Published in final edited form as:

Cancer Res. 2009 February 15; 69(4): 1643–1650. doi:10.1158/0008-5472.CAN-08-3187.

Protein Kinase C β Is an Effective Target for Chemoprevention of Colon Cancer

Alan P. Fields¹, Shelly R. Calcagno¹, Murli Krishna², Sofija Rak¹, Michael Leitges³, and Nicole R. Murray¹

¹Department of Cancer Biology, Mayo Clinic College of Medicine, Jacksonville, Florida

²Department of Pathology, Mayo Clinic College of Medicine, Jacksonville, Florida

³Department of Biotechnology Centre of Oslo, University of Oslo, Oslo, Norway

Abstract

Colon cancer develops over a period of 10 to 15 years, providing a window of opportunity for chemoprevention and early intervention. However, few molecular targets for effective colon cancer chemoprevention have been characterized and validated. Protein kinase C β II (PKC β II) plays a requisite role in the initiation of colon carcinogenesis in a preclinical mouse model by promoting proliferation and increased β -catenin accumulation. In this study, we test the hypothesis that PKC β II is an effective target for colon cancer chemoprevention using enzastaurin (LY317615), a PKC β -selective inhibitor, in a mouse model of colon carcinogenesis. We find that enzastaurin potently reduces azoxymethane-induced colon tumor initiation and progression by inhibiting PKC β II-mediated tumor cell proliferation and β -catenin accumulation. Biochemically, enzastaurin reduces expression of the PKC β II- and β -catenin/T-cell factor-regulated genes *PKC β II*, *cyclooxygenase II*, and *vascular endothelial growth factor*, three genes implicated in colon carcinogenesis. Our results show that enzastaurin is an effective chemopreventive agent in a mouse model of sporadic colon cancer that significantly reduces both tumor initiation and progression by inhibiting expression of proliferative genes. Thus, PKC β II is an important target for colon cancer chemoprevention and the PKC β -selective inhibitor enzastaurin may represent an effective chemopreventive agent in patients at high risk for colon cancer.

Introduction

Colon cancer is one of the leading causes of cancer-related deaths in the United States (1). Numerous molecular alterations in colon cancer have been identified, providing potential targets for colon cancer therapy (2). However, few molecularly targeted therapies have progressed to clinical trials for the treatment of colon cancer. The time course of colon cancer development (10-15 years), coupled with the relative accessibility of the organ to serial observation and sampling via endoscopy, has allowed the identification and characterization of precursor lesions that can be monitored to evaluate the effects of molecularly targeted, early interventional therapy. For these reasons, colon cancer is well suited for implementation of chemoprevention strategies.

Requests for reprints: Nicole R. Murray, Department of Cancer Biology, Mayo Clinic College of Medicine-Jacksonville, Room 213, Griffin Cancer Building, 4500 San Pablo Road, Jacksonville, FL 32224. Phone: 904-953-6108; Fax: 904-953-6233; E-mail: murray.nicole@mayo.edu.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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

Our previous work showed that PKC β II plays a critical role in colon carcinogenesis (3-5). PKC β II mRNA abundance and protein expression are highly induced in aberrant crypt foci and subsequent colon tumors in the well-characterized azoxymethane mouse model of sporadic colon carcinogenesis (3). Transgenic mice expressing elevated PKC β II in the colonic epithelium (transgenic PKC β II mice) are more susceptible to azoxymethane-induced colon cancer than their nontransgenic littermates (5), whereas PKC $\beta^{-/-}$ mice are resistant to azoxymethane-mediated colon carcinogenesis (4,6), demonstrating the requisite role of PKC β II in colon carcinogenesis.

Molecular and biochemical analysis of transgenic PKC β II mice revealed that their cancer-prone phenotype results from PKC β II-mediated hyperproliferation of the colonic epithelium, as characterized by an increased proliferative index and resultant hypercellularity of colonic crypts (5,7). This hyperproliferation is mediated by reduced glycogen synthase kinase 3- β (GSK-3 β) activity and increased β -catenin expression in the colonic epithelium of transgenic PKC β II mice (5). Thus, PKC β II induces colonic epithelial hyperproliferation and enhanced susceptibility to colon carcinogenesis likely through activation of a PKC β II/ β -catenin/T-cell factor (TCF; Wnt signaling) axis (5). Interestingly, we have shown that dietary ω -3 fatty acids mediate their chemopreventive effects through inhibition of PKC β II-mediated hyperproliferation in the colonic epithelium (7). These studies define a procarcinogenic role for PKC β II in the early stages of colon carcinogenesis and directly implicate PKC β II as a target for chemoprevention.

Enzastaurin (LY317615), a macrocyclic bisinolyimide, is an ATP-competitive inhibitor of serine/threonine kinases with high selectivity for PKC β (8). Enzastaurin has been shown to inhibit proliferation and induce apoptosis in cancer cell lines and human xenograft tumors (8-10). Other preclinical studies showed that oral administration of enzastaurin decreases plasma vascular endothelial cell growth factor (VEGF) levels and intratumoral blood vessel formation (8,11). Enzastaurin was found to be well tolerated with few toxic side effects in a phase I study (12), and encouraging results have been obtained in phase II studies of enzastaurin in patients with diffuse large B-cell lymphomas, relapsed/refractory mantle cell lymphoma, and high-grade gliomas (13-15). Although enzastaurin has shown promising results as a chemotherapeutic in clinical trials, it has not been evaluated in a chemoprevention setting.

In this study, we tested the hypothesis that PKC β II is an effective target for colon cancer chemoprevention using enzastaurin. We find that enzastaurin inhibits azoxymethane-induced colon tumor initiation and progression and suppresses tumor cell proliferation. This is likely due to repression of expression of several PKC β II/ β -catenin-regulated, proliferative genes. Our data show that PKC β II is an effective target for colon cancer chemoprevention and that enzastaurin may be useful in a chemopreventive setting in high-risk colon cancer patients.

Materials and Methods

Mice

Female FVB/N mice were obtained from The Jackson Laboratory. PKC $\beta^{-/-}$ mice on a C57Bl/6 background (16) and control nontransgenic C57Bl/6 mice (originally purchased from The Jackson Laboratory) were used for analysis of colonic epithelial cell proliferation. All animals were housed in microisolator cages in a pathogen-free barrier facility and maintained at a constant temperature and humidity on a 12-h light/12-h dark cycle with free access to food and filtered water. All of the animal experiments and procedures performed in this study were approved by the Mayo Institutional Animal Care and Use Committee.

Enzastaurin administration and tissue isolation

Mice were fed pelleted, control diet (AIN-76A), or control diet with increasing concentrations of enzastaurin *ad libitum* throughout the experiments. Food consumption was monitored by weighing food upon addition to cage and at removal of unconsumed diet (twice weekly). All defined animal diet used in these studies was prepared by Research Diets, Inc. After being fed experimental diets for 2 wk, mice were euthanized by CO₂ asphyxiation. All mice were harvested between 9:00 and 11:00 a.m. to reduce diurnal variations. Colons were excised from cecum to rectum, flushed with cold PBS, and slit longitudinally. Colon tissue (1.5 cm) was isolated from the distal and proximal ends of the colon and fixed in 10% buffered formalin for histology. After 4 h, the colons were washed in cold PBS and stored in 70% ethanol at 4°C until processing for histology. Purified colonic crypts were isolated from the remaining colon using our previously characterized isolation procedure (4).

Plasma collection and analysis of enzastaurin concentration

At the time of harvest, blood was isolated by cardiac puncture into heparinized tubes. Blood was kept on ice until centrifuged at 2,000 rpm for 15 min at 4°C, and then the plasma was transferred to a separate tube and stored at -20°C until liquid chromatography/tandem mass spectrometry analysis of enzastaurin concentration.

Carcinogenesis protocol

Female FVB/N mice (6 wk old) were enrolled in our previously described carcinogen protocol (3). One week before azoxymethane injections, mice were randomly assigned to control (AIN-76A) or enzastaurin-containing (AIN-76A with 0.272% enzastaurin) diets (30 and 33 mice, respectively). Azoxymethane was purchased from the National Cancer Institute's Chemical Carcinogen Reference Standards Repository (operated under contract by Midwest Research Institute, no. N02-CB-07008). Mice were injected i.p. with 10 mg/kg azoxymethane once a week for 4 wk. All mice were sacrificed 22 wk after the last azoxymethane injection. Colons were excised from cecum to rectum, flushed with cold PBS, slit longitudinally, and fixed flat in 10% buffered formalin. The left kidney and liver were removed, weighed, and fixed in 10% buffered formalin for histologic analysis.

Tumor analysis and image capture

Fixed colons were stained briefly with 0.5% methylene blue and evaluated for the presence of colon tumors. The location and size of each colon lesion was recorded. Tumors were isolated, along with adjacent normal epithelium, and processed for histopathologic examination that was performed by a board-certified pathologist who was blinded to the treatment status of the mice. All stained slides were scanned using the T2 ScanScope console (Aperio Technologies) and images were captured using Aperio ImageScope software.

Proliferation, apoptosis, and angiogenesis

One hour before harvest, mice were injected i.p. with 50 µg/kg 5' bromodeoxyuridine (BrdUrd). Formalin-fixed colonic epithelium was analyzed for proliferation as determined by detection of incorporation of BrdUrd (7,17). Images of slides of colon tumors stained to detect BrdUrd incorporation were captured using the T2 ScanScope console (Aperio Technologies). Automated proliferative analysis was performed by quantitating BrdUrd-positive cells using an automated thresholding algorithm specific for nuclear staining (provided with the ImageScope analysis software; ref. 18). The proliferative index was calculated for each colon tumor as the ratio of BrdUrd-positive nuclei to the sum of all nuclei. A minimum of 2,000 nuclei were evaluated for BrdUrd staining in each tumor.

Apoptosis was detected in azoxymethane-induced tumors by TdT-mediated dUTP-biotin nick end labeling (TUNEL) of fragmented DNA in formalin-fixed colon tumors using the DeadEnd Colorimetric TUNEL System (Promega). Using the Aperio algorithm for nuclear staining, the apoptotic index was calculated as the ratio of TUNEL-positive nuclei to the sum of all nuclei.

Angiogenesis was characterized in azoxymethane-induced tumors by quantitative analysis of immunohistochemical detection of CD31 (PECAM-1) expression using an anti-CD31 antibody (Santa Cruz Biotechnology, Inc.) as previously described (19,20). Using the Aperio image analysis software, an algorithm for positive pixel labeling was performed in which the relative area of blood vessels was calculated as the ratio of CD31-positive pixels to the sum of all pixels.

Immunoblot and quantitative reverse transcription-mediated real-time PCR analysis

Purified colonic epithelial cells were isolated as colonic crypts and subjected to immunoblot analysis for PKC β II, GSK-3 β , phospho-GSK-3 β ^{Ser9}, extracellular signal-regulated kinase 1/2 (ERK1/2), and phospho-ERK1/2 using antibodies purchased from Santa Cruz Biotechnology as previously described (4,17,21). Densitometric analysis of immunoblots was performed using the Kodak Molecular Imaging System.

Total RNA was isolated from purified colonic epithelial cells and subjected to quantitative reverse transcription-mediated real-time PCR (qPCR) analysis as previously described (4). All primers and probes were purchased from Applied Biosystems. Data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA abundance to control for RNA concentration.

Immunohistochemical detection of β -catenin

Formalin-fixed control and enzastaurin-treated colon tumors of all histologic grades were analyzed for β -catenin expression and localization by immunohistochemical analysis using an antibody specific for β -catenin (BD Transduction Laboratories) and DAKO Envision Dual Link + detection system (DAKO). Tissues were counterstained with hematoxylin. Azoxymethane-induced colon tumors were scored on a scale of 0 to 4 based on the percent of tumor cells that exhibited predominantly cytoplasmic and nuclear localization of β -catenin (0 = none, 1 = 0-25%, 2 = 26-50%, 3 = 51-75%, and 4 = 76-100%). This analysis was performed by an investigator blinded to the treatment status of the sample (A.P.F.).

Statistical analysis

Fisher's exact analysis was used to compare tumor incidence and distribution of tumor stage between treatment groups. One-way ANOVA was used to compare the means of tumor volume, tumor burden, and average tumor number (per tumor-bearing mouse) between experimental groups. ANOVA analysis was used to compare the means of the BrdUrd labeling index, apoptotic index, CD31 staining, β -catenin mislocalization score, and mRNA abundance between experimental groups.

Results and Discussion

Establishing a physiologically relevant dose of enzastaurin

Published pharmacologic studies predicted that steady-state plasma concentrations of enzastaurin would be achieved within 2 weeks of daily oral administration (22). Therefore, mice were fed a pelleted, purified rodent diet (AIN-76A, control diet) supplemented with 0.034%, 0.068%, 0.136%, and 0.272% enzastaurin by weight for 2 weeks. Food consumption and enzastaurin exposure were determined as described in Materials and Methods (Supplementary Table). Enzastaurin plasma concentrations were analyzed by liquid chromatography/tandem mass spectrometry (Supplementary Table). The highest dose of

enzastaurin (0.272% by weight) yielded an average plasma concentration of 4.8 ± 2.5 $\mu\text{mol/L}$ (Supplementary Table), which is similar to the plasma concentration of enzastaurin that inhibits xenograft tumor formation in mice (8). Therefore, we chose to administer enzastaurin at this concentration to evaluate its chemopreventive effects in azoxymethane-induced tumorigenesis.

Enzastaurin inhibits azoxymethane-induced tumor initiation and progression

To assess the efficacy of enzastaurin as a chemopreventive agent, mice were given either control diet or control diet supplemented with 0.272% enzastaurin beginning 1 week before carcinogen administration (see Supplementary Fig. S1 for timeline). The mice were maintained on these diets until 22 weeks after the last azoxymethane injection, at which time the mice were euthanized and evaluated for tumor formation. Enzastaurin treatment had no detectable toxicity at the dose administered, as determined by a lack of significant effect on mouse weight gain (Fig. 1A), survival (26 of 30 for control versus 32 of 33 for enzastaurin treatment), or liver and kidney histology (data not shown).

Eighty percent (21 of 26) of control mice developed colon tumors during the experimental period, whereas only 50% (16 of 32) of the enzastaurin-treated mice developed colon tumors (Fig. 1B). Enzastaurin treatment also caused a decrease in tumor volume and tumor burden; however, these effects did not reach statistical significance (Table 1). To assess the effect of enzastaurin on tumor progression, colon tumors were characterized as low-grade adenoma, high-grade adenoma, or carcinoma (23). The majority of the tumors from mice in both treatment groups were characterized as low-grade adenomas (Fig. 1C and D). However, enzastaurin treatment resulted in a significant increase in the percentage of tumors that were low-grade adenoma (88%, versus 61% in the control diet group, $P = 0.041$; Fig. 1C). A trend toward decreased average tumor number per tumor-bearing mouse (tumor multiplicity) was also observed in enzastaurin-treated mice (Table 1). Moreover, when average tumor number was evaluated based on histologic grade, a statistically significant decrease in average tumor number of high-grade adenomas and carcinomas was observed in enzastaurin-treated mice (Table 1). Taken together, these data show that enzastaurin inhibits both colon tumor initiation and progression, consistent with the requirement for PKC β II for tumor formation (4,6).

Enzastaurin administration inhibits tumor cell proliferation

Transgenic PKC β II mice exhibit hyperproliferation of the colonic epithelium and increased susceptibility to colon carcinogenesis, indicating that PKC β II drives carcinogenesis via hyperproliferation (4,5). Therefore, we evaluated the effect of enzastaurin on colon tumor cell proliferation (Fig. 2). Enzastaurin significantly reduced cellular proliferation in azoxymethane-induced tumors (Fig. 2A and B). In contrast, enzastaurin had no significant effect on basal proliferation of mouse colonic epithelial cells (Fig. 2C). Likewise, genetic knockout of PKC β did not significantly alter basal colonic epithelial cell proliferation (Fig. 2D). Therefore, enzastaurin selectively inhibits tumor cell proliferation, but not proliferation of nontransformed colonic epithelial cells, consistent with a mechanism of action involving PKC β II inhibition, because PKC β II overexpression in the colonic epithelium promotes hyperproliferation (5) but inhibition of PKC β expression does not inhibit proliferation of nontransformed colonic epithelial cells (Fig. 2D). Selective inhibition of tumor cell proliferation suggests that enzastaurin would be an effective chemopreventive agent.

Enzastaurin has been reported to induce apoptosis in human tumor cell lines (8,9) and inhibit angiogenesis in xenograft tumors (10,24). We evaluated the effect of enzastaurin on tumor cell apoptosis and angiogenesis. Enzastaurin treatment did not significantly alter TUNEL labeling (apoptosis) or CD31 expression (angiogenesis) in azoxymethane-induced colon tumors (Fig. 3). The lack of detectable effect of enzastaurin on colon tumor apoptosis may be due to the inherent differences in susceptibility to apoptosis of tumor cell lines *in vitro* and in the xenograft

tumor models, compared with endogenous colon tumors developing *in situ* (8-10). Our data indicate that the major mechanism by which enzastaurin prevents tumor formation and progression is by inhibiting tumor cell proliferation.

Enzastaurin blocks PKC β II-mediated signaling in the colonic epithelium

PKC β II promotes proliferation and susceptibility to carcinogenesis by regulating expression of pro-proliferative genes in mouse colonic epithelium (4,25). Among the targets of PKC β II is PKC β II itself, which is up-regulated through an autocrine mechanism *in vitro* and in the colonic epithelium *in vivo* (4). Because PKC β II regulates its own expression, we assessed whether enzastaurin-mediated inhibition of PKC β II leads to reduced PKC β II expression in the colonic epithelium, as a specific measure of inhibition of PKC β II signaling. As expected, enzastaurin dramatically decreased expression of PKC β II mRNA (Fig. 4A) and protein (Fig. 4B) in the colonic epithelium, indicating that enzastaurin inhibits PKC β II-mediated signaling in the colonic epithelium.

We next evaluated the effect of enzastaurin on PKC β II-driven oncogenic pathways in the colonic epithelium. We have previously shown that PKC β II activates two signaling pathways critical to colon cancer development, the APC/ β -catenin/TCF (5) and Ras-PKC α /Rac1-Mek (26) pathways. Aberrant activation of the APC/ β -catenin/TCF signaling pathway occurs in a majority of both mouse and human colon tumors, resulting in the stabilization and nuclear accumulation of β -catenin (27,28). GSK-3 β is a key negative regulator of APC/ β -catenin/TCF signaling (29). Over-expression of PKC β II in mouse colonic epithelium decreases GSK-3 β activity *in vivo* (5) and PKC β directly phosphorylates and inhibits GSK-3 β *in vitro* (30). Enzastaurin has been previously shown to reduce GSK-3 β ^{ser9} phosphorylation in cancer cells *in vitro* and in xenograft tumors in mice (8-10). Thus, we assessed the status of GSK-3 β phosphorylation in the colonic epithelium of enzastaurin-treated mice. We found that enzastaurin significantly reduced GSK-3 β ^{ser9} phosphorylation in the colonic epithelium without altering the expression of GSK-3 β (Fig. 4C and D).

GSK-3 β mediates its negative effect on APC/ β -catenin/TCF signaling by phosphorylating β -catenin and targeting it for ubiquitination and subsequent degradation (29). Overexpression of PKC β II in the colonic epithelium drives increased expression of β -catenin, likely through inhibition of GSK-3 β (5). Because PKC β II overexpression increases β -catenin in the colonic epithelium (5), we predicted that enzastaurin treatment would reduce β -catenin expression. Therefore, we assessed the effect of enzastaurin on the expression and subcellular localization of β -catenin (Fig. 5A). β -Catenin exhibits highly restricted localization at the basolateral membrane of normal intestinal epithelial cells (Fig. 5A, *top images*). Enzastaurin treatment had no significant effect on the expression or subcellular localization of β -catenin in the normal colonic epithelium (Fig. 5A, *top images*). Azoxymethane-induced colon tumors exhibited substantial elevation of β -catenin expression (Fig. 5A, *bottom left*). This increase in β -catenin expression was accompanied by a redistribution of β -catenin to both the cytoplasm and nucleus (Fig. 5A, *bottom left*). In contrast, tumors from enzastaurin-treated mice expressed lower levels of β -catenin than tumors from control mice, with most tumor cells exhibiting primarily membrane localization of β -catenin (Fig. 5A, *bottom right*). A significant reduction in nuclear and cytoplasmic β -catenin was observed in tumors from enzastaurin-treated mice (2.1 ± 1.2 for enzastaurin-treated tumors versus 2.8 ± 1.1 for control tumors, $P = 0.045$; see Materials and Methods for a detailed description of the analysis). The reduction in β -catenin mislocalization was most dramatic in low-grade adenomas from enzastaurin-treated mice (1.5 ± 0.5 versus 3.3 ± 1.0 for control tumors $P = 5.45 \times 10^{-5}$). The level of mislocalized β -catenin in high-grade adenomas (3.3 ± 1.5 for enzastaurin-treated tumors versus 2.5 ± 1.1 for control tumors) and carcinomas (3.0 ± 1.4 for enzastaurin-treated tumors versus 2.6 ± 1.1 for control tumors) was not significantly altered by enzastaurin treatment. The lack of an observed effect

of enzastaurin on β -catenin mislocalization in higher-grade tumors may be due to the lower number of tumors available for analysis (see Table 1) or may reflect the fact that the likely target of enzastaurin, PKC β II, plays a critical role in the early stages of colon carcinogenesis, whereas genetic mutations acquired later in the carcinogenic process, including those in APC or β -catenin, may overcome the requirement for PKC β II in colon carcinogenesis. Genetic knockout of PKC β has no significant effect on intestinal tumorigenesis in *Apc*^{Min/+} mice, supporting this conclusion (6). Because PKC β II expression in the colonic epithelium is required for azoxymethane-induced colon carcinogenesis (4,6) and PKC β II overexpression induces β -catenin accumulation in the colonic epithelium (5), our current data suggest that enzastaurin blocks tumor proliferation by inhibiting PKC β II-mediated inhibition of GSK-3 β , thereby preventing stabilization of β -catenin and transcriptional up-regulation of proproliferative genes.

A second procarcinogenic signaling pathway is frequently activated in colon cancer through mutational activation of the *K-ras* proto-oncogene (31,32). Oncogenic *K-ras* promotes hyperproliferation in the colonic epithelium through Mek activation (33). PKC β II has been implicated as a regulator of K-ras signaling *in vitro* (26). We therefore evaluated the effect of enzastaurin treatment on the activation status of the downstream effector of the Ras-Mek pathway, ERK1/2. Whereas some variability within the treatment groups was observed (Fig. 4C and D), no significant difference was detected in the level of ERK1/2 phosphorylation in control and enzastaurin-treated colonic epithelium (Fig. 4C and D). These data suggest that enzastaurin does not significantly alter Mek-ERK signaling.

Enzastaurin inhibits expression of proproliferative, procarcinogenic genes

VEGF-A is a transcriptional target of β -catenin/TCF known to promote tumor growth and metastasis by stimulating endothelial cell proliferation and migration necessary for angiogenesis (34,35). A relationship between PKC β II and VEGF expression has clearly been established in the kidney, as well as in tumor xenograft models (11,36,37). Genetic knockout of *PKC β* , as well as pharmacologic inhibition, using the PKC β inhibitor, ruboxistaurin, blocked VEGF-A expression in the mouse kidney (11,36,37). However, the effect of enzastaurin on VEGF-A expression in the colon has not been evaluated. We assessed VEGF-A expression in the colonic epithelial cells from enzastaurin-treated mice. Enzastaurin significantly reduced the expression of VEGF-A mRNA in mouse colonic epithelial cells *in vivo* (Fig. 5B). Despite a significant decrease in VEGF-A expression in the colonic epithelial cells, we did not observe inhibition of tumor angiogenesis (as measured by CD31 staining) by enzastaurin in azoxymethane-induced colon tumors (Fig. 3B). Although VEGF promotes angiogenesis through its effects on endothelial cells, colon cancer cells as well as human tumors also express VEGFR (38,39). Likewise, *in vitro* evidence suggests that VEGF promotes colon cancer cell proliferation (38). Therefore, another possible mechanism of enzastaurin-mediated inhibition of proliferation of azoxymethane-induced colon tumors may be the reduced expression of a tumor-specific autocrine growth factor (VEGF).

Cyclooxygenase 2 (Cox-2) expression is induced in human colon tumors and is a prognostic indicator in colorectal cancer (40). Cox-2 is expressed at a very low level in normal mouse epithelium and is significantly increased in azoxymethane-induced colon tumors in rodents (41,42). Cancer-prone transgenic PKC β II mice exhibit increased Cox-2 expression in the colonic epithelium similar to levels that occurs in early colon carcinogenesis (3,25). Therefore, we evaluated the effect of enzastaurin treatment on Cox-2 expression in the colonic epithelium. Enzastaurin significantly repressed Cox-2 expression (Fig. 5C). Pharmacologic inhibition of Cox-2 also significantly reduces azoxymethane-induced tumorigenesis in rodents (42,43), suggesting that enzastaurin-mediated repression of Cox-2 expression may be critical to its inhibition of azoxymethane-induced tumor proliferation.

The ability of enzastaurin to repress Cox-2 expression is of particular interest in the context of colon cancer chemoprevention because clinical and epidemiologic studies have shown the efficacy of Cox-2 inhibitors [primarily nonsteroidal anti-inflammatory drugs (NSAID)] in the prevention of human colon cancer (reviewed in ref. 44). However, recent reports of cardiovascular complications resulting from long-term NSAID use in humans have dampened enthusiasm for the use of Cox-2 inhibitors for colon cancer chemoprevention (45,46). This is due to the fact that the requirements for an effective cancer chemopreventive agent are quite different than for a cancer therapeutic, as a chemopreventive drug will be administered for a longer duration and therefore the cumulative exposure will be much greater. In addition, because a chemopreventive drug will be administered to a population who is only at risk for a disease, the tolerance for adverse side effects is much lower. In this regard, enzastaurin is orally available, making it amenable to longer-term dosing, and has been found to be well tolerated and associated with minimal toxicities in several phase II clinical trials (13,15). Our results suggest that enzastaurin may confer the ability to down-regulate Cox-2 in a chemopreventive setting, without the side effects associated with Cox-2 inhibitors.

In this report, we evaluated the PKC β -selective inhibitor enzastaurin as a potential colon cancer chemotherapeutic agent. We determined that enzastaurin significantly reduced azoxymethane-mediated colon tumor initiation and progression. A primary mechanism by which enzastaurin reduced tumorigenesis was via selective inhibition of proliferation in tumor cells but not nontransformed colonic epithelial cells. At the dose used, PKC β II is clearly not the only kinase inhibited by enzastaurin (8); however, many of the mechanistic and gene expression changes affected by enzastaurin in this model have been shown to be mediated by PKC β II (4,5,7,25). Given that overexpression of PKC β II in the colonic epithelium induces hyperproliferation and increased susceptibility to azoxymethane-induced colon carcinogenesis (5), whereas genetic inhibition of PKC β expression has no effect on proliferation of nontransformed colonic epithelium, but blocks azoxymethane-induced ACF and tumor formation (4,6), the inhibitory effect of enzastaurin on tumor formation and proliferation is likely mediated by inhibition of PKC β II. Enzastaurin reduces inhibitory phosphorylation of GSK-3 β , reduces tumor-associated increased β -catenin expression and mislocalization, and promotes a more “normal” subcellular distribution of β -catenin in colon tumors. Whereas numerous studies have detected an enzastaurin-mediated decrease in GSK-3 β phosphorylation (which should result in increased GSK-3 β activity; refs. 8,9), this is the first report of enzastaurin reducing the increased expression and altered subcellular localization of β -catenin normally observed in colon tumors.

It is interesting that enzastaurin represses expression of both Cox-2 and VEGF-A in the normal colonic epithelium, but only inhibits proliferation of tumor cells. Whereas Cox-2 and VEGF clearly play an important role in colon carcinogenesis and angiogenesis (34,42,44), our results suggest that these genes either do not play a role in basal proliferation of the colonic epithelium or are not sufficiently reduced in expression to significantly affect proliferation of normal colonic epithelial cells. Cox-2 has been characterized to be up-regulated by β -catenin/TCF signaling and PKC β II overexpression in the colonic epithelium (25,47). Conversely, overexpression of Cox-2 leads to accumulation of prostaglandin E₂, which can inactivate GSK-3 β in colon cancer cells, inducing β -catenin/TCF signaling and increased VEGF expression (48,49). The exact mechanism by which enzastaurin represses Cox-2 expression and inhibits tumor-associated increased β -catenin expression and translocation will require further study; however, our data suggest that enzastaurin may be uniquely effective in colon cancer chemoprevention, selectively suppressing tumor-associated hyperproliferation, resulting in reduced colon tumor initiation and progression. Taken together with currently available clinical data regarding safety and bioavailability, our data strongly suggest that enzastaurin may be an effective chemopreventive agent for patients at high risk for colon cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant support: NIH grants CA117476 (N.R. Murray) and CA081436 (A.P. Fields) and The Mayo Clinic Foundation.

We thank Pamela Kreinest and Brandy Edenfield of the Mayo Clinic Jacksonville Tissue Facility for excellent technical support, Michael Clay of Eli Lilly for excellent technical support, Dr. Donald Thorton of Eli Lilly for providing the enzastaurin and thoughtful discussion, and Dr. Andras Khor for pathologic evaluation of tissues. This project was performed using azoxymethane provided by the National Cancer Institute's Chemical Carcinogen Reference Standards Repository, operated under contract by Midwest Research Institute (Kansas City, MO) no. N02-CB-07008.

References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. *CA Cancer J Clin* 2007;57:43–66. [PubMed: 17237035]
2. Baranda J, Williamson S. The new paradigm in the treatment of colorectal cancer: are we hitting the right target? *Expert Opin Investig Drugs* 2007;16:311–24.
3. Gokmen-Polar Y, Murray NR, Velasco MA, Gatalica Z, Fields AP. Elevated protein kinase C β II is an early promotive event in colon carcinogenesis. *Cancer Res* 2001;61:1375–81. [PubMed: 11245437]
4. Liu Y, Su W, Thompson EA, Leitges M, Murray NR, Fields AP. Protein kinase C β II regulates its own expression in rat intestinal epithelial cells and the colonic epithelium *in vivo*. *J Biol Chem* 2004;279:45556–63. [PubMed: 15322124]
5. Murray NR, Davidson LA, Chapkin RS, Clay Gustafson W, Schattenberg DG, Fields AP. Overexpression of protein kinase C β II induces colonic hyperproliferation and increased sensitivity to colon carcinogenesis. *J Cell Biol* 1999;145:699–711. [PubMed: 10330400]
6. Murray NR, Weems J, Braun U, Leitges M, Fields AP. Protein kinase C β II and PKC γ/λ : collaborating partners in colon cancer promotion and progression. *Cancer Res* 2009;69:656–62. [PubMed: 19147581]
7. Murray NR, Weems C, Chen L, et al. Protein kinase C β II and TGF β RII in ω -3 fatty acid-mediated inhibition of colon carcinogenesis. *J Cell Biol* 2002;157:915–20. [PubMed: 12058013]
8. Graff JR, McNulty AM, Hanna KR, et al. The protein kinase C β -selective inhibitor, enzastaurin (LY317615.HCl), suppresses signaling through the AKT pathway, induces apoptosis, and suppresses growth of human colon cancer and glioblastoma xenografts. *Cancer Res* 2005;65:7462–9. [PubMed: 16103100]
9. Moreau AS, Jia X, Ngo HT, et al. Protein kinase C inhibitor enzastaurin induces *in vitro* and *in vivo* antitumor activity in Waldenstrom macroglobulinemia. *Blood* 2007;109:4964–72. [PubMed: 17284528]
10. Podar K, Raab MS, Zhang J, et al. Targeting PKC in multiple myeloma: *in vitro* and *in vivo* effects of the novel, orally available small-molecule inhibitor enzastaurin (LY317615.HCl). *Blood* 2007;109:1669–77. [PubMed: 17023575]
11. Keyes KA, Mann L, Sherman M, et al. LY317615 decreases plasma VEGF levels in human tumor xenograft-bearing mice. *Cancer Chemother Pharmacol* 2004;53:133–40. [PubMed: 14593497]
12. Carducci MA, Musib L, Kies MS, et al. Phase I dose escalation and pharmacokinetic study of enzastaurin, an oral protein kinase C β inhibitor, in patients with advanced cancer. *J Clin Oncol* 2006;24:4092–9. [PubMed: 16943527]
13. Robertson MJ, Kahl BS, Vose JM, et al. Phase II study of enzastaurin, a protein kinase C β inhibitor, in patients with relapsed or refractory diffuse large B-cell lymphoma. *J Clin Oncol* 2007;25:1741–6. [PubMed: 17389337]
14. Fine HA, Kim L, Royce C, et al. Results from phase II trial of Enzastaurin (LY317615) in patients with recurrent high grade gliomas. *Journal of Clinical Oncology ASCO Annual Meeting Proceedings* 2005:1504.

15. Morschhauser F, Seymour JF, Kluin-Nelemans HC, et al. A phase II study of enzastaurin, a protein kinase C β inhibitor, in patients with relapsed or refractory mantle cell lymphoma. *Ann Oncol* 2008;19:247–53. [PubMed: 17906297]
16. Leitges M, Schmedt C, Guinamard R, et al. Immuno-deficiency in protein kinase c β -deficient mice. *Science* 1996;273:788–91. [PubMed: 8670417]
17. Calcagno SR, Li S, Colon M, et al. Oncogenic K-ras promotes early carcinogenesis in the mouse proximal colon. *Int J Cancer* 2008;122:2462–70. [PubMed: 18271008]
18. Whiteford CC, Bilke S, Greer BT, et al. Credentialing preclinical pediatric xenograft models using gene expression and tissue microarray analysis. *Cancer Res* 2007;67:32–40. [PubMed: 17210681]
19. Regala RP, Thompson EA, Fields AP. Atypical protein kinase C ι expression and aurothiomalate sensitivity in human lung cancer cells. *Cancer Res* 2008;68:5888–95. [PubMed: 18632643]
20. Regala RP, Weems C, Jamieson L, Copland JA, Thompson EA, Fields AP. Atypical protein kinase C ι plays a critical role in human lung cancer cell growth and tumorigenicity. *J Biol Chem* 2005;280:31109–15. [PubMed: 15994303]
21. Su W, Bush CR, Necela BM, et al. Differential expression, distribution, and function of PPAR $\{\gamma\}$ in the proximal and distal colon. *Physiol Genomics* 2007;30:342–53. [PubMed: 17519361]
22. Welch PA, Sinha VP, Cleverly AL, Darstein C, Flanagan SD, Musib LC. Safety, tolerability, QTc evaluation, and pharmacokinetics of single and multiple doses of enzastaurin HCl (LY317615), a protein kinase C- β inhibitor, in healthy subjects. *J Clin Pharmacol* 2007;47:1138–51. [PubMed: 17766700]
23. Guindi M, Riddell RH. The pathology of epithelial pre-malignancy of the gastrointestinal tract. *Best Pract Res Clin Gastroenterol* 2001;15:191–210. [PubMed: 11355911]
24. Dudek AZ, Zwolak P, Jasinski P, et al. Protein kinase C- β inhibitor enzastaurin (LY317615.HCl) enhances radiation control of murine breast cancer in an orthotopic model of bone metastasis. *Invest New Drugs* 2008;26:13–24. [PubMed: 17805485]
25. Yu W, Murray NR, Weems C, et al. Role of cyclooxygenase 2 in protein kinase C β II-mediated colon carcinogenesis. *J Biol Chem* 2003;278:11167–74. [PubMed: 12480928]
26. Zhang J, Anastasiadis PZ, Liu Y, Thompson EA, Fields AP. Protein kinase C (PKC) β II induces cell invasion through a Ras/Mek-, PKC γ /Rac 1-dependent signaling pathway. *J Biol Chem* 2004;279:22118–23. [PubMed: 15037605]
27. Takahashi M, Nakatsugi S, Sugimura T, Wakabayashi K. Frequent mutations of the β -catenin gene in mouse colon tumors induced by azoxymethane. *Carcinogenesis* 2000;21:1117–20. [PubMed: 10836998]
28. Pennisi E. How a growth control path takes a wrong turn to cancer. *Science* 1998;281:1438–9. 41. [PubMed: 9750112]
29. Hart MJ, de los Santos R, Albert IN, Rubinfeld B, Polakis P. Downregulation of β -catenin by human Axin and its association with the APC tumor suppressor, β -catenin and GSK3 β . *Curr Biol* 1998;8:573–81. [PubMed: 9601641]
30. Goode N, Hughes K, Woodgett JR, Parker PJ. Differential regulation of glycogen synthase kinase-3 β by protein kinase C isotypes. *J Biol Chem* 1992;267:16878–82. [PubMed: 1324914]
31. Takayama T, Ohi M, Hayashi T, et al. Analysis of K-ras, APC, β -catenin in aberrant crypt foci in sporadic adenoma, cancer, and familial adenomatous polyposis. *Gastroenterology* 2001;121:599–611. [PubMed: 11522744]
32. Slattery ML, Anderson K, Curtin K, et al. Lifestyle factors and Ki-ras mutations in colon cancer tumors. *Mutat Res* 2001;483:73–81. [PubMed: 11600135]
33. Haigis KM, Kendall KR, Wang Y, et al. Differential effects of oncogenic K-Ras and N-Ras on proliferation, differentiation and tumor progression in the colon. *Nat Genet* 2008;40:600–8. [PubMed: 18372904]
34. Verheul HM, Pinedo HM. The role of vascular endothelial growth factor (VEGF) in tumor angiogenesis and early clinical development of VEGF-receptor kinase inhibitors. *Clin Breast Cancer* 2000;1(Suppl 1):S80–4. [PubMed: 11970755]
35. Easwaran V, Lee SH, Inge L, et al. β -catenin regulates vascular endothelial growth factor expression in colon cancer. *Cancer Res* 2003;63:3145–53. [PubMed: 12810642]

36. Kelly DJ, Buck D, Cox AJ, Zhang Y, Gilbert RE. Effects on protein kinase C- β inhibition on glomerular vascular endothelial growth factor expression and endothelial cells in advanced experimental diabetic nephropathy. *Am J Physiol Renal Physiol* 2007;293:F565–74. [PubMed: 17522264]
37. Ohshiro Y, Ma RC, Yasuda Y, et al. Reduction of diabetes-induced oxidative stress, fibrotic cytokine expression, and renal dysfunction in protein kinase C β -null mice. *Diabetes* 2006;55:3112–20. [PubMed: 17065350]
38. Mulkeen AL, Silva T, Yoo PS, et al. Short interfering RNA-mediated gene silencing of vascular endothelial growth factor: effects on cellular proliferation in colon cancer cells. *Arch Surg* 2006;141:367–74. [PubMed: 16618894]discussion 74
39. Giatromanolaki A, Koukourakis MI, Sivridis E, et al. Activated VEGFR2/KDR pathway in tumour cells and tumour associated vessels of colorectal cancer. *Eur J Clin Invest* 2007;37:878–86. [PubMed: 17883421]
40. Soumaoro LT, Uetake H, Higuchi T, Takagi Y, Enomoto M, Sugihara K. Cyclooxygenase-2 expression: a significant prognostic indicator for patients with colorectal cancer. *Clin Cancer Res* 2004;10:8465–71. [PubMed: 15623626]
41. Shao J, Sheng H, Aramandla R, et al. Coordinate regulation of cyclooxygenase-2 and TGF- β 1 in replication error-positive colon cancer and azoxymethane-induced rat colonic tumors. *Carcinogenesis* 1999;20:185–91. [PubMed: 10069452]
42. Fukutake M, Nakatsugi S, Isoi T, et al. Suppressive effects of nimesulide, a selective inhibitor of cyclooxygenase-2, on azoxymethane-induced colon carcinogenesis in mice. *Carcinogenesis* 1998;19:1939–42. [PubMed: 9855006]
43. Reddy BS, Hirose Y, Lubet R, et al. Chemoprevention of colon cancer by specific cyclooxygenase-2 inhibitor, celecoxib, administered during different stages of carcinogenesis. *Cancer Res* 2000;60:293–7. [PubMed: 10667579]
44. Koehne CH, Dubois RN. COX-2 inhibition and colorectal cancer. *Semin Oncol* 2004;31:12–21. [PubMed: 15252926]
45. Chan AT, Manson JE, Albert CM, et al. Nonsteroidal antiinflammatory drugs, acetaminophen, and the risk of cardiovascular events. *Circulation* 2006;113:1578–87. [PubMed: 16534006]
46. Solomon SD, Pfeffer MA, McMurray JJ, et al. Effect of celecoxib on cardiovascular events and blood pressure in two trials for the prevention of colorectal adenomas. *Circulation* 2006;114:1028–35. [PubMed: 16943394]
47. Araki Y, Okamura S, Hussain SP, et al. Regulation of cyclooxygenase-2 expression by the Wnt and ras pathways. *Cancer Res* 2003;63:728–34. [PubMed: 12566320]
48. Shao J, Jung C, Liu C, Sheng H. Prostaglandin E2 stimulates the β -catenin/T cell factor-dependent transcription in colon cancer. *J Biol Chem* 2005;280:26565–72. [PubMed: 15899904]
49. Castellone MD, Teramoto H, Williams BO, Druey KM, Gutkind JS. Prostaglandin E2 promotes colon cancer cell growth through a Gs- α - β -catenin signaling axis. *Science* 2005;310:1504–10. [PubMed: 16293724]

Figure 1.

Enzastaurin inhibits azoxymethane-induced colon tumor initiation and progression. *A*, enzastaurin has no effect on mouse weight gain. *Points*, mean ($n = 25$ – 33 mice per treatment group); *bars*, SD. *B*, the incidence of colon tumor formation at 22 wk after the last azoxymethane injection is presented for each treatment group ($n = 21$ – 32 mice per treatment group). *C*, all colon tumors were isolated and a H&E-stained slide of each tumor was evaluated for tumor stage by a pathologist blinded to the treatment status of the mice. Data are plotted as the percent of tumors of each histologic grade. *D*, representative H&E-stained colon tumors of each histologic grade.

Figure 2.

Enzastaurin inhibits colon tumor proliferation. BrdUrd incorporation was analyzed and quantitated as described in Materials and Methods. *A*, detection of BrdUrd incorporation in colon tumors from control or enzastaurin-treated mice. *B*, the proliferative index (% BrdUrd-labeled cells) is plotted for colon tumors from control and enzastaurin-treated mice ($n = 17-19$ mice per treatment group). *C*, the proliferative index is plotted for normal, distal colonic epithelium from control (*Cont*), or enzastaurin-treated (*Enz*) mice ($n = 8-10$ mice per treatment group). *D*, the proliferative index is plotted for normal, distal colonic epithelium from nontransgenic (*NTG*), and $PKC\beta^{-/-}$ mice ($n = 8-10$ mice per genotype).

Figure 3.

Enzastaurin does not alter colon tumor apoptosis or angiogenesis. *A*, colon tumor cells undergoing apoptosis were quantitated by immunohistochemical detection of TUNEL staining. Apoptotic index (% TUNEL-labeled cells) is plotted ($n = 19$ mice per treatment group). Tonsil is included as a positive control for detection of TUNEL-labeled cells (+ *Cont*). *B*, angiogenesis in colon tumors was detected by immunohistochemical analysis of CD31 expression and quantitated as described in Materials and Methods. Results are presented as relative area of blood vessels, calculated as the ratio of CD31-positive pixels to the sum of all pixels ($n = 19$ – 21 mice per treatment group).

Figure 4.

Enzastaurin blocks PKC β II expression and GSK-3 β^{ser9} phosphorylation. Enzastaurin treatment reduces (A) PKC β II mRNA and (B) PKC β II protein expression in normal colonic epithelium. A, PKC β II mRNA abundance was determined by qPCR analysis of mRNA isolated from colonic epithelial cells of control or enzastaurin-treated mice. mRNA abundance was normalized to GAPDH and plotted as the mean ($n = 8-10$ mice per treatment group) $\pm$ SE. B, immunoblot analysis of PKC β II protein expression in isolated colonic epithelial cells of control or enzastaurin-treated mice (*inset*) was quantitated and plotted as the mean $\pm$ SE. Actin protein expression is shown as a loading control ($n = 4$ mice per treatment group). C, immunoblot analysis of phospho-GSK-3 β^{ser9} , total GSK-3 β , phospho-ERK1/2, and total ERK1/2 was performed on purified colonic epithelium isolated from control or enzastaurin-treated mice. Actin protein expression is shown as a control for protein loading. Gel lane labels: C, control; E, enzastaurin. D, the ratio of phospho-GSK-3 β^{ser9} to total GSK-3 β (*left*) and phospho-ERK1/2 to total ERK1/2 (*right*) detected by immunoblot analysis is plotted. Data are presented as the mean ($n = 4$ per group) $\pm$ SE.

Figure 5.

Enzastaurin reduces tumor-associated increased expression and nuclear/cytoplasmic localization of β-catenin in colon tumors and inhibits expression of VEGF-A and Cox-2. A, immunohistochemical detection of β-catenin was performed on normal colonic epithelium (top panels) and colon tumors (bottom panels) from control mice (left panels) and enzastaurin-treated mice (right panels). Representative images of β-catenin immunostaining are shown for each treatment group. VEGF-A (B) and Cox-2 (C) mRNA abundance was determined by qPCR analysis of mRNA isolated from colonic epithelial cells from control (Cont) or enzastaurin-treated (Enz) mice. mRNA abundance was normalized to GAPDH and plotted as the mean ($n = 8-10$ mice per treatment group) $\pm$ SE.

Table 1

Tumor parameters

Tumor parameter	Control	Enzastaurin	<i>P</i>
Tumor volume (mm ³)	7.8 ± 3.7	3.7 ± 1.6	0.11
Tumor burden (mm ³)	21.9 ± 15.0	8.2 ± 3.3	0.12
Average tumor number (all stages)	3.0 ± 0.9	2.2 ± 0.6	0.15
Average tumor number (HG adenoma)	0.9 ± 0.4	0.3 ± 0.2	0.046
Average tumor number (HG and carcinoma)	1.2 ± 0.5	0.4 ± 0.6	0.018

NOTE: Values are mean ± 95% confidence interval.

Abbreviation: HG, high grade.