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

Alan P. Fields,¹ Shelly R. Calcagno,¹ Murli Krishna,² Sofija Rak,¹ Michael Leitges,³ and Nicole R. Murray¹

Introduction

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 Cβ (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 proliferative 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]

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 Cβ (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 proliferative 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]
β-catenin–regulated, proliferative genes. Our data show that PKC\[\beta\]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\[\beta\]II– 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 animals were 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 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.

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.
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

### Table 1. Tumor parameters

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

NOTE: Values are mean \( \pm \) 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-enzastaurin on colon tumor cell proliferation (Fig. 2). Enzastaurin proliferation (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 and B). In contrast, enzastaurin had no significant effect on basal proliferation of mouse colonic epithelial cells (Fig. 2C). Likewise, genetic knockout of PKCβ 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 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. 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 colony 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. 4A) and protein (Fig. 4B) 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-PKCιota/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-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-3βSer9 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-3βSer9 phosphorylation in the colonic epithelium without altering the expression of GSK-3β (Fig. 4C 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. 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.
Enzastaurin-treated tumors versus control. A significant reduction in nuclear and cytoplasmic β-catenin expression was observed in tumors from enzastaurin-treated mice (2.1fold, 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 (2.1fold, P= 0.045 vs 3.3fold for control tumors). The level of mislocalized β-catenin in high-grade adenomas (3.3fold) 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 ApcMin-/- 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). PKCbetaII 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 colon 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 PKCβII and VEGF expression has clearly been established in the kidney, as well as in tumor xenograft models (11, 36, 37). Genetic knockout of PKCβII, 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. 5B). However, the effect of enzastaurin on VEGF-A expression in the colon has not been evaluated. 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 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. 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 PKCα-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, PKCαII 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 PKCαII (4, 5, 7, 25). Given that overexpression of PKCαII in the colonic epithelium induces hyperproliferation and increased susceptibility to azoxymethane-induced colon carcinogenesis (5), whereas genetic inhibition of PKCα 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 PKCαII. 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 PKCαII overexpression in the colonic epithelium (25, 47). Conversely, overexpression of Cox-2 leads to accumulation of prosta-glandin E2, 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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