Modulation of 4E-BP1 Function as a Critical Determinant of Enzastaurin-Induced Apoptosis

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Abstract

Enzastaurin (LY317615.HCl) is currently in a phase III registration trial for diffuse large B-Cell lymphoma and numerous phase II clinical trials. Enzastaurin suppresses angiogenesis and induces apoptosis in multiple human tumor cell lines by inhibiting protein kinase C (PKC) and phosphoinositide 3-kinase (PI3K)/AKT pathway signaling. PI3K/AKT pathway signaling liberates eukaryotic translation initiation factor 4E (eIF4E) through the hierarchical phosphorylation of eIF4E binding proteins (4E-BP). When hypophosphorylated, 4E-BPs associate with eIF4E, preventing eIF4E from binding eIF4G, blocking the formation of the eIF4F translation initiation complex. Herein, we show that enzastaurin treatment impacts signaling throughout the AKT/mTOR pathway leading to hypophosphorylation of 4E-BP1 in cancer cells of diverse lineages (glioblastoma, colon carcinoma, and B-cell lymphoma). Accordingly, enzastaurin treatment increases the amount of eIF4E bound to 4E-BP1 and decreases association of eIF4E with eIF4G, thereby reducing eIF4F translation initiation complex levels. We therefore chose to evaluate whether this effect on 4E-BP1 was involved in enzastaurin-induced apoptosis. Remarkably, enzastaurin-induced apoptosis was blocked in cancer cells depleted of 4E-BP1 by siRNAs, or in 4EBP1/2 knockout murine embryonic fibroblasts cells. Furthermore, eIF4E expression was increased and 4E-BP1 expression was decreased in cancer cells selected for reduced sensitivity to enzastaurin-induced apoptosis. These data highlight the importance of modulating 4E-BP1 function, and eIF4F complex levels, in the direct antitumor effect of enzastaurin and suggest that 4E-BP1 function may serve as a promising determinant of enzastaurin activity. Mol Cancer Ther; 9(12); 3158–63. ©2010 AACR.
variety of cancers including prostate (9), lung adenocarcinoma (10), gastric (11), breast (12), head and neck (13), and others (7). elf4E activity is attenuated by binding to the 4E-BPs. Experimental models show that the 4E-BPs are involved in tumorigenesis (14–16) and that 4E-BP1 overexpression suppresses tumor formation and growth (17, 18). Additionally, clinical data indicate increased phosphorylation of 4E-BP1 is associated with disease progression and poor patient survival in prostate (9), breast (19), and ovarian (20) cancers. Collectively, these data reveal that activation of elf4E (either by direct overexpression or release from the 4E-BPs) promotes tumor formation and malignancy in experimental models and is related to reduced patient survival in numerous human cancers. Accordingly, 4E-BP1 has recently been shown to play a crucial role in transformation and that targeting elf4E or the elf4E complex provides an attractive, novel anticancer strategy (21–24).

We sought to understand more fully the critical determinants for the anticancer activity of enzastaurin. We now show that enzastaurin inhibits multiple phosphorylation events in the AKT/mTOR pathway leading to the hypophosphorylation of 4E-BP1. Consequently, enzastaurin increases the binding of elf4E to 4E-BP1 and decreases the association of elf4E with elf4G. These data indicate enzastaurin treatment reduces the levels of the elf4E/translation initiation complex. Moreover, elf4E expression is upregulated and 4E-BP1 expression is downregulated in cells selected for reduced sensitivity to enzastaurin-induced apoptosis. Importantly, 4E-BP1 reduction or 4E-BP1/2 knockout blocks enzastaurin-induced apoptosis. These data indicate that the modulation of 4E-BP is functionally important for enzastaurin-induced apoptosis and may serve as a critical determinant of enzastaurin activity.

Material and Methods

Cell culture

4E-BP1 and 4E-BP2 knockout (4E-BP1/2 KO) and wild-type (4E-BP1/2 WT) murine embryonic fibroblasts (MEF) were derived as previously described (14, 25). CRL2611 human glioma cells, HCT116 human colon cancer cells, and Farage B-cell lymphoma cells were obtained from the American Type Culture Collection. Cells lines were tested for mycoplasma and other pathogens and were authenticated using short tandem repeat genotyping in accordance with the referenced American Type Culture Collection genotype when available. CRL2611 and MEF cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, 11995) supplemented with 10% heat inactivated fetal bovine serum (HI FBS; Gibco, 10082). HCT116 cells were maintained in McCoy’s 5A media supplemented with 1% HI FBS, 5% sodium pyruvate (Gibco, 11360), 5% sodium bicarbonate (Gibco, 25080), and 5% D-(-)-glucose (Sigma Aldrich, G8769). Cells were routinely treated in media with 1% HI FBS as indicated (1). HCT116 cells were maintained in McCoy’s 5A media supplemented with 1% HI FBS and 1 μmol/L enzastaurin for more than 100 passages to select for cells with reduced sensitivity to enzastaurin-induced apoptosis.

Western blotting

Western blots were done as previously described (1). The antibodies to 4E-BP1, p4E-BP1Thr37/46, p4E-BP1Ser65, p4E-BP1Thr70, AKTThr308, AKTThr451, elf4G, mTOR, pMTORSer2448 were used at 1:500 dilution (Cell Signaling), elf4E and AKT were used at 1:500 dilution (BD Biosciences), β-actin at 1:20,000 dilution (Sigma Aldrich). All Western blots were incubated in 5% BSA in Tris-buffered saline Tween-20 (TBST) with 0.1% Tween-20. Secondary antibodies were used at 1:5,000 dilution (Santa Cruz) and incubated in 5% milk and 0.1% Tween-20 in PBS. 7-Methyl-GTP-sepharose (m7GTP) chromatography was used to assess the interaction of elf4E with either elf4G or 4E-BP1 from cell extracts as described (9).

siRNA transfection

A total of 1,000 cells (CRL2611 or HCT116) was plated in 96-well poly-D-lysine plates (Becton Dickinson) and transfected with 5 to 20 nmol/L 4E-BP1 siRNA (Ambion, s223471), 20nmol/L pooled Dharmacon ON-target plus Duplex 4E-BP1 siRNA (D-003005-11,12,13,14), or negative control siRNA no. 2 (AM4637, Ambion) in Oligofectamine (Invitrogen, 12252-011). Transfections were carried out in 100 μL DMEM 11995 for CRL2611 cells or McCoy’s 5A supplemented with 1% FBS for HCT116 cells for 72 hours. After the 72 hour transfection, 100 μL DMEM 11995 for CRL2611 cells or McCoy’s 5A supplemented with 1% FBS for HCT116 cells for 72 hours. The 72 hour transfection, 100 μL of 2x dimethyl sulfoxide (DMSO) or enzastaurin was added for 48 to 72 hours.

Apoptosis assays

Induction of apoptosis was measured by oligonucleosomal fragmentation for 48 to 72 hours as indicated (Cell Death Detection Elisa; Roche Applied Science) or by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining for 48 hours, as described (1). Absorbance values were normalized to DMSO controls (error bars represent ±SEM).

Results and Discussion

Enzastaurin suppresses AKT/mTOR pathway phosphorylation

Enzastaurin inhibits signaling through the PKCβ and P38K/ AKT pathways specifically blocking the phosphorylation of GSK3β, rpS6, and AKT (1). We sought to determine to what extent enzastaurin treatment...
would inhibit signaling throughout this pathway in cancer cell lines representing diverse cellular lineages (colon carcinoma, glioma, B-cell lymphoma). The chosen clinical dose for enzastaurin (500 or 525 mg per day) routinely yields 3 μmol/L mean plasma concentrations of total drug, reaching 8 μmol/L in some patients (26, 27). We therefore evaluated enzastaurin at 1 to 4 μmol/L.

At 16 and 24 hours, enzastaurin suppressed the phosphorylation of rpS6 (S240/244), AKT (S473), AKT (T308), AKT (T308), mTOR (S2448), p70S6K (T389), 4E-BP1 (T37/46), and β-actin. A, HCT116 colon cancer cells; B, CRL2611 glioblastoma cells; C, Farage B-cell lymphoma cells.

Enzastaurin reduces the levels of the eIF4F translation initiation complex

Enzastaurin inhibits 4E-BP1 phosphorylation at multiple phosphorylation sites including T37/46, S65, and T70 (Fig. 1A–C). As the hypophosphorylation of 4E-BP1 increases the binding of 4E-BP1 to eIF4E (5), we sought to determine whether enzastaurin would increase this association using m7GTP cap analog beads to capture eIF4E and to pull down associated proteins. Enzastaurin treatment substantially increased the amount of 4E-BP1 bound to eIF4E in all 3 cell lines at all concentration levels [Fig. 2A (inset), B (left), and C (left)]. To confirm that this increased binding was not a reflection of changes in total eIF4E or 4E-BP1 levels we analyzed total levels of eIF4E and 4E-BP1 [Fig. 2B (right) and C (right)].

Figure 1. Enzastaurin suppresses cell signaling downstream of the AKT/mTOR pathway. Cancer cells were incubated with 0 to 4μmol/L enzastaurin in media supplemented with 1% HI FBS for 16 and 24 hours. Western blot analyses were done for total 4E-BP1, 4E-BP1 Thr37/46, 4E-BP1 Ser65, 4E-BP1 Thr70, AKT Ser473, AKT Thr308, mTOR, mTOR Ser2448, p70S6K Thr389, rpS6 Ser240/244, rpS6, AKT, and β-actin. A, HCT116 colon cancer cells; B, CRL2611 glioblastoma cells; C, Farage B-cell lymphoma cells.

Reducing 4E-BP1 expression suppresses enzastaurin-induced apoptosis

Enzastaurin induces apoptosis in a wide variety of human cancer cell lines including glioblastoma multiforme (1, 28), cutaneous T-cell lymphoma (29), colon (1), gastric (30), multiple myeloma (31, 32), and Waldenström’s macroglobulinemia (33). Our data...
Enzastaurin-induced apoptosis requires 4E-BP1

Figure 2. Enzastaurin decreases eIF4F complex levels. Cancer cells were incubated with 0 to 4 μmol/L enzastaurin in media supplemented with 1% HI FBS for 24 hours. Western blot analyses were done for total eIF4G, eIF4E, and 4E-BP1 from m7GTP pull-down lysates and whole cell lysates. Whole lysates were additionally probed for β-actin to control for loading and transfer. Graph represents eIF4G normalized to eIF4E divided by 4E-BP1 normalized to eIF4E [(eIF4G/eIF4E)/(4E-BP1/eIF4E)]. Data represent the mean of 3 separate experiments (±SEM). A, HCT116 colon cancer cells; B, Farage B-cell lymphoma cells; C, CRL2611 glioblastoma cells; D, percentage of apoptotic cells following enzastaurin treatment in HCT116 cells and in HCT116 colon cancer cells selected for reduced sensitivity (HCT116res) to enzastaurin as measured by TUNEL staining. Inset shows the levels of eIF4E and 4E-BP1 by Western blot from whole cell lysates. β-Actin was assessed to control for loading and transfer.

indicate that enzastaurin treatment blocks 4E-BP1 phosphorylation and increases the association of eIF4E with 4E-BP1. We therefore examined whether manipulating 4E-BP1 levels would impact enzastaurin-induced apoptosis. Total 4E-BP1 expression was reduced 70% to 80%, 72 hours after 4E-BP1 siRNA transfection in HCT116 and CRL2611 cells (Fig. 3A). Enzastaurin was added after 4E-BP1 knockdown and assayed for the induction of apoptosis by oligonucleosomal fragmentation 48 to 72 hours thereafter. Apoptosis was evident in both HCT116 and CRL2611 cells treated for 48 hours with 1 to 2 μmol/L enzastaurin, a concentration routinely achieved clinically [Fig. 3B (black bars) and C (black bars)]. Depletion of 4E-BP1 by siRNA transfection blocked enzastaurin-induced apoptosis in both cell lines [Fig. 3B (white bars) and C (white bars)]. These data indicate that enzastaurin-induced apoptosis can be significantly inhibited when 4E-BP levels are reduced.

To address the potential off-target effects of 4E-BP1 siRNA transfection and to confirm and extend these siRNA data, we also sought to determine whether complete knockout of 4E-BP1 and 4E-BP2 (4E-BP1/2 KO) would affect apoptosis and eIF4F complex levels after enzastaurin treatment. In wild-type (4E-BP1/2 WT) MEFs, enzastaurin-induced apoptosis (Fig. 4A, black bars) and increased the association of 4E-BPs with eIF4E, concomitant with 4E-BP hypophosphorylation (Fig. 4B). By contrast, in the 4E-BP1/2 knockout (4E-BP1 KO) MEFs, enzastaurin-induced apoptosis was blocked (Fig. 4A, white bars). To quantify the reduction in eIF4F complex levels, we normalized the levels of cocaptured 4E-BP1 and eIF4G to eIF4E and then divided the normalized eIF4G values by the normalized 4E-BP1 (Fig. 4C). The 4E-BP1/2 KO MEFs showed higher eIF4F assembly (i.e., eIF4G bound to eIF4E) than the 4E-BP1/2 WT MEFs. Further, the 4E-BP1/2 KO MEFs showed dose-dependent reduction of eIF4F levels after enzastaurin treatment (Fig. 4C). These data, along with the 4E-BP1 siRNA depletion data in cancer cells, further support a functional role for the 4E-BPs in enzastaurin-induced apoptosis.

Enzastaurin was originally advanced into the clinic as an antiangiogenic therapy and has subsequently been shown to induce apoptosis directly in cancer cells (1). The data in this article represent a continued effort to understand the critical effectors for the anticancer effect of enzastaurin. These data confirm and extend previously published data showing that enzastaurin inhibits multiple, key phosphorylation events throughout the PI3K/akt pathway impacting phosphorylation not only of GSK3β, rpS6, and AKT (1) but also of mTOR, p70S6K, and 4E-BP1. These data show that, by augment-
ing eIF4E:4E-BP1 binding and decreasing eIF4E:eIF4G binding, enzastaurin substantially decreases levels of the eIF4F translation initiation complex. Importantly, knockout or depletion of 4E-BP obviates the apoptotic effect of enzastaurin on these cells highlighting that 4E-BP1 function is a critical determinant of enzastaurin activity in cancer cells of diverse lineage.

**Disclosure of Potential Conflicts of Interest**

All authors are employees of Eli Lilly and Company. N. Sonenberg is a consultant for Eli Lilly and Company.

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Enzastaurin-Induced Apoptosis Requires 4E-BP1

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