REVIEW

Cyclooxygenase-2 (COX-2)–Independent Anticarcinogenic Effects of Selective COX-2 Inhibitors

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Nonsteroidal antiinflammatory drugs (NSAIDs) appear to reduce the risk of developing cancer. One mechanism through which NSAIDs act to reduce carcinogenesis is to inhibit the activity of cyclooxygenase-2 (COX-2), an enzyme that is overexpressed in various cancer tissues. Overexpression of COX-2 increases cell proliferation and inhibits apoptosis. However, selective COX-2 inhibitors can also act through COX-independent mechanisms. In this review, we describe the COX-2–independent molecular targets of these COX-2 inhibitors and discuss how these targets may be involved in the anticarcinogenic activities of these selective COX-2 inhibitors. We also compare the concentrations of these inhibitors used in in vitro and in vivo experiments and discuss the implications of the in vitro studies for clinical management of cancer with these drugs. [J Natl Cancer Inst 2006;98:736–47]

More than 20 years ago, epidemiological studies found that treatment with nonsteroidal antiinflammatory drugs (NSAIDs) was associated with a reduced risk for colon cancer *(1,2)*. Since then, the anticarcinogenic effects of NSAIDs have been evaluated in many randomized clinical trials *(3–11)*.

Traditional NSAIDs are nonselective inhibitors of both cyclooxygenase-1 (COX-1) and COX-2. Although COX-1 is constitutively expressed in many tissues, COX-2 is detected negligibly in most tissues but can generally be induced by cytokines and stress in various tissues. Cyclooxygenases convert arachidonic acid to prostaglandin H_2 in a two-step reaction. Prostaglandin H_2 is further converted by specific prostaglandin synthases to prostaglandin E_2, prostaglandin $F_{2\alpha}$, or prostaglandin D_2. In many types of cancer, the regulation of COX-2 and the expression of microsomal prostaglandin E synthase 1 are abolished, so that both enzymes are overexpressed, leading to an increase in prostaglandin E_2 production in these cells *(12–26)*. Overexpression of both proteins occurs in the early adenoma stage of colon cancer, and this event is therefore one of the first steps in the development of colon cancer *(27)*. Increased COX-2 expression appears to be involved in the development of cancer by promoting cell division *(28,29)*, inhibiting apoptosis *(30–33)*, altering cell adhesion and enhancing metastasis *(34–38)*, and stimulating neovascularization *(39,40)* (Fig. 1). The inhibition of COX-2 activity by traditional NSAIDs blocks these activities and thus may account for the anticarcinogenic activity of these drugs [for review, see *(41–45)*]. However, in addition to mechanisms that involve the inhibition of COX-2, mechanisms independent of COX-2 also participate in the anticarcinogenic activities of traditional NSAIDs, and each traditional NSAID appears to have its own, more or less specific, COX-independent target. Overviews of these studies have been presented by Tegeder et al. *(46)* and by Soh and Weinstein *(47)*.

Anticarcinogenic treatment regimens are usually carried out over long periods (months to years), and so determination of a reasonable benefit–risk ratio for these treatments is important. However, long-term use of traditional NSAIDs, which inhibit both COX-1 and COX-2, is associated with serious gastrointestinal side effects, such as ulceration and perforation of the gastric mucosa. These side effects have been attributed to the inhibition of COX-1, which mediates gastroprotective prostaglandin production. To circumvent the side effects associated with COX-1 inhibition, selective COX-2 inhibitors, such as celecoxib and rofecoxib, were developed *(48–51)*. Clinical studies, indeed, found that the selective COX-2 inhibitors had fewer gastrointestinal side effects than traditional NSAIDs but had antiinflammatory activities that were similar to those of traditional NSAIDs *(49,50,52–54)*. Nevertheless, the group of COX-2 inhibitors, including celecoxib, rofecoxib, valdecoxib, etoricoxib, and lumiracoxib, is currently under critical investigation because of the increased risk of cardiovascular side effects (such as an increase in blood pressure, stroke, and myocardial infarction) that have appeared after long-term use of rofecoxib and of valdecoxib *(55–58)*. These cardiovascular side effects prompted Merck to withdraw rofecoxib from the pharmaceutical market in September 2004, and they prompted the Food and Drug Administration (FDA) to ask Pfizer to withdraw valdecoxib in April 2005.

Results for celecoxib are somewhat different. Although one study found that cardiovascular side effects, such as myocardial infarction, stroke, or heart failure, were associated with prolonged treatment with celecoxib *(56)*, a similar clinical trail, the Prevention of Spontaneous Adenomatomous Polyps Trial, did not find that use of celecoxib was associated with increased cardiovascular risk. Thus, the FDA has allowed celecoxib to remain on the market but has required that it carry a modified label to acknowledge the cardiovascular risk (as well as the gastrointestinal and cutaneous risks). The cardiovascular risks of COX-2 inhibitors and of NSAIDs in general are discussed in more detail elsewhere *(59–62)*.

Celecoxib is the only NSAID that has been approved by the FDA (in December 1999) for adjuvant treatment of patients with
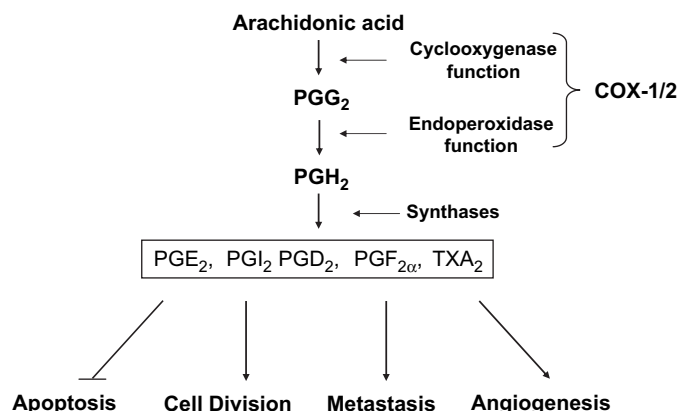
Arachidonic acid

↓ ← Cyclooxygenase function

PGG_2

↓ ← Endoperoxidase function ⎱ COX-1/2

PGH_2

↓ ← Synthases

PGE_2, PGI_2 PGD_2, $PGF_{2\alpha}$, TXA_2

Apoptosis   Cell Division   Metastasis   Angiogenesis

Fig. 1. Cyclooxygenase-2 (COX-2) pathway in cancer development. COX-2 catalyzes the conversion of arachidonic acid to prostaglandin H_2 (PGH_2), which is further converted by different prostaglandin synthases to PGE_2, PGD_2, $PGF_{2\alpha}$, and thromboxane A_2 (TXA_2). These prostaglandins promote cell division, metastasis, and angiogenesis, and they inhibit apoptosis, which leads to increased tumor growth.

familial adenomatous polyposis. Since the introduction of celecoxib in 1998 and rofecoxib in 1999, more than 3000 studies have investigated the molecular targets and clinical effects of these drugs. This review discusses the anticarcinogenic molecular mechanisms associated with selective COX-2 inhibitors (in particular, celecoxib and rofecoxib) and their COX-independent mechanisms of action. In general, the anticarcinogenic mechanisms of both celecoxib and rofecoxib involve blocking cell cycle progression and angiogenesis and inducing apoptosis.

Inhibition of Cell Cycle Progression

Transitions between the various phases of the cell cycle are controlled by various cyclins, cyclin-dependent kinases (CDKs), and cell cycle inhibitors. Treatment of various tumor cell lines with celecoxib induces G_1-phase arrest, which is accompanied by the decreased expression of cyclins A, B, and D; the increased expression of cell cycle inhibitors p21[waf1] and p27[kip1]; and the loss of CDK activity (Table 1) (63–66). These activities appear to be, in part, independent of COX-2 inhibition for the following reasons: The concentration of celecoxib required to inhibit cell proliferation is higher than that required to inhibit COX-2 activity (Table 2); these effects could not be reversed by the addition of prostaglandin E_2 or mimicked by the inhibition of COX-2 activity (67); and rofecoxib, a COX-2 inhibitor that is more potent than celecoxib, did not consistently produce similar effects.

Molecular mechanisms involved in cell cycle arrest induced by celecoxib treatment are at least partly understood. Celecoxib can inhibit protein kinase B (PKB/Akt) or its upstream kinase phosphoinositide-dependent kinase 1 (PDK-1) (68–72). In cell-free assays, PDK-1 appears to be a direct target of celecoxib. It, however, is only a weak PDK-1 inhibitor; the concentration required for 50% inhibition (IC_{50}) is 48 μM. Celecoxib, by means of its 4-methylphenyl moiety, inhibits PDK-1 by competing with ATP at the ATP binding site of PDK-1 (71). Although many studies (70,73) have reported that PKB is inhibited by celecoxib, whether celecoxib binds directly to PKB or acts by means of another celecoxib target, such as PDK-1, is unclear. However, 4-[5-(2,5-dimethylphenyl)-3(trifluoromethyl)-1H-pyrazol-1-yl]benzene sulfonamide, which is a celecoxib analogue that lacks COX-2 inhibitory activity, also inhibits PDK-1 and PKB activity. Thus, these inhibitory effects may be independent of COX-2 (72).

PKB regulates cell cycle progression by its ability to phosphorylate, and thereby inactivate, the CDK inhibitors p21[waf1] and p27[kip1]. Inactivation of the CDK inhibitors leads to the activation of various cyclin–CDK complexes and to the activation of proliferating cell nuclear antigen; these activities promote DNA replication and cell proliferation (74–78). PKB can also induce activation of the cell cycle regulator E2F in T lymphocytes (79) and increase the expression of cyclin D in various cancer cells (80). Other targets of PKB involved in cell cycle regulation have been

Table 1. Celecoxib and the expression of proteins involved in apoptosis, cell cycle regulation, and angiogenesis/metastasis*

Apoptosis (ref)	Cell cycle (ref)	Angiogenesis or metastasis (ref)	Kinases (ref)	Transcription factors (ref)	Others (ref)
+ Caspase 3 (100,101)	− Cyclin D1 (64)	− VEGF (140)	− PDK-1 activity (70,71)	− NF-κB (128,129)	− COX-2 activity (48)
+ Caspase 8 (96,102)	− Cyclin A (63,65)	− Phospho-EGFR (96)	− AKT/PKB activity (70,72,100,105)	− SP1 (141)	− Ca^{2+}-ATPase activity (113)
+ Caspase 9 (96,101,102)	− Cyclin B (63,65)	− MMP-1 (145)	− Phospho-SAPK (98)	− Egr-1 (84)	− CA I (117)
− Bcl-2 (97,100)	+ p21 (64,65)	− MMP-2 (145)		− c-Fos (84)	− CA II (117)
− Bcl-xL (97)	+ p27 (64,65)	− MMP-3 (145)		+ NF-κB (102)	− CA IV (117)
− Survivin (100)	+ Rb (64)	− MMP-9 (144)		+ PPARγ (64)	− CA IX (117)
− Mcl-1 (31,98)					
+ 15-LOX-1 (126)	− ODC (84)			− Nuclear β-catenin (152)	+ Cytosolic β-catenin (152)
					− Membranous β-catenin (152)
+ Ceramide (82)	− pRb (68)				
+ Bad (99)	− CDK2/cyclin E activity (68)				
+ DR5 (104)	− CDK1/cyclin B1 activity (68)				

*+ = Increased expression or activity; − = decreased expression or activity; LOX = lipoxygenase; DR5 = death receptor 5; Rb = retinoblastoma; ODC = ornithine decarboxylase; pRb = phosphorylated retinoblastoma; CDK = cyclin-dependent kinase; VEGF = vascular endothelial growth factor; EGFR = epithelial growth factor receptor; MMP = matrix metalloprotease; PDK-1 = phosphoinositide-dependent kinase 1; PKB = protein kinase B; SAPK = stress-activated protein kinase; NF-κB = nuclear factor κB; SP1 = transcription factor SP1; Egr-1 = early growth response factor; c-Fos = protooncogene protein c-FOS; PPAR = peroxisome proliferator activated receptor; COX-2 = cyclooxygenase-2; CA = carbonic anhydrase.

Table 2. Celecoxib and rofecoxib concentrations used in in vitro experiments to achieve anticarcinogenic effects*

Effect	Drug concentration	(Ref)
Mechanisms involved in inhibition of cell cycle progression		
Proteins decreasing expression	10–40 μM Celecoxib	(64)
of cell cycle–promoting proteins	100 μM Celecoxib	(65)
and increasing expression of cell	40–100 μM Celecoxib	(66)
cycle–inhibiting proteins	10–30 μM Celecoxib	(68)
	12.5 μM Rofecoxib	(88)
	10–200 μM Rofecoxib	(90)
	10–100 μM Rofecoxib	(87)
Inhibition of PKB	10–60 μM Celecoxib	(72)
	(PKB IC$_{50}$ = 28 μM)	
	10–50 μM Celecoxib	(68)
	25–75 μM Rofecoxib	(91)
	50 μM Rofecoxib	(73)
Inhibition of PDK-1	25–100 μM Celecoxib	(70)
	10–100 μM Celecoxib	(71)
	(PDK-1 IC$_{50}$ = 48 μM)	
	50 μM Celecoxib	(92)
Increase in ceramide	25–50 μM Celecoxib	(82)
Inhibition of ODC	2.5–50 μM Celecoxib	(84)
Mechanisms involved in induction of apoptosis		
Decreased expression of	10 μM Celecoxib	(31)
antiapoptotic proteins	25–100 μM Celecoxib	(98)
Increased expression of	10 μM Celecoxib	(99)
proapoptotic proteins		
Activation of intrinsic	10–40 μM Celecoxib	(64)
apoptotic pathway	75–100 μM Celecoxib	(96)
	50 μM Celecoxib	(100)
	50–100 μM Celecoxib	(101)
	1–10 μM Rofecoxib	(105)
	0.1–2.5 μM Rofecoxib	(106)
Activation of extrinsic	3–100 μM Celecoxib	(102)
apoptotic pathway	25–75 μM Celecoxib	(104)
Inhibition of endoplasmic	10–100 μM Celecoxib	(113)
reticulum Ca^{2+} ATPase	100 μM Rofecoxib	(113)
Inhibition of CA	Nanomolar concentrations	(117)
	of celecoxib	
Increased expression of 15-LOX-1	12.5 μM Celecoxib	(126)
Effects on NF-κB	3–100 μM Celecoxib	(102)
	10–40 μM Celecoxib	(64)
	75–100 μM Celecoxib	(131)
	1–50 μM Celecoxib	(132)
	10–100 μM Rofecoxib	(133)
Inhibition of angiogenesis and metastasis		
Inhibition of Egr-1	2.5–50 μM Celecoxib	(84)
Suppression of VEGF	10–30 μM Celecoxib	(141)
Antiproliferative effects on	50–100 μM Celecoxib	(68)
HUVECs or HMVECs	1–50 μM Celecoxib	(87)
	10–100 μM Rofecoxib	(87)
	500 nM–10 μM Rofecoxib	(143)
Inhibition of MMPs	Rofecoxib at 20–50 mg/kg/	(89)
	day in vivo	
	10–25 μM Celecoxib	(144)
	3–30 μM Celecoxib	(145)
Effects on the APC–β-catenin	0–100 μM Celecoxib	(152)
pathway	100 μM Rofecoxib	(152)
	10–200 μM Rofecoxib	(90)

*The underlined concentrations are those predominantly used in the experiments. PKB = protein kinase B; IC$_{50}$ = concentration required for 50% inhibition; PDK-1 = phosphoinositide-dependent kinase 1; ODC = ornithine decarboxylase; CA = carbonic anhydrase; LOX = lipoxygenase; NF-κB = nuclear factor κB; Egr-1 = early growth response protein 1; VEGF = vascular endothelial growth factor; HUVEC = human umbilical vein endothelial cell; HMVEC = human dermal microvascular endothelial cell; MMP = matrix metalloprotease; APC = adenomatous polyposis coli.

summarized by Hanada et al. (81). Inhibition of PKB by celecoxib prevents the cell proliferation–promoting effects of PKB and could be one mechanism by which celecoxib induces a cell cycle block.

Furthermore, celecoxib inhibits various CDK–cyclin complexes in cell-free assay systems (68), leading to reduced phosphorylated retinoblastoma and arrest cells in G$_1$ phase of the cell cycle (68). However, expression levels of various cyclins (cyclins A, B1, D1, and E), cyclin-dependent inhibitors (p21 and p27), and CDKs (CDK1, CDK2, CDK4, and CDK6) in human umbilical vein endothelial cells are not affected by celecoxib treatment (68), and celecoxib does not affect the level of phosphorylation of CDK2 at threonine-160, which excludes the involvement of the CDK-activating kinase in inhibiting CDK2 kinase activity (68). Thus, the mechanism of CDK–cyclin complex inhibition by celecoxib is still unclear.

Also, celecoxib increases the level of ceramide in mammary tumor cells, and the level of ceramide appears to be associated with the arrest of cells in G$_1$ phase (82). Ceramide and other sphingolipids are important signaling molecules in the cells that regulate diverse cellular processes including cell proliferation, cell senescence, apoptosis, and cell cycle. Ceramide has been shown to activate several enzymes involved in stress signaling cascades, including protein kinases (jun kinases, kinase suppressor of Ras, and protein kinase C isoforms) and protein phosphatases (protein phosphatase 1 and protein phosphatase 2A) [for review, see (83)]. Thus, changes in ceramide concentrations after celecoxib treatment may also play a role in celecoxib-induced growth inhibition.

Ornithine decarboxylase is another enzyme that is inhibited by celecoxib (84). This enzyme converts L-ornithine to the polyamine putrescine. Increased polyamine levels are associated with increased cell proliferation, decreased apoptosis, and increased expression of genes affecting tumor invasion and metastasis. However, although polyamines seem to be associated with many cellular processes, a key criticism of polyamine research has been that the specific mechanisms underlying their modes of action—including those in cancer cells—have not been defined. Over the past few years, polyamines have been shown to affect specific gene expression through both transcriptional and posttranscriptional processes (85). The activity of ornithine decarboxylase is 10- to 15-fold higher in colon cancer tissue than in normal colon epithelium (85,86). Inhibition of ornithine decarboxylase activity by celecoxib may thus contribute to the G$_1$-phase arrest. Although the mechanism by which celecoxib inhibits ornithine decarboxylase activity is currently unclear, it may result from both downstream effects of COX-2 inhibition and COX-independent mechanisms that have yet to be elucidated (84).

The effects of rofecoxib on cell cycle regulation are controversial because rofecoxib had no effect on cell proliferation in human umbilical vein endothelial cells (87) but it decreased cyclin D1 expression, increased the expression of cell cycle inhibitors p21^{waf1} and p33, and increased the expression of the growth-arrest DNA damage–inducible genes (GADD) 34 and GADD45 in human pancreatic cancer cells (87–90) (Table 3). Thus, the effects of rofecoxib appear to be more cell type specific than those of celecoxib. Inhibition of PKB appears not to be involved, because rofecoxib inhibits PKB only marginally (72,73,91).

Induction of Apoptosis

Many studies have shown that celecoxib exerts its anticarcinogenic effect in various cancer cell lines by inducing apoptosis (65,70,73,92). Apoptosis, or programmed cell death, can be

Table 3. Rofecoxib and expression of proteins involved in apoptosis, cell cycle regulation, and angiogenesis/metastasis*

Apoptosis (ref)	Cell cycle (ref)	Angiogenesis/metastasis (ref)	Transcription factors (ref)	Others (ref)
+ Caspase 3 (4)	− Cyclin D1 (88,89)	− VEGF (89)	+ PPARγ (4)	− COX-2 activity (41)
+ Bcl-2 (4)	+ p21 (88)	− pFGF (142)		− Cytosolic β-catenin (89)
− Survivin (4)	+ p33 (88)	− MMP-2 (89)		− Membranous β-catenin (162)
+ Bax (4)	+ GADD34 (88)	− MMP-9 (89)		− IL-10 (89)
	+ GADD45 (88)			+ IL-12 (89)

*GADD = growth arrest, DNA damage–inducible gene; VEGF = vascular endothelial growth factor; pFGF = phosphorylated fibroblast growth factor; PPAR = peroxisome proliferator–activated receptor; FGF = fibroblast growth factor; MMP = matrix metalloprotease; COX-2 = cyclooxygenase-2; IL = interleukin.

induced by the extrinsic pathway through activation of death receptors or by the intrinsic pathway by means of the release of cytochrome c from the mitochondria (93,94). Both pathways require the activation of various caspases, which cleave various proteins and activate DNases, leading to DNA fragmentation (95). Evidence that the intrinsic apoptotic pathway appears to be activated by celecoxib (96) includes the observations that expression of the antiapoptotic proteins Bcl-2, Bcl-xL, Mcl-1, and survivin decreases after treatment of cancer cells with celecoxib, whereas expression of the proapoptotic protein Bad increases (31,97–99), and rapid release of cytochrome c from mitochondria and the activation of Apaf-1 and caspases 3, 8, and 9 are observed (64,96,100,101) (Table 1). Evidence that the extrinsic apoptotic pathway is activated after celecoxib treatment includes the increased expression of the death receptor DR5 in celecoxib-treated human non–small-cell lung carcinoma cells and activation of the FAS–FADD pathway in celecoxib-treated cervical carcinoma cells (102–104). However, these effects were not observed in all cells tested. For example, in the human cervix carcinoma cell lines HeLa and CaSki, the FADD-mediated death pathway was not induced (102). Thus, the importance of these findings is unclear. Rofecoxib has also been reported to induce apoptosis in various carcinoma cells (4,105–107), but little information about the molecular mechanisms associated with apoptosis is available.

One target for selective COX-2 inhibitors is PDK-1 or its downstream substrate PKB/AKT (68,70–73,92). PKB induces antiapoptotic effects by phosphorylating and then inactivating the proapoptotic protein BAD (i.e., the Bcl-2 or Bcl-X antagonist), by phosphorylating procaspase 9 to prevent its cleavage to active caspase 9, or by phosphorylating the apoptosis signal–regulating kinase 1, which inhibits the stress-activated protein kinase pathway and other kinases [for reviews, see Handa et al. (81) and Datta et al. (108)]. Inhibition of PKB by celecoxib reduces all of these activities and promotes apoptosis. Rofecoxib, in contrast, has only marginal PKB inhibitory activity (72,73,91), so that PKB does not appear to be involved in rofecoxib-induced apoptosis.

Another target of celecoxib seems to be the sphingolipid pathway. Celecoxib treatment increases the level of ceramide in the murine mammary tumor cell line 66.1 (82). As discussed above, an increase in ceramide is also associated with the induction of apoptosis [(109–111) and for review, see (83)]. For instance, ceramides play a major regulatory role in apoptosis by inducing the release of proapoptotic proteins from the mitochondria that may occur via the formation of ceramide channels in the mitochondrial outer membrane (112). Information about the relation of rofecoxib and ceramide levels, however, is lacking.

Celecoxib treatment also inhibits the activity of the Ca^{2+} ATPase (IC_{50} = 35 μM) located in the endoplasmic reticulum of PC-3 human prostate cancer cells, so that the reuptake of Ca^{2+} from the cytosol is prevented, which elevates the free intracellular concentration of Ca^{2+} (113). This activity is highly specific for celecoxib and is not associated with other COX inhibitors, including rofecoxib (113). By use of microsome and plasma membrane preparations from human prostate cancer cells, only Ca^{2+} ATPases located in the endoplasmic reticulum have been shown to be direct targets of celecoxib (113). The concentration of Ca^{2+} plays a central role in apoptosis, because it is involved in the activation of Ca^{2+}-sensitive proteases, endonucleases, and caspases. Moreover, opening the mitochondrial permeability transition pores, which releases cytochrome c, is sensitive to the concentration of Ca^{2+} (113,114). Consequently, celecoxib-induced inhibition of Ca^{2+} ATPases in the endoplasmic reticulum may provide a plausible link with the apoptosis inducing activity of celecoxib.

Celecoxib inhibits the activity of carbonic anhydrases I, II, IV, and IX. Carbonic anhydrases are widespread enzymes that catalyze the reversible hydration of carbon dioxide. Several isozymes have been identified; some are cytosolic (carbonic anhydrases I, II, III, VII, and XIII), and others are membrane bound (carbonic anhydrases IV, IX, XII, and XIV). The expression of carbonic anhydrase IX is elevated by hypoxia and is involved in acidification of hypoxic tumors that are characterized as having more aggressive behavior and poorer prognosis than tumors without such expression (115). Furthermore, carbonic anhydrase IX is also involved in cell–cell adhesion and cell proliferation (116). At nanomolar concentrations, celecoxib or valdecoxib specifically inhibits various carbonic anhydrase isozymes (carbonic anhydrases I, II, IV, and IX) (117). The crystal structure of the carbonic anhydrase II–celecoxib complex indicates that the sulfonamide group of celecoxib binds to the catalytic zinc of carbonic anhydrase II (117), whereas rofecoxib, which contains a methyl sulfone group, does not inhibit carbonic anhydrase activity (117). Carbonic anhydrases II and IX appear to play a role in tumor growth and development (118,119) and are potential biomarkers for various tumor types (e.g., biliary tumors, colorectal tumors, gastric tumors, and renal clear-cell carcinoma) (120–123). However, to our knowledge, no study clearly shows that inhibition of carbonic anhydrases plays a role for the anticarcinogenic activity of celecoxib.

One mechanism by which cancer cells escape normal growth regulation is by inhibiting the production of proapoptotic molecules, such as 13-S-hydroxyoctadecadienoic acid (13-S-HODE), which is produced by the 15-lipoxygenase 1 pathway. Expression of 15-lipoxygenase 1 and the production of its product 13-S-HODE are lower in human colon cancer, esophageal cancer, and

colonic cell lines than in normal control tissues. Adding 13-*S*-HODE to human colon or esophageal cancer cell lines inhibited cell proliferation and induced apoptosis *(124,125)*. Celecoxib induced apoptosis in the human colonic cell line RKO, which was accompanied by the induction of 15-lipoxygenase 1 expression and by increased production of 13-*S*-HODE *(126)*. Overexpression of an antisense construct for 15-lipoxygenase 1 in RKO cells prevented the increased production of 13-*S*-HODE and apoptosis in celecoxib-treated cells *(126)*. The effects of celecoxib on the lipoxygenase pathway appear to be independent of COX-2 because they occur also in colon cancer cell lines deficient in COX-2. These effects have not, however, been investigated with rofecoxib *(126,127)*.

Celecoxib alters the DNA-binding activity of the transcription factor nuclear factor κB (NF-κB). NF-κB is inducible or overexpressed in various cancer cells, and its activation leads to cell proliferation and neoplastic transformation or to differentiation and apoptosis, depending on the cell type and the stimuli *(128–130)*. Treatment of cervical cancer cells with celecoxib leads to the binding of NF-κB to DNA and to apoptosis *(102)*. In contrast, chemically induced activation of NF-κB by cigarette smoke is inhibited by celecoxib treatment in human non–small-cell lung carcinoma; later, the expression of various NF-κB–regulated genes that are involved in inflammation, proliferation, and carcinogenesis (such as COX-2, cyclin D1, and matrix metalloproteinase 9) is reduced *(64,131,132)*.

Rofecoxib inhibited NF-κB binding activity in various inflammation model systems *(133)*, but whether this mechanism is also related to its anticarcinogenic effect is unknown. Konturek et al. *(4)* have shown that the treatment of gastric cancer patients with 25 mg of rofecoxib twice a day increased the expression of the peroxisome proliferator–activated receptor γ and of the proapoptotic proteins Bax and caspase 3 in tumor tissues but decreased the expression of the antiapoptotic proteins Bcl-2 and survivin (Table 3). The molecular targets responsible for this effect are yet unknown. Most studies *(90,91)* have detected no change in the expression level or activity of the various proteins involved in apoptosis after rofecoxib treatment, and these results concur with those *(73,87,91,134–136)* that found rofecoxib, at comparable concentrations, to have statistically significantly lower antiproliferative efficacy in various cancer cells than that of celecoxib.

Inhibition of Angiogenesis and Metastasis

Early tumor growth can be divided into two stages: one in which malignant cells form small tumors of limited size because of an inadequate supply of oxygen (hypoxia) and the other in which hypoxia triggers a dramatic change in gene expression, leading to the formation of new blood vessels and a switch in energy metabolism, from respiration to glycolysis *(119)*. Overexpression of COX-2 in tumor cells affects angiogenesis by the production of COX-2–derived eicosanoids (i.e., thromboxane A_2 and prostaglandins I_2 and E_2), which stimulate endothelial cell migration and angiogenesis by increasing the expression of vascular endothelial growth factor (VEGF) and stimulating endothelial cell proliferation *(35,39)*. Both mechanisms contribute to the formation of new blood vessels. Inhibition of COX-2 activity by celecoxib or rofecoxib reduces all these effects and leads to inhibition of angiogenesis and decreased tumor growth *(137)*.

COX-2–independent mechanisms that contribute to the antiangiogenic effects of celecoxib have also been described. Celecoxib inhibits the activation of the early growth response factor Egr-1 in rat hepatoma cells *(84)* (Table 1). Egr-1 is a transcription factor that is rapidly activated by many extracellular agonists (such as growth factors and cytokines) and environmental stress such as hypoxia, vascular injury, and UV radiation *(138)*. Egr-1 plays a role in the transcriptional regulation of the fibroblast growth factor and various cytokines and receptors that are involved in angiogenesis and promotion of tumor development *(139,140)*. Thus, inhibition of Egr-1 gene activation by celecoxib counteracts the different proangiogenic stimuli and inhibits angiogenesis.

Inhibition of angiogenesis after celecoxib treatment is also associated with decreased expression of VEGF in human pancreatic cancer cells that was attributed to the inhibition of transcription factor Sp1 *(141)*. Celecoxib treatment reduced both the DNA binding activity of Sp1 and the transactivating activity of Sp1 in these cells, a result that corresponded with reduced Sp1 protein expression and its phosphorylation *(141)*. The promoter region of VEGF contains an Sp1-binding site that appears to be crucial for VEGF expression *(142)*. Thus, inhibition of Sp1 in tumor cells might be another COX-2–independent anticarcinogenic mechanism for celecoxib.

In addition to its effects on tumor cells, celecoxib also directly inhibits the proliferation of endothelial cells. Lin et al. *(68)* have shown that the antiproliferative effect of celecoxib on human umbilical vein endothelial cells (HUVECs) depends on inhibition of PKB/AKT and CDKs. These effects are associated with decreased phosphorylation of the retinoblastoma protein and a G_1-phase block. Niederberger et al. *(87)* reported that HUVECs initiated apoptosis after celecoxib treatment and that apoptosis was accompanied by an increased concentration of intracellular Ca^{2+} and the activation of caspases. Rofecoxib treatment neither affected cell cycle progression nor induced apoptosis in HUVECs, indicating that these effects are specific for celecoxib and independent of COX-2 inhibition *(87)*.

By contrast, Woods et al. *(143)* demonstrated that rofecoxib inhibits the migration and tube formation of human dermal microvascular endothelial cells (HMVECs). They found that this effect depended on the inhibition of cell proliferation induced by phorbol 12-myristate 13-acetate and was correlated with the reduced expression of the proangiogenic factor, basic fibroblast growth factor, in cocultured RA synovial fibroblasts *(143)*. Yao et al. *(89)* detected statistically significant decreased expression of VEGF in mouse colorectal carcinoma tissues after rofecoxib treatment. They found that rofecoxib induced expression of matrix metalloprotease 2, COX-2, cyclin D1, β-catenin, and the tumor suppressive interleukin 10; increased expression of antineoplastic interleukin 12 (Table 3); and slightly decreased the expression of matrix metalloprotease 9 *(89)*. These effects are associated with growth inhibition and attenuation of the metastatic potential of cancer cells and are exclusively explained by COX-2 inhibition by rofecoxib.

As for rofecoxib, celecoxib also alters the expression and activity of matrix metalloproteases. Treatment with celecoxib inhibited the activity of matrix metalloprotease 9 in lung adenocarcinoma cells cultured in conditioned medium *(144)* and decreased the secretion of matrix metalloproteases 1–3 from fibroblast-like synoviocytes *(145)*. Matrix metalloproteases play a role in tissue invasion, metastasis, and angiogenesis. Matrix metalloproteases 2 and 9 are the principal enzymes for degrading

type IV collagen, the major component of basement membranes, and are believed to play a role in cancer invasion and metastasis *(146)*. The expression of matrix metalloprotease 2 depends on activated PKB in human glioblastoma cells, and inhibition of PKB phosphorylation by celecoxib leads to inhibition of the invasion by these cells *(147)*. Thus, celecoxib may exert its antiangiogenic and antiinvasive effects by inhibiting PKB/AKT.

Effects on Adenomatous Polyposis Coli–β-Catenin Pathway

The adenomatous polyposis coli (APC)–β-catenin pathway is involved in the development of various cancer types, especially colon cancer. Mutations in the APC or β-catenin gene appear in approximately 80% of all patients with sporadic colon cancer, and germline mutations in these genes are responsible for hereditary familial adenomatous polyposis, which is characterized by the formation of many adenomas with increased expression of β-catenin. Such adenomas are obligate precursors for colorectal cancer. Furthermore, deregulation of the APC–β-catenin pathway is also common in other human gastrointestinal and extragastrointestinal cancers *(148–150)*. Steinbach et al. *(151)* reported that, in patients with familial adenomatous polyposis, treatment with 400 mg of celecoxib twice a day was statistically significantly associated with decreased polyp size and with the presence of fewer polyps. These results provided the first hint that celecoxib may affect the APC–β-catenin pathway. After treatment of human colon carcinoma cells with celecoxib, β-catenin protein is released from the membrane to the cytoplasm; in the cytoplasm, its level first increases in a time-dependent manner and then decreases as the protein is degraded. β-Catenin is degraded by both proteasomes and caspases *(152)*. The change in cellular location of β-catenin depends on its phosphorylation by glycogen synthase kinase 3β (GSK-3β) *(152)*, which is directly regulated by PKB. Activated PKB phosphorylates GSK-3β and thereby inactivates it *(76,153)*. Inhibition of PKB/AKT by celecoxib may inhibit this PKB–GSK-3β pathway and contribute to the activation of GSK-3β. To perform its function as a transcription factor, the unphosphorylated nuclear β-catenin protein binds to the transcription factor T-cell factor (TCF)–Lef complex and activates transcription of target genes, such as c-Myc and the genes for cyclin D1, VEGF, and matrix metalloprotease 7 *(154–158)*. After celecoxib treatment, the expression of nuclear β-catenin protein increases but its DNA-binding activity decreases, indicating that the transcriptional activity of the β-catenin–TCF–Lef complex is reduced *(152)*. Neither rofecoxib nor R-flurbiprofen, an anticarcinogenic non–COX-inhibiting NSAID *(159–161)*, affected the expression of β-catenin in vitro, indicating that the decrease in β-catenin expression after celecoxib treatment is not generally attributed to all NSAIDs and is thus independent of COX-2 inhibition *(152)*.

In in vivo experiments, Oshima et al. *(162)* showed that treatment of APC$^{\Delta716}$ mice (in which the APC gene has been mutated by heterologous recombination) with rofecoxib reduced the number and size of intestinal and colonic polyps. This effect was accompanied by the increased expression of membrane-bound β-catenin in the polyps but not the expression of nuclear β-catenin. This result was confirmed by Gardner et al. *(90)*, who found no effect on nuclear β-catenin expression in human colorectal cancer cells after rofecoxib treatment. Thus, the impact of rofecoxib on the APC–β-catenin pathway remains questionable.

Concentrations of Celecoxib in Cell Culture Experiments and in Plasma of Patients

The concentration of celecoxib used in most cell culture experiments is higher than that achieved in the plasma of patients in clinical studies to obtain anticarcinogenic effects (Table 2). When we compared the concentrations of celecoxib required in most cell culture experiments (i.e., 40–100 μM) to induce apoptosis with the maximum human plasma concentrations after administration of one 400-mg dose (approximately 3 μM), we found that the concentration of celecoxib needed to induce apoptosis in vitro was five- to 20-fold higher than that in vivo *(66,132)*. This discrepancy may be explained by the fact that tumor regression in patients or animals requires weeks or months of drug treatment, whereas antiproliferative effects in cultured cells are observed after only a few hours *(152)*. This possibility is supported by Patel et al. *(163)* who reported that celecoxib has an antiproliferative effect in PC3 and LNCaP human prostate cancer cells at clinically achievable concentrations (i.e., 2.5–10 μM) when the treatment period was extended to 96 hours. These authors used human prostate cancer cell lines that express only COX-1, not COX-2, and they found that rofecoxib had no effect at the same concentrations *(163)*. Thus, it is possible to achieve anticarcinogenic effects in vitro with low concentrations of celecoxib when an increased treatment period is used, and these activities can be independent of COX-2. Also, the proliferation of cultured cells occurs under optimized conditions, which are not comparable to in vivo conditions. The response to antitumor therapy in vivo includes responses to the cumulative effects of chemotherapy or radiation therapy because of the repeated treatment of cancer patients over weeks or months *(164)*.

Possibilities for Clinical Management

Various clinical trials have investigated the anticarcinogenic effects of rofecoxib or celecoxib in different tumor types. Because celecoxib is the only NSAID that is currently approved by the FDA for the treatment of familial adenomatous polyposis, most clinical trials have used celecoxib. Increasing attention was focused on therapies that combine standard chemotherapy with celecoxib *(6,9,165–171)*. Combination therapies are generally well tolerated; however, some celecoxib treatment regimens had no clear effect or had increased toxic effects *(7,172–174)*. Rofecoxib has also been tested in combination therapies *(175–177)*, and the observed side effects were generally mild. However, one study *(178)* that investigated the combination of rofecoxib with 5-fluorouracil and leucovorin was interrupted because of increased gastrointestinal toxicity. Because of the recent information on the cardiovascular risks associated with COX-2–selective inhibitors, the use of such inhibitors in clinical practice has changed dramatically. After the withdrawal of rofecoxib and valdecoxib from the pharmaceutical market, other COX-2 inhibitors and NSAIDs in general are now being critically evaluated. In December 2004, the National Cancer Institute canceled the Adenoma Prevention with Celebrex Study because of a statistically significantly increased number of cardiovascular events in the group of patients treated with celecoxib. This decision prompted Pfizer to delay the launch of celecoxib in the European Union for the treatment of intestinal polyps in patients with familial adenomatous polyposis. By contrast, in two large ongoing trials with celecoxib, the Prevention of Spontaneous Adenomatomous Polyps Trial and the Alzheimer's
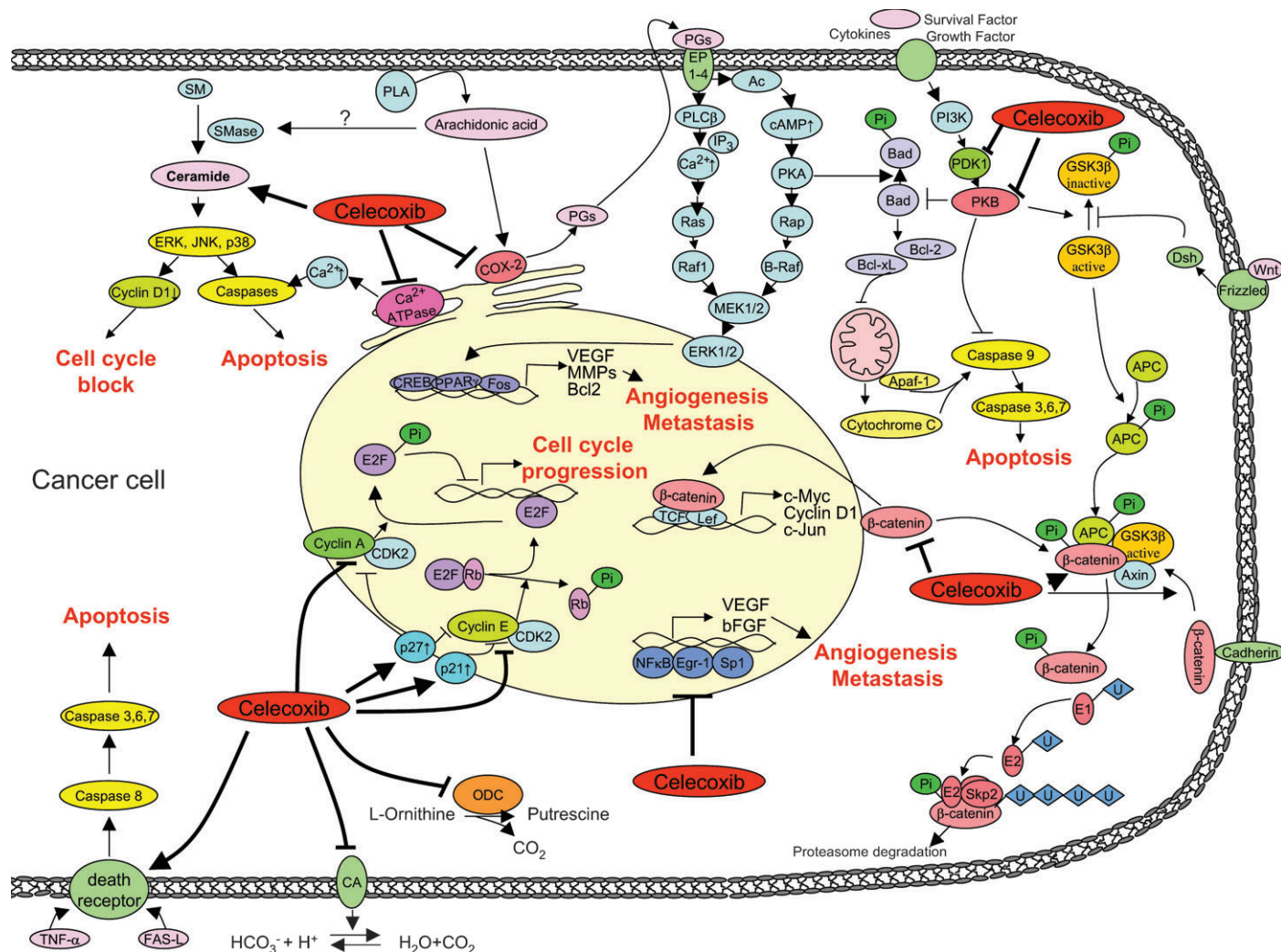
Fig. 2. Molecular mechanisms of celecoxib and its anticarcinogenic effects. Direct targets of celecoxib are cyclooxygenase-2 (COX-2), Ca^{2+} ATPase, protein-dependent kinase 1 (PDK-1), cyclin-dependent kinases (CDKs) in complex with cyclins, and carbonic anhydrase (CA), all of which are inhibited by celecoxib and are the main components in various pathways. Inhibition of these proteins leads to the induction of apoptosis or to the inhibition of cell cycle progression, angiogenesis, and metastasis, which are the main mechanisms by which celecoxib exerts its anticarcinogenic activity. SM = sphingomyelin; SMase = sphingomyelinase; ERK = extracellular signal–regulated kinase; JNK = c-Jun N-terminal kinase; PLA = phospholipase A; PGs = prostaglandins; EP = prostaglandin receptor; Ac = adenylate cyclase; PLCβ = phospholipase Cβ; IP_3 = inositol-1,4,5-trisphosphate; MEK1/2 = mitogen-activated protein kinase kinase 1/2; PKA = protein kinase A; cAMP = cyclic adenosine monophosphate; Rap = Ras-associated protein; Raf = Raf protooncogene serine/threonine–protein kinase; B-Raf = B-raf protooncogene serine/threonine–protein kinase; BAD = Bcl2-antagonist of cell death; Bcl-2 = apoptosis regulator BCL-2; Bcl-xL = apoptosis regulator Bcl-xL; Apaf-1 = apoptotic protease activating factor; PKB = protein kinase B; PI3K = phosphatidylinositol 3-kinase; PDK-1 = phosphoinositide-dependent kinase 1; GSK-3β = glycogen synthase kinase 3β; Dsh = disheveled; APC = adenomatous polyposis coli; TCF = T-cell factor; Lef = lymphoid enhancer factor 1; VEGF = vascular endothelial growth factor; bFGF = basic fibroblast growth factor; Rb = retinoblastoma; CDK = cyclin-dependent kinase; MMP = matrix metalloprotease; E2F = transcription factor E2F; Myc = myc protooncogene protein; PPAR = peroxisome proliferator activated receptor; Fos = protooncogene protein c-fos; NF-κB = nuclear factor κB; SP1 = transcription factor SP1; EGR-1 = early growth response protein 1; U = ubiquitin.

Disease Antiinflammatory Prevention Trial, which were also evaluated by data safety monitoring boards, no increased risk of cardiovascular side effects have been associated with celecoxib. Thus, the cardiovascular side effects associated with COX-2 inhibitor treatment may not be attributable solely to COX-2 inhibition, so that both COX-dependent and COX-independent molecular mechanisms of COX-2 inhibitors and their association with cardiovascular side effects should be investigated further.

Prospects

It will be important to determine which direct COX-2–independent targets for celecoxib and other COX-2 inhibitors participate in the anticarcinogenic effects of these drugs so that

new drugs without gastrointestinal or cardiovascular side effects can be developed for these targets. Currently, celecoxib derivatives have been developed to inhibit PKB/AKT or to disrupt the mitochondrial membrane potential and to have anticarcinogenic activity without inhibiting cyclooxygenases *(71,179)*. By analyzing these derivatives, it may be possible to identify the chemical moieties that are required for the anticarcinogenic effect of celecoxib and to determine whether modification of these moieties can produce more potent anticarcinogenic drugs.

There are many COX-2–independent mechanisms used by celecoxib to mediate its anticarcinogenic effects (Fig. 2). Inhibition of PDK-1 and its downstream substrate PKB/AKT appears to play a central role in the induction of apoptosis and the inhibition of cell cycle arrest, angiogenesis, and metastasis. Such a central

target has not yet been described for rofecoxib, and so the COX-2-independent molecular mechanism of rofecoxib remains unclear. Some of the observed anticarcinogenic effects of celecoxib and rofecoxib could result from the inhibition of COX-2 and downstream components. Moreover, most in vitro effects occurred at celecoxib or rofecoxib concentrations that were higher than the concentrations needed for the inhibition of COX-2 activity. There are also discrepancies between the anticarcinogenic effects of celecoxib and rofecoxib, although rofecoxib is a more potent COX-2 inhibitor than celecoxib. However, unpublished results from our group (Schiffmann S. Maier T.J., Janssen A., Schmidt R., Corban-Wilhelm H., Angioni C., Geisslinger G., Grösch S., unpublished results) indicate that celecoxib and methylcelecoxib, a celecoxib derivative without COX-2–inhibiting activity, had strong anticarcinogenic activity, whereas all other COX-2 inhibitors (including rofecoxib, valdecoxib, etoricoxib, and lumiracoxib) had either no antiproliferative activity or only weak antiproliferative activity on various human colon carcinoma cell lines in vitro and in vivo. The anticarcinogenic potency of celecoxib thus may be unique among selective COX-2 inhibitors; in addition to COX-2 inhibition, celecoxib must also target other COX-2–independent proteins. The reasons for these discrepancies are still poorly understood. All COX-2 inhibitors are selective inhibitors of COX-2, but the chemical structures of these compounds are different. Thus, the sulfonamide and the 4-methylphenyl moieties of celecoxib may be particularly important because these chemical moieties also target other proteins, such as the carbonic anhydrases or PDK-1, respectively, as previously discussed.

The many in vivo and in vitro studies that have demonstrated the benefit of COX-2 inhibitors in cancer therapy has prompted various researchers to investigate the structural basis of these activities further. New antineoplastic agents that show a higher apoptosis-inducing activity and fewer gastric and cardiovascular side effects (such as ulcerations, stroke, and myocardial infarction) may represent a new class of compounds suitable for tumor prevention and chemotherapy (71,72,179,180).

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