6-C-(E-phenylethenyl)-Naringenin Suppresses Colorectal Cancer Growth by Inhibiting Cyclooxygenase-1

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Abstract
Recent clinical trials raised concerns regarding the cardiovascular toxicity of selective cyclooxygenase-2 (COX-2) inhibitors and cyclooxygenase-1 (COX-1) is now being reconsidered as a target for chemoprevention. Our aims were to determine whether selective COX-1 inhibition could delay or prevent cancer development and also clarify the underlying mechanisms. Data clearly showed that COX-1 was required for maintenance of malignant characteristics of colon cancer cells or tumor promoter-induced transformation of preneoplastic cells. We also successfully applied a ligand-docking computational method to identify a novel selective COX-1 inhibitor, 6-C-(E-phenylethenyl)-naringenin (designated herein as 6CEPN). 6CEPN could bind to COX-1 and specifically inhibited its activity both in vitro and ex vivo. In colorectal cancer cells, it potently suppressed anchorage-independent growth by inhibiting COX-1 activity. 6CEPN also effectively suppressed tumor growth in a 28-day colon cancer xenograft model without any obvious systemic toxicity. Taken together, COX-1 plays a critical role in human colorectal carcinogenesis, and this specific COX-1 inhibitor merits further investigation as a potential preventive agent against colorectal cancer. Cancer Res; 74(1); 243–52. ©2013 AACR.

Introduction
Colorectal cancer is the third most common noncutaneous malignancy and also the third leading cause of cancer-related death in the United States (1). Fortunately, during colorectal carcinogenesis, the transition from normal mucosa to adenoma and final carcinoma is a protracted event that offers opportunities for preventive interventions. Chemoprevention by targeting cyclooxygenase-2 (COX-2) has been used successfully and seems to be a promising strategy for prevention of colorectal cancer (2–4). However, recent clinical trials raised concerns regarding the cardiovascular toxicity of selective COX-2 inhibitors (5, 6). Animal model studies further revealed that COX-2 plays a crucial role in cardioprotection (7–10), and thus persistent COX-2 inhibition might not be an ideal chemopreventive strategy.

Although evidence implicates a crucial role of COX-2 in colorectal cancer development, the idea that COX-2 is the only COX isofrom involved in carcinogenesis has been challenged. For example, aspirin at low doses (81 mg per day) is widely accepted to be able to provide both cardioprotective and colon cancer chemopreventive effects (11–13). However, pharmacokinetic data analysis revealed that low doses of aspirin mainly target COX-1 rather than COX-2 (14, 15). Genetic disruption of ptgs-1 [gene encoding for cyclooxygenase-1 (COX-1)] or ptgs-2 (gene encoding for COX-2) reduces intestinal polyposis to a similar extent (16, 17). Importantly, targeting COX-1 was effective in preventing not only colon cancer but also other tumor types such as skin cancer and ovarian cancer (17, 18). Deficiency of COX-1 reduced mouse skin tumorigenesis by 75%, whereas a COX-1 inhibitor (SC560) effectively attenuated epithelial ovarian tumor growth in multiple genetically engineered mouse models.

Thus, COX-1 is now being reconsidered as a target for chemoprevention (19, 20). To gain a deeper insight into the role of COX-1 in human cancer development, we used a ligand-docking computational method to identify a novel selective COX-1 inhibitor, 6-C-(E-phenylethenyl)-naringenin (i.e., 6CEPN). We then evaluated its chemopreventive activity against colon cancer both in vitro and in vivo.

Materials and Methods
Reagents and chemicals
6CEPN was chemically synthesized as described previously (21). CNBr-Sepharose 4B beads were purchased from Amersham Pharmacia Biotech. All primary antibodies were purchased from Cell Signaling Technology. All other chemicals were obtained from Sigma-Aldrich unless otherwise specified.

Cell culture
All cell lines used in this study were obtained from the American Type Culture Collection (ATCC) and maintained following ATCC instructions. Cells were cytogenetically tested and authenticated before being frozen. Each vial of frozen cells was thawed and maintained for a maximum of 20 passages.
Cell transfection

For either transient or lentiviral transfection in adherent cells, the jetPEI reagent (Qbiogen, Inc.) was used following the manufacturer's instructions. Full-length cDNAs for COX-1 and COX-2 (pCMV-SPORT6-COX-1 and pCMV-SPORT6-COX-2) and the 29-mer small hairpin RNA (shRNA) constructs against COX-1 and COX-2 were from Open Biosystems, Inc.

Cell growth assay

Cells were seeded (1 x 10^5 cells per well) in 96-well plates. After incubation for various times, 20 μL of CellTiter96 Aqueous One Solution (Invitrogen) were added. Cells were further incubated for 1 hour at 37°C. Finally, the optical density was determined at 492 nm.

Anchorage-independent cell growth

In each well of a 6-well plate, cells (8 x 10^3) were suspended in Basal Medium Eagle (BME) medium (1 mL, with 10% FBS and 0.33% agar) and plated over a layer of solidified BME (3 mL, with 10% FBS and 0.5% agar). The cultures were incubated in a 37°C, 5% CO₂ incubator for 7 days and colonies in soft agar were counted under a microscope equipped with the Image-Pro Plus software program (vs. 6; Media Cybernetics).

In vitro pull-down assay

Recombinant COX-1 and COX-2 (0.5 μg) or endogenous cell lysates (500 μg) were incubated with 6CEPN-Sepharose 4B beads (100 μL, 50% slurry) in reaction buffer (50 mmol/L Tris pH 7.5, 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 0.01% NP40, 2 μg/mL bovine serum albumin, 0.02 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml protease inhibitor mixture). Incubation with gentle rocking was performed overnight at 4°C. The beads were then washed a total of 5 times with washing buffer and proteins bound to the beads were analyzed by Western blotting.

In vitro COX enzyme assay

The effect of 6CEPN on COX activity was evaluated using a COX Inhibitor Screening Kit (Cayman Chemical Company) according to the manufacturer’s instructions.

Prostaglandin E₂ (PGE₂), thromboxane B₂ (TXB₂), and 6-keto prostaglandin F₁α (6-keto PGF₁α) determination

The measurement of prostaglandins in the cell-culture supernatant fraction was designated as serum. The targeted protein bands were visualized using an enhanced chemiluminescence reagent (Amersham Corporation) after hybridization with a secondary antibody conjugated with horseradish peroxidase.

Xenograft mouse model

Athymic mice [C57BL/6J (Lewis), NIH Swiss nude, 6- to 9-week old] were obtained from Charles River and maintained under "specific pathogen-free" conditions based on the guidelines established by the University of Minnesota Institutional Animal Care and Use Committee (Austin, MN). Mice were divided into 4 groups (n = 6 in each group). HT29 colon cancer cells (2 x 10^6 cells/100 μL) were suspended in serum-free McCoy's 5A medium and injected subcutaneously into the right flank of each mouse. 6CEPN dissolved in 5% (v/v) dimethyl sulfoxide (DMSO)/PEG400 was given to the mice by gavage every other day for total of 6 weeks. Tumor volume and body weight were measured every other day. Venous blood was collected from mice postmortem by suctioning from the right ventricle using a syringe containing sodium citrate. Blood samples were then centrifuged at 2,000 x g for 15 minutes, and the resulting supernatant fraction was designated as serum.

Molecular modeling

The three-dimensional structures of COX-1 and COX-2 were directly downloaded from the Protein Data Bank (PDB) for docking studies. COX-1 (PDB code 3KK6) is an X-ray diffraction structure with a resolution of 2.75 Å and COX-2 (PDB code 1PXX) is an X-ray diffraction structure with a resolution of 2.9 Å. The proteins were prepared for docking following the standard procedure outlined with the Protein Preparation Wizard in Schrödinger Suite 2012. All crystallographic waters were deleted and a 30-Å grid was generated on both COX-1 and COX-2 active sites to define the protein receptor for docking following the standard procedure outlined in Schrödinger's GLIDE docking package. The Zinc natural database, FDA-approved drug database, Traditional Chinese Medicine, a flavonoid compound database, and our in-house library of compounds were each used in the virtual screening.

Statistical analysis

All cell line experiments were performed independently at least 3 times. Statistical analysis was performed using the Prism statistical package. Turkey t test was used to compare data between 2 groups. One-way ANOVA and the Bonferroni correction were used to compare data between 3 or more groups. Values are expressed as means ± SEM unless otherwise indicated. A P value of < 0.05 was considered statistically significant.

Results

COX-1 is required for maintaining colorectal cancer cell malignant characteristics

To determine whether COX-1 is directly associated with the tumorigenic properties of colon cancer cells, we first examined COX expression in normal and colon cancer cells (Fig. 1A). Consistent with previous reports (22, 23), COX-1 is present both in normal and malignant colon cancer cells, whereas
COX-2 is overexpressed only in colorectal cancer cells. Among the cell lines tested, HT29, HCT115, and DLD1 cells expressed relatively higher COX-1 levels, and were therefore chosen for subsequent studies.

Anchorage-independent growth ability is an ex vivo indicator and a key characteristic of the transformed cell phenotype (24). We thus questioned whether COX-1 inhibition would affect colon cancer cell growth under anchorage-independent conditions. Our results revealed that knocking down COX-1 or COX-2 in colon cancer cells was analyzed by Western blot. Mock and knockdown cells were then subjected to anchorage-dependent growth, anchorage-independent growth, and PGE2 production assays as described in Materials and Methods. Cell growth was evaluated by MTS assay. Data are presented as means ± SEM (n = 4). Anchorage-independent cell growth was evaluated by colony formation in soft agar. Data are presented as means ± SEM from 3 independent experiments. Production of PGE2 in supernatant fractions was measured by ELISA. Data are presented as means ± SEM (n = 4). ***, significant (P < 0.001) difference compared with Mock group.

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We also compared the function of COX-1 with COX-2 in human colon cancer cells. Our results revealed that knocking down the expression of either COX-1 or COX-2 delayed cell growth, reduced the number of colonies formed in soft agar, and decreased PGE2 production in HT29 cells (Fig. 1C). Consistent with our findings, a previous animal study also indicated that both COX-1 and COX-2 contribute to PGE2 production in polyp formation (16).
Involvement of COX-1 in neoplastic transformation

Based on the finding showing that knockdown of COX-1 greatly abrogated anchorage-independent cell growth, we hypothesized that COX-1 might also be involved in neoplastic transformation. The JB6 CI41 cell model is a promotion sensitive (P+)-mouse epidermal skin cell line that provides a unique cell model to characterize the role of COX-1 in preneoplastic cells (25, 26). We established 2 stable JB6 CI41 clones that express an shRNA-targeting mouse COX-1 (Fig. 2A), and then tested the effects of COX-1 inhibition on tumor promoter (TPA or EGF)–induced cell transformation. Results indicated that either EGF- or TPA-induced cell transformation was markedly attenuated by knockdown of COX-1 (Fig. 2B). Additional results indicated that with EGF stimulation, the epidermal growth factor receptor (EGFR) downstream signaling cascades were substantially suppressed in the absence of COX-1 (Fig. 2C).

Taken together, these findings indicated that COX-1 is required for both the maintenance of cancer cell malignant characteristics and neoplastic transformation.

The predicted binding mode of 6CEPN with COX-1

Although COX-1 is now being reconsidered as a target for colorectal cancer chemoprevention, only a few selective COX-1 inhibitors have been found (19). To identify a novel potent selective COX-1 inhibitor, we conducted an intensive molecular-docking analysis using Glide v5.7 (16). We screened several libraries of compounds and 6CEPN (Fig. 3A) was identified as a potential selective COX-1 inhibitor based on its docking scores against COX-1 and COX-2, respectively. Our computational modeling data clearly showed that 6CEPN could only bind to the COX-1 active site by forming 3 hydrogen bonds with Tyr355, Phe518, and Ser530 (Fig. 3B). This compound failed to bind to the COX-2 catalytic pocket because of a potential steric-hindrance effect—a phenomenon in which the enzyme is inaccessible to substrates with an improper molecular size as well as shape (Fig. 3C, left). However, even though 6CEPN failed to occupy the active pocket of COX-2, it still might bind to COX-2 in another region (i.e., His 207 and His388; Fig. 3C, right).

To validate the computational prediction, we performed an in vitro pull-down assay using 6CEPN-conjugated Sepharose 4B beads (Fig. 3D). Results revealed that both recombinant COX-1 and COX-2 bind with 6CEPN-Sepharose 4B beads, but not with Sepharose 4B beads alone in vitro. We then examined the potential inhibitory effect of 6CEPN against COX-1 and COX-2 enzyme activity using a COX Inhibitor Screening Assay Kit. Our data confirmed that 6CEPN selectively inhibited COX-1, but not COX-2, activity in vitro (Fig. 3E). These results clearly support our hypothesis that 6CEPN is a selective COX-1 inhibitor.

6CEPN suppresses human colorectal cancer cell growth

Next, we determined whether 6CEPN could selectively inhibit COX-1 activity in cells. Human embryonic kidney HEK293T cells were transiently transfected with a COX-1 or COX-2 plasmid (Fig. 4A, left) and then treated with 6CEPN. Data regarding PGE2 release in supernatant fractions clearly indicated that 6CEPN selectively inhibited COX-1 activity rather than COX-2, especially at low doses (Fig. 4A, right). We also confirmed that 6CEPN binds to endogenous COX-1 (Fig. 4B).
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4B, left) and lowers PGE₂ production in colon cancer cells (Fig. 4B, right). Moreover, 6CEPN potently inhibited anchorage-independent growth in a dose-dependent manner (Fig. 4C) in 3 colon cancer cell lines. 6CEPN at 2.5 or 5 μmol/L caused a decrease of more than 70% to 90% compared with untreated control HT29 cells, which highly express COX-1.

Evaluation of cardiovascular toxicity of 6CEPN

Next, we evaluated the potential cardiovascular toxicity of 6CEPN in an in vitro model using human umbilical vein endothelial cells (HUVEC). Although undetectable under normal physiological conditions, COX-2 is markedly induced and exerts a cardiac protective role under pathophysiological conditions such as during cardiac ischemia or reperfusion injury. The imbalance between COX-1–derived prothrombotic thromboxane A₂ (TXA₂) and COX-2–related antithrombotic prostacyclin (PGI₂) production is suspected to contribute to the cardiovascular side effects of selective COX-2 inhibitors. In addition, the ratio of TXB₂ (i.e., the stable breakdown product of TXA₂) to 6-keto-PGF₁α (i.e., the hydrolysis product of PGI₂)
has been used as one of the biomarkers for COX-2 inhibition-related cardiovascular toxicity (7, 9, 10, 19, 20, 27, 28). To mimic proinflammatory conditions, HUVECs were treated with IL-1β, an inflammatory cytokine implicated in vascular diseases. IL-1β stimulation resulted in a remarkable increase in COX-2 expression as well as 6-keto-PGF1α synthesis (Fig. 5A). 6CPEN, but not celecoxib, had a modest but significant inhibitory effect on TXB2 synthesis (Fig. 5B, middle). In contrast, celecoxib, but not 6CPEN, could potently suppress 6-keto-PGF1α synthesis (Fig. 5B, lower). More importantly, the ratio of TXB2/6-keto-PGF1α was significantly increased by the selective COX-2 inhibitor celecoxib even at very low doses (e.g., 0.1 µmol/L), but only weakly disturbed by 6CPEN (Fig. 5B, upper), suggesting that cardiovascular toxicity is caused by celecoxib but not by 6CPEN.

6CPEN suppresses tumor growth by inhibiting COX-1 activity in vivo

Based on both in vitro and ex vivo data, we determined whether 6CPEN could suppress tumor growth in vivo. Results (Fig. 6A) indicated that 28 days of continuous 6CPEN treatment (10 or 40 mg/kg body weight) by gavage significantly reduced tumor volume by 31% or 59%, respectively. Similar inhibitory effects were observed on final tumor mass (Fig. 6B). Classic COX-1 inhibitors such as aspirin and ibuprofen are known to exert their cardioprotective activity by inhibiting COX-1 activity, but the cardioprotective effects of 6CPEN are distinct from those of aspirin and ibuprofen.
platelet COX-1 activity, resulting in decreased synthesis of TXA2, but not PGI2 (13, 19, 27, 28). Similar results were obtained with 6CEPN treatment in this study (Fig. 6C). Importantly, the ratio of TXB2 to 6-keto-PGF1α was greatly attenuated by continuous 6CEPN treatment, suggesting that 6CEPN might provide cardioprotective effects. Moreover, based on body weight data (Fig. 6D), general appearance, and organ histology, 6CEPN was well tolerated in mice and no obvious systemic toxicity (e.g., diarrhea or bleeding in the digestive tract) was observed during the entire period of drug treatment.

Discussion

In this study, we confirmed a critical role for COX-1 in colorectal cancer. Phenotypically, COX-1 knockdown or catalytic inactivation in colon cancer cells resulted in an obvious reduction of malignant characteristics, including anchorage-dependent and -independent cell growth as well as in the production of endogenous PGE2. Importantly, COX-1 was also required for genotoxic carcinogen-induced malignant transformation in preneoplastic cells. All of these findings provided an explanation as to why genetic disruption of ptgs-1 reduces cancer incidence both in skin and colon.

Although more attention has been given to COX-2 as a key player in the development of various cancers, accumulating evidence indicates that COX-1 is equally as important as COX-2 for carcinogenesis, especially in skin and colon (16, 17). The immediate-early phase of prostaglandin production is reportedly mediated by constitutive expression of COX-1, whereas...
Figure 6. Chemopreventive activity of 6CEPN in an HT29 colon cancer xenograft model. A, effect of 6CEPN on tumor growth. B, effect of 6CEPN on tumor mass. C, effect of continuous 6CEPN treatment on the ratio of TXB₂ to 6-keto-PGF₁α. D, effect of 6CEPN on body weight of mice. Chemopreventive activity of 6CEPN was evaluated in a 28-day colon cancer xenograft model. Before cell injection, mice were pretreated with 6CEPN for 14 days. 6CEPN dissolved in 5% (v/v) dimethyl sulfoxide/PEG400 was given to the mice by gavage every other day for a total of 42 days. Data are presented as means ± SEM (n = 6 mice). The asterisks indicate a significant (*, P < 0.05; **, P < 0.001) difference compared with vehicle control group.
the later phase of prostaglandin production is dependent on the induction of COX-2 (29). However, PGE₂ can transactivate the EGFR kinase cascade in colon cancer cells, which is dependent on the extracellular release of an EGF-like ligand (30), whereas activation of EGFR might conversely stimulate COX-2 biosynthesis (31). Overall, our data suggest the possibility that both COX-1 and COX-2 contribute to colon cancer development by cooperating with the EGFR signaling pathway, which modulates tumorigenesis through multiple biological effects including anchorage-independent cell growth (32). Therefore, although COX-1 is generally described as constitutively expressed both in malignant and normal colon tissues, this might be an oversimplification because the COX enzymes are known to function in the production of prostaglandins, which is either because of increased protein expression, catalytic activity, or both (27).

In this study, we successfully applied a ligand-docking computational method and discovered a novel selective COX-1 inhibitor (6CEPN). Notably, 6CEPN, chemically seems to be a natural product-based compound, a hybrid molecule of stilbene with a flavonoid structure. Compared with other known COX-1 inhibitors, it has a unique carbon skeleton that might present a new leading COX-1 inhibitor (19, 27, 33). To identify compounds that show higher selective COX-1 inhibition, more structure-activity studies are needed.

Another question to be addressed is whether selective COX-1 inhibition causes gastrointestinal toxicity. Conventional NSAIDs are known to normally produce much stronger gastrointestinal toxicity than selective COX-2 inhibitors. Considering its high expression in the gastrointestinal tract, COX-1 is commonly believed to protect the gastrointestinal tract, and thus selective COX-1 inhibition is still a controversial issue (19, 20, 27). However, no direct evidence exists to support selective inhibition of COX-1 as the cause of gastrointestinal side effects. Notably, homozygous p53/– (genes coding for COX-1) mutant mice do not exhibit gastric lesions even though their PGE₂ production in the gastrointestinal tract is just 1% that of wild-type mice (16). Pharmacological inhibition of COX further suggested that inhibition of both COX-1 and COX-2 was required for NSAIDs-induced gastrointestinal toxicity. In the Wistar rat, neither a selective COX-1 inhibitor (SC560) nor a COX-2 inhibitor (celecoxib) could cause obvious gastric damage. However, their combination results in severe gastrointestinal side effects (34). In this study, our COX-1 inhibitor, 6CEPN, was also well tolerated in mice, and no obvious gastrointestinal toxicity (e.g., diarrhea and bleeding in digestive tract) was observed during the entire period of drug treatment.

Although our findings in this study are promising, several significant questions remain unanswered. For example, whether the inhibitory potential of 6CEPN in vivo was because of a direct suppression of COX-1 activity (not COX-2) is still unclear. Addressing this question is challenging with current technologies because COX-1 and COX-2 normally share the same substrate (arachidonic acid) and yield the same product (PGH₂) in vivo. Thus, differentiating between their respective activities is not possible at this time. Another issue is the relevance of the JB6 cell model to colon carcinogenesis. JB6 CH1 cells are a promotion sensitive (P–) mouse epidermal cell line. This cell line enables the study of genetic susceptibility to promotion of transformation, and thus might provide a unique cell model to characterize activated COX-1 in preneoplastic cells. TXA₂ production in vivo is known to be associated with COX-1. 6CEPN could markedly lower the serum levels of TXA₂ in mice. However, we did not collect pharmacokinetic data in this study, and therefore whether 6CEPN reached tumors directly or only inhibited COX-1 activity pharmacologically is still unknown.

In summary, this study was conducted to characterize the role of COX-1 in colorectal cancer and evaluate the clinical potential of a selective COX-1 inhibitor in chemoprevention. This work together with previous studies by others should provide insight into the potential application of selective COX-1 inhibitors in colorectal cancer chemoprevention (16, 17, 19, 34, 35).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: F. Zhu, H. Chen, Z. Dong
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Li, F. Zhu, K.W. Cheng, N. Oi, R.A. Lubet, Z. Dong
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