Protein Kinase Cβ Is an Effective Target for Chemoprevention of Colon Cancer

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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 CβII (PKCβII) 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 PKCβII is an effective target for colon cancer chemoprevention using enzastaurin (LY317615), a PKCβ-selective inhibitor, in a mouse model of colon carcinogenesis. We find that enzastaurin potently reduces azoxymethane-induced colon tumor initiation and progression by inhibiting PKCβII-mediated tumor cell proliferation and β-catenin accumulation. Biochemically, enzastaurin reduces expression of the PKCβII- and β-catenin/T-cell factor–regulated genes *PKCβII, 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 proproliferative genes. Thus, PKCβII is an important target for colon cancer chemoprevention and the PKCβ-selective inhibitor enzastaurin may represent an effective chemopreventive agent in patients at high risk for colon cancer. [Cancer Res 2009;69(4):1643–50]

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.

Our previous work showed that PKCβII plays a critical role in colon carcinogenesis (3–5). PKCβII 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 PKCβII in the colonic epithelium (transgenic PKCβII 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 PKCβII in colon carcinogenesis.

Molecular and biochemical analysis of transgenic PKCβII mice revealed that their cancer-prone phenotype results from PKCβII-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-3β) activity and increased β-catenin expression in the colonic epithelium of transgenic PKCβII mice (5). Thus, PKCβII induces colonic epithelial hyperproliferation and enhanced susceptibility to colon carcinogenesis likely through activation of a PKCβII/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 PKCβII-mediated hyperproliferation in the colonic epithelium (7). These studies define a procarcinogenic role for PKCβII in the early stages of colon carcinogenesis and directly implicate PKCβII as a target for chemoprevention.

Enzastaurin (LY317615), a macrocyclic bisinolymaleimide, is an ATP-competitive inhibitor of serine/threonine kinases with high selectivity for PKCβ (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 PKCβII 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 PKCβII/

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β-catenin–regulated, proproliferative genes. Our data show that PKCβII 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. *PKCβ*$^{-/-}$ 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 CO_2 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 (provide 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 apopototic 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 PKCβII, GSK-3β, phospho-GSK-3β^{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 glyeraldehyde-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 μmol/L (Supplementary Table), which is similar to the plasma concentration of enzastaurin that inhibits xenograft tumor formation in mice

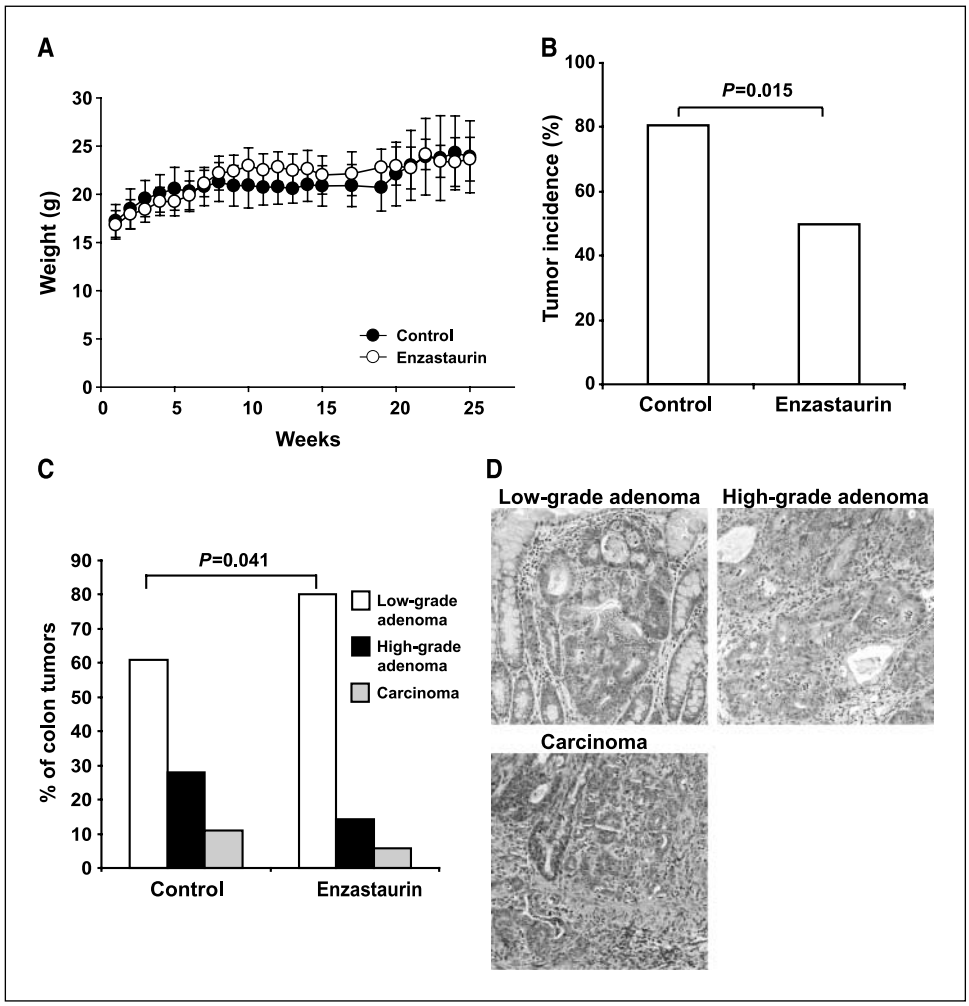

Figure 1. Enzastaurin inhibits azoxymethane-induced colon tumor initiation and progression. *A,* enzastaurin has no effect on mouse weight gain. *Points,* mean (n = 25–33 mice per treatment group); *bars,* SD. *B,* the incidence of colon tumor formation at 22 wk after the last azoxymethane injection 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.

(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. 1*A*), 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. 1*B*). 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. 1*C* 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. 1*C*). 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

Table 1. Tumor parameters

Tumor parameter	Control	Enzastaurin	P
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.

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 PKCβII for tumor formation (4, 6).

Enzastaurin administration inhibits tumor cell proliferation. Transgenic PKCβII mice exhibit hyperproliferation of the colonic epithelium and increased susceptibility to colon carcinogenesis, indicating that PKCβII 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. 2*A* and *B*). In contrast, enzastaurin had no significant effect on basal proliferation of mouse colonic epithelial cells (Fig. 2*C*). Likewise, genetic knockout of PKCβ did not significantly alter basal colonic epithelial cell proliferation (Fig. 2*D*). Therefore, enzastaurin selectively inhibits tumor cell proliferation, but not proliferation of nontransformed colonic epithelial cells, consistent with a mechanism of action involving PKCβII inhibition, because PKCβII overexpression in the colonic epithelium promotes hyperproliferation (5) but inhibition of PKCβ expression does not inhibit proliferation of nontransformed colonic epithelial cells (Fig. 2*D*). 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 PKCβII-mediated signaling in the colonic epithelium. PKCβII promotes proliferation and susceptibility to carcinogenesis by regulating expression of pro-proliferative genes in mouse colonic epithelium (4, 25). Among the targets of PKCβII is PKCβII itself, which is up-regulated through an autocrine mechanism *in vitro* and in the colonic epithelium *in vivo* (4). Because PKCβII regulates its own expression, we assessed whether enzastaurin-mediated inhibition of PKCβII leads to reduced PKCβII expression in the colonic epithelium, as a specific measure of inhibition of PKCβII signaling. As expected, enzastaurin dramatically decreased expression of PKCβII mRNA (Fig. 4*A*) and protein (Fig. 4*B*) in the colonic epithelium, indicating that enzastaurin inhibits PKCβII-mediated signaling in the colonic epithelium.

We next evaluated the effect of enzastaurin on PKCβII-driven oncogenic pathways in the colonic epithelium. We have previously shown that PKCβII activates two signaling pathways critical to colon cancer development, the APC/β-catenin/TCF (5) and Ras-PKCiota/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

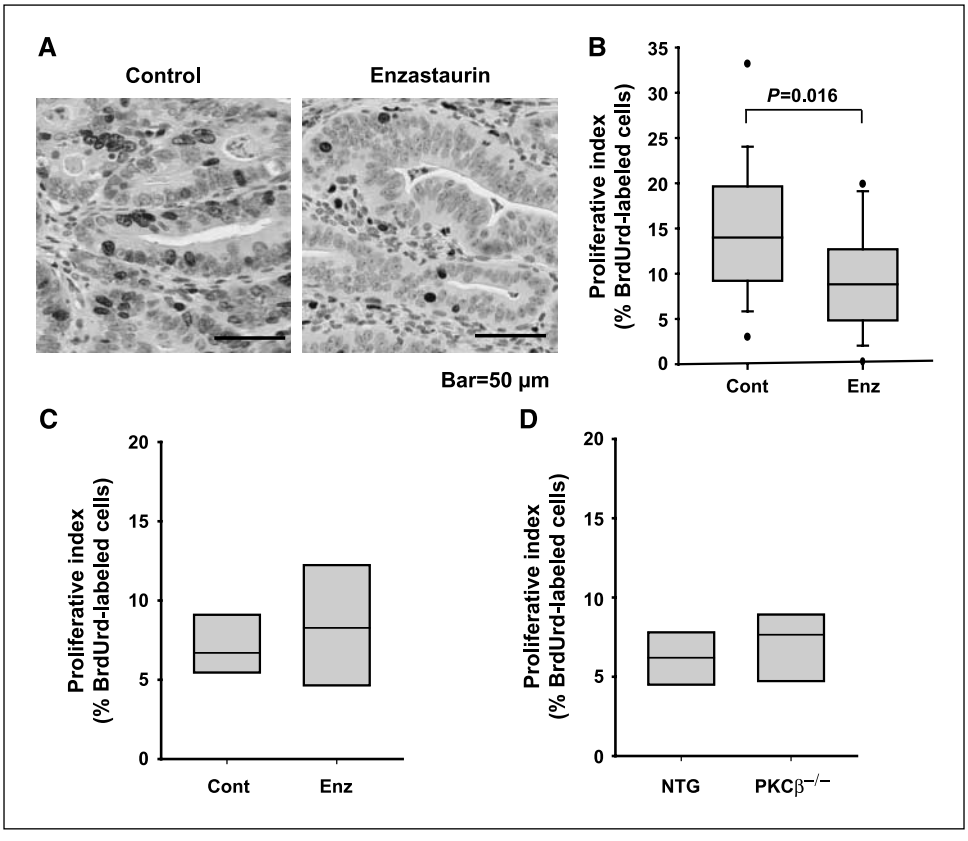

Figure 2. Enzastaurin inhibits colon tumor proliferation. BrdUrd incorporation was analyzed and quantitated as described in Materials and Methods. *A,* detection of BrdUrd incorporation in colon tumors from control or enzastaurin-treated mice. *B,* the proliferative index (% BrdUrd-labeled cells) is 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 *PKCβ*$^{-/-}$ mice (n = 8–10 mice per genotype).

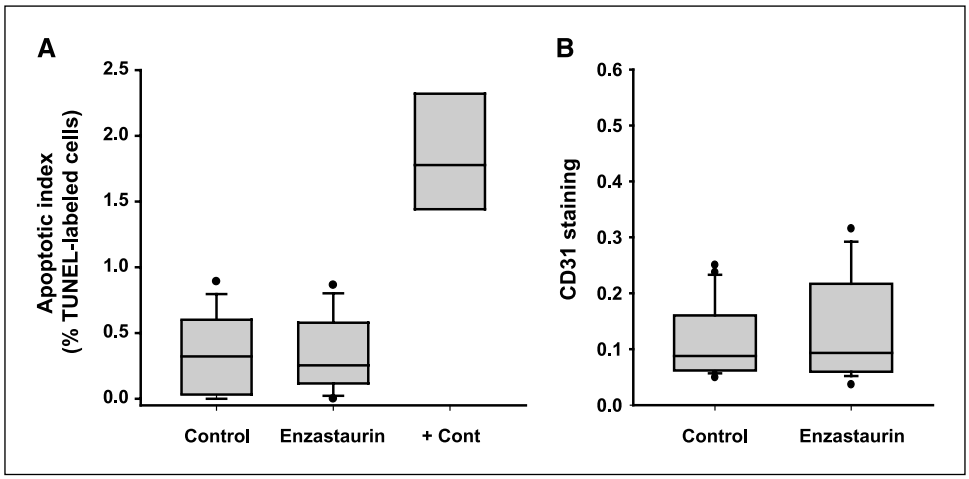

Figure 3. Enzastaurin does not alter colon tumor apoptosis or angiogenesis. *A,* colon tumor cells undergoing apoptosis were quantitated by immunohistochemical detection of TUNEL staining. Apoptotic index (% TUNEL-labeled cells) is plotted (n = 19 mice per treatment group). Tonsil is included as a positive 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).

nuclear accumulation of β-catenin (27, 28). GSK-3β is a key negative regulator of APC/β-catenin/TCF signaling (29). Overexpression of PKCβII in mouse colonic epithelium decreases GSK-3β activity *in vivo* (5) and PKCβ directly phosphorylates and inhibits GSK-3β *in vitro* (30). Enzastaurin has been previously shown to reduce GSK-3β^{ser9} phosphorylation in cancer cells *in vitro* and in xenograft tumors in mice (8–10). Thus, we assessed the status of GSK-3β phosphorylation in the colonic epithelium of enzastaurin-treated mice. We found that enzastaurin significantly reduced GSK-3β^{ser9} phosphorylation in the colonic epithelium without altering the expression of GSK-3β (Fig. 4*C* and *D*).

GSK-3β 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 PKCβII in the colonic epithelium drives increased expression of β-catenin, likely through inhibition of GSK-3β (5). Because PKCβII 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. 5*A*). β-Catenin exhibits highly restricted localization at the basolateral membrane of normal intestinal epithelial cells (Fig. 5*A, top images*). Enzastaurin treatment had no significant effect on the expression or subcellular localization of β-catenin in the normal colonic epithelium (Fig. 5*A, top images*). Azoxymethane-induced colon tumors exhibited substantial elevation of β-catenin expression

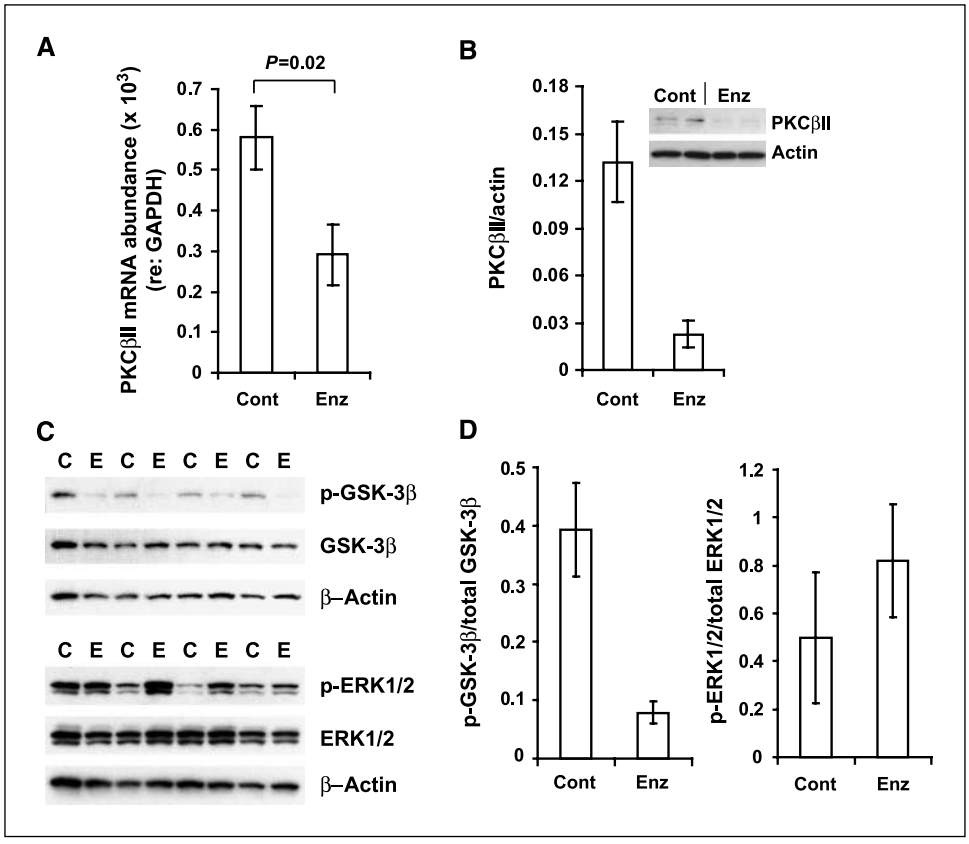

Figure 4. Enzastaurin blocks PKCβII expression and GSK-3β^{ser9} phosphorylation. Enzastaurin treatment reduces (*A*) PKCβII mRNA and (*B*) PKCβII protein expression in normal colonic epithelium. *A,* PKCβII mRNA abundance was determined by qPCR analysis of mRNA isolated from colonic epithelial cells of control or enzastaurin-treated mice. mRNA abundance was normalized to GAPDH and plotted as the mean (n = 8–10 mice per treatment group) ± SE. *B,* immunoblot analysis of PKCβII protein expression in isolated colonic epithelial cells of control or enzastaurin-treated mice (*inset*) was quantitated and plotted as the mean ± SE. Actin protein expression is shown as a loading control (n = 4 mice per treatment group). *C,* immunoblot analysis of phospho-GSK-3β^{ser9}, total GSK-3β, 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-3β^{ser9} to total GSK-3β (*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) ± SE.

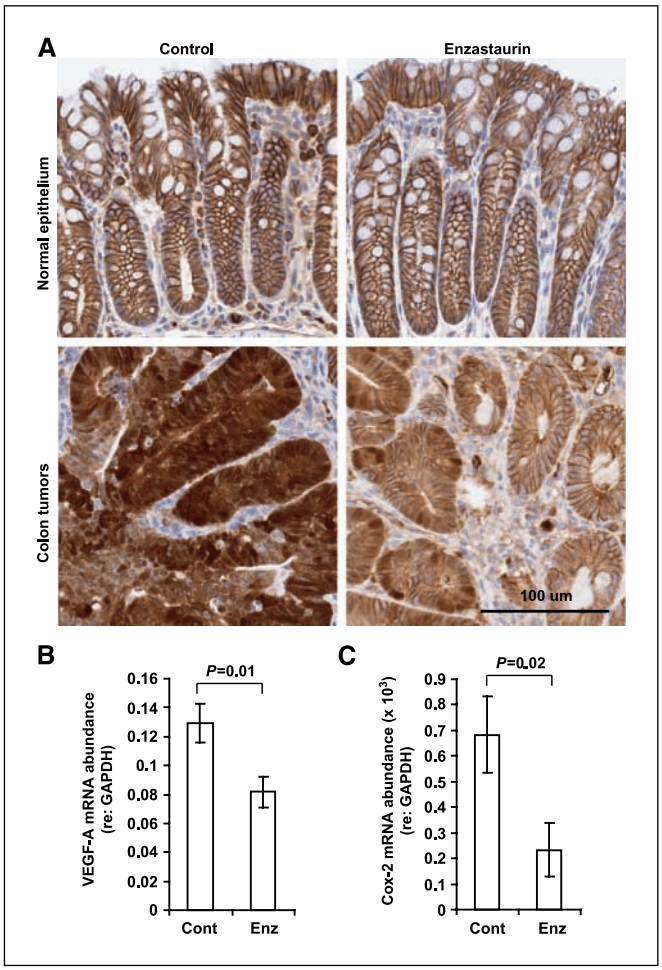

Figure 5. Enzastaurin reduces tumor-associated increased expression and nuclear/cytoplasmic localization of β-catenin in colon tumors and inhibits expression of VEGF-A and Cox-2. *A,* immunohistochemical detection of β-catenin was performed on normal colonic epithelium (*top panels*) and colon tumors (*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) ± SE.

(Fig. 5*A, bottom left*). This increase in β-catenin expression was accompanied by a redistribution of β-catenin to both the cytoplasm and nucleus (Fig. 5*A, 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. 5*A, 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, PKCβII, 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 PKCβII in colon carcinogenesis. Genetic knockout of PKCβ has no significant effect on intestinal tumorigenesis in $Apc^{Min/+}$ mice, supporting this conclusion (6). Because PKCβII expression in the colonic epithelium is required for azoxymethane-induced colon carcinogenesis (4, 6) and PKCβII overexpression induces β-catenin accumulation in the colonic epithelium (5), our current data suggest that enzastaurin blocks tumor proliferation by inhibiting PKCβII-mediated inhibition of GSK-3β, 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). PKCβII 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. 4*C* and *D*), no significant difference was detected in the level of ERK1/2 phosphorylation in control and enzastaurin-treated colonic epithelium (Fig. 4*C* 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 PKCβII and VEGF expression has clearly been established in the kidney, as well as in tumor xenograft models (11, 36, 37). Genetic knockout of *PKCβ,* as well as pharmacologic inhibition, using the PKCβ 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. 5*B*). 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. 3*B*). 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 PKCβII 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. 5*C*). 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 PKCβ-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, PKCβII 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 PKCβII (4, 5, 7, 25). Given that overexpression of PKCβII in the colonic epithelium induces hyperproliferation and increased susceptibility to azoxymethane-induced colon carcinogenesis (5), whereas genetic inhibition of PKCβ 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 PKCβII. Enzastaurin reduces inhibitory phosphorylation of GSK-3β, 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-3β phosphorylation (which should result in increased GSK-3β 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 PKCβII overexpression in the colonic epithelium (25, 47). Conversely, overexpression of Cox-2 leads to accumulation of prostaglandin E_2, which can inactivate GSK-3β 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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