Cyclooxygenase-2: a target for the prevention and treatment of breast cancer

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Abstract

Cyclooxygenase-2 (COX-2), an inducible prostaglandin synthase, is normally expressed in parts of the kidney and brain. Aberrant COX-2 expression was first reported in colorectal carcinomas and adenomas, and has now been detected in various human cancers, including those of the breast. Strikingly, COX-2 overexpression in murine mammary gland is sufficient to cause tumour formation. To date, the role of COX-2 in tumorigenesis has been most intensively studied in the colon. Thus, the relationship between COX-2 and neoplasia can best be illustrated with reference to intestinal tumorigenesis. Here we consider the potential utility of selective COX-2 inhibitors for the prevention and treatment of breast cancer. Data for cancers of the colon and breast are compared where possible. In addition, the mechanisms by which COX-2 is upregulated in cancers and contributes to tumorigenesis are discussed. Importantly, several recent studies of mammary tumorigenesis in animal models have found selective COX-2 inhibitors to be effective in the prevention and treatment of breast cancer. Clinical trials will be needed to determine whether COX-2 inhibition represents a useful approach to preventing or treating human breast cancer.

Introduction

Cyclooxygenase-2 (COX-2) is emerging as an increasingly promising pharmacological target for the prevention and treatment of many human cancers. COX-1 and COX-2 are prostaglandin (PG) synthases which catalyse sequential synthesis of prostaglandin \(G_2\) (PG\(G_2\)) and PG\(H_2\) from arachidonic acid by virtue of intrinsic cyclooxygenase and peroxidase activities (Fig. 1). PG\(H_2\) is then converted by specific isomerases to other eicosanoids, including PGs, thromboxane (Tx) and prostacyclin. Cyclooxygenase-derived prostanoids contribute to many normal physiological processes including haemostasis, platelet aggregation, kidney and gastric function, reproduction, pain and fever. Despite the similar enzymatic activities of COX-1 and COX-2, the COX-1 and COX-2 genes have distinct properties, and differing expression patterns (Table 1). While COX-1 is constitutively expressed, COX-2 is upregulated in response to growth factors, tumour promoters and cytokines (reviewed by Herschman 1996). Additionally, COX-2 is responsive to several oncoproteins, including v-src, v-Ha-ras, HER-2/neu and Wnt genes (Xie & Herschman 1995, Subbaramaiah et al. 1996, Sheng et al. 1998b, Howe et al. 1999, Vadlamudi et al. 1999, Haertel-Wiesmann et al. 2000). Thus, increased PG synthesis is detected in inflamed and neoplastic tissues. Analysis of COX-2-deficient mice suggests that COX-2 is normally important for post-natal renal development and multiple female reproductive processes including ovulation, fertilisation, implantation and decidualisation (Dinchuk et al. 1995, Morham et al. 1995, Lim et al. 1997, 1999a). Aberrant COX-2 expression has been detected in multiple human cancers, as shown in Table 2. Together, a weight of epidemiological, pharmacological, genetic and expression data combine to suggest an important role for COX-2 in tumorigenesis, particularly in colorectal cancer. There is recent evidence that COX-2 may also represent a novel target for the prevention and treatment of breast cancer.

Cyclooxygenase activity is inhibited by nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and sulindac, which are most commonly administered for the relief of pain and inflammation. However, adverse side effects including peptic ulcer disease are associated with the use of such compounds, which are nonselective inhibitors of
Figure 1 Biosynthesis of prostaglandins. Arachidonic acid, released from membrane phospholipids by phospholipase A2 action (reaction 1), is metabolised by cyclooxygenases to PGH₂ in two steps. PGG₂ is generated by cyclooxygenase activity (reaction 2), then converted to PGH₂ by the peroxidase activity (reaction 3); both enzyme activities are intrinsic to COX-1 and COX-2. PGH₂ can be converted to several eicosanoids by specific isomerases. Additionally, MDA (malondialdehyde) can be produced enzymatically or by degradation of PGH₂. TxA₂, thromboxane A₂.

Table 1 Properties of COX-1 and COX-2.

<table>
<thead>
<tr>
<th></th>
<th>COX-1</th>
<th>COX-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expression</td>
<td>Constitutive</td>
<td>Inducible</td>
</tr>
<tr>
<td>Size of gene</td>
<td>22 kb</td>
<td>8.3 kb</td>
</tr>
<tr>
<td>mRNA transcript</td>
<td>2.7 kb</td>
<td>4.5 kb, with multiple Shaw-Kamen sequences</td>
</tr>
<tr>
<td>Size of protein</td>
<td>72 kDa</td>
<td>72/74 kDa doublet</td>
</tr>
<tr>
<td>Localisation</td>
<td>Endoplasmic reticulum, nuclear envelope</td>
<td>Endoplasmic reticulum, nuclear envelope</td>
</tr>
<tr>
<td>Expression pattern</td>
<td>Most tissues, including stomach, kidney, colon and platelets</td>
<td>Regions of brain and kidney, activated macrophages, synoviocytes during inflammation, malignant epithelial cells. Expression stimulated by cytokines, growth factors, oncogenes and tumour promoters</td>
</tr>
</tbody>
</table>

COX-1 and COX-2. In fact, prior to the development of selective COX-2 inhibitors, there were an estimated 100 000 hospitalisations and 16 500 deaths per year in the United States related to NSAID use (Singh 1998). Toxicity associated with the use of nonselective NSAIDs was the major stimulus to develop selective COX-2 inhibitors. Endoscopically controlled studies show that selective COX-2 inhibitors are far less ulcerogenic than classical NSAIDs (Langman et al. 1999, Simon et al. 1999). Since selective COX-2 inhibitors appear sufficiently safe to allow large scale administration on a chronic basis to healthy individuals, they represent potentially useful agents for cancer chemoprevention.

**COX-2 and cancer: epidemiology and expression**

**Colon cancer**

One of the first clues that cyclooxygenase inhibition might be an effective approach to preventing cancer came from
Table 2 COX-2 overexpression in human tumours.

<table>
<thead>
<tr>
<th>Organ site</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cancer</td>
<td>Parrett et al. (1997), Hwang et al.</td>
</tr>
<tr>
<td></td>
<td>(1998), Masferrer et al. (2000),</td>
</tr>
<tr>
<td></td>
<td>Subbaramaiah et al. (1999b),</td>
</tr>
<tr>
<td></td>
<td>Soslow et al. (2000)</td>
</tr>
<tr>
<td>Cervical dysplasia and cancer</td>
<td>Kulkarni et al. (2001)</td>
</tr>
<tr>
<td>Prostate carcinoma</td>
<td>Gupta et al. (2000), Yoshimura et</td>
</tr>
<tr>
<td></td>
<td>al. (2000)</td>
</tr>
<tr>
<td>Bladder transitional cell carcinoma</td>
<td>Mohammed et al. (1999)</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>Koga et al. (1999)</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>Molina et al. (1999), Okami et al.</td>
</tr>
<tr>
<td></td>
<td>(1999), Tucker et al. (1999)</td>
</tr>
<tr>
<td>Skin cancer</td>
<td>Buckman et al. (1998)</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>Hida et al. (1998), Wolff et al.</td>
</tr>
<tr>
<td>Head and neck cancer</td>
<td>Chan et al. (1999)</td>
</tr>
<tr>
<td>Colorectal adenomas and carcinomas</td>
<td>Eberhart et al. (1994), Kargman et</td>
</tr>
<tr>
<td></td>
<td>al. (1995), Sano et al. (1995),</td>
</tr>
<tr>
<td></td>
<td>Kutcher et al. (1996)</td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>Ristimaki et al. (1997)</td>
</tr>
<tr>
<td>Barrett’s oesophagus and oesophageal cancer</td>
<td>Wilson et al. (1998)</td>
</tr>
</tbody>
</table>

epidemiological studies. Several studies reported an inverse correlation between colon cancer incidence and regular use of NSAIDs including aspirin (Thu et al. 1991, Greenberg et al. 1993, Logan et al. 1993, Suh et al. 1993, Reeves et al. 1996). Since NSAIDs are known to function, at least in part, by inhibiting cyclooxygenase enzyme activity, these observations suggested that aberrant PG biosynthesis might contribute to colorectal neoplasia. This led to an analysis of COX expression in colorectal neoplasms. Levels of COX-1 were not increased in colorectal carcinomas relative to adjacent normal mucosa (Eberhart et al. 1994, Kargman et al. 1995, Sano et al. 1995, Kutcher et al. 1996). In contrast, striking COX-2 upregulation was observed in colon carcinomas compared with the virtually undetectable expression in normal mucosa (Eberhart et al. 1994, Kargman et al. 1995, Sano et al. 1995, Kutcher et al. 1996). Eberhart et al. (1994) also detected COX-2 expression in 9 of 20 adenomas examined. In carcinomas, COX-2 protein localised predominantly to the epithelial component, but could also be detected in tumour-associated fibroblasts, vascular endothelial cells, and inflammatory mononuclear cells (Sano et al. 1995, Kutcher et al. 1996). COX-2 expression has also been detected in intestinal adenomas from rodent models of intestinal tumorigenesis (Boobol et al. 1996, Dubois et al. 1996a, Williams et al. 1996, Singh et al. 1997).

Together, these epidemiological and expression studies suggested a role for COX-2 in colorectal tumorigenesis. This idea is supported by the results of clinical trials. Treatment with the NSAID sulindac or with celecoxib, a selective COX-2 inhibitor, causes a decrease in the size and number of polyps in familial adenomatous polyposis patients (Giardiello et al. 1993, Steinbach et al. 2000). Thus, overexpression of COX-2 appears to contribute to colorectal cancer and cyclooxygenase inhibitors are likely to be useful chemopreventive agents.

Breast cancer

In contrast to colon cancer, the role of COX-2 in breast cancer is less clear. Epidemiological studies conducted to investigate the relationship between NSAID use and breast cancer incidence have reported conflicting findings. Several studies have failed to find a significant relationship between aspirin use and breast cancer risk (Paganini-Hill et al. 1989, Thun et al. 1991, Egan et al. 1996). However, other analyses have revealed an association between NSAID consumption and decreased breast cancer incidence. Friedman & Ury (1980) found significantly reduced breast cancer incidence in 4867 women who used indomethacin, compared with age-matched controls. Harris and colleagues (1996) compared NSAID use in 511 women with newly diagnosed breast cancer with 1534 population control subjects, and found that the relative risk of breast cancer was reduced to 66% in women using NSAIDs at least 3 times per week for at least one year. Two additional studies also found that NSAIDs protected against breast cancer (Schreinemachers & Everson 1994, Sharpe et al. 2000). The basis for the lack of consistency among different studies is unclear. One potential explanation is that some NSAIDs may have restricted bioavailability in breast tissue. Thus, conflicting data obtained in separate studies may reflect the usage of different NSAIDs in the populations examined. Another potential complication is that significant COX-2 overexpression may be limited to a subset of human breast cancers, which could certainly confound epidemiological analyses. Approximately 85% of human colorectal adenocarcinomas overexpress COX-2 (Eberhart et al. 1994, Kargman et al. 1995, Sano et al. 1995, Kutcher et al. 1996). This could account for the strong correlation between regular NSAID use and reduced cancer incidence (Thu et al. 1991, Greenberg et al. 1993, Logan et al. 1993, Suh et al. 1993, Reeves et al. 1996). In contrast, as discussed below, COX-2 is not abundantly overexpressed in the majority of human breast cancers (Hwang et al. 1998, Subbaramaiah et al. 1999b). With this in mind, it is predictable that the results of epidemiological studies would be less clear-cut for breast than colon cancer even if NSAIDs were active against COX-2-positive breast cancers.

Enhanced COX expression in breast cancer was first suggested by reports of elevated PG levels in breast tumours (Tan et al. 1974, Bennett et al. 1977, Rolland et al. 1980). PG production was increased in human breast cancers, particularly in those from patients with metastatic disease (Bennett et al. 1977).
COX-2 is expressed in intestinal and mammary tumours in rodents

Rodent models of intestinal tumorigenesis can be divided into carcinogen-induced tumour models, and those in which tumour formation is induced by introduction of germline mutations into tumour suppressor genes such as Apc. In humans, germline mutation of the APC gene is responsible for familial adenomatous polyposis (FAP), in which individuals develop numerous adenomatous colorectal polyps, which predispose to colorectal carcinomas. In addition, APC is mutated in approximately 85% of sporadic colorectal carcinomas. Several mouse strains have been developed which harbour mutations in one Apc allele, including the Min mouse (Moser et al. 1990), Apc\(^{1638N}\) (Oshima et al. 1995), Apc1638N (Fodde et al. 1994), and Apc\(^{A772}\) (Sasai et al. 2000). These mice consistently develop intestinal adenomas, although these are more prevalent in the small intestine than in the colon. Analysis of adenomatous polyps from Min mice revealed increased COX-2 expression relative to normal mucosa (Williams et al. 1996). Elevated expression of COX-2 was also detected in colonic mucosa and tumours from rats treated with azoxymethane (AOM) (DuBois et al. 1996a, Singh et al. 1997). Thus COX-2 is commonly overexpressed in both human colorectal cancers and animal models of colorectal cancer. The cellular localisation of COX-2 in both human and rodent tumours continues to be investigated.

Rodent models have also been used to examine COX-2 expression in mammary tumours. In the rat, COX-1 is ubiquitously expressed in virgin, pregnant, lactating, and post-lactational mammary glands, but COX-2 is only detectable in the mammary glands of lactating animals (Badawi et al. 1999). Treatment of ovariectomised animals with oestradiol and progesterone causes induction of COX-2 and PG synthesis (Badawi & Archer 1998, Badawi et al. 1999), suggesting that COX-2 transcription is susceptible to hormonal regulation. COX-2 protein has been detected in rat mammary tumours induced by various carcinogens, including N-nitrosomethyl urea (NMU), dimethylbenz(a)anthracene (DMBA) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (Robertson et al. 1998, Hamid et al. 1999, Nakatsugi et al. 2000). Based on immunohistochemical analyses, COX-2 protein was observed in the epithelial cells within the mammary tumours (Robertson et al. 1998, Nakatsugi et al. 2000). Interestingly, dietary administration of n-6 polyunsaturated fatty acids (PUFAs) in the form of safflower oil stimulated COX-2 expression in rat mammary glands, suggesting a potential mechanism by which n-6-PUFAs may contribute to mammary tumorigenesis (Badawi et al. 1998).

In addition to these rat studies, COX-2 protein levels have also been examined in mammary tissues from transgenic mice strains that develop mammary tumours due to mammary-targeted oncogene expression. Significant amounts of COX-2 protein were detected in mammary tumours from mice overexpressing neu (K Subbaramaiah and A J Dannenberg, unpublished observations), consistent with our findings in HER-2/neu-overexpressing human
breast cancers (Subbaramaiah et al. 1999b). We have also found increased COX-2 protein in mammary tumours from Wnt-1 transgenic mice, relative to the levels in normal mammary gland (Fig. 2; Howe et al. 2001). Consistent with this, COX-2 is transcriptionally upregulated in mouse mammary epithelial cell lines engineered to express Wnt-1 (Howe et al. 1999), and expression is also increased in response to transformation by other oncogenes (Subbaramaiah et al. 1996).

**Mechanisms of COX-2 upregulation**

There is evidence that COX-2 is upregulated in both neoplastic and stromal cells within tumours. Hence, multiple mechanisms are likely to account for overexpression of COX-2 in these different cell types. It is relevant, therefore, to evaluate the effects of different stimuli in various cell types. COX-2 expression is normally regulated at both transcriptional and post-transcriptional levels, and can also be regulated by the rate of protein synthesis and/or degradation. The human COX-2 promoter contains multiple transcription factor binding sites, including a cAMP response element (CRE), and potential binding sites for Myb, nuclear factor interleukin-6 (NF-IL6), nuclear factor κB (NF-κB), and Ets factors (Genbank Accession Number 505116). Of these, the sites proximal to the transcription start site (Fig. 3) have been shown to be differentially responsive to various stimuli. Induction of COX-2 by v-src, serum, platelet-derived growth factor (PDGF) and ceramide requires activation of both Ras/Raf-1/ERK and Ras/MEKK1/JNK signal transduction pathways and is predominantly mediated via the CRE (Xie & Herschman 1995, 1996, Subbaramaiah et al. 1998a). In contrast, the NF-IL6 and NF-κB sites are required for induction of COX-2 in response to tumour necrosis factor (TNF) in osteoblasts (Yamamoto et al. 1995). The NF-IL6 and CRE sites have been identified as being critical for the induction of COX-2 in response to other stimuli, including lipopolysaccharide (LPS) and immunoglobulin E receptor aggregation (Inoue et al. 1995, Reddy et al. 2000b, Wadleigh et al. 2000). Other studies have implicated the NF-κB site as being important for LPS- and benzo[a]pyrene-mediated induction of COX-2 (Hwang et al. 1997, Yan et al. 2000). The expression of COX-2 can also be increased by stabilisation of the COX-2 transcript (Ristimaki et al. 1994, Sheng et al. 1998b). The 3′ untranslated region of COX-2 mRNA contains a 116 nucleotide AU-rich sequence element (ARE) which can negatively regulate transcript stability and modulate translation (Dixon et al. 2000).

During tumorigenesis, increased expression of COX-2 is likely to be a consequence of multiple effects. For example, transcriptional activation is likely to occur in response to growth factors and oncogenes. Moreover, since wildtype p53 decreases COX-2 transcription, loss-of-function p53 mutations may contribute to COX-2 upregulation (Subbaramaiah et al. 1999a). Dixon et al. (2000) speculated that ARE-binding proteins which normally negatively regulate transcript stability may be defective in tumour cells. This, too, could result in increased levels of COX-2. The relative importance of these different factors is likely to vary in different tissues. In mouse skin carcinogenesis, promoter activation by upstream stimulatory factor (USF) and CCAAT/enhancer binding proteins (C/EBPs) appears to be important (Kim & Fischer 1998). By contrast, bile acids, which have been implicated in the promotion of

![Figure 2](image_url)  
**Figure 2** COX-2 protein is increased in Wnt-1-expressing mammary tumours. COX-2 protein was analysed in lysates prepared from mammary tumours from three Wnt-1 transgenic female mice (lanes 4–6) and from mammary glands (MG) isolated from three strain-matched wildtype female mice (lanes 1–3). Lysates were prepared from 10 mg of each tissue sample. COX-2 protein was immunoprecipitated, and immunoprecipitates were analysed for COX-2 by Western blotting. The arrow indicates the position of a COX-2 standard. Little COX-2 protein was detectable in the wildtype mammary glands (lanes 1–3). In contrast, appreciable COX-2 protein was observed in all three tumour samples (lanes 4–6). Adapted and reproduced with permission from Howe et al. (2001).
Evidence from rodent models that COX-2 contributes to cancer

Genetic evidence for a role for COX-2 in tumour formation

Definitive evidence linking cyclooxygenases to tumorigenesis was first provided by studies using mice with targeted disruptions of the COX-1 or COX-2 genes. Oshima et al. (1996) pioneered these experiments by generating Apc<sup>−/−</sup>, COX-2-null mice. Intestinal adenoma incidence was reduced by 86% in COX-2 knockout mice, and by 66% in COX-2 heterozygotes, relative to COX-2 wildtype mice carrying the Apc mutation (Oshima et al. 1996). Tumour size was also significantly reduced in COX-2-deficient mice. COX-2 deficiency also protects against chemically induced papilloma formation in mouse skin (Tiano et al. 1997), and COX-2-null embryonic stem cells have a dramatically reduced ability to form teratomas when injected into syngeneic mice (Zhang et al. 2000a). Interestingly, disruption of either COX-1 or COX-2 caused similar reductions in tumour multiplicity in the Min mouse (Chulada et al. 2000), suggesting that both enzymes can impact on tumorigenesis. The results of similar studies to determine the effects of COX-2 deficiency on the incidence of mammary cancer are eagerly awaited. However, results from the converse experiment designed to address the consequence of COX-2 overexpression in mammary gland have recently been reported (Liu et al. 2001). Liu and colleagues overexpressed human COX-2 from the mouse mammary tumor virus (MMTV) promoter, and demonstrated that COX-2 overexpression was sufficient to cause breast tumour formation in more than 85% of multiparous mice. Virgin females did not develop tumours, but exhibited precocious lobuloalveolar differentiation and enhanced expression of the milk protein ß-casein. MMTV-driven COX-2 expression increased during pregnancy, suggesting a basis for the failure of virgin animals to develop tumours. Interestingly, mammary gland involution was delayed in COX-2 transgenic mice, with a decrease in the apoptotic index of mammary epithelial cells, and COX-2-induced tumour tissue expressed

Figure 3 Human COX-2 promoter schematic. The transcription start site is indicated by an arrow, the TATA box at −31/−25 is shown as a white rectangle, and three transcription factor binding sites lying between −327/+59 of the human COX-2 promoter are depicted as black ovals.
Pharmacological studies in rodent models of intestinal tumorigenesis

In addition to genetic evidence implicating cyclooxygenases in intestinal tumorigenesis, there are complementary pharmacological data. Many animal-based studies have been performed to investigate the utility of cyclooxygenase inhibitors for prevention or treatment of intestinal tumours. The prevention studies have predominantly examined either AOM-induced lesions in rat colon (aberrant crypt foci or carcinomas) or intestinal adenomas in Apc-deficient mice. A consistent finding has been that tumour incidence and multiplicity are reduced by both nonselective NSAIDs (Reddy et al. 1993, Rao et al. 1995, Boolbol et al. 1996, Jacoby et al. 1996, 2000a), and selective COX-2 inhibitors (Table 3). In addition, those tumours that do develop in drug-treated animals tend to be reduced in size relative to those in control animals (Nakatsugi et al. 1997, Fukutake et al. 1998, Jacoby et al. 2000a,b, Reddy et al. 2000a). It is notable that selective COX-2 inhibitors appear to be at least as effective in preventing tumours as nonselective NSAIDs. This result has important clinical implications, given the enhanced safety profile of selective COX-2 inhibitors versus traditional NSAIDs.

In addition to these prevention studies, cyclooxygenase inhibitors are also being evaluated as therapeutic agents for pre-existing tumours. Reduction in growth of colon cancer xenografts has been achieved by treatment with meloxicam, SC-58125 and celecoxib (Sheng et al. 1998, Goldman et al. 1998, Williams et al. 2000b). Celecoxib also decreased tumour multiplicity in Min mice by 52%, when administered after adenomas had been established (Jacoby et al. 2000b).

### Table 3 Chemoprevention of intestinal tumorigenesis in rodents using selective COX-2 inhibitors.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Animal</th>
<th>Model</th>
<th>Tumour type</th>
<th>Drug</th>
<th>Effect on tumour multiplicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oshima et al. (1996)</td>
<td>Mouse</td>
<td>Apc&lt;sup&gt;Min&lt;/sup&gt;</td>
<td>Adenoma</td>
<td>MF tricyclic</td>
<td>62% inhibition</td>
</tr>
<tr>
<td>Nakatsugi et al. (1997)</td>
<td>Mouse</td>
<td>Apc</td>
<td>Adenoma</td>
<td>Nimesulide</td>
<td>48% inhibition</td>
</tr>
<tr>
<td>Kawamori et al. (1998)</td>
<td>Rat</td>
<td>AOM</td>
<td>Colon carcinoma</td>
<td>Celecoxib</td>
<td>97% inhibition</td>
</tr>
<tr>
<td>Fukutake et al. (1998)</td>
<td>Mouse</td>
<td>AOM</td>
<td>Colon carcinoma</td>
<td>Nimesulide</td>
<td>81% inhibition</td>
</tr>
<tr>
<td>Reddy et al. (2000a)</td>
<td>Rat</td>
<td>AOM</td>
<td>Colon carcinoma</td>
<td>Celecoxib</td>
<td>84% inhibition</td>
</tr>
<tr>
<td>Sasai et al. (2000)</td>
<td>Mouse</td>
<td>Apc&lt;sup&gt;Min&lt;/sup&gt;</td>
<td>Adenoma</td>
<td>JTE-522</td>
<td>32% inhibition</td>
</tr>
<tr>
<td>Jacoby et al. (2000b)</td>
<td>Mouse</td>
<td>Apc</td>
<td>Adenoma</td>
<td>Celecoxib</td>
<td>71% inhibition</td>
</tr>
</tbody>
</table>

Several of these studies tested a range of drug concentrations. The effect on tumour multiplicity (number of tumours per animal) reported in this table was that achieved at the highest drug dose tested. In addition to inhibition of tumour multiplicity, these agents also caused reduced tumour incidence (proportion of animals with tumours). Note that individual studies examined different endpoints – carcinomas or adenomas.

Pharmacological studies in rat breast cancer models

Carcinogen-induced rat mammary tumours have been used as a model system to test various NSAIDs and, more recently, selective COX-2 inhibitors for their chemopreventive potential (Table 4). In general, indomethacin was found to reduce the incidence and multiplicity of DMBA-induced tumours (Carter et al. 1983, 1989, McCormick et al. 1985, Noguchi et al. 1991). Because the incidence of breast cancer may be affected by dietary fat, some of these studies have compared NSAID effects in cohorts of animals fed low-versus high-fat diets. Carter et al. (1983) found that indomethacin reduced tumour incidence in DMBA-treated animals fed 18% corn oil to the level observed in DMBA-treated animals fed 5% corn oil, but did not see an effect on incidence in the low-fat cohort. In contrast, two other studies found that the inhibitory effect of indomethacin was not confined to rats fed high-fat diets (McCormick et al. 1985, Noguchi et al. 1991). Interestingly, McCormick et al. (1985) found that indomethacin treatment from 2 weeks before to 1 week after DMBA administration primarily targeted benign tumours. However, when treatment with indomethacin was initiated 1 week after DMBA and continued until the end of the trial, the multiplicity of malignant tumours was also significantly reduced. Conflicting data were obtained by Abou-el-Ela et al. (1989) who found no inhibition of mammary tumorigenesis by indomethacin. The basis for these discrepant observations is unclear. Two additional NSAIDs, flurbiprofen and aspirin, are also capable of reducing carcinogen-induced mammary tumorigenesis (McCormick & Moon 1983, Suzui et al. 1997, Mori et al. 1999), although piroxicam was not found to be effective in one study (Kitagawa & Noguchi 1994).

Two recent studies evaluated the effects of selective COX-2 inhibitors on mammary tumorigenesis. As shown in Fig. 4A, treatment with celecoxib significantly delayed tumour onset in DMBA-treated rats, and was more effective than ibuprofen (Harris et al. 2000). Dietary administration...
Table 4 Chemoprevention of mammary tumorigenesis in rats using cyclooxygenase inhibitors

<table>
<thead>
<tr>
<th>Reference</th>
<th>Tumour induction</th>
<th>Drug</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carter et al. (1983)</td>
<td>DMBA/18% corn oil</td>
<td>Indomethacin</td>
<td>54% inhibition of tumour multiplicity; reduction in tumour incidence</td>
</tr>
<tr>
<td>McCormick &amp; Moon (1983)</td>
<td>NMU</td>
<td>Flurbiprofen</td>
<td>Reduction in tumour incidence and multiplicity at low NMU dose</td>
</tr>
<tr>
<td>McCormick et al. (1985)</td>
<td>DMBA</td>
<td>Indomethacin</td>
<td>Reduction in benign or malignant tumours according to period of drug administration</td>
</tr>
<tr>
<td>Abou-el-Ela et al. (1989)</td>
<td>DMBA/20% fat</td>
<td>Indomethacin</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Carter et al. (1989)</td>
<td>DMBA/20% fat</td>
<td>Indomethacin</td>
<td>Inhibition of tumorigenesis in animals fed 4 or 12% linoleate</td>
</tr>
<tr>
<td>Noguchi et al. (1991)</td>
<td>DMBA/20% corn oil</td>
<td>Indomethacin</td>
<td>61% inhibition of tumour multiplicity; reduction in tumour incidence</td>
</tr>
<tr>
<td>Kitagawa &amp; Noguchi (1994)</td>
<td>DMBA/20% soybean oil</td>
<td>Piroxicam</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Suzuki et al. (1997)</td>
<td>PhIP/high corn oil</td>
<td>Aspirin</td>
<td>44% inhibition of tumour multiplicity</td>
</tr>
<tr>
<td>Mori et al. (1999)</td>
<td>PhIP/high fat</td>
<td>Aspirin</td>
<td>Inhibited tumour multiplicity</td>
</tr>
<tr>
<td>Harris et al. (2000)</td>
<td>DMBA</td>
<td>Celecoxib</td>
<td>86% inhibition of tumour multiplicity; 68% reduction in tumour incidence</td>
</tr>
<tr>
<td>Nakatsugi et al. (2000)</td>
<td>PhIP/24% corn oil</td>
<td>Nimesulide</td>
<td>54% inhibition of tumour multiplicity; 28% reduction in tumour incidence</td>
</tr>
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of celecoxib reduced incidence, multiplicity and volume of malignant breast tumours by 68%, 86% and 81% respectively relative to the control group. The chemopreventive properties of another COX-2 inhibitor, nimesulide, was tested in rats in which the environmental carcinogen PhIP, together with a 24% corn oil diet, was used to induce COX-2 expression and mammary tumours (Nakatsugi et al. 2000). A small reduction in tumour incidence was achieved by administration of 400 parts per million nimesulide (Table 5). In addition, both size and multiplicity of tumours were significantly reduced in the nimesulide-treated animals. Together, these studies represent the first direct evidence that selective COX-2 inhibitors can protect against experimental breast cancer.

Two additional studies suggest that cyclooxygenase inhibition may be a useful strategy for treating breast cancer. Robertson et al. (1998) measured tumour size in rats that were maintained for 100 days post DMBA treatment then fed a control or an ibuprofen-containing diet for an additional 5 weeks prior to necropsy. Tumours from the control animals increased in volume by approximately 180%. In contrast, those from the ibuprofen-treated cohort decreased in volume by almost 40%. More recently, a similar study was conducted in which the effects of the selective COX-2 inhibitor celecoxib were investigated (Alshafie et al. 2000). In this study, rats were maintained for 4 months post DMBA treatment to induce tumours. Subsequently, the rats were given a control or a celecoxib-containing diet for an additional 6 weeks. The mean tumour volume increased by 518% in control animals, but decreased by 32% in the group fed celecoxib (Fig. 4B). In addition, the total tumour number continued to increase in the control animals, but was reduced in the celecoxib cohort. This report of regression of mammary tumours in vivo by a selective COX-2 inhibitor is consistent with earlier studies showing that various NSAIDs reduced the growth of mammary tumour xenografts (Fulton 1984, Karmali & Marsh 1986). Together, these observations suggest that COX-2 inhibition may represent a strategy not only for prevention but also for treatment of human breast cancer.

The ability of COX-2 inhibitors to significantly reduce tumour multiplicity strongly suggests that COX-2 contributes to tumorigenesis. However, COX-independent effects of NSAIDs have also been described (see below), raising the possibility that the observed inhibition may not necessarily be ascribed solely to effects on COX-2. Nevertheless, taken together the pharmacological and genetic studies provide overwhelming support for a role for COX-2 in tumorigenesis. Definitive evidence has now been provided by the recent demonstration that COX-2 overexpression is sufficient to induce mammary tumor formation in transgenic mice (Liu et al. 2001).

How does COX-2 contribute to cancer?

Prostaglandins stimulate proliferation and mediate immune suppression

Since COX-2 is a PG synthase, the most obvious consequence of COX-2 overexpression is increased PG production, and indeed high PG levels have been detected in many cancers. Enhanced PG synthesis may contribute to carcinogenesis in several ways, including direct stimulation of cell growth. PGE$_{20}$ and PGF$_{2\alpha}$ can both stimulate mitogenesis in Balb/c 3T3 fibroblasts in synergy with epidermal growth factor (EGF) (Nolan et al. 1988), and PGF$_{2\alpha}$ is also mitogenic for Swiss 3T3 cells and osteoblasts.
Figure 4. Celecoxib is effective for breast cancer prevention and treatment. (A) Rats were assigned to a control diet, or a diet containing 1500 ppm ibuprofen or 1500 ppm celecoxib 7 days prior to a single intragastric dose of DMBA, and tumour incidence was measured for 16 weeks. This figure is reproduced with permission from Harris et al. (2000). (B) Rats were maintained for four months after a single intragastric dose of DMBA to allow palpable tumour development, then assigned to a control diet or a diet containing 1500 ppm celecoxib, and tumour size was monitored for 6 weeks. This figure is reproduced with permission from Alshafie et al. (2000).
This table is reproduced with permission from Nakatsugi et al. PGE2 has been demonstrated to increase aromatase activity CYP19. However, in adipose tissue adjacent to breast tumours, aromatase is normally expressed from promoter I.4. Which distinct transcripts are generated. In adipose tissue, oestrogen synthesis, has three promoters, I.4, I.3 and II, from CYP19. Huang et al. (1999). The aromatase gene (Huang et al. 1996). Both PGE1 and PGE2 stimulate proliferation of mammary epithelial cells in the presence of EGF (Bandyopadhyay et al. 1987). Thus, inappropriate stimulation of cellular proliferation by PGs may contribute to tumorigenesis. However, PGs do not act as mitogens for all cell types, and in fact depress proliferation of some cells, particularly those of the immune system (Marnett 1992).

Antiproliferative effects may contribute to the immune suppression associated with PGs. PGE2 inhibits T and B cell proliferation and cytokine synthesis, and diminishes the cytotoxic activity of natural killer cells. PGE2 also inhibits the production of TNFα while inducing interleukin-10 production, which itself has immunosuppressive effects (Huang et al. 1996). PGs may also inhibit antigen processing by dendritic cells (Stolina et al. 2000). Thus, PG-mediated immune suppression may contribute to tumorigenesis, since this may allow tumours to avoid immune surveillance that might otherwise limit their growth.

In breast tissue, PGs may also stimulate proliferation indirectly by increasing oestrogen biosynthesis (Harris et al. 1999). The aromatase gene CYP19, which is responsible for oestrogen synthesis, has three promoters, I.4, I.3 and II, from which distinct transcripts are generated. In adipose tissue, aromatase is normally expressed from promoter I.4. However, in adipose tissue adjacent to breast tumours, CYP19 tends to be expressed from promoter II. Recently, PGE2 has been demonstrated to increase aromatase activity (Zhao et al. 1996, Purohit et al. 1999) and cause CYP19 promoter switching to promoter II in adipose stromal cells (Zhao et al. 1996). These data suggest that PG overproduction can induce aromatase, leading to increased oestrogen synthesis. Consistent with this, a positive correlation has been observed between CYP19 and COX expression in human breast cancer specimens (Brueggemeier et al. 1999). Thus it is possible that PG-mediated oestrogen overproduction may be an important organ site-specific consequence of COX-2 upregulation in breast cancer.

### Cyclooxygenase-mediated production of mutagens

Thus far, the potential contributions of PG overproduction to tumorigenesis, including increased cellular proliferation and diminished immune surveillance, have been discussed. However, COX-2 overexpression may also have PG-independent consequences. In particular, COX-2 overexpression may result in increased production of mutagens. Malondialdehyde (MDA) can be produced by isomerisation of PGH2, both enzymatically and non-enzymatically (Fig. 1). Therefore, MDA production may be elevated due to increased availability of the precursor molecule PGH2. MDA forms adducts with deoxynucleosides and induces frame-shifts and base-pair substitutions, and thus has potent mutagenic activity (Marnett 1992). Additional carcinogens can be formed by oxidation of aromatic amines, heterocyclic amines, and dihydrodiol derivatives of polycyclic hydrocarbons (Wiese et al. 2001). This oxidation step is catalysed by the peroxidase activity of cyclooxygenase, which requires a reductant to convert PGG2 to PGH2. Thus, COX-2 overexpression may lead to DNA damage, thereby contributing to carcinogenesis. Consistent with this hypothesis, the selective COX-2 inhibitor nimesulide decreases formation of the mutagen 8-oxo-7,8-dihydro-2′-deoxyguanosine in the colonic mucosa (Tardieu et al. 2000).

### Effects on angiogenesis

Recently, it has become apparent that cyclooxygenases are involved in angiogenesis (reviewed by Gately 2000). This is a crucial facet of tumorigenesis since neovascularisation is required for tumours to grow beyond 2–3 mm in size. Experiments in the 1980s showed that xenograft vascularisation was significantly reduced by the NSAIDs indomethacin, diclofenac and aspirin (Peterson 1983). More recently, COX-2 has been specifically implicated. In vitro, selective COX-2 inhibitors decrease endothelial tubule formation (Tsujii et al. 1998, Jones et al. 1999), while, in vivo, selective COX-2 inhibitors reduce angiogenesis in several models (Majima et al. 1997, Daniel et al. 1999, Sawaoka et al. 1999, Yamada et al. 1999, Masferrer et al. 2000). A representative illustration of celecoxib-mediated inhibition of corneal angiogenesis is shown in Fig. 5.

In an interesting corollary, Lewis lung carcinoma xenografts showed marked attenuation of growth when implanted in COX-2-null mice, but grew normally in COX-1-deficient mice (Williams et al. 2000a). The tumours from COX-2 knockout mice exhibited 30% decreased vascular density, implicating host COX-2 in tumour neovascularisation. It seems likely that COX-2 in epithelial cells, endothelial cells and fibroblasts may all contribute to the angiogenic process, although there are some

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**Table 5** Effects of nimesulide on the incidence, multiplicity and volume of mammary carcinomas induced by PhIP in Sprague-Dawley rats. Results are means ± S.E.

<table>
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<th>Control diet</th>
<th>400 ppm nimesulide</th>
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<tr>
<td>Tumour incidence (% rats with cancers)</td>
<td>30/42 (71%)</td>
<td>19/37 (51%)</td>
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<tr>
<td>Multiplicity (no. of cancers/rat)</td>
<td>2.6 ± 0.5</td>
<td>1.2 ± 0.2*</td>
</tr>
<tr>
<td>Cancer volume/rat (cm³)</td>
<td>4.1 ± 1.3</td>
<td>1.1 ± 0.4*</td>
</tr>
<tr>
<td>Effective no. of rats</td>
<td>42</td>
<td>37</td>
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*Significantly different from the control diet group by Welch’s t test (P >0.05).

This table is reproduced with permission from Nakatsugi et al. (2000).
discrepancies between observations made in vivo and in vitro (Majima et al. 1997, Tsujii et al. 1998, Daniel et al. 1999, Masferrer et al. 2000, Williams et al. 2000a). COX-2 apparently contributes to the production of pro-angiogenic factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor-1, PDGF, and endothelin-1. NS-398 treatment of a COX-2-overexpressing colorectal cancer cell line diminishes secretion of these factors (Tsujii et al. 1998), and COX-2 (−/−) fibroblasts have a 94% reduction in the ability to produce VEGF relative to wild-type fibroblasts (Williams et al. 2000a). However, the molecular mechanisms underlying COX-2-mediated production of pro-angiogenic factors remain to be defined.

COX-1 can also contribute to angiogenesis. Nonselective NSAIDs decrease the vascularisation of xenografts comprised of cells not expressing COX-2 (Sawaoka et al. 1999). Moreover, NSAIDs inhibit endothelial tubule formation even when cells do not express COX-2 (Tsujii et al. 1998, Jones et al. 1999). Thus both COX-1 and COX-2 are likely to contribute to tumour vascularisation. The possibility that COX-2 inhibitors diminish tumorigenesis...
partly by preventing angiogenesis further enhances their attractiveness as potential anti-cancer agents.

Effects of COX-2 overexpression on cell invasiveness and apoptosis

The potential consequences of COX-2 overexpression have been addressed in vitro by generation of cell lines overexpressing COX-2. In particular, rat intestinal epithelial cells stably overexpressing COX-2 show several altered characteristics, including increased adhesion to extracellular matrix, resistance to butyrate-induced apoptosis and a delayed transit through the G1 phase of the cell cycle (Tsujii & DuBois 1995, DuBois et al. 1996b). Additionally, stable COX-2 expression in Caco-2 cells or in the breast cancer cell line Hs578T increases expression or activity of enzymes capable of digesting the basement membrane, presumably contributing to the observed increase in ability to invade through a layer of Matrigel (Tsujii et al. 1997, Takahashi et al. 1999). All of these characteristics may contribute to tumorigenicity, although the molecular mechanism(s) by which COX-2 causes these effects is unknown.

Much interest has centred on the ability of COX-2 to suppress apoptosis. Diminished apoptosis is thought to favour carcinogenesis by permitting survival of cells that have acquired mutations, and thus is viewed as one of the central mechanisms of tumorigenesis. Conversely, many NSAIDs enhance apoptotic cell death, although this is unlikely to be solely due to inhibition of cyclooxygenase activity (see below). Several hypotheses have been advanced to account for suppression of apoptosis in response to COX-2 overexpression. The ability of PGE₂ to inhibit apoptosis caused by a selective COX-2 inhibitor, and concomitantly to induce Bcl-2, suggests that PG-mediated upregulation of Bcl-2 may suppress apoptosis (Sheng et al. 1998a). Alternatively, since arachidonic acid stimulates apoptosis, enhanced COX-2 expression could inhibit apoptosis by increasing the conversion of arachidonic acid to PG (Chan et al. 1998, Cao et al. 2000). Kinzler and colleagues propose that arachidonic acid stimulates the conversion of sphingomyelin to ceramide, which then causes apoptosis (Chan et al. 1998). They further suggest that the apoptosis-promoting effect of NSAIDs such as sulindac is due to NSAID-induced accumulation of arachidonic acid. In contrast, although Prescott and co-workers also consider arachidonate to be a key determinant of apoptosis, they do not observe increased levels of ceramide in response to exogenous administration of arachidonic acid (Cao et al. 2000).

Clearly, the suppression of apoptosis associated with COX-2 overexpression could be an important factor in tumorigenesis, although the precise mechanistic basis remains uncertain. Interestingly, an apoptosis-related protein was found in a two-hybrid screen designed to identify proteins that interact with cyclooxygenases (Ballif et al. 1996). Nucleobindin associates with DNA from apoptotic cells, and can itself promote apoptosis. The interaction of COX-1 and COX-2 with nucleobindin may contribute to COX-mediated suppression of apoptosis, potentially via sequestration of nucleobindin, but further studies are required to fully understand the significance of the interaction.

As mentioned above, multiple NSAIDs, including selective COX-2 inhibitors, induce apoptosis in a variety of cells (Lu et al. 1995, Hara et al. 1997, Sheng et al. 1998a, Ding et al. 2000, Hida et al. 2000, Li et al. 2000). The simplest interpretation of this phenomenon is that, since COX-2 overexpression suppresses apoptosis, inhibition of COX-2 activity is sufficient to induce apoptosis. However, NSAID-induced apoptosis has also been demonstrated in cell lines that do not express COX-2, including COX-2-null mouse embryo fibroblasts (Hanif et al. 1996, Elder et al. 1997, Zhang et al. 1999). Additionally, non-cyclooxygenase-inhibiting sulindac metabolites such as sulindac sulphone retain the ability to induce apoptosis (Piazza et al. 1997, Lim et al. 1999b). Thus, NSAIDs most likely stimulate apoptosis via both COX-dependent and -independent mechanisms (Rigas & Shiff 2000), including inhibition of the protein kinase Akt (Hsu et al. 1996, Elder et al. 1997, Yamamoto et al. 2000, Hida et al. 2000). The weight of evidence implicating COX-2 in colorectal cancer has stimulated clinical trials to investigate the efficacy of selective COX-2 inhibitors in individuals at risk for colorectal cancer. Treatment with celecoxib has been shown to reduce the size and number of polyps in FAP patients (Steinbach et al. 2000), and is currently being evaluated for efficacy in preventing sporadic colorectal adenomas. Undoubtedly the potential use of selective COX-2 inhibitors for the treatment of colorectal cancer will also be investigated.

Clinical prospects for COX-2 inhibitors and breast cancer

The weight of evidence implicating COX-2 in colorectal cancer has stimulated clinical trials to investigate the efficacy of selective COX-2 inhibitors in individuals at risk for colorectal cancer. Treatment with celecoxib has been shown to reduce the size and number of polyps in FAP patients (Steinbach et al. 2000), and is currently being evaluated for efficacy in preventing sporadic colorectal adenomas. Undoubtedly the potential use of selective COX-2 inhibitors for the treatment of colorectal cancer will also be investigated.

Here we have reviewed evidence that aberrant COX-2 expression is also associated with breast cancer, both in rodent models and in the human disease. Selective COX-2 inhibitors have proved effective in preventing experimental breast cancer (Harris et al. 2000, Nakatsugi et al. 2000). Whether COX-2 inhibitors will also be useful for preventing breast cancer in high-risk individuals needs to be investigated. In addition, selective COX-2 inhibitors may have a role in the treatment of breast cancer (Alshafie et al. 2000). Since COX-2 is overexpressed in HER-2/neu-positive breast cancers (Subbaramaiah et al. 1999b), selective COX-2 inhibitors should be evaluated as therapy in this patient population. Because COX-2-derived PGs may enhance aromatase activity, a therapeutic regimen combining a selective COX-2 inhibitor with an aromatase inhibitor should be considered. There is also recent evidence that
microtubule-interfering agents, including taxol, stimulate COX-2 transcription (Subbaramaiah et al. 2000). This could decrease the efficacy of this class of drugs. Thus, coadministration of a selective COX-2 inhibitor with drugs such as taxol might enhance their anti-cancer activity. Finally, a number of natural substances have been identified that inhibit the transcriptional activation of COX-2. Examples include retinoids, triterpenoids, antioxidants and resorcinols (Mestre et al. 1997a,b, Chinery et al. 1998, Subbaramaiah et al. 1998b, Suh et al. 1998, Mutoh et al. 2000). Some of these compounds also inhibit experimental breast cancer. Hence, it is possible that studies of COX-2 will provide insights that will prove useful in developing dietary recommendations to decrease cancer risk.

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