Back to the Future: COX-2 Inhibitors for Chemoprevention and Cancer Therapy

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Abstract: For more than a century, inhibition of prostaglandin biosynthesis *via* inhibition of the fatty acid cyclooxygenase (COX) has been achieved by non-steroidal anti-inflammatory drugs (NSAIDs), which targets both COX-1 and COX-2 and as such could be responsible for causing gastrointestinal (GI) toxicity. COX-2 is an inducible enzyme produced by many cell types in response to multiple stimuli. Recently, COX-2 over-expression has been found in several types of human cancers such as colon, breast, prostate and pancreas and appears to control many cellular processes. Because of its role in carcinogenesis, apoptosis, and angiogenesis, it is an excellent target for developing new drugs with selectivity for prevention and/or treatment of human cancers. Development of selective COX-2 inhibitors has been successfully documented and as such showed less toxicity to GI tract as compared to conventional NSAIDs. However, the long term use of COX-2 selective inhibitors showed cardiovascular toxicity, and thus their utilization for cancer prevention and therapy is currently questionable, suggesting that further development of novel COX-2 selective agents are needed. Among many solid tumors, pancreatic cancer has the worst prognosis, and inflammation has been identified as a significant factor in the development of pancreatic malignancy. Several cytokines, reactive oxygen species (ROS) and mediators of inflammatory pathway such as activation of nuclear factor-kappaB (NF-κB) and COX-2 leads to an increase in cell proliferation, survival, and inhibition of pro-apoptotic pathway, ultimately resulting in tumor angiogenesis, invasion and metastasis. In this brief review, we summarize the role of COX-2 and discuss some of the experimental data linking inflammation with the development of pancreatic cancer. In addition, we provide further evidence regarding the state of our knowledge toward the development of novel COX-2 targeting agents for the prevention and/or treatment of human cancers especially pancreatic cancer.

INTRODUCTION

1. Inflammation and Cancer Development

The possible casual relationship between inflammation and cancer has been observed in a number of malignancies; however, the exact link between chronic inflammation and carcinogenesis remains unclear. During the inflammatory process, the formation of potential carcinogens produced may increase the probability of the initiation of carcinogenesis. The inflammation in the tissues leads to a high concentration of growth factors and cytokines in the microenvironment and may lead to proliferation of the initiated cells. Such changes take place over several years and, therefore, interruption of the inflammatory pathway associated with procarcinogenesis could prevent the onset of tumor formation. Certain signal transduction pathways, including AP-1 and NF-κB, are known to be activated by reactive oxygen species (ROS) and lead to transcription of several genes involved in cell growth regulatory pathways [1, 2]. Recent reviews have suggested a clear link between inflammation and cancer associated with the activation of NF-κB [3-5]. Several studies have focused on the genetic changes in the development of pancreatic cancer; however, a few have investigated the role of inflammation. Until now, a number of human cancers such as colorectal, gastric, ovarian, cervical, and esophageal and lung cancers have been associated with inflammatory origins. However, recurrent inflammation observed in patients with hereditary pancreatitis suggests that certain inflammatory mediators may also play a role in the progression of chronic pancreatitis to subsequent cancer development [6].

Pancreatic cancer has the worst prognosis and is the fourth leading cause of all cancer deaths in the United States [7]. The high rate of mortality clearly reflects the aggressiveness of the disease and lack of effective therapies. Complete surgical resection is the only potential curative treatment for the disease [8]. However, only about 15% of the all cases present with resectable disease. Many of the patients develop metastatic infiltration even after curative surgical resection [9]. The malignant transformation of pancreatic cells is strongly favored due to initiation of inflammatory events, followed by generation of ROS, release of cytokines and high expression of inflammatory mediators such as NF-κB, which leads to the expression of its downstream signaling molecules such as COX-2, MMP-9, uPA and VEGF (Fig. **1**) that are known to be associated with invasion, angiogenesis and metastases [10-12] and lead to poor prognosis [12, 13]. The importance of these signaling pathways and their crosstalk is depicted in Fig. (**1**). In addition, the process of inflammation is also associated with overall genomic damage, cellular proliferation, loss of tumor suppressor function such as p53 and mutation of oncogenes such as K-Ras as found in majority of pancreatic adenocarcinomas [14].

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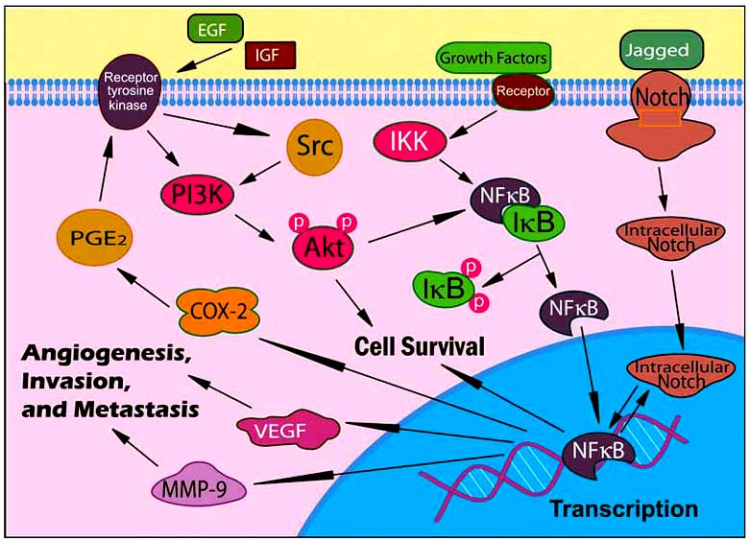

Fig. (1).

During the normal healing process, growth factors are released leading to increased cell proliferation. In chronic pancreatitis, various cytokines such as tumor necrosis factor α (TNFα), interleukin-6 (IL-6), interleukin-8 (IL-8) and interferon-γ, are released along with ROS causing acute to chronic inflammation and cellular damage leads to fibrosis, scarring and, in turn, persistent inflammation [15-17]. TNFα up-regulates epidermal growth factor receptor (EGFR) and its activating ligand, transforming growth factor (TGF-α) as well as platelet-derived growth factor (PDGF) expression in cultured pancreatic cancer cells and PDGF is known to strongly stimulate fibrogenesis [16]. These results suggest that the activation of multiple signaling molecules and their crosstalk is responsible for the sustained inflammatory microenvironment which eventually leads to pancreatic carcinogenesis among other solid tumors.

NF-κB, a heterodimer composed of two subunits p65/RelA and p50 [18], is an ubiquitous transcription factor involved in many disease processes and controls various genes thus playing an important role in inflammation, apoptosis, and carcinogenesis [3-5]. In its inactive state, during normal homeostasis, NF-κB remains bound to the inhibitor of kappa B (IκB) in the cytoplasm. Phosphorylation and subsequent degradation of IκB releases NF-κB and allows the active NF-κB dimer to translocate to the nucleus where binding to a specific consensus sequence found in the promoter region of various growth regulatory genes leads to further transcription and the vicious cycles of inflammation continues throughout the process of carcinogenesis [3-5]. In pancreatic cancer, up-regulated expression of NF-κB stimulates the expression of both IL-1β and TNFα, amplifying the inflammatory response, thus triggering transformed cells to survive with loss of apoptotic mechanisms [19, 20]. NF-κB also stimulates at least two genes [Cox-2 and nitric oxide synthase (NOS)] whose metabolic products are known mediators of inflammatory processes [21, 22]. The promoter region of mouse COX-2 contains consensus sequences for the binding of NF-κB, the nuclear factor for IL-6 expression, as well as a cyclic AMP response element (CRE) integrated in an E-box [23, 24] whereas NOS-2 has a 1.7 kb fragment of the flanking region of the gene which contains at least 24 consensus sequences for the binding of transcription factors regulated by cytokines, including motifs activated in response to IκB and two NF-κB binding sites which are critical for the expression of NOS-2 in response to a wide variety of stimuli [25-27].

Abrogation of NF-κB in cultured cells appears to be a likely mechanism for decreasing the corresponding levels of COX-2 and NOS-2 in trophoblasts [22]. Previously, several groups have suggested that reactive oxygen intermediates (ROI) and ROS may be responsible for the activation of the IκB kinase (IKK) and the degradation of the inhibitory IκB proteins from the cytosolic NF-κB complex [28, 29]. This may have physiological importance associated with the mechanism of activation of NF-κB in cancer cells. NF-κB also activates cyclin D1 expression, a regulatory protein that promotes cell cycle activity and is up-regulated in several pancreatic cancers [21, 30]. Although the development of pancreatic cancers are partly due to chronic inflammatory processes, these observations clearly point out an urgent need for developing effective and selective therapeutic agents directed at the inflammatory mechanisms in order to improve the prognosis for patients diagnosed with pancreatic cancers.

2. COX Cascade and Relevance to Cancer

The relationship between cyclooxygenase-2 (COX-2) and cancer was initially discovered in colon cancer [31, 32] and now has been explored in several human cancers including breast, lung, gastric and esophageal, prostate and pancreatic cancers [33] which have been shown to exhibit up-regulated expression of COX-2 (Fig. **1**), the rate limiting enzyme in the biosynthesis of prostaglandins from arachidonic acid (AA). Interestingly, COX-2 is over expressed in the majority of human primary pancreatic carcinomas irrespective of histology and grade [10, 34]. In contrast, benign pancreatic tumors do not express this enzyme. These studies suggest that COX-2 expression may represent an important hallmark for the

malignant characteristic of pancreatic cancer and, as such, provide important clues for the rational development of an anti-COX-2 agent for the prevention and/or treatment of this deadly disease.

So far three enzyme isoforms, COX-1, COX-2 and COX-3, have been identified. COX-1 is a constitutive 'housekeeping' gene, involved in the maintenance of tissue homeostasis and is responsible for platelet aggregation, renal blood flow and maintenance of gastric mucosa [35]. The traditional NSAIDs such as aspirin [36, 37] and sulindac [38] reversibly or irreversibly inhibit the COX-1 enzyme. The inducible isoform COX-2 [39], constitutively expressed in human kidney and brain, is found in inflammed and neoplastic tissues. Selective COX-2 inhibitors have been designed to inhibit COX-2 enzymatic activity [40] whereas all NSAIDs are COX-1 or mixed-COX inhibitors. Recently, a third isoform, COX-3 [41], has been identified as a spliced variant of COX-1, which is present mainly in the brain and spinal cord.

3. STRUCTURE OF THE COX ISOENZYMES

COX-1 and COX-2 are membrane associated enzymes that show very similar homology consisting of a long narrow channel with a hairpin bend at the end. The two enzymes are homo-dimers, each monomer consisting of three sites, viz an epidermal growth factor-like domain, a membrane binding domain and a catalytic domain that contains both the cyclooxygenase and peroxidase active sites. The membrane binding domain of COX-1 and COX-2 is incorporated in the inner layer of the plasma membrane bilayer which allows liberated AA access to the cyclooxygenase active site. Both enzymes show a channel extending from the center of the catalytic domain to the outer surface of the membrane-binding site. Eight amino acid residues play an important role for the substrate and inhibitor binding in the cyclooxygenase channel. When cell membranes are damaged, AA is released and is pulled inside the hydrophobic pocket of the enzyme twisted around the hairpin bend, where it interacts with the residue present at the active pocket. Both isoforms possess a polar arginine at the position 120. There is a single amino acid difference between both isoforms where an isoleucine molecule is at the position 523 in COX-1 and a valine residue at the same position in COX-2. This leaves a gap in the channel wall of COX-2 creating a side pocket where many selective drugs bind whereas the bulkier isoleucine at 523 in COX-1 blocks access to the side pocket. In the presence of molecular oxygen, the COX (PGH_2) pathway produces unstable intermediate PGG_2, which is rapidly converted into PGH_2 by the peroxidase activity of PGH_2 synthase [42]. Further specific isomerases convert PGH_2 to various biologically active prostaglandins (PGs) PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2 and TxA_2 (collectively called prostanoids), which act on rhopdopsin-like G-protein-coupled receptors (GPCRs). Among many products of COX-2, PGE_2 appears to play an important role in carcionogenesis [43-46] and is an important player of cellular signaling by its crosstalk with other cell signaling processes, including the activation of EGFR [47] as depicted in Fig. (**1**).

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

Prostanoids are involved in a very broad range of physiological and pathophysiological responses and have both autocrine and paracrine functions. These bioactive lipids are often considered to be local hormones. In the cardiovascular system, PGD_2, PGE_2 and PGI_2 act as potent vasodilators while PGI_2 specifically exhibits anticoagulant properties. TxA_2 plays a major role in the induction of platelet aggregation while acting as a potent vasoconstrictor. In the gastrointestinal tract, PGE_2, $PGF_{2\alpha}$ and PGI_2 have been found to protect the gastric mucosa. In the inflammatory cascade, PGE_2 and PGI_2 are potent vasodilators and act synergistically with other autocoids such as histamine and bradykinin. This leads to increased blood flow (redness) and characteristic swelling due to increased vascular permeability at acute inflammatory regions. Recently, it has been demonstrated by Guay *et al.*, [49] that PGE_2 is the most prevalent prostaglandin in the cerebrospinal fluid and spinal cord. Finally, PGE_2 acts on neurons and contributes to fever, fatigue and hypersensitivity to pain [50].

PGE_2 acts *via* the IP receptor, and the physiological effects of PGE_2 are mediated by coupling to four subtypes of G-protein coupled receptors which have been classified as EP1, EP2, EP3 and EP4 [51-53]. These receptors are often over expressed in the same cell and utilize alternate and sometimes opposing intracellular signaling pathways. EPs are encoded by distinct genes and have divergent amino-acid sequences, but all bind PGE_2 with higher affinity than other prostanoids. Hence, drug interactions should discriminate between different actions of PGE_2. The EP1 receptor increases intracellular calcium through phospholipase C (PLC)/ inositol triphosphate signaling, and protein kinase C (PKC) activity [51, 52]. Among these, EP2 and EP4 are principal receptors implicated in mediating tumor progression through their ability to induce pro-angiogenic factor and/or tumor cell invasiveness [54]. The first evidence supporting the existence of a functional prostaglandin E2 receptor that shares the pharmacological features of EP4 receptor was provided by Pelletier, *et al.,* [55] in guinea-pig tracheal epithelial cells. These receptors modulate cyclic AMP formation as well as endothelin-1 (ET-1) production/secretion in these cells. It has been shown that EP2 and EP4 stimulate adenylate cyclase, leading to the production of adenosine 3',5'-monophosphate (cyclic AMP, cAMP), which then activates the cAMP-dependent protein kinase (PKA) [56], whereas Pino, *et al.,* have demonstrated in human pancreatic cancer cells that PGE_2 increases the cAMP concentration thereby leading to activation of protein kinase A (PKA) which, in turn, phosphorylates the cAMP response element binding protein (CREB), leading to interaction of cAMP response element in the promoter region of COX-2 gene [57]. PGE_2-EP3 signaling appears critical for tumor-associated angiogenesis, tumor progression and tumor growth in a mouse tumor implanta-

tion model [58]. The EP3 receptor protein has multiple splice variants causing more complexity to EP3-mediated signaling and is likely to signal through G-protein-Rho interactions [53]. Recently it has been reported that EP3 plays an important role in the appearance of the malignant phenotype of lung adenocarcinomas as indicated by the PGE_2 activation of Src signaling [59]. To understand the function of individual prostanoid receptors, several groups have developed prostanoid receptor deficient mice [60, 61] and studies are continuing. Thus, the pleiotropic effects of PGE_2 arise due to the diversity of the receptors and the possible co-expression of one or more isoform in the same pathological processes.

Along with COX-2, the most relevant prostanoids are PGE_2 and PGI_2 for induction of inflammation and cell proliferation leading to carcinogenesis [49, 62]. So far, inhibition of COX activity is the only approach routinely utilized to target the prostanoids pathway. Recently, researchers have identified signaling elements downstream of COX, such as the prostaglandin E synthases and prostaglandin receptors, and their physiological role in cancer progression makes them good targets for the development of novel agents. Three prostaglandin synthases have been identified which produce PGE_2 from PGH_2: two membrane-bound forms, called microsomal PGE synthase-1 (mPGES-1) and mPGES-2 and one cytosolic form cPGES [63]. The cPGES and mPGES-2 are constitutively expressed in most tissue and cPGES metabolizes the PGH_2 generated by COX-1 [64] whereas mPGES-2 shows no preference towards COX-1 or COX-2. The mPGES-1 is closely associated with COX-2 in many tissues [65] and it is the primary enzyme that metabolizes PGH_2 generated by COX-2. A recent study demonstrated that a series of derivatives based on 5-lipoxygenase-actvating protein (FLAP) inhibitor MK-886 specifically inhibits mPGES-1 at very low nanomolar concentrations [66]. While determining alternate targets for the development of selective drugs, several problems are encountered especially generation of unwanted side effects, accumulation of pharmacologically active metabolite and shift to alternative signaling pathways by cancer cells.

COX-2 over-expression in cancer development has been debated by several researchers and it was concluded that the over-expression of COX-2 may not be the major contributor to carcinogenesis but occurs as a result of tumor development. Initially it was proposed that COX-1 over-expression in intestinal tumorigenesis may mark it as a chemotherapeutic target for NSAIDs [67]. However, a counter argument was reported where studies show that over-expression of COX-1 along with highly elevated PGE_2 was not sufficient to induce lung tumors [68]. Interestingly, it was demonstrated that COX-1, COX-2 and mPGES are co-localized in the stromal fibroblasts in the polyps of $APCm^{\Delta 716}$ mice, where COX-2 was induced only in polyps > 1 mm in diameter, but COX-1 was found in polyps of any size [69]. These observations suggest that COX-1 expression secures the basal level of PGE_2 to initiate polyp formation while simultaneous expression of COX-2 and mPGES increase PGE_2 production to cause increased development of multiple polyps. Further, EP2 has been shown to play a role in the acceleration of intestinal polyposis in $Apc^{\Delta 716}$ knockout mice [70]. Thus, such accumulating evidence suggests a link between inflammation and cancer and shifting the focus on inflammatory pathway may help develop novel and new targeting agents for specific targets that contribute to the progression of gastrointestinal (GI) and especially pancreatic cancer [12, 45, 71, 72].

ROLE OF NSAIDS AS CHEMOPREVENTIVE AGENTS

In the early 1970s, Piper and Vane demonstrated that NSAIDs prevented prostaglandin production [73]. With the elucidation of the COX cascade and the role of prostaglandins, expression of several prostaglandin receptors and prostaglandin enzymes in inflammatory pathways, the role of NSAIDs and the selective COX-2 inhibitors (Coxibs) targeting inflammatory pathways during carcinogenesis has been re-investigated due to their protective effects against a variety of human malignancies.

NSAID compounds (Fig. **2**) are classified into the following subtypes depending upon their preferential and selective actions on the cycloxygenase isoforms: Selective COX-1 inhibitors (**1** and **2**), Non-selective COX inhibitors (**3**), and Preferential COX-2 inhibitors (**4**). A selective COX-1 inhibitor, Aspirin (**1**), which is an acetyl derivative of salicylic acid differs from the rest of the NSAIDs due of its action wherein it acetylates the serine group in the active pocket of the cycloxygenase enzyme which loses the activity of converting arachidonic acid into the respective prostaglandins. Other derivatives (**5** to **12**) have been identified as NSAIDs and their structures are shown in Fig. **2**. Several epidemiological studies have shown that a substantial decrease of 30-50 % in risk of death from colon cancers is associated with the use of Aspirin and other NSAIDs [48]. In the case of some NSAIDs, like aspirin, inhibition of platelet function is found to lead to coagulation problems and bleeding.

The evidence of NSAIDs causing apoptotic cell death has been provided by recent studies [48] which showed that indomethacin (**10**) and sodium diclofenac (**11**) cause apoptosis in rat gastric mucosal cells. These studies indicated the involvement of caspases rather than inhibition of prostaglandin synthesis. In 2003, Paraskeva *et al.*, showed that Nimesulide (**12**) which is a preferential COX-2 inhibitor plays important role in cancer prevention [74]. Badwai, *et al*. [75, 76] have studied the effects of acetaminophen, aspirin, naproxen (**7**) and ibuprofen (**8**) on cell survival, cell cycle and induction of apoptosis in LnCaP human prostate cells. Ibuprofen was significantly more effective against the prostate cancer cells *in vitro*. Cell cycle analysis indicated that ibuprofen caused the LnCaP cells to shift from the S and G_2/M phase to G_0/G_1 phase.

In 1983, through a double blind, placebo-controlled study, Waddell and Loughry showed that the NSAID sulindac (**9**) reduces both the number and the size of colorectal adenomas in the patients [77]. Sulindac has two primary metabolites – sulindac sulfide (the active form of sulindac) and sulindac sulfone. Sulindac is thought to be activated to sulindac sulfide by the intestinal flora, which becomes concentrated in the enterohepatic circulation. Thus, the colon is exposed to higher concentrations of the drug than is present in the systemic circulation. Treated patients experienced a reduction of nearly 60% in the number of tumors developed, compared to patients taking placebo. Non-selective COX

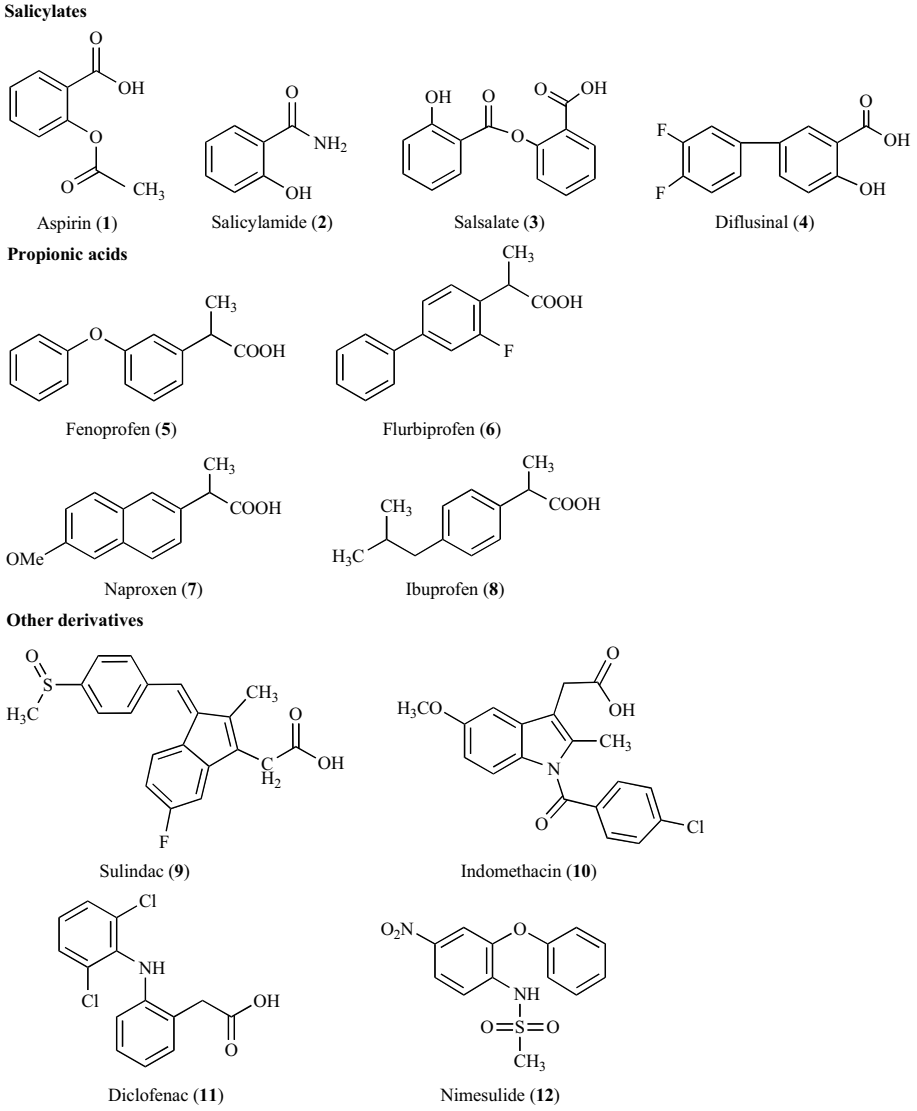

Fig. (2).

inhibitors such as sulindac [78] have been reported to produce a dose-dependent inhibition of growth of pancreatic cancer cells independent of the levels of COX isoforms in the cell lines. The indolic derivative, indomethacin, has been reported to inhibit cell growth in both COX-2 positive and COX negative human pancreatic tumor cells [79]. Treatment with another COX inhibitor, Piroxicam, in some patients has shown complete regression of adenomas. Similarly selective COX-2 inhibitor, NS-398 was reported to suppress cell growth in COX positive and COX negative cell line [80] whereas its structural analogue, Nimesulide has antimitogenic action in human pancreatic cancer cell lines independent of COX-2 expression [81].

Recent reports have shown that selective COX-2 inhibitors, NS-398 and nimesulide, also play a role in angiogenesis and growth in pancreatic cancer [82, 83]. Experimental studies have shown that administration of aspirin, piroxicam and sulindac inhibits chemically induced colon carconigenesis [84-87] and Coxibs have been shown to suppress tumor growth in animal models and reduce the risk of developing polyps and colon cancer in patients with familial adenomatous polyposis (FAP) [88, 89].

After treatment with these compounds, colon-cancer cells contract, form micronuclei and develop membrane blebs, all of which are markers of apoptotic activation. These effects can be blocked by drugs that inhibit corresponding gene expression, suggesting that the cell death induced by NSAID treatment is a *bona fide* programmed cell death and not necrotic cell death caused by general toxic effects of the drugs. The observation that NSAIDs cause apoptosis in colorectal cancer cells *in vitro* suggests that the chemopreventive effects of NSAIDs are cell autonomous, at least in part.

Studies with animals have provided valuable insights into the chemopreventive properties of NSAIDs. The *APC*$^{Min/+}$ mice harbor germline mutations of the mouse homologue of

APC gene and develops intestinal adenomas similar to those in patients with familial adenomatous polyposis (FAP). $APC^{Min/+}$ mice have been used widely as an experimental model for FAP. Administration of sulindac to $APC^{Min/+}$ mice causes a dramatic reduction in tumor burden. Other NSAIDs, including Aspirin, Piroxicam, Rofecoxib, Flurbiprofen and Indomethacin, are also effective in reducing, and in some cases nearly abrogating, the tumor burden in these mice. Furthermore, in rats with chemically induced colon cancer, various NSAIDs can prevent tumorigenesis or dramatically decrease tumor load. Recent studies have shown that combinations of chemopreventive drugs may also hold promise for preventing the development of tumors. When $APC^{Min/+}$ mice are treated with sulindac or EGF-receptor inhibitors, such an EKI-785, they develop about 50–70% fewer tumors [90]. Moreover, when combined with a dose of sulindac that would be too low to prevent disease progression on its own, the frequency of polyp formation was reduced by more than 95%. Similarly in a recent double blind, placebo-controlled study, Steinbach and colleagues have noted that patients with FAP who received Celecoxib, a COX-2 specific NSAID, developed 30% fewer polyps [88].

MECHANISMS OF NSAIDS-MEDIATED APOPTOSIS

1. COX Dependent Mechanisms

Analysis of COX expression shows that COX-2 is increased in up to 90% of sporadic carcinomas and 40% of adenomas, but not in normal colonic mucosa. In the adenomas of patients with FAP, and in rats with experimentally induced colon tumors, higher than normal concentrations of COX-2 but not COX-1, prostaglandins, or both COX-2 and prostaglandins are seen. Furthermore, the concentration of prostaglandin E_2 (PGE_2) is particularly high in human colon cancers. These findings support the idea that COX-2 over expression is important during colorectal carcinogenesis. The molecular mechanism of colorectal cancer progression is known to involve several key events and the mutation of a number of crucial genes. Although an increase in the size of colorectal tumors is associated with an increase in concentration of COX-2, it is not yet clear where COX-2 deregulation occurs in the multistep progression of colon cancers. However, the observation that young, small adenomas overexpress COX-2, and that NSAIDs can prevent adenoma formation, imply that COX-2 deregulation happens early in tumor formation.

Several studies have now provided strong evidence for the theory that NSAIDs cause apoptosis in colon-cancer cells by inhibition of COX-2. Tsujii and DuBois [91] found that rat intestinal epithelial cells, modified to increase expression of COX-2, were resistant to apoptosis. Apoptosis in the colonic epithelium appears to be progressively inhibited during colonic carcinogenesis. One of the ways in which NSAIDs prevent cancer and exert their chemopreventive effects in the colon may be through induction of apoptosis in the colonic mucosa. Relatively high (micromolar rather than nanomolar) concentrations of NSAIDs are required to evoke apoptosis in both *in-vitro* and *in-vivo* systems. AA has been shown to be a critical signal for apoptosis, with NSAIDs triggering apoptosis by inhibiting the metabolism of AA and allowing its accumulation. Researchers have, however, questioned whether the chemopreventive and tumor regression effects of NSAIDs in colorectal patients are the same biological phenomenon.

Over-expression of COX-2 has also been found to lead to increased production of PGE_2, increased adhesion to the extracellular matrix, increased concentrations of Bcl-2, reduced $TGF\beta_2$ receptor expression and the absence of E-cadherin protein, respectively. All these changes suggest increased tumorigenic potential and support the notion that COX-2 over-expression alters the biology of intestinal cells, which affects the transformation process. Treatment of such cells with sulindac sulfide has been found to block COX-2 activity and restore the apoptotic response, which adds further support to the idea that the anti-neoplastic activity of NSAIDs involves inhibition of COX-2. Direct genetic evidence for the role of COX-2 in colon cancer has been provided by Oshima and colleagues [92, 93] using *APC* knock-out mice, which develop polyps in their intestinal tracts because of a truncation mutation in the *APC* gene.

The question of how COX-2 inhibition leads to apoptosis have been debated. Several studies have suggested that decreased COX-2 activity leads to a reduction of eicosanoids, such as the prostaglandins, and this lack of prostaglandins, in turn, affects cell proliferation and apoptosis. So far, there has been no definitive evidence to support the existence of a signaling pathway through which prostaglandins can directly affect apoptosis. AA may also provide a mechanism for COX-2 dependent induction of apoptosis. Treatment of colorectal carcinoma cells with various NSAIDs results in inhibition of COX-2 and a dramatic increase in the concentration of AA which stimulates the enzyme sphingomyelinase to convert sphingomyelin to ceramide, a potent inducer of apoptosis. Several NSAIDs cause such a dramatic increase in ceramide and subsequent activation of apoptosis in colon cancer cells. It is possible that the pro-apoptotic effects of NSAIDs are the result of such induction of the ceramide-induced apoptosis. Other studies have shown that intracellular increases in AA can signal apoptosis and that the cellular concentration of un-esterified AA is a general mechanism by which apoptosis is regulated. It seems that AA can alter mitochondrial permeability and cause cytochrome C release, leading to apoptosis.

2. COX Independent Mechanisms

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

Another potential COX-independent mechanism of NSAID-mediated apoptosis involves the peroxisome-proliferator-activated receptor δ (PPARδ), which is a growth-promoting protein. It has recently been reported that NSAIDs such as sulindac can bind to and inhibit PPARδ [94]. Normally, when colon-cancer cells are treated with sulindac, they undergo apoptosis. When PPARδ is over-expressed in colon-cancer cells, the cells are partially protected from NSAID-induced apoptosis. Furthermore, PPARδ is over-expressed in colon cancers. They also observed that sulindac can cause the PPARδ gene product (a transcription factor) to dissociate from DNA [94]. As a result, the cell is left unable to transcribe the genes necessary for its survival. It is particularly interesting that PPARδ is suppressed by *APC*. When *APC* is mutated in colorectal cancers, one would therefore expect that PPARδ would be higher than in normal cells, promoting unchecked cell growth.

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

Angiogenesis is essential for tumor growth beyond 1-2 mm in size. Dubois *et al.*, have shown that PGE_2 synthesized by over-expressed COX-2 in CaCo-2 colon carcinoma cells can induce secretion of the angiogenic factors like VEGF, TGF and bFGF leading to stimulation of vascular tube formation. Sulindac and COX-2 selective inhibitors can inhibit angiogenesis by inhibition of these angiogenic factors by tumor cells. The mechanisms by which NSAIDs exert their chemopreventive effects are currently an area of debate. The COX-dependent and COX-independent mechanisms are not mutually exclusive and it is likely that they act in both ways, which could be important for chemopreventive mechanisms of action of NSAIDs. Understanding the underlying mechanism of these compounds can lead to the rational development of superior cytotoxic agents.

ROLE OF COX ENZYME IN PANCREATIC CANCER PROGRESSION

Pancreatic cancer is characterized as one of the deadliest malignancies and its treatment is a great challenge to clinical oncologists. Cyclooxygenase-2 (COX-2) plays a critical role in the development and growth of human malignancies. Expression of COX-2 is detectable in 75% of pancreatic cancers with over expression found in 50% of these cases. The inducible COX-2 enzyme and its metabolic product prostaglandin E2 (PGE_2) are involved in inflammatory response and inhibition of apoptosis in cancer cells, suggesting that COX-2 is a favorable target for anti-cancer drug design (Fig. **1**). From a mechanistic point of view, several molecular pathways have been identified such as Ras-Raf-MEK-ERK, PI3K/Akt and NF-κB pathway that are believed to be important in the development, progression and metastasis of pancreatic cancer (Fig. **1**). In addition, the involvement of cyclooxygenase and lipoxygenase family of enzymes in the development and progression of pancreatic cancer has been recently appreciated. The cyclooxygenase (COX) family of enzymes catalyzes the rate-limiting step in conversion of arachidonic acid into prostaglandins. Three isoforms of COX enzyme have been reported - COX-1, COX-2 and COX-3. COX-1 is constitutively expressed in many tissues where it serves as house keeping gene whereas COX-2 is an inducible enzyme that is rapidly transcribed, perhaps regulated by NF-κB and other transcription factors in response to inflammation, growth factors, cytokines and tumor promoters. The expression of COX-2 is largely undetectable in most tissues under normal conditions with the exception of brain, kidney and testis.

The potential role of COX-2 in tumorigenesis and tumor progression includes decreased apoptosis, increased angiogenesis and increased tumor invasiveness. Mounting evidence derived from clinically proven biopsy samples showed that COX-2 is up-regulated in human pancreatic tumor tissues as compared to normal adjacent pancreatic tissues. Moreover, COX-2 inhibitors inhibit cell growth with greater efficacy in cell lines with stronger COX-2 expression compared to weakly COX-2 expressing pancreatic cancer cell lines (14, 16). Further studies have shown that genetic deletion of COX-2 abrogates tumorigenesis as well as intestinal polyposis in mouse model of familial adenomatous polyposis $APC^{\Delta 716}$ compared to wild type animal. Studies from our laboratory have also shown over expression of COX-2 in pancreatic ductal adenocarcinoma, which was associated with increased perineural invasion.

Despite numerous advances in our understanding of the pathophysiology and molecular biology of pancreatic cancer, currently available standard therapeutic approaches for pancreatic cancer show limited benefit in improving the survival of patients diagnosed with this deadly disease. NSAIDS are a well studied class of chemopreventive agents and have been shown to act through both COX dependent and independent pathways. Rofecoxib and celeocoxib were introduced into the market as selective COX-2 inhibitors with non-gastrointestinal side effects, but their prolonged use has raised concern about their adverse cardiovascular effects. Since the use of these agents for cancer therapy has not been fully evaluated, understanding the underlying mechanisms of these agents can lead to further development of novel therapeutic agents for pancreatic cancer utilizing the non-steroidal scaffolds such as COX-2 inhibitors.

NOVEL SYNTHETIC METAL DERIVATIVES OF COX-2 INHIBITORS FOR TREATMENT OF PANCREATIC CANCER

Recently, we have reported that the 3-benzoyl-α-methyl benzene acetic acid (ketoprofen) can be easily appended with pharmacophores such as thiosemicarbazone bearing amino groups to yield Schiff base compounds with transition metals such as copper to yield potent anticancer agents against breast cancer cells [95]. Our design strategy was based on a non steroidal motif such as 3-benzoyl-α-methyl benzene acetic acid (ketoprofen) having a ketone functionality (Fig. **3**). We carried out the synthesis of the ketoprofen-salicylhydrazone (FPA-301) by reacting ketoprofen and salicylhydrazide in 1:1 molar ratio in methanol. The reaction mixture was

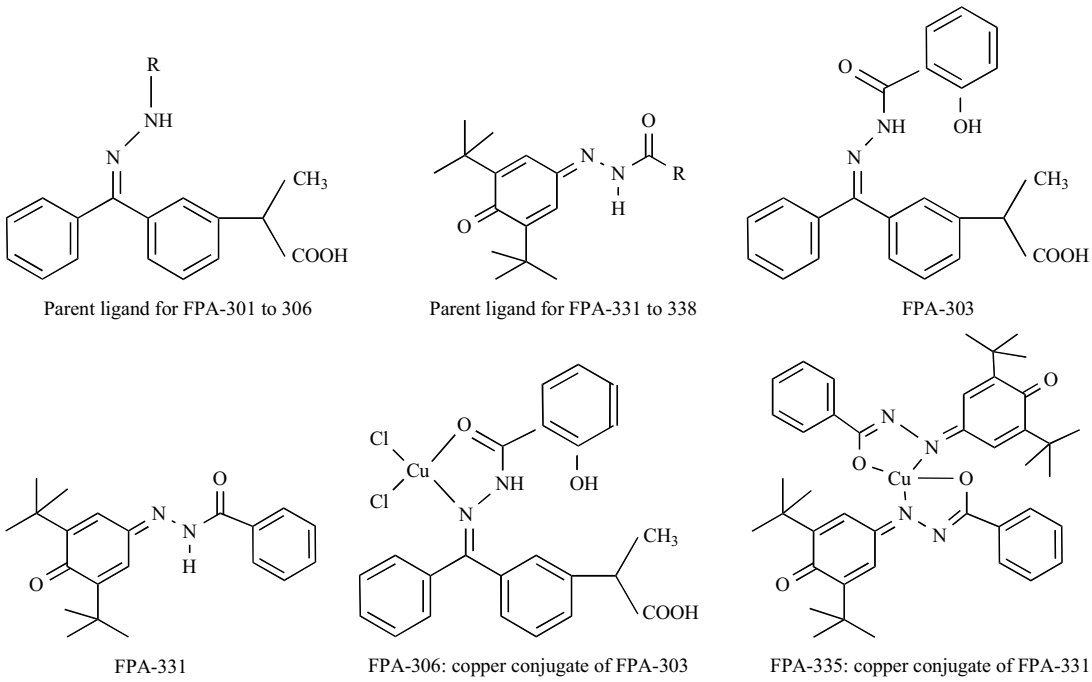

Fig. (3).

maintained at 60°C on water bath with constant stirring for 6 h and completion of the reaction was checked by TLC using $CHCl_3$-Methanol (9:1 v/v) as developing solvent. Subsequently solvent was stripped off on the rotavapor, and the precipitated FPA-301 ligand was washed with cold methanol and dried in vacuum over $CaCl_2$. FPA-306 was prepared by mixing methanolic solution of FPA-301 and copper chloride in 1:1 stoichiometric ratio and refluxing the reaction mixture for 3 hrs. The precipitated complex was washed with water and cold acetone and dried under vacuum over $CaCl_2$. Both compounds were further characterized by elemental analysis, infrared and electronic spectroscopy, magnetism and epr study and also by cyclic voltammetric studies as described earlier [95]. The copper conjugate FPA-306 was found to be a neutral, monomeric compound having square planar geometry and redox potential of +0.47 V due to facile Cu^{+2}/ Cu^{+1} couple which may be relevant to its biological activity.

We have performed biological characteristics and molecular modeling of this novel synthetic derivative of ketoprofen, FPA-306, as a selective COX-2 inhibitor in pancreatic cancer cell lines. Firstly, we measured the levels of PGE_2 which reflects the activity of COX-2 in these cell lines and observed that BxPC-3 and COLO-357 cells have higher levels of PGE_2 secretion while low COX-2 expressing cell line MIAPaCa has an almost undetectable level of PGE_2 secretion. Then, the biological activity of FPA-306 was compared with the parent molecule 3-benzoyl -α methyl benzene acetic acid (ketoprofen) and well known COX-2 inhibitor celecoxib in pancreatic BxPC-3, MIAPaCa and COLO357 cancer cell lines. Our results revealed that FPA-306 had potent antiproliferative activity in high COX-2 and PGE_2 expressing cell lines, BxPC-3 and COLO357 cells (IC_{50} = 10 μmol/L and 25 μmol/L, respectively) as compared to celecoxib (35 μmol/L for BxPC-3 and COLO357) and ketoprofen (80 μmol/L) whereas both celecoxib and FPA-306 were ineffective in MIAPaCa, a low COX-2 and PGE_2 expressing cell line as described in our publication [95]. The efficacy of COX-2 inhibitor is dependent upon the levels of COX-2 expression in pancreatic cancer, and thus greater suppression of growth was observed in COX-2 positive cell line compared to COX-2 negative cell line, consistent with the findings that FPA-306 is more effective in the inhibition of cell proliferation and induction of apoptosis in COX-2 positive cell line (BxPC-3) compared to COX-2 negative cell line (MIAPaCa).

Inhibitory potency and selectivity of conventional NSAIDS (aspirin, ibuprofen, indomethacin, ketoprofen, ibuprofen) and coxibs (celecoxib, rofecoxib) for COX-1 and COX-2 enzymes vary greatly. Novel coxibs such as valdecoxib, etoricoxib, parecoxib with enhanced biochemical COX-2 selectivity over that of rofecoxib and celecoxib have been recently developed with the potential advantage to spare COX-1 activity, thus reducing gastrointestinal toxicity, even when administered at high doses to improve efficacy. Inhibitory effects of NSAIDS on gastric PGE_2 synthesis correlate with COX-1 inhibitory potency in blood and with COX-1 selectivity but not with COX-2 inhibitory potency. However, even COX-2 selective NSAIDS still have sufficient anti COX-1 activity to cause potent inhibition of gastric PGE_2. Thus, at therapeutic concentrations, none of the currently marketed NSAIDS spare gastric COX-1 activity, thus causing the well known renal and GI side effects.

Molecular modeling studies of FPA-306 in the active site of COX-2 enzyme showed that 3-benzoyl-α-methyl benzene acetic acid (ketoprofen) scaffold presents the key structural requirements, such as the methyl and acetic acid group pre-

sent on the aromatic ring, necessary for interaction with important aminoacids such as Arg 106 and Tyr 341 for hydrogen bonding. Further, van der Waals and hydrophobic interactions with phenyl and benzoyl groups also helped to stabilize the compound FPA-306 within the COX-2 pocket. FPA-306 occupies the same cavity space within the COX-2 active site as SC-558, a selective COX-2 inhibitor, thus confirming our *in vitro* results as observed in COX-2 positive pancreatic cancer cells. These results further suggest that these compounds may be Cox-2 selective inhibitors as suggested in our published report [95].

Selective COX-2 inhibitor celecoxib has been reported to inhibit NF-κB, an ubiquitous nuclear transcription factor. NF-κB is constitutively activated in pancreatic cancer and controls various genes involved in tumorigenesis, apoptosis, metastasis and inflammation. We also tested the NF-κB DNA binding status in BxPC-3 cells treated with FPA-306. We have found a significant reduction in the DNA binding activity with FPA-306 treatment. NF-κB has been shown to inhibit apoptosis in response to chemotherapeutic agents and to promote transcription of the COX-2 gene. Thus, inhibition of NF-κB by FPA-306 may lead to the induction of apoptosis in cancer cells, which may represent a rational method of treating pancreatic cancer.

It has been known that NF-κB transcriptionally regulates two important anti-apoptotic proteins, Bcl-2 and survivin, which stabilize the mitochondrial membrane integrity and prevent release of cytochrome c thereby withholding caspase activation and PARP cleavage. We have tested these two proteins and found down-regulation of Bcl-2 and complete inhibition of survivin in FPA-306 (60 μmol/L) treated cells [95]. Over expression of Bcl-2 protein in pancreatic cancer seems to be responsible for the resistance to chemotherapy and radiotherapy and enhances the tumor metastatic potential; therefore, its down-regulation upon treatment with FPA-306 is an important finding which could be further exploited in designing a rational combination chemotherapy regimen.

Our reported studies [95] have shown that conversion of a bi-aryl ring motif such as 3-benzoyl-α-methyl benzene acetic acid (ketoprofen) into triaryl ring systems (Fig. **3**) yields molecules with potent COX-2 inhibitory activity. FPA-306 has a potent antiproliferative and proapoptotic activity in COX-2 positive cells. However, further escalation of dose other than the one needed for inhibition of COX-2 activity, indicates other non COX-2 dependent pathways may also be involved. A similar speculation has been proposed in previous studies reporting the ability of NSAIDs to inhibit growth of colorectal cancer cell line that lack COX-1 and COX-2 enzymatic expression [96]. This is further substantiated by the observation that induction of apoptosis was accompanied by a decrease in the COX-2 mRNA and protein with FPA-306, and these results are comparable to our previous observation with celecoxib showing down-regulation of COX-2 mRNA levels in pancreatic BxPC-3 cell lines. Collectively, these results suggest that FPA-306 inhibits NF-κB activation leading to the inhibition of COX-2 expression and its activity, thereby inhibiting cell growth and inducing apoptotic cell death. However further preclinical animal experiments are warranted to test the anti-tumor activity of FPA-306 and other compounds of this series in future studies.

CONCLUSIONS AND PERSPECTIVE

New and novel COX-2 inhibitors offer great potential in the fight against cancer in general and pancreatic cancer in particular because COX-2 inhibitors will not only inhibit COX-2 but will results in the inhibition of other signaling pathways that crosstalk with COX-2 signaling pathways and considered important in pancreatic cancer. In addition to the role of COX-2 inhibitors for cancer therapy, these inhibitors may also play important role for the prevention of cancers and thus innovative research is urgently needed for the evaluation of novel COX-2 inhibitors in various animal tumor models and in human clinical trials. In the coming years, it seems likely that specific COX-2 inhibitors will take center stage for their anti-tumor activity without any adverse side effects and as such should provide opportunities to further develop novel and selective COX-2 targeted synthetic small molecule inhibitors toward customize approaches for the prevention and/or treatment of cancers in general and pancreatic cancer in particular.

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