecoxib and celecoxib were associated with increased incidence of myocardial infarction, chiefly attributable to a higher-dose daily drug intake (Kearney et al, 2006). In fact, certain coxibs were withdrawn from the market although the real reasons of cardiovascular (CV) complications attributed to the therapy remained unclear. Because the long-term use of coxibs is associated with CV risks, the role of COX-2 inhibitors for inhibition of cancer remains an open question (Liao et al, 2007).

Celecoxib has been evaluated in patients with FAP, which represents a precancerous condition. In patients with FAP, celecoxib 400 mg b.i.d. for 6 months decreased the size and number of colorectal and duodenal polyps by 28% (P=0.003 for comparison with placebo) leading to the approval for the treatment of this condition (Steinbach et al, 2000; Phillips et al, 2002). Currently, celecoxib is the only approved coxib as an adjunct to endoscopic surveillance in patients with FAP (Shi and Koltz, 2008).

Several clinical trials have shown the effectiveness of celecoxib in the chemoprevention of colorectal, lung and breast cancer. The Adenoma Prevention with Celecoxib (APC) and the Prevention of Spontaneous Adenomatous Polyps (PreSAP) trials were randomized, placebo controlled trials evaluating for 3 years for the efficacy of celecoxib for prevention of colorectal adenomas (Bertagnolli et al, 2006; Arber et al, 2006). The cumulative incidence of adenomas detected through 3 year of the APC trial was 43.2% for patients taking celecoxib 200 mg b.i.d. [relative risk (RR) 0.67, 95% CI 0.59-0.77, P<0.001] and 37.5% for those receiving 400 mg b.i.d. (RR 0.55, 95% CI 0.48-0.64, P<0.001) compared to 60.7% for those receiving placebo (Bertagnolli et al, 2006).

In the PreSAP trial, the cumulative rate of adenomas was 33.6% in the celecoxib group and 49.3% in the placebo group (RR 0.64, 95% CI 0.56-0.75, P<0.001). The cumulative rate of advanced adenomas was 5.3% in the celecoxib group and 10.4% in the placebo group (RR 0.49, 95% CI 0.33-0.73, P<0.001) (Arber et al, 2006). Comparable results were calculated in a meta-analysis (RR 0.72, 95%
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CI 0.68-0.77) (Rostom et al, 2007). Meanwhile, all these studies found significant increased CV events with celecoxib (Bertagnolli et al, 2006; Arber et al, 2006; Rostom et al, 2007). Thus, the balance of benefits to risks does not decidedly favor chemoprevention with coxibs in the general population.

However, in a recent case control study, the use of celecoxib or rofecoxib for more than 1 year resulted in a significant reduction (60%) in the risk of lung cancer (Harris et al, 2007). Various studies demonstrated a significant reduction in the risk as well as in the treatment of different cancers by coxibs (Harris et al, 2006; Jimeno et al, 2006; Smith et al, 2006). Furthermore, etoricoxib, the latest among the new age coxibs, has been found to be effective in prevention of rat mammary carcinogenesis in a recently reported study (Orendas et al, 2007). Taken together, these results thus strongly indicate that coxibs have the potential for chemoprevention of cancer, although surely there are needs for further detailed animal and clinical studies.