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CG100649, a novel COX-2 inhibitor, inhibits colorectal adenoma and tumor growth in mouse models

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Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) and selective cyclooxygenase-2 (COX-2) inhibitors (COXIBs) can reduce the risk of developing colorectal cancer (CRC) and are being considered for use as adjuvant therapy for treatment of CRC patients. However, long-term use of most NSAIDs, except aspirin, increases cardiovascular risk, hampering use of these drugs in CRC prevention and possibly for treatment. CG100649 is a new member of the COXIB family, which is proposed to inhibit both COX-2 and carbonic anhydrase-I/-II (CA-I/-II) activity. Using mouse models, we show here that CG100649 inhibits premalignant and malignant colorectal lesions in mouse models, partly through inhibiting tumor cell proliferation. These pre-clinical findings suggest a need for further exploration of CG100649 for CRC prevention and treatment. The long-term safety profile of CG100649, particularly regarding its effect on cardiovascular risk, is yet to be determined.

Keywords

colorectal cancer; COX-2; CG100649; celecoxib; PGE₂; carbonic anhydrase-I/-II

Introduction

The link between inflammation and cancer has been investigated for decades, leading to the recent addition of inflammation as an enabling characteristic facilitating acquisition of the hallmarks of cancer [1]. Colorectal cancer (CRC) in particular is associated with a pro-inflammatory environment [2]. It is well recognized that a significant risk factor for CRC includes a personal history of inflammatory bowel disease (IBD). The link between inflammation and cancer was the foundation for testing nonsteroidal anti-inflammatory drugs (NSAIDs) for their efficacy in preventing and/or treating CRC. One of mechanisms by

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Conflict of Interest

All authors declare that they have no conflict of interest.

which NSAIDs exert their antitumor effect is through inhibition of cyclooxygenase-2 (COX-2). Three large randomized controlled trials (RCTs), namely APC, APPROVe and PreSAP, have clearly shown that COX-2 selective inhibitors (COXIBs) reduce the risk of adenoma recurrence in patients with a recent history of colorectal adenomas or CRC [3-5]. The importance of NSAIDs in CRC treatment was also demonstrated in an observational study showing that regular aspirin use after the diagnosis of CRC at stages I-III improves overall survival, especially among individuals with tumors that overexpress COX-2 [6]. Another observational study showed that aspirin could reduce the incidence of COX-2 overexpressing CRCs, but not those with a weak or absent expression of COX-2 [7]. Our group was the first to demonstrate the presence of COX-2 expression in colorectal adenomas and carcinomas [8]. Now it has been well established that COX-2 plays a major role in inflammation and cancer [9]. However, COXIB use is associated with an increased risk of cardiovascular and thrombotic events, including myocardial infarctions and ischemic cerebrovascular events, shown in 2 out of 4 of the clinical trials [10,11], particularly for patients with preexisting atherosclerotic heart disease [12]. One theory indicates that the deleterious cardiovascular side effects are attributed to suppression of COX-2-dependent production of the cardioprotective prostaglandin I₂ (PGI₂ or prostacyclin) [13-15]. However, recent evidence indicates that non-selective NSAIDs have equivalent or worse cardiovascular side effects [16], bringing into question the PGI₂ theory. Another, less harmful, side effect of COXIB use is a mild elevation in blood pressure (BP) [17], which may be also clinically significant in relation to increased cardiovascular risk. This COXIB-related elevation in BP may be linked to suppression of PGI₂ as well [15]. Therefore, a clinical need exists for COXIBs with fewer cardiovascular side effects. CG100649 is a novel member of the COXIB family, which is proposed to inhibit both COX-2 and carbonic anhydrase-I/-II (CA-I/-II) (Fig. 1). The postulated inhibition of CA-I/-II is hypothesized to maintain BP at base-line levels, thereby possibly reducing cardiovascular risk. In terms of anti-inflammatory and analgesic effects, a recent short-term phase IIb clinical trial demonstrated non-inferiority of CG100649 compared to celecoxib in treating osteoarthritis patients, while there were no indications that CG100649 had a negative impact on heart function, ECG profiles, or blood pressure (www.cgxinc.com). Notably, a recent study addressed the impact of CG100649 on prostanoid biosynthesis and CA-I/-II function using healthy volunteers. This study showed that CG100649 also suppresses PGI₂, in an even more sustained pattern than that of celecoxib [18]. In addition, no signs of CA-I/-II inhibition were detected in the CG100649-treated group [18]. The purpose of the present study was to evaluate the efficacy of CG100649 in inhibiting colonic adenomas and carcinomas using mouse models.

Material and Methods

Cell culture

Colon cancer cell lines, HT-29 and HCA-7, were maintained in McCoy's 5A medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin in a 5% CO₂ atmosphere.

COX-1 and COX-2 activity assays and prostaglandin E₂ (PGE₂) measurement

To determine the specificity of CG100649 (CrystalGenomics, Korea) on cyclooxygenase-1 (COX-1) or COX-2 enzymatic activity *in vitro*, the production of PGE₂ was measured using cloned COX-1 (ovine) or COX-2 (human recombinant) enzymes (Cayman Chemical Co.). Briefly, CG100649 was incubated for 10 minutes prior to addition of 50 μ M arachidonic acid with enzymes (15 U) in 0.1 M Tris-HCl buffer, pH 8.0, containing 5 mM EDTA, 2 mM phenol, and 1 μ M hematin. The reaction was stopped by addition of 1N citric acid. PGE₂ was then extracted and analyzed as previously described [19]. To measure the effect of CG100649 on COX-2 activity in cells, HT-29 or HCA-7 cells (1×10^6) were plated in 100 mm culture dishes in complete McCoy's 5A medium and were allowed to attach overnight. Cells were then treated with various concentrations of CG100649 for 24 hours. Culture medium was then collected and subjected to PGE₂ measurement.

Animal experiments

For the premalignant mouse model, C57BL/6J-*Apc*^{Min/+} male mice were obtained from Jackson Laboratories at five weeks of age. Mice were housed and fed with standard mouse diet. At the age of 6 or 12 weeks, mice were randomly grouped to receive either vehicle or 7 mg/kg of CG100649. After 8 weeks of treatment, mice were sacrificed and entire intestinal tissues were flushed with ice-cold phosphate-buffered saline (PBS) and filled with 10% neutral buffered formalin for 24 hours. Intestines were transferred to 70% ethanol for 24 hours, opened longitudinally, and examined under a dissecting microscope. Polyp number and size were measured using Living Image software version 3.0.

For the subcutaneous xenograft mouse model, HCA-7 cells (2×10^6 , dissolved in 100 μ l) were injected subcutaneously into the dorsal flanks of athymic nude mice (Harlan Laboratories). Tumor size was measured starting from 13 to 28 days after injection using a digital caliper. After tumors were established, mice were randomized into four different treatment groups (vehicle, 7 mg/kg or 15 mg/kg of CG100649, and 500 mg/kg of celecoxib).

For the orthotopic xenograft mouse model, luciferase labeled HT-29 (HT-29-Luc) cells (0.5×10^6 , dissolved in 30 μ l) were injected into the cecal wall of athymic *nu/nu* mice. Fourteen days post-injection mice were divided to the same treatment groups as in the xenograft model experiment, and treated for 8 weeks. Tumor volume was measured using the Xenogen IVIS 200 bioluminescence imaging system (Caliper Life Sciences).

All mice were housed and treated in accordance with protocols approved by the Institutional Animal Care and Use Committee at The University of Texas MD Anderson Cancer Center.

Treatment regimen

CG100649 was given daily by oral gavage, at 7 mg/kg or 15 mg/kg concentrations (obtained from CrystalGenomics, Inc., Seoul, Korea), dissolved in 0.5% methylcellulose. Celecoxib was mixed with the diet at the concentration of 500 mg/kg and given *ad libitum*.

Proliferation assay

Assessment of proliferation in the epithelial cells of the intestinal mucosa was performed using the dual-targeted immunofluorescence tyramide signal amplification (TSA) kit (Perkin Elmer, Boston, MA), enabling simultaneous detection of 5-bromo-2-deoxyuridine (BrdU) and E-cadherin, coupled with 4',6-diamidino-2-phenylindole (DAPI) staining of the nuclei. For BrdU staining, mice were injected with BrdU solution (BD Pharmingen) 2 hours before sacrifice. Sections were incubated with biotinylated anti-BrdU primary antibody (1:50; BD cat# 51-75512L), followed by staining with the ABC staining kit (Vector Laboratories). For E-cadherin staining, sections were incubated with mouse anti-E-cadherin primary antibody (1:1000; BD cat# 610958), followed by incubation with the Alexa Fluor 594 anti-mouse secondary antibody (Invitrogen).

Statistical analysis

Statistical significance was determined using Student's t-test or two-way analysis of variance (ANOVA), where applicable. $p < 0.05$ (*) and $p < 0.01$ (**) were considered statistically significant. For analyzing the data of the tumor volume of subcutaneous xenograft mouse model, ANOVA was performed with repeated measures. The within subject factor was time and the between subject factor was group. Post-hoc analysis was Tukey's Method for Multiple comparisons.

Results

CG100649 inhibits COX-2 activity and PGE₂ production in human colon cancer cell lines

To examine CG100649 inhibition of COX-2, we performed *in vitro* COX-1 and COX-2 activity assays. CG100649 inhibits COX-2 activity with an IC₅₀ around 0.1 $\mu\text{g/ml}$, whereas a higher concentration was needed to suppress COX-1 activity (Fig. 2a), demonstrating CG100649 is a COX-2 selective inhibitor. Next, we compared the inhibitory potency of CG100649 and celecoxib in HCA-7 and HT-29 colon carcinoma cell lines, which both express COX-2 protein [20,21]. CG100649 at a concentration of 0.01 $\mu\text{g/ml}$ suppressed over 95% of PGE₂ production in HCA-7 cells, compared to only 61% inhibition by celecoxib at the same concentration (Fig. 2b) indicating a difference in potency. Similarly, in HT-29 cells, CG100649 and celecoxib at a concentration of 0.001 $\mu\text{g/ml}$ inhibited 90% and 45% of PGE₂ production, respectively (Fig. 2c). These results show that CG100649 can inhibit COX-2 activity and PGE₂ production in human colon cancer cells, at lower concentrations compared to celecoxib.

CG100649 suppresses intestinal polyp formation in *Apc*^{Min/+} mice

We utilized the *Apc*^{Min/+} mouse model to determine efficacy in polyp reduction, which spontaneously develops intestinal polyps, caused by a germ-line mutation in the adenomatous polyposis coli (*Apc*) gene [22]. *APC* is a key tumor-suppressor gene mutated in familial adenomatous polyposis (FAP) syndrome, as well as in the vast majority of sporadic CRC cases. Since polyp formation in the *Apc*^{Min/+} mouse model starts around the age of 8 weeks, we initiated treatment with CG100649 at either 6 or 12 weeks of age, to assess its role in prevention and in treatment of intestinal polyps. Treatment of CG100649

effectively reduced polyp number and size in both the small and large intestine (Fig. 3a, b) in a prevention study. In treatment experiments, CG100649 significantly suppressed established polyp growth in the small intestine (Fig. 3c) and reduced polyp growth in the colon, as evident by the reduction in number of polyps >2 mm (Fig. 3d). However, no effect was noted on total number of polyps in the colon (Fig. 3d). These results indicate that CG100649 can be used effectively for prevention and treatment of polyps in *Apc*^{Min/+} mice.

CG100649 suppresses human colorectal carcinoma growth in subcutaneous and orthotopic xenograft mouse models

To further examine the possible use of CG100649 in treating colon carcinomas, we carried out studies in subcutaneous and orthotopic xenograft mouse models. Treatment of either CG100649 or celecoxib reduced subcutaneous tumor growth (Fig. 4a, b). CG100649 reduced tumor volume and tumor weight by 58% and 48%, respectively, compared to a 48% and 36% reduction following treatment with celecoxib (Fig. 4a, b). Similarly, CG100649 inhibited CRC growth in an orthotopic xenograft mouse model, reducing tumor weight by 70% using 7 mg/kg or by 83% using 15 mg/kg, compared to a similar 70% reduction following treatment with 500 mg/kg of celecoxib (Fig. 4c, d). Taken together, these results indicate that CG100649 can be used to treat both premalignant (polyps) and malignant colonic lesions in mouse models.

CG100649 exerts its *in vivo* antitumor effect partly via inhibiting proliferation

To further delineate how CG100649 exerts its antitumor effect, we evaluated cell proliferation following CG100649 treatment in *Apc*^{Min/+} mice and orthotopic xenograft mouse models. Treatment with CG100649 of *Apc*^{Min/+} mice and nude mice orthotopically injected with HT-29 cells resulted in a significant decrease in numbers of BrdU-stained cells compared to the control group, indicating an inhibition of proliferation (Fig. 5a, b). Inhibition of proliferation occurred mainly in epithelial cells, as evident by the co-staining with the E-cadherin epithelial cell marker (Fig. 5b). Collectively, these results show for the first time that CG100649 can effectively inhibit premalignant and malignant colonic lesions in mouse models by, at least in part, inhibiting tumor cell proliferation.

Discussion

Selective COX-2 inhibitors have been shown to be efficient in preventing colonic adenomas [3-5], but their use is currently not recommended due to increased cardiovascular risk in a small subset of patients [10,11]. In an attempt to avoid the harmful side effects while still preserving the beneficial effect of COXIBs, several studies were aimed at identifying the specific population that will be at risk due to COXIB use, such as those with preexisting atherosclerotic disease [12] or those with high levels of C-reactive protein [23]. Simultaneously, other research has focused on downstream targets of COX-2, mainly PGE₂, hoping to achieve the same clinical benefit with fewer side effects [9,24]. In addition, efforts have been undertaken to develop novel COXIBs with fewer side effects. One such attempt is the development of CG100649, a new member of the COXIB family, which is proposed to inhibit both COX-2 and CA-I/-II. In this study we show that CG100649 can attenuate colonic adenomas and carcinomas in mouse models, at least in part due to decreased

proliferation. Our results suggest a possible use of CG100649 in treatment and prevention of CRC.

CG100649 is suggested to be more potent than celecoxib in inhibiting COX-2 activity *in vitro*, in HCA-7 and HT-29 CRC cells, as determined by PGE₂ levels (Fig. 2b, c). CG100649 resulted in inhibition of COX-2 activity in CRC cells at concentrations 10-1000 fold lower than that of celecoxib, while their molecular weight is similar (361.38 for CG100649, 381.38 for celecoxib).

CG100649 is proposed to bind mainly to CA-I/-II, and not COX-2, in the cardiovascular and gastrointestinal systems, according to unpublished preclinical studies, performed by the manufacturer of the drug (CrystalGenomics, Inc.; www.cgxinc.com). Here we show a clear inhibitory effect of CG100649 on premalignant and malignant lesions in the gastrointestinal tract, presumably through inhibition of the COX-2 pathway. This might be explained by the different patterns of expression of COX-2 and CA-I/-II in normal vs. tumor tissues. COX-2 is upregulated in approximately 50% of adenomas and 85% of carcinomas, compared to normal colon tissue [8,25-27]. On the contrary, CA-II is downregulated in colonic polyps compared to normal colon tissue [28]. Therefore, the shift in balance towards COX-2 expression occurring in colonic lesions may explain the therapeutic effect on these lesions seen by CG100649 administration if CA-II is shown to be a target *in vivo*.

In summary, our findings show that CG100649 effectively inhibits colonic adenomas and carcinomas *in vivo*, partly through attenuating proliferation. Therefore, CG100649 has some potential for further investigation as an agent for use in CRC prevention and/or treatment. However, additional studies are needed to examine the safety profile of CG100649, mainly with regard to cardiovascular-related risk. As we know from previous studies, the safety profile of selective COX-2 inhibitors must be studied over several years of exposure to the drug on a continuous basis to understand the real toxicity.

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Figure 1.
The chemical structure of CG100649

Figure 2. CG100649 inhibits COX-2 activity *in vitro* and *in vivo*

a the inhibitory effect of CG100649 and celecoxib on COX-1 and COX-2 activity was determined by *In vitro* enzyme activity assay. **b, c** HCA-7 and HT-29 cells were treated with different concentrations of CG100649 and celecoxib. 24 hours after treatment, PGE₂ levels were measured in the culture media by mass spectrometry.

Figure 3. CG100649-induced inhibition of intestinal polyps in *Apc*^{Min/+} mice

Apc^{Min/+} mice were treated with 7 mg/kg of CG100649 from 6 to 14 weeks of age (**a, b**), or from 12 to 20 weeks of age (**c, d**). Polyp numbers in small intestine and colon are shown, subdivided by polyp size.

Figure 4. CG100649 inhibits CRC growth in subcutaneous and orthotopic xenograft mouse models

a HCA-7 cells were injected subcutaneously into the flank of *nu/nu* mice. Mice were randomized into the following treatment groups: control, CG100649-7 (7mg/kg), CG100649-15 (15mg/kg) or celecoxib (500 mg/kg). Mice were treated from day 27 post-injection to day 111 and tumor size was measured with an external caliper from day 20 to day 111. ANOVA and Tukey's test showed a statistically significant difference between either the CG100649 groups versus the control group ($p < 0.05$). **b** tumor weight at the end of the experiment. **c** HT-29-Luc cells were injected into the cecal wall of *nu/nu* mice and treatment began on day 14 and continued for 8 weeks. Tumor size was estimated based on bioluminescence signal intensity. **d** tumor weight at the end of the experiment.

Figure 5. CG100649 effect on cellular proliferation *in vivo*

a *Apc*^{Min/+} mice were treated with 7 mg/kg of CG100649 (CG) from 12 to 20 weeks of age. Small intestines were harvested and stained for BrdU (Brown). Shown are two representative sections (out of five specimens examined). **b** HT-29-Luc cells were injected into the cecal wall of *nu/nu* mice and treatment with 7 mg/kg of CG100649 (CG) began on day 14 and was continued for 8 weeks. Tumors were harvested and co-immunostained for BrdU (green) and E-cadherin (red). Nuclei were stained with DAPI (blue).