Epithelial-to-Mesenchymal Transition and Oncogenic Ras Expression in Resistance to the Protein Kinase Cβ Inhibitor Enzastaurin in Colon Cancer Cells

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Abstract

Identifying molecular factors of sensitivity and resistance of cancer cells to enzastaurin, a drug inhibiting protein kinase C (PKC) β, remains a major challenge to improve its clinical development. Investigating the cellular effects of enzastaurin in a panel of 20 human cancer cell lines, we found that most cells displaying oncogenic K-Ras mutations also display resistance to enzastaurin. Wild-type (WT) K-Ras cancer cells displaying high sensitivity to enzastaurin also expressed high mRNA levels of epithelial markers, such as E-cadherin (CDH1), and low mRNA expressions of mesenchymal markers, such as vimentin, N-cadherin (CDH2), and other genes frequently expressed in mesenchymal transition such as ZEB1, TWIST, SLUG, SNAIL, and TGFβ. WT K-Ras enzastaurin-resistant cells also expressed high levels of mesenchymal markers. Based on this observation, the effects of enzastaurin were investigated in epithelial colon COLO205-S cells that expressed WT Ras/Raf and its derived COLO205-R mesenchymal counterpart selected for resistance to most PKC modulators and displaying oncogenic K-Ras (G13D/exon 2). In COLO205-S cells, inhibition of phosphorylated PKCβ led to the inactivation of AKT and glycogen synthase kinase 3β and was associated with apoptosis without significant effect on cell cycle progression. In COLO205-R cells, enzastaurin induced mainly necrosis at high concentrations. In COLO205-R cells, a strong activation of extracellular signal-regulated kinase 1/2 possibly due to oncogenic K-Ras was predominantly associated with transcription of potent antiapoptotic genes, such as BCL2, GADD45B, and CDKN1A, as well as the multidrug resistance gene ABCB1. From this study, colon cancer cells undergoing apoptosis under enzastaurin exposure seem to frequently express a WT Ras and an epithelial phenotype. Mol Cancer Ther; 9(5); 1308–17. ©2010 AACR.

Introduction

The protein kinase C (PKC) family comprises 12 different serine-threonine kinase isoforms that contribute to the control of several essential steps for malignant progression, proliferation, survival, and apoptosis (1). The best-characterized and first discovered isoforms were conventional PKCs including PKCα, two alternatively spliced variants PKCβI and PKCβII, and PKCγ (1, 2). PKCβ has been implicated in malignant progression of colorectal carcinomas (3). Several lines of evidence have suggested that PKCβ contributes to cancer cell proliferation and survival, eliciting antiapoptotic effects in carcinoma cells (1). Signaling pathways affected by PKCβ activation and contributing to its antiapoptotic effects remain unclear. PKC activation is acknowledged triggering signaling through the Ras/extracellular signal-regulated kinase (ERK) pathway, which may be involved in controlling cellular proliferation and apoptosis (4). PKCβII activity may also play a role in reducing the sensitivity of intestinal epithelia to the growth-suppressive effects of transforming growth factor β (TGFβ); ref. 5). Recent works have linked PKC activity to the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, a major regulatory pathway governing the apoptotic response in many carcinomas (6). In some models, PKC activation was triggered by the phosphatidylinositol-dependent kinase-1, a kinase activated immediately downstream of PI3K (6). PKCβ can also directly activate AKTSer473. Moreover, both PKC and AKT can phosphorylate glycogen synthase kinase 3β (GSK3β) at Ser9 (7). Based on those observations, PKC...
interplay with the PI3K/AKT pathway was proposed as a mechanism modulating the apoptotic response in response to growth factors and/or cellular stress (8).

Enzastaurin (LY317615-HCI, Eli Lilly), an acyclic bisindolylmaleimide, is a potent and selective competitive inhibitor of serine-threonine kinases including PKCβ (9–11). Albeit its antiangiogenic effects are shown in several xenograft models, enzastaurin also induces direct effects in cancer cells. In preclinical studies, enzastaurin induces apoptosis and suppresses the proliferation of cultured cancer cells (8). Enzastaurin treatment also suppresses the phosphorylation of GSK3β in several xenograft models, enzastaurin also induces apoptosis in wild-type (WT) cancer cells that express either K-Ras or epithelial-mesenchymal markers seem more resistant to enzastaurin. Conversely, enzastaurin has both direct tumor cell death effects (apoptosis) and indirect effects by blocking tumor angiogenesis.

Based on promising effects in preclinical models, clinical trials have been initiated in several tumor types. Despite evidence of sporadic antitumor activity, the lack of biological tumor markers allowing identifying subsets of patients with enzastaurin-sensitive or enzastaurin-resistant tumors remains a major limiting factor for enzastaurin clinical development. Mechanisms by which cancer cells may become resistant to drugs that target PKC are still poorly understood and may require further investigations to help improving patient selection in clinical trials (1, 12). In previous experiments, we showed that resistance to PKC inhibitors was associated with changes in cellular morphology, invasion, and gene expression that were reminiscent of a mesenchymal phenotype (13, 14). In this study, we further investigated the respective roles of oncogenic K-Ras and epithelial-mesenchymal transition (EMT) in the sensitivity and resistance of cancer cells to enzastaurin. We show that enzastaurin may readily induce apoptosis in wild-type (WT) Ras colon cancer cells expressing high levels of epithelial markers, such as E-cadherin, and low levels of mesenchymal markers, such as vimentin. Conversely, cancer cells that express either K-Ras mutations or mesenchymal markers seem more resistant to enzastaurin. Those data provide new insight on mechanisms associated with sensitivity and resistance to this novel anticancer agent.

**Materials and Methods**

**Materials.** Enzastaurin was supplied by Eli-Lilly. Staurosporine was purchased from Sigma.

**Cell lines.** SCC61, SKBR3, MCF7, SKOV3, HEF2, SQ20B, CAK11, MIAFAC2, HT29, OVCAR3, DU145, and PC3 cell lines were obtained from the American Type Culture Collection. HCT116, COLO205-S, HCC2998, HOP62, HOP92, IGROV1, and MDA-MB-435 cell lines were obtained from the National Cancer Institute collection. COLO205-R cells were developed in our laboratory.

Cells were grown as monolayers in RPMI 1640 supplemented with 10% FCS (Invitrogen), 2 mmol/L glucose, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified 5% CO2 atmosphere and regularly checked for the absence of Mycoplasma.

**Cell cytotoxicity assay.** Cell viability was determined using the MTT assay (Sigma). The conversion of yellow water-soluble tetrazolium MTT into purple insoluble formazan is catalyzed by mitochondrial dehydrogenases and is used to estimate the number of viable cells. In brief, cells were seeded in 96-well tissue culture plates at a density of 2 × 103 per well. After 72-hour incubation with drug followed by 48-hour postincubation in drug-free medium, cells were incubated with 0.4 mg/mL MTT for 4 hours at 37°C. After incubation, the supernatant was discarded, insoluble formazan precipitates were dissolved in 0.1 mL of DMSO, and the absorbance was measured at 560 nm by use of a microplate reader (Thermo). Wells with untreated cells or with drug-containing medium without cells were used as positive and negative controls, respectively. Growth inhibition curves were plotted as the percentage of untreated control cells.

**Western blot analysis.** Cells were lysed in buffer containing 50 mmol/L HEPES (pH 7.6), 150 mmol/L NaCl, 1% Triton X-100, 2 mmol/L sodium vanadate, 100 mmol/L NaF, and 0.4 mg/mL phenylmethylsulfonyl fluoride. Equal amounts of protein (20 μg/lane) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk or 5% bovine serum albumin in 0.01% Tween 20/PBS and then incubated with the primary antibody overnight. Membranes were then washed and incubated with the secondary antibody conjugated to horseradish peroxidase. Bands were visualized by using the enhanced chemiluminescence Western blotting detection system. Densitometric analysis was done under conditions that yielded a linear response. The following antibodies were used: anti-PKCα, anti-PKCβ, anti-PKCδ, anti-phospho-p38 (BD Biosciences), anti-phospho-ERK1/2 (p-ERK1/2), anti–cleaved caspase-3, anti–phospho-AKT (p-AKT), anti–phospho-S6 kinase, anti–phospho-GSK3β (p-GSK3β), anti–phospho-PKCα (p-PKCα), anti–PKCδ, and anti–phospho-Raf (p-Raf; Cell Signaling). All antibodies were used at a 1:1,000 dilution, except for anti-PKCβ, which was used at a 1:500 dilution.

**Cell cycle analysis and apoptosis assays.** Cell cycle analysis and the measurement of the percentage of apoptotic cells were assessed by flow cytometry. In brief, cells were seeded onto 25 cm² flasks and treated with various concentrations of enzastaurin. At various time points, adherent and nonadherent cells were recovered, washed with PBS, fixed in 70% ethanol, and stored at 4°C until use. Cells were rehydrated in PBS and incubated for 20 minutes at room temperature with 250 μg/mL RNase A and for 20 minutes at 4°C with 50 μg/mL propidium iodide in the dark. The cell cycle distribution and percentage of apoptotic cells were determined with a
flow cytometer (FACSCalibur and CellQuest Pro software (BD Biosciences). Apoptosis was confirmed using the Annexin V-FITC Apoptosis Detection kit (Sigma).

**Real-time reverse transcription-PCR.** The theoretical and practical aspects of real-time quantitative reverse transcription-PCR (qRT-PCR) using the ABI Prism 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems) have been described in detail elsewhere (15). Results were expressed as n-fold differences in target gene expression relative to the TBP gene (an endogenous RNA control) and relative to a calibrator (1× sample), consisting of the cell line sample from our tested series that contained the smallest amount of target gene mRNA. Experiments were done in duplicate.

**DNA extraction and mutation screening.** High-molecular weight DNA was prepared by standard proteinase K digestion followed by phenol-chloroform extraction. Genomic DNA from the cell lines was amplified with primers specific for KRAS (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue; NM_004985) and RAF1 (v-raf-1 murine leukemia viral oncogene homologue 1; NM_002880). The primer oligonucleotide sequences and PCR conditions are available on demand. PCR was done with the Taqman PCR Core Reagent kit. Mutation screening was done using bidirectional DNA sequencing of purified PCR products with the ABI BigDye Terminator Sequencing kit on an ABI Prism 3130 automatic DNA sequencer. Sequences were aligned with SeqScape analysis software and compared with the corresponding reference sequences for genomic DNA (Perkin-Elmer Applied Biosystems).

**Results**

Resistance to enzastaurin is frequently associated with K-Ras mutations. The antiproliferative effect of
72-hour exposure to enzastaurin was assessed in a panel of 20 human cancer cell lines of different origins using MTT assay. As shown in Fig. 1A, the antiproliferative effects (IC50) of 72-hour exposure to enzastaurin were determined in HCC2998 (2 ± 0.7 μmol/L), SCC61 (6 ± 1.7 μmol/L), MDA-MB-435 (10 ± 3.0 μmol/L), SKBR3 (12 ± 3.0 μmol/L), COLO205-S (14 ± 5.0 μmol/L), IGROV1 (24 ± 6.0 μmol/L), MCF7 (25 ± 7.0 μmol/L), SKOV3 (42 ± 15 μmol/L), HEP2 (50 ± 18 μmol/L), SQ20B (65 ± 21 μmol/L), COLO205-R (75 ± 28 μmol/L), CAKI1 (120 ± 20 μmol/L), MIAPACA2 (150 ± 42 μmol/L), HCT116 (160 ± 45 μmol/L), HT29 (170 ± 51 μmol/L), HOP92 (175 ± 61 μmol/L), OVCAR3 (190 ± 98 μmol/L), DU145 (240 ± 81 μmol/L), PC3 (320 ± 87 μmol/L), and HOP62 cells (450 ± 130 μmol/L). In our panel, enzastaurin and staurosporine displayed dissimilar cytotoxicity profiles (data not shown). Enzastaurin being known as a drug that target PKCs, protein expressions of several PKC isoforms were determined in cancer cells. Western blot analysis aiming correlating PKCα, PKCβ, PKCδ, and PKCe protein expression in eight cell lines of our panel with sensitivity to enzastaurin showed no significant correlation (data not shown). Oncogenic K-Ras and B-Raf have been described as major survival factors driving resistance of cancer cells to several targeted agents (16). We therefore investigated the presence of K-Ras and B-Raf mutations in our panel of cancer cells. Activating mutations of K-Ras (exon 2) and B-Raf (exon 15) were detected in five and two cancer cell lines, respectively (Table 1). No Ras mutation on exon 3 was detected (data not shown). Only two cancer cell lines harbored B-Raf mutations: MDA-MB-435 being sensitive, whereas HT29 was resistant to enzastaurin. Interestingly, most of cancer cells with K-Ras mutations also display significant resistance to enzastaurin (Fig. 1B).

Enzastaurin-resistant cells express mesenchymal markers. Oncogenic Ras was previously reported as a key factor activating several signaling pathways triggering EMT in human cancer cells (13, 17–19). Furthermore, we and others showed that resistance to PKC modulators was associated with expression of mesenchymal transition markers (13, 20, 21). Therefore, we investigated the expression of several EMT markers in cancer cells sensitive and resistant to enzastaurin. The two cancer cell lines with B-Raf mutations were excluded from this analysis. Cancer cells displaying no K-Ras mutation and considered sensitive to enzastaurin (IC50 < 30 μmol/L) were shown to express high levels of the epithelial marker CDH1 (E-cadherin) or ERBB3 and low levels of most

<table>
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<th>Cancer cells</th>
<th>K-Ras mutation exon 2</th>
<th>B-Raf mutation exon 15</th>
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<tr>
<td>MDA-MB-435</td>
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<td>V600E</td>
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<tr>
<td>COLO205-R</td>
<td>G13D</td>
<td>WT</td>
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<td>MIAPACA2</td>
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<td>HCT116</td>
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<tr>
<td>OVCAR3</td>
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<td>WT</td>
</tr>
<tr>
<td>HOP62</td>
<td>G12C</td>
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Figure 2. mRNA expression profile of selected genes involved in EMT in enzastaurin-sensitive and enzastaurin-resistant cell lines. mRNA expression profile of genes (qRT-PCR) observed in enzastaurin-sensitive cancer cells with no K-Ras mutation (A; n = 7), enzastaurin-resistant cancer cells with no K-Ras mutation (B; n = 8), and enzastaurin-resistant cancer cells with oncogenic K-Ras (C; n = 5).
mesenchymal markers, including VIM (vimentin), CDH2 (N-cadherin), ZEB1, and TWIST (Fig. 2A). Conversely, cancer cells that were considered resistant to enzastaurin with and without K-Ras mutations expressed very different patterns with higher levels of several mesenchymal markers (Fig. 2B and C). Indeed, enzastaurin-resistant cancer cells expressed significant higher mRNA levels of VIM, ZEB1, SLUG, TGFB1-2, and FGFR1 compared with sensitive cancer cells (two-sided P < 0.05). Consistently, CDH1 was significantly lower in cancer cells resistant to enzastaurin than in sensitive cells. Patterns of EMT gene expression were similar in enzastaurin-resistant cancer cells regardless of K-Ras mutations (Fig. 2B and C). Those data strongly suggested that enzastaurin-sensitive cancer cells mostly expressed epithelial markers and no Ras mutations, whereas expression of mesenchymal markers either or not associated with K-Ras mutation is a common feature in enzastaurin-resistant cells. Oncogenic Ras and EMT were previously shown to be frequently associated with resistance to anticancer drugs by the transcriptional activation of either antiapoptotic genes or genes involved in multidrug resistance (22, 23). Consistently, mRNA levels of BCL2 and ABCB1 were expressed at significantly higher levels in enzastaurin-resistant cells harboring EMT independently of K-Ras mutations.

K-Ras and EMT markers in enzastaurin-resistant colon cancer cells. To examine the role of EMT and K-Ras mutations in sensitivity to enzastaurin, we further investigated the drug effects in four colon cancer cell lines from our panel. As illustrated in Fig. 3A, mRNA expression of TGFB1-2, TWIST, and SLUG was significantly

![Graph A](image1.png)

**Figure 3.** Mesenchymal K-Ras–mutated colon cancer cells displaying resistance to enzastaurin. qRT-PCR analysis showed that mRNA levels of TGFB1-2, ZEB, SIP1, TWIST, SLUG, SNAIL, and HMGA2 are overexpressed in G13D exon 2 K-Ras–mutated HCT116 and COLO205-R colon cancer cells. A, B-Raf–mutated (HT29) and WT Ras (COLO205-S) colon cancer cells expressed high CDH1 mRNA levels and low levels of mesenchymal markers. B, an overexpression of several genes involved in EMT and a down-expression of epithelial markers were found in COLO205-R compared with COLO205-S cells. COLO205-R cells were ∼10-fold more resistant to enzastaurin than COLO205-S cells in MTT assay. C, exposure to enzastaurin for 48 to 72 hours induced 50% and 100% growth inhibition at concentrations of 3 and 10 μmol/L in COLO205-S cells and at concentrations of 10 and ≥30 μmol/L in COLO205-R cells, respectively.
lower in the enzastaurin-sensitive COLO205-S cells compared with enzastaurin-resistant and K-Ras-mutated COLO205-R and HCT116 cancer cells. The isogenic COLO205-S and COLO205-R cells that we previously identified as a relevant model for studying resistance to PKC modulators (13, 14) were used in further experiments. Here, we observed that epithelial COLO205-S cells displayed WT Ras, whereas mesenchymal COLO205-R cells selected for resistance to several PKC modulators harbored the G13D activating K-Ras mutation on exon 2. As shown in Fig. 3B, the mRNA expressions of most mesenchymal markers were higher in COLO205-R than in COLO205-S cells. Conversely, mRNA expression of epithelial markers such as MUC1 and CDH1 was lower in COLO205-R cells. Differences of expression of CDH1 and vimentin in COLO205-S and COLO205-R cells were confirmed at the protein level using Western blot (data not shown). Interestingly, PROM1 (Prominin1, CD133), which has been widely used as a marker for cancer stem cells, was higher in COLO205-R cells with K-Ras mutation than in COLO205-S cells with normal K-Ras, suggesting that these cells at least partially may acquire stem cell–like characteristics. Cellular effects of enzastaurin were investigated using cell survival and growth inhibition assays (Fig. 3C). We observed that mesenchymal and K-Ras-mutated COLO205-R cells were significantly more resistant that parental COLO205-S cells.

Enzastaurin modulates PKC signaling in COLO205 colon cancer cells. Both COLO205-S and COLO205-R cells expressed PKCα, PKCβ, and PKCδ; the PKCα protein expression being lower in COLO205-R than in COLO205-S cells (Fig. 4A). The effects on cell signaling of enzastaurin concentrations inhibiting cell growth at 48 hours was investigated in COLO205-S and COLO205-R cells (Fig. 4B). COLO205-S and COLO205-R cells displayed similar levels of p-Raf at baseline and under exposure to enzastaurin. In COLO205-S cells, 10 μmol/L enzastaurin reduced p-PKCβ, p-AKT, and p-GSK3β but increased p-ERK1/2 and phospho-PTEN (p-PTEN). In COLO205-R cells, no p-PKCβ was detectable either at baseline or after exposure to enzastaurin. The concentration of 50 μmol/L enzastaurin increased p-ERK1/2, p-PTEN, and p-GSK3β and decreased p-AKT in COLO205-R cells at 48 hours. Those data suggested that enzastaurin may modulate cell signaling by reducing p-PKCβ in COLO205-S cells but may still have effects on cell signaling in the absence of effects on PKCβ phosphorylation in COLO205-R cells.

Enzastaurin-induced apoptosis in COLO205-S and necrosis in COLO205-R cells. Concentrations corresponding to 50% and 100% growth inhibitions at day 4 (see Fig. 3C and D) in COLO205-S and COLO205-R cells were used for cell cycle analysis. In COLO205-S cells, increasing concentrations of enzastaurin were shown in Fig. 5A to increase the percent of cells in the sub-G1 phase of the cell cycle, the difference being significant at 3 μmol/L (two-sided P < 0.01). The cell cycle distribution in COLO205-R cells showed a slight increase in the number of cells in sub-G1 phase of the cell cycle, the difference being significant at 3 μmol/L (two-sided P < 0.01). The cell cycle distribution in COLO205-R cells showed a slight increase in the number of cells in G0-G1 compared with COLO205-S cells. In COLO205-R cells, the number of cells in sub-G1 significantly increased only for high enzastaurin concentrations (Fig. 5B). Annexin V immunostaining showed that apoptosis increased in COLO205-S cells at concentrations ≥3 μmol/L with almost no necrosis. Conversely, at 10 μmol/L enzastaurin, a limited number of COLO205-R cells underwent apoptosis up to 50 μmol/L enzastaurin, a concentration that was also associated with significant necrosis induction (Fig. 5C). We further investigated mRNA expressions of proapoptotic (IER3, TNFAIP3, NOXA, and PUMA) and antiapoptotic (BCL2, BCL2L1, BCL2L2, BCL2L3, BCL2L4, BCL2L5, BCL2L6, and BCL2L7) genes.
GADD45B, and CDKN1A/p21cip1 genes as well as cell cycle–related (CDC25B) and multidrug resistance protein 1 (ABCB1/mdr1) genes in COLO205-S and COLO205-R cells. Transcripts of both proapoptotic and antiapoptotic genes were increased in COLO205-R compared with COLO205-S cells, with gene upregulation affecting predominantly antiapoptotic genes in COLO205-R cells. Moreover, mRNA expression of CDC25B was reduced in COLO205-R cells that also overexpressed ABCB1 mRNA gene expression. Altogether, those data indicate that overexpression of antiapoptotic genes along with that of MDR1 may prompt resistance of COLO205-R cells to several anticancer agents. Cleaved caspase-3 and poly(ADP-ribose) polymerase (PARP) were investigated in COLO205-S and COLO205-R cells in time course experiments (Fig. 6B). A concentration of 10 μmol/L enzastaurin induced cleavage of caspase-3 after 48-hour exposure in COLO205-S (Fig. 6B) but not in COLO205-R cells (data not shown). When using concentrations corresponding to 100% growth inhibition in COLO205-S and COLO205-R (10 and 50 μmol/L, respectively), an increase in cleaved PARP was detected in both cell lines after 48-hour exposure (Fig. 6B). From those experiments, we showed that enzastaurin may trigger apoptosis in both COLO205-S and COLO205-R cells, although unspecific necrosis may be prominent in COLO205-R cells exposed to enzastaurin at high concentrations.

Discussion

The important role of PKCβ in vascular endothelial growth factor–dependent tumor angiogenesis has driven the clinical development of enzastaurin toward its use against established tumor angiogenesis. In recent years, additional studies have revealed that enzastaurin may also yield potent and direct antiproliferative

Figure 5. Cellular effects induced by enzastaurin in COLO205-S and COLO205-R cells. A, cell cycle distributions of COLO205-S cells exposed to 3 and 10 μmol/L enzastaurin. B, cell cycle distributions of COLO205-R cells exposed to 10 and 50 μmol/L enzastaurin. C, Annexin V immunostaining of COLO205-S and COLO205-R cells exposed to increasing concentrations of enzastaurin.
and proapoptotic effects in cancer cells (8, 24–26). Thus far, mechanisms that render cells exquisitely sensitive or resistant to enzastaurin remain poorly understood. In this study, we tested the cytotoxic effects of enzastaurin in a panel of human cancer cell lines. As reported previously, PKCβ protein expression was found in most cancer cell lines but did not predict sensitivity and resistance to enzastaurin (8, 24–26). Both oncogenic K-Ras and EMT were previously reported as playing a major role in the resistance to several cytotoxic agents and targeted therapies (19–21). Interestingly, seeking for genetic abnormalities in our panel of cell lines, we found that most cancer cells harboring resistance to enzastaurin also displayed oncogenic exon 2 K-Ras mutations. Several factors, such as TGFβ, HMGA2, and TWIST, were shown to cooperate with oncogenic Ras to trigger and maintain EMT (21–23). In this study, we considered that cells with IC50 of >30 μmol/L enzastaurin may be regarded as resistant to that drug because such plasma concentrations may be barely reachable in cancer patients. Ras- and Raf-mutated cells were shown to express high levels of vimentin and CDH2. Similarly, enzastaurin-resistant cancer cells without K-Ras mutation also display relatively high expression of mesenchymal markers, including vimentin, CDH2, TWIST, SLUG, and TGFBI. Conversely, enzastaurin-sensitive cancer cells were found expressing WT K-Ras, epithelial markers such as CDHI, and low levels of mesenchymal markers such as vimentin and CDH2. Those data strongly suggest that EMT, either alone or associated with activating K-Ras mutation, may lead to primary resistance to the cytotoxic effects of enzastaurin in cancer cells. To further examine the role of EMT in resistance to enzastaurin, we investigated the effects of the drug in COLO205-S cells and its derived, COLO205-R, counterpart selected for a pleiotropic resistance to PKC modulators, including PEP005, staurosporine, bistratene A, bryostatin, and phorbol 12-myristate 13-acetate (13). Experiments showed that COLO205-R cells displayed also resistance to the cytotoxic and cytostatic effects of enzastaurin. Whereas COLO205-S cells displayed an epithelial phenotype and expressed epithelial markers, COLO205-R cells displayed a mesenchymal phenotype, expressed most of mesenchymal markers, and were found more invasive in Matrigel assay and human xenografts. Previous studies showed no PKCα and PKCδ mutation in COLO205-R cells. In this study, we found that, unlike COLO205-S, COLO205-R cells

![Figure 6. Differential expression of apoptotic markers in COLO205-S and COLO205-R cells.](image-url)
expressed an activating K-Ras mutation, further suggesting that expression of oncogenic Ras and EMT may cooperate to induce resistance to enzastaurin as well as other PKC modulators. Considering that K-Ras is mutational activated in ~20% of all solid tumors and that EMT is increasingly found associated with resistance to several anticancer agents (16), substantial efforts will have to be made to identify alternative or new drugs counteracting the effects of oncogenic Ras and EMT in those subsets of otherwise pharmacologically intractable and clinically refractory human cancers.

As illustrated in our preclinical trial, enzastaurin may induce inhibition of cellular proliferation through direct induction of apoptosis and without significantly affecting cell cycle progression in COLO205 cancer cells. This effect was previously reported by others who also found that enzastaurin-induced apoptosis was dependent of the inhibition of AKT and/or GSK3β signaling, activating the proapoptotic protein BAD (12, 24–31). In COLO205-S cells, we also found that enzastaurin inhibited the activation of PKCβ and downstream effectors AKT and GSK3β at concentrations that were also effective for inducing apoptosis. Thereby, it is likely that AKT and GSK3β inhibition may have participated to enzastaurin-induced toxicity in this cell line. Thus far, few studies have been dedicated to investigate mechanisms associated with resistance to enzastaurin. We took the opportunity of COLO205-R cells, a well-characterized cell line showing resistance to multiple PKC modulators, to investigate the effects of enzastaurin on the phosphorylation of proteins involved in apoptosis. We found that COLO205-R cells had no baseline activation of PKCβ, although some degrees of inhibition of AKT and GSK3β phosphorylation were detectable. It is likely that at high enzastaurin concentrations, other signaling proteins than PKCβ such as PKCα, PKCγ, and PKCε may have been affected, inducing downstream effects usually restricted to PKCβ in more sensitive cell lines. Interestingly, ERK1/2 was strongly activated by enzastaurin in this cell line possibly by the acquired mutational status of Ras. Activation of the Ras/mitogen-activated protein/ERK kinase/ERK pathway has been shown to activate the transcriptional machinery and genes involved in the control of cell cycle and apoptosis (32, 33). In our study, we found an overexpression of several genes involved in EMT and resistance to targeted therapies. Some of those genes, such as SNAIL, SLUG, and TWIST, may participate in the control of apoptosis. In addition, although both proapoptotic and antiapoptotic genes were expressed in COLO205-R cells, genes with antiapoptotic functions, such as BCL2, GADD45B, and CDKNA1/p21, were predominantly overexpressed. However, gene upregulation affected predominantly antiapoptotic genes in COLO205-R cells, suggesting that the balance favoring antiapoptotic gene may explain, at least in part, the resistance of those cells to enzastaurin-induced apoptosis observed in COLO205S cells. Consistently, when exposed to enzastaurin, COLO205-R cells were more resistant to apoptosis, cellular effects observed at high concentrations being mainly related to unspecific necrosis induction.

We have found that MDR1/ABCB1 mRNA was overexpressed in COLO205-R compared with COLO205-S cells. Previous in vitro data indicated that enzastaurin inhibits MDR1 (IC50, 2.7 μmol/L), whereas the major metabolite LSN326020 is a much weaker inhibitor of MDR1 (IC50, 18.7 μmol/L). In vivo studies with MDR1 knockout mice also suggest that LSN326020 (but not necessarily enzastaurin itself) is a substrate of MDR1 (data on file, Eli Lilly). Thus, the preclinical data suggest a possible role of MDR1 in enzastaurin cellular effects.

Taken together, our data provide new insights on mechanisms associated with sensitivity and/or resistance to enzastaurin in colon cancer cells and could supply valuable information for the ongoing clinical development of personalized medicine using this drug in patients with colon cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


2. Ghoul A, Serova M, Benhadji KA, et al. Protein kinase C α and δ are members of a large kinase family of high potential for novel anticancer targeted therapy. Targeted Oncol 2005;1:34–47.


6. Balendran A, Hare GR, Kielo A, Williams MR, Alessi DR. Further evidence that 3-phosphoinositide-dependent protein kinase-1
EMT and K-Ras Cooperate for Enzastaurin Resistance


Molecular Cancer Therapeutics

Epithelial-to-Mesenchymal Transition and Oncogenic Ras Expression in Resistance to the Protein Kinase C β Inhibitor Enzastaurin in Colon Cancer Cells

Maria Serova, Lucile Astorgues-Xerri, Ivan Bieche, et al.


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