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Novel sulindac derivatives for colorectal cancer chemoprevention that target cGMP phosphodiesterases to suppress Wnt/β-catenin transcriptional activity

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ABSTRACT

Approximately 28 million individuals in the United States face the risk of developing precancerous colonic adenomas (polyps) and potentially progressing to colorectal cancer (CRC). While a promising strategy for CRC prevention involves pharmacological intervention, such as cancer chemoprevention or interception, currently, there are no FDA-approved drugs capable of preventing the formation or progression of adenomas to adenocarcinoma. Numerous clinical, epidemiological, and preclinical studies have offered compelling evidence supporting the efficacy of nonsteroidal anti-inflammatory drugs (NSAIDs) in CRC chemoprevention. However, the prolonged use of NSAIDs is not FDA-approved due to potential life-threatening toxicities resulting from cyclooxygenase (COX) inhibition and the depletion of physiological prostaglandins. Despite indications that the COX inhibitory activity of NSAIDs may not be essential for their antineoplastic effects, the absence of a well-defined target impeded the development of derivatives that do not inhibit COX. Earlier research suggests that the inhibition of cyclic guanosine monophosphate phosphodiesterase (cGMP PDE) may be responsible, at least in part, for the antineoplastic activity of the NSAID sulindac. This could potentially offer a novel target for CRC chemoprevention. To identify the cGMP PDE isozyme(s) contributing to the antineoplastic activity of sulindac, we synthesized a chemically diverse library of over 1500 compounds, all sharing the indene scaffold of sulindac. Subsequently, we screened these compounds for their impact on cancer cell growth and PDE inhibitory activity. From this screening, a series of lead compounds emerged. These compounds lacked COX-1 and COX-2 inhibitory activity, surpassing sulindac in potency to inhibit CRC cell growth. Importantly, they demonstrated greater selectivity by not affecting normal cell growth. Through chemical optimization, we identified several development candidates that selectively inhibit PDE5 and/or PDE10. These compounds activate cGMP/PKG signaling, suppressing Wnt/β-catenin transcription. This action counters the growth advantages resulting from APC or CTNNB1 mutations, which are responsible for most human CRCs. This review delves into the scientific literature supporting PDE5 and/or PDE10 as potential targets for CRC chemoprevention or interception. Our findings suggest a promising avenue for developing drugs that may effectively intervene in the progression of colorectal cancer, offering hope for improved preventive strategies in the future.

KEYWORDS

cGMP; chemoprevention; colorectal cancer; cyclooxygenase; NSAIDs; phosphodiesterase; PKG; sulindac; Wnt; β-catenin

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1. Introduction

NSAIDs are among the most frequently prescribed drugs worldwide for the treatment of pain, fever, or inflammation. Epidemiologic studies have demonstrated that long-term use of NSAIDs, including aspirin and other non-prescription NSAIDs, can significantly reduce the risk of developing CRC and other cancers [1-6]. In addition, randomized clinical trials involving patients with a rare hereditary precancerous condition, familial adenomatous polyposis (FAP), demonstrated that two prescription NSAIDs, sulindac, a non-selective cyclooxygenase (COX) inhibitor [7-11], and celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor [12] could suppress the formation and/or cause the regression of precancerous adenomatous polyps. The cancer chemopreventive activity of NSAIDs and COX-2 inhibitors is also supported by multiple lines of evidence from preclinical studies using various experimental models.

Although NSAIDs have shown promising cancer chemopreventive activity, none are FDA-approved for long-term use because of potentially fatal toxicities associated with the inhibition of COX-1 and/or COX-2 enzymes that catalyze the conversion of arachidonic acid to prostaglandins and other eicosanoids. Prostaglandins are well known to have an essential role in normal physiological processes, including gastric protection and kidney function, but are also implicated in colon tumorigenesis by altering cell adhesion, impeding apoptosis, and promoting angiogenesis [13, 14]. In addition, prostaglandin levels are elevated in adenomas and adenocarcinomas [15] due to COX-2 overexpression, which supports the rationale to target COX-2 for CRC chemoprevention. The ApcΔ716 mouse model of CRC provides further evidence for the role of COX-2 in the development of intestinal adenomas because its adenomatous phenotype can be partially suppressed by COX-2 knockdown [16].

While the cancer chemopreventive activity of NSAIDs and COX-2 inhibitors is commonly attributed to COX-2 inhibition, multiple lines of evidence suggest that an off-target effect may partially or fully account for their antineoplastic activity. For example, dosages exceeding those required for COX inhibition are required to inhibit CRC cell growth. Perhaps the most compelling evidence for a COX-independent mechanism comes from studies reporting that the non-COX inhibitory sulfone metabolite of sulindac has cancer chemopreventive activity in multiple rodent models of chemical-induced carcinogenesis [10, 17-22]. Hence, in addition to COX-1 and COX-2, multiple other targets, along with accompanying signaling pathways, have been suggested to be responsible for the antineoplastic activity of NSAIDs and COX-2 inhibitors including cGMP PDE, RAS, PPARδ, and NF-ƙB to name a few [23-29]. This review focuses on cGMP PDE isozymes as novel targets for CRC, which, as illustrated in Figure 1 can be inhibited by sulindac, other NSAIDs, sulindac sulfone, and certain non-COX inhibitory derivatives to induce cGMP/PKG signaling. Activated PKG can phosphorylate β-catenin, triggering ubiquitination and proteasomal degradation of β-catenin to block TCF/LEF transcriptional activity, thereby countering the stabilization of β-catenin resulting from mutations in APC or β-catenin responsible for the majority of CRC cases[19, 30-33].

2. FAP and NSAIDs

FAP is an autosomal dominant syndrome, which is the second most common inherited CRC syndrome with an incidence of 1 in 10,000 [34] characterized by a high prevalence of adenomas or “polyps”. Clinical management of this disease involves frequent colonoscopies that are expensive, uncomfortable, and potentially fatal because of accidental perforation of the gastrointestinal tract. Surgical resection of the colon is often performed to prevent malignant progression in FAP patients at an early age. Pharmacological intervention has the potential to minimize the frequency of colonoscopies and delay surgical intervention. Medications effective for preventing colon adenomas would also be expected to benefit individuals with sporadic adenomatous polyposis and be particularly valuable in developing countries where screening by routine colonoscopy is not easily accessible. Based on experimental and clinical studies, the reduction of polyp burden by a cancer chemopreventive drug would also

reduce the risk of developing CRC in these high-risk populations.

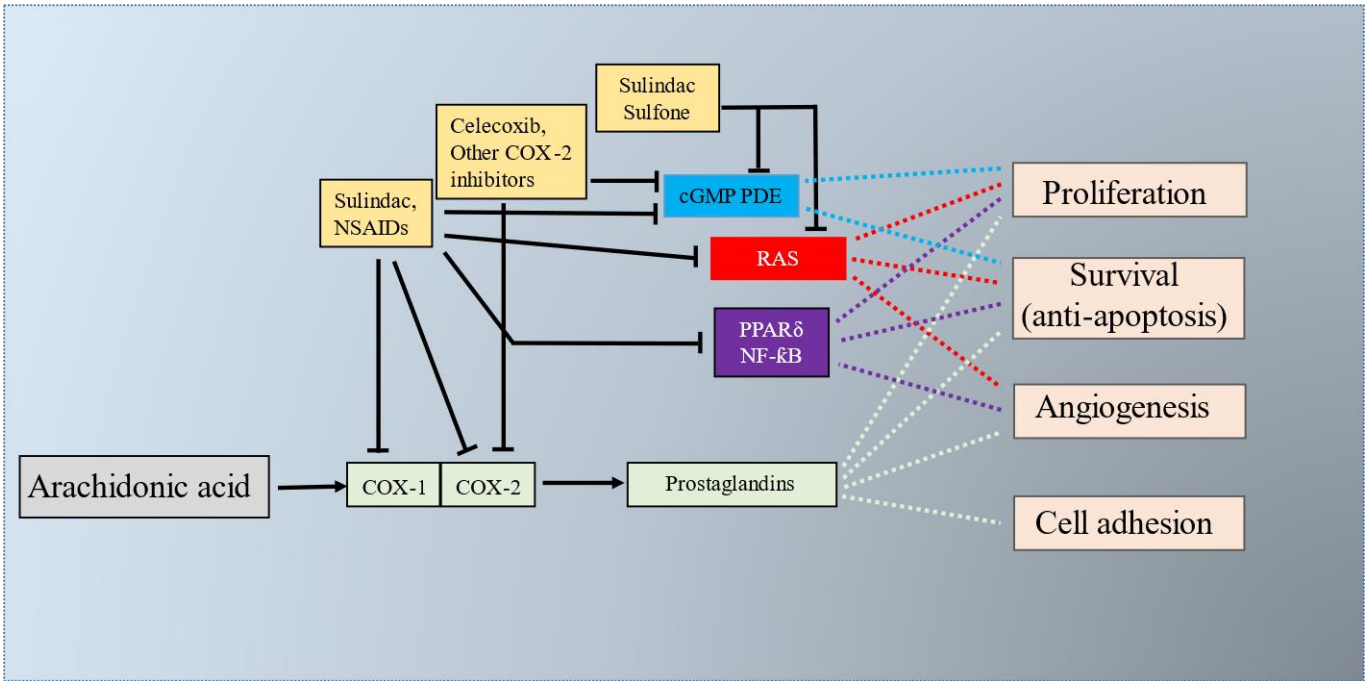

Figure 1. Potential COX-dependent and independent targets responsible for the cancer chemopreventive activity of NSAIDs, COX-2 inhibitors, sulindac sulfone, and derivatives. Blocking (indicated by ⊣ symbol) of molecular targets results in modulation (indicated by dashed lines **---**) of the pathologic and physiologic processes.

FAP is attributed to loss-of-function mutations in adenomatous polyposis coli (APC), a tumor suppressor gene, which plays a pivotal role in regulating β-catenin transcriptional activity. The stability of cytoplasmic β-catenin is controlled by a molecular complex commonly referred to as the "destruction complex" comprised of APC, axin, glycogen synthase kinase 3 (GSK3), and casein kinase 1 (CK1) (Figure 2) [35]. The complex serves to regulate cytoplasmic levels of β-catenin by ubiquitin-dependent proteasomal degradation. Loss of APC tumor suppressor function resulting from APC gene mutations stabilizes β-catenin and promotes translocation to the nucleus to activate TCF/LEF mediated transcription of multiple growth-promoting and survival genes resulting in dysplasia and the formation of adenomas [36, 37]. Additional mutations in genes encoding for p53 and K-RAS and other oncoproteins arise that provide proliferation and survival advantages to precancerous cells, which have the potential to advance from dysplasia to adenomas to adenocarcinoma [38].

Randomized controlled clinical trials have shown that sulindac can significantly reduce the number and size of colon adenomas in FAP patients [8]. The efficacy of celecoxib, a selective COX-2 inhibitor, was also demonstrated in FAP patients [12, 39, 40], although at dosages appreciably higher than sulindac. The FDA previously approved celecoxib for the treatment of FAP; however, the approval was later withdrawn because of the increased risk of mortality from cardiovascular toxicity associated with COX-2 inhibitors and other NSAIDs [41].

2.1. COX-independent antitumor activity of NSAIDs

COX-1 and COX-2 isoforms have similar enzymatic activity but differ in how their expression is regulated and in their sensitivity to inhibitors [42]. COX-1 is constitutively expressed in a wide range of tissues, while the expression of COX-2 is generally low in normal tissues but induced by cytokines or stress in various tissues. COX-1 maintains prostaglandin levels essential for various physiological processes, including gastric protection and kidney function. As such, the depletion of prostaglandins by non-selective COX inhibitors can cause ulceration of the gastric mucosa, leading to gastrointestinal perforation or vasoconstriction at the glomerular level to reduce renal plasma

flow, resulting in kidney damage. By contrast, COX-2 is an inducible enzyme in which its expression is regulated at the transcriptional level [43]. COX-2 expression can be induced by cytokines under inflammatory conditions and is elevated in cancer cells to promote tumor growth [44], inhibit apoptosis [45], and induce angiogenesis [13, 46]. Consequently, COX-2 was assumed to be an attractive target for inflammation and cancer prevention whereby COX-2 inhibitors would have selective anti-inflammatory and antitumor activities. However, clinical trials of the COX-2 inhibitor rofecoxib (Vioxx) revealed an unexpected risk of cardiovascular toxicity that led to its withdrawal from the market and increased scrutiny for the long-term use of other COX-2 inhibitors, as well as non-selective NSAIDs, for cancer chemoprevention [41, 47, 48].

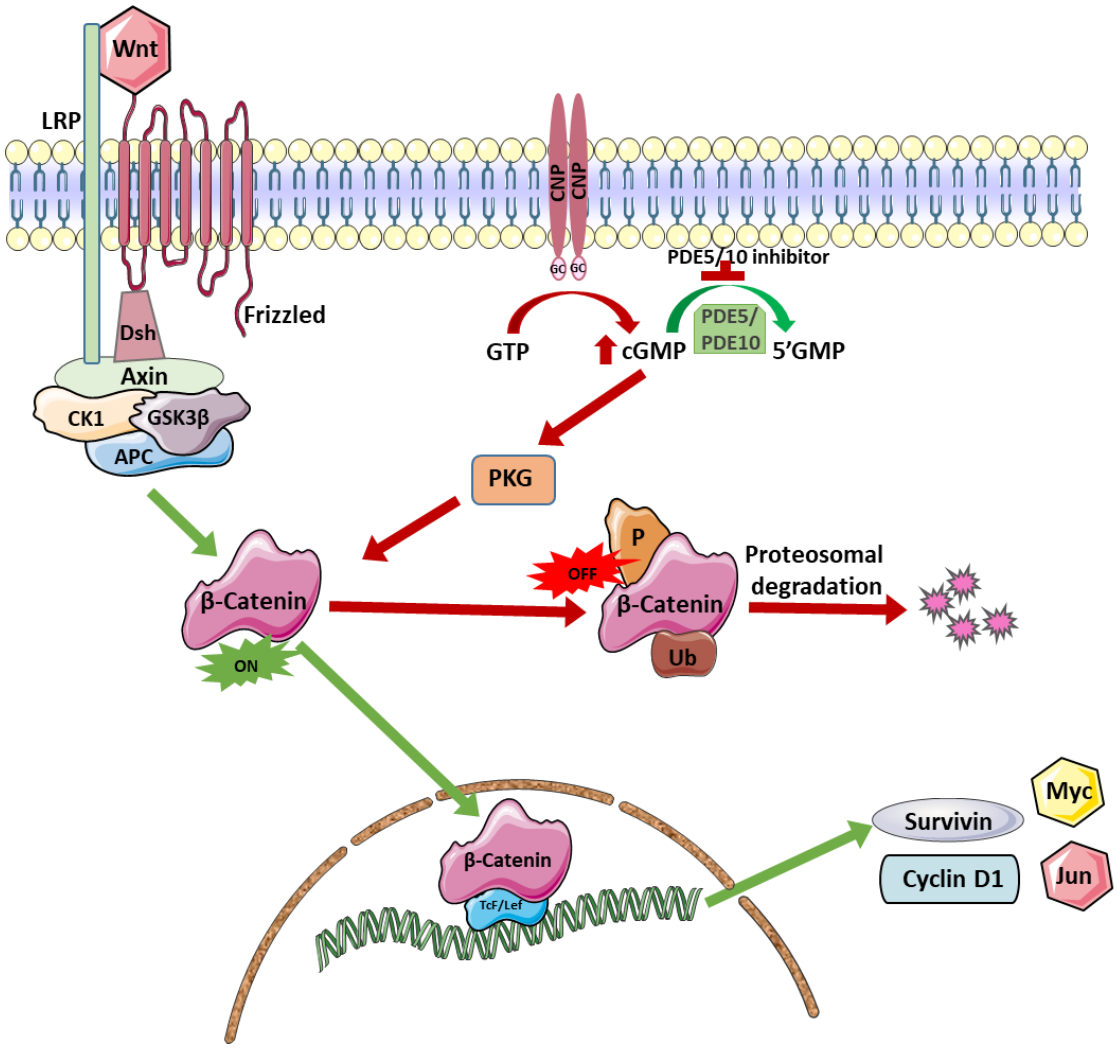

Figure 2. Loss of function mutations in the tumor suppressor gene, *APC,* or gain-in-function mutations in the oncogene, *CTNNB1* encode for proteins that result in the aberrant stabilization of β-catenin in the cytoplasm and translocation to the nucleus to activate TCF/LEF mediated transcription of proteins essential for colon tumorigenesis. PDE5 and PDE10 are overexpressed during the early stages of colon tumorigenesis and function to regulate intracellular cGMP levels, whereby low levels appear essential for the proliferation and survival of neoplastic cells. PDE5 and PDE10 inhibitors increase intracellular cGMP levels to activate PKG that can phosphorylate β-catenin on amino acid residues that induce ubiquitination and proteasomal degradation of β-catenin. Targeting PDE5 and/or PDE10 with non-COX inhibitory sulindac derivatives provides a strategy to develop drugs for CRC chemoprevention given that the proliferation and survival of neoplastic cells is dependent on TCF/LEF transcription factors that encode for oncogenic proteins such as cyclin D, survivin, Jun and Myc.

Elder and colleagues originally suggested evidence for a COX-2 independent mechanism responsible for the antineoplastic activity of COX-2 selective inhibitors, reporting that their apoptosis-inducing activity is unrelated to COX-2 expression levels [49]. Other investigators showed that supplementation with prostaglandins does not reverse the growth-inhibitory activity of NSAIDs [50, 51]. In addition, the rank order of potency among a series of chemically distinct NSAIDs and COX-2 inhibitors to inhibit CRC cell growth and COX-2 does not match: and that inhibition of CRC cell growth typically requires appreciably higher dosages than those required to inhibit prostaglandin synthesis [52-54].

Other investigators reported that sulindac sulfone, a non-COX inhibitory metabolite of sulindac, inhibits tumor formation in a variety of preclinical rodent models of chemical-induced carcinogenesis, providing additional evidence for a COX-independent mechanism of action [10, 17-22]. Because of reduced likelihood for gastrointestinal and renal toxicity resulting from the depletion of prostaglandins, sulindac sulfone (exisulind) was advanced to clinical trials in FAP patients by Cell Pathways Inc. Exisulind demonstrated promising inhibition or regression of colon adenomas in early clinical trials [55], but did not receive FDA approval because of hepatotoxicity observed in a Phase III registration trial in addition to a low response rate. A 12-month multicenter randomized double-blind placebo-controlled phase 3 dose-response study involving subjects with sporadic polyps also reported modest efficacy of exisulind by the decrease in median polyp size which was significantly greater in patients who received exisulind compared with those who received placebo. [56]. The limited efficacy of exisulind as observed in clinical trials, was likely attributable to the low potency [57]. A more potent analog of exisulind, CP-461, was later developed by Cell Pathways Inc., but poor oral bioavailability halted its clinical development [58]. Despite these failures, mechanistic studies of exisulind, CP-461, and other highly potent derivatives (e.g., CP-248) revealed a strong association between their growth inhibitory activity in CRC cells and the inhibition of cGMP PDE activity [32], although it was unclear which PDE isozyme(s) were required for colon cancer growth that might provide a vulnerability to selectively target for CRC chemoprevention.

2.2. Sulindac derivatives lacking COX-inhibitory activities

An initial attempt to develop novel sulindac derivatives involved molecular modeling using crystal structures of COX-1 and COX-2 to interfere with COX-1 and COX-2 inhibition. These studies revealed an essential role of the carboxylic acid moiety of sulindac sulfide to bind both COX-1 and COX-2, which guided the design of sulindac sulfide amide (SSA) that was appreciably more potent than sulindac sulfide to inhibit CRC growth [59]. The positively charged N, N-dimethyl ethyl ammonium moiety in SSA that replaced the negatively charged carboxylic acid moiety in sulindac was sufficient to eliminate both COX-1 and COX-2 inhibitory activity while improving potency to inhibit CRC cell growth. SSA was well tolerated and demonstrated in vivo antitumor activity in rodent models of colon, breast, and prostate cancer, although with modest potency that was attributed to limited oral bioavailability [59-61].

Further chemical optimization with adaptations and substitutions to different parts of the indene scaffold was performed to improve potency and oral bioavailability. This effort resulted in a custom library of over 1500 indenes that were screened for growth inhibition using human CRC cell lines HT29, HCT116, and SW480, while NCM-460 normal colon mucosa cells were used to assess cancer cell selectivity. Testing a wide range of concentrations allowed the measurement of accurate IC50 values to define specific chemical properties necessary for potency and cancer cell selectivity. Most of the compounds in the library were significantly more potent than sulindac sulfide with low micromolar IC50 values to inhibit growth but with poorly defined structure-activity relationships (SAR). However, a discrete set of compounds with well-defined SAR and high potency and selectivity for CRC cell growth inhibition were identified by counter–screening in enzymatic assays that evaluated cGMP PDE inhibitory activity using tumor cell lysates. Compounds of interest were further screened using recombinant PDE isozymes to assess isozyme

selectivity. This chemical-biology approach aimed to identify specific PDE isozymes that represent potential targets for CRC chemoprevention.

3. Targeting PDE5 and PDE10 for CRC chemoprevention

3.1. Role of PDE5 in regulating CRC growth

Previous studies suggested that cGMP PDE inhibition is closely associated with the activity of sulindac silfide and sulfone to inhibit CRC cell growth [32, 53, 62]. In these experiments, cancer cell growth measured using multiple cancer cell lines, while cGMP PDE enzymatic activity was measured using recombinant PDE isozymes. This methodology and other experimental procedures revealed a novel mechanism by which PDE5 inhibition can selectively inhibit CRC cell growth. As illustrated in Figure 2, guanylyl cyclases (GCs), which are widely distributed and activated in response to nitric oxide or natriuretic peptides from the tumor microenvironment, regulate physiologically cGMP levels in cells. The effects of endogenous GC activators can be augmented by cGMP PDE inhibition, thus increasing intracellular cGMP levels and PKG activation. The oncoprotein β-catenin appears to be an essential substrate of PKG. PKG-mediated phosphorylation of β-catenin can cause ubiquitination and proteasomal degradation of the "oncogenic" (nonphosphorylated, stable) pool of β-catenin and inhibit Wnt-induced Tcf/Lef transcription of genes involved in cancer cell survival and proliferation, including those encoding survivin and cyclin D. In addition to providing insight to the mechanism of growth suppression from PDE5 inhibition, these studies also revealed that cAMP PDE inhibitors/PKA activators do not appreciably affect CRC cell growth, while inhibitors of cGMP PDE isozymes could suppress CRC cell growth. PDE5 was implicated in the growth inhibitory activity of sulindac sulfide and sulfone based on observations showing overexpression in CRC cells compared to cells from normal colon mucosa [53, 63]. Sulindac sulfide also directly inhibited recombinant PDE5 at concentrations comparable to those that inhibit CRC cell growth [53, 64]. Furthermore, a novel sulindac derivative, sulindac benzylamine (SBA) was identified which lacked significant COX-1 and COX-2 inhibitory action but inhibited PDE5 and CRC cell growth, and, was able to suppress β-catenin transcriptional activity [63].

Consistent with the growth and PDE5 inhibitory activity of sulindac sulfide and SBA, genetic silencing of PDE5 in CRC cells was found to suppress CRC cell growth by activating cGMP/PKG signaling and inhibiting β-catenin transcriptional activity [63, 65]. In support of these observations implicating PDE5 as a novel anticancer target, other investigators reported that the highly specific PDE5 inhibitor, sildenafil, can inhibit CRC cell growth and tumor formation *in vitro* and *in vivo*, respectively, although at higher concentrations than those required to inhibit recombinant PDE5 [66]. The high dosages of sildenafil required to inhibit CRC cell growth may be attributed to efflux and low intracellular levels, given that PDE5 inhibitors can act as substrates for specific ABC transport proteins that may be overexpressed in cancer cells [67].

In support of the role of PDE5 in CRC, sildenafil was reported to inhibit inflammation-induced colon carcinogenesis in animal models by two independent groups using pharmacologically relevant concentrations [68, 69]. Recent epidemiological studies showing that PDE5 inhibitors can reduce the risk of developing CRC provides further evidence that PDE5 is an essential target for CRC chemoprevention [70]. Another consideration is the potential involvement of PDE5 in suppressing tumor immunity, as demonstrated by publications reporting that tadalafil, a PDE5 inhibitor, can activate mechanisms of antitumor immunity [64, 71-73]. Thus, PDE5 may be a promising molecular target for the chemoprevention of CRC, but further investigation is needed to understand better if the mechanism involves a direct effect on neoplastic cells or an indirect effect involving the activation of antitumor immunity.

3.2. Role of PDE10 in regulating CRC cell growth

PDE10 is a dual cAMP and cGMP degrading PDE isozyme that has limited expression in peripheral tissues compared with other PDE isozymes [74-76], but is highly expressed in the brain and localized in the medium spiny neurons of the striatum [76, 77]. PDE10 is known to regulate glutamatergic stimulation through the striatal pathway [78]; however, there is no known physiological role of PDE10 outside of the central nervous system. Preclinical studies showed that PDE10 inhibitors may affect cognition, behavior, and motor functions, as demonstrated in rodent models and genetic knockout experiments [79], and were developed for treating schizophrenia and Huntington's disease. PDE10 also has distinct kinetic properties from other PDE isozymes with higher affinity but lower Vmax for cAMP than cGMP [80].

Despite low expression in most normal peripheral tissues, PDE10 was reported to be overexpressed in colon adenomas, adenocarcinomas, and metastatic lesions compared to normal colonic mucosa [81-83]. An essential role of PDE10 in CRC cell growth was first reported by Li and colleagues [81]. For example, known highly specific PDE10 inhibitors were found to selectively inhibit the proliferation and induce apoptosis of CRC cells with reduced effects on the growth of normal colonocytes, which correlated with differences in PDE10 expression. Consistent with the effect of PDE10 inhibitors, PDE10 siRNA also selectivity inhibited growth and induced apoptosis of CRC cells with reduced effects on normal colonocytes. Conversely, ectopic expression of PDE10 in normal colonocytes or cells derived from colon adenomas increased the growth rate [81]. PDE10 inhibitors and siRNA knockdown of PDE10 also increased intracellular cGMP levels, activated PKG and suppressed β-catenin transcriptional activity in a manner as described above for PDE5 inhibitors.

A novel non-COX inhibitory derivative of sulindac with PDE10 isozyme selectivity, ADT-061, was developed that potently inhibits the growth of CRC cells expressing PDE10, but not the growth of normal colonocytes lacking PDE10 expression. PDE10 inhibition by ADT-061 was associated with the activation of cGMP/PKG signaling and the phosphorylation and degradation of β-catenin, thereby reducing nuclear levels of β-catenin and TCF/LEF transcriptional activity [84]. In addition to suppressing β-catenin transcriptional activity, PDE10 inhibitors have been reported to activate dendritic cells and increase T-cell tumor invasion, suggesting the potential to generate anticancer immune responses [85, 86]. ADT-061 (aka MCI-030) was also reported to inhibit the growth of ovarian cancer cells by suppressing Wnt/β-catenin and RAS/MAPK signaling [87], both of which may result from its PDE10 inhibitory activity as previously reported for known PDE10 inhibitors [82], possibly by a cGMP/PKG dependent mechanism to block RAS activation [88]. Alternatively, the inhibitory effect on RAS/MAPK signaling may result from a distinct effect on RAS, as suggested by previous studies of sulindac [19, 26].

3.3. Non-COX-inhibitory sulindac derivatives that inhibit both PDE5 and PDE10

Since CRC cells co-express PDE5 and PDE10 to regulate intracellular cGMP levels, dual PDE5/10 inhibitors would be expected to have more significant growth inhibitory activity than selective inhibitors of PDE5 or PDE10, given the possibility that the uninhibited isozyme remains active to diminish sensitivity to an isozyme specific inhibitor. The co-expression of PDE5 and PDE10 in CRC cells may also explain why high doses of the PDE5 selective inhibitor, sildenafil, are required to inhibit CRC cell growth *in vitro* and tumor growth in a subcutaneous mouse cancer model [66]. For example, uninhibited PDE10 could rapidly degrade cGMP that results from a PDE5 specific inhibitor, necessitating high dosages to non-selectively inhibit PDE10. Conversely, PDE5 co-expression with PDE10 may contribute to resistance to PDE10 selective inhibitors (e.g., Pf2545920), as uninhibited PDE5 could rapidly degrade cGMP generated by a particular PDE10 inhibitor. Supporting the possibility that a dual inhibitor might have advantages, we reported that genetic knockdown of both PDE5 and PDE10 in CRC cells or treatment with a dual PDE5/PDE10 inhibitor, ADT-094, resulted in more significant growth inhibitory activity compared with single

knockdown of PDE5 or PDE10 or selective PDE5 or PDE10 inhibitors [89]. The mechanism by which ADT-094 inhibits CRC cell growth involves binding to both PDE5 and PDE10 to activate cGMP/PKG signaling, which suppresses Wnt-induced β-catenin/Tcf-mediated transcriptional activity by phosphorylation of amino acid residues known to, induce ubiquitination and proteasomal degradation of β-catenin.

4. Conclusion

Multiple lines of evidence from preclinical, epidemiological, and clinical trials report that NSAIDs have robust antineoplastic activity, but their long-term use for cancer chemoprevention is not approved because of potentially fatal toxicities resulting from COX inhibition and suppression of physiological prostaglandins. While numerous investigators have concluded that the antineoplastic activity of sulindac and other NSAIDs is unrelated to their COX inhibitory activity, the potential to develop safer and more efficacious derivatives for CRC chemoprevention rests on identifying the underlying mechanism of action. This review focused on non-COX inhibitory sulindac derivatives that can selectively suppress the growth of CRC cells by inhibiting cGMP PDE isozymes, PDE5 or PDE10, resulting in the activation of cGMP/PKG signaling and suppression of Wnt/β-catenin transcriptional activity, to block the expression of proteins essential for the proliferation and survival of cancer cells.

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Conflict of interest

All the authors claim that the manuscript is entirely original. Drs. Chen, Keeton, and Piazza are co-founders and co-owners of ADT Pharmaceuticals LLC. All other authors declare no conflict of interest.

Author contributions

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