Enzastaurin (LY317615), a protein kinase Cβ inhibitor, inhibits the AKT pathway and induces apoptosis in multiple myeloma cell lines

Mujahid A. Rizvi,[1,2] Kulsoom Ghias,[2] Katharine M. Davies,[2] Chunguang Ma,[2] Frank Weinberg,[2] Hidayatullah G. Munshi,[1,2] Nancy L. Krett,[2] and Steven T. Rosen[1,2]

[1]Division of Hematology/Oncology, Department of Medicine, Northwestern University Feinberg School of Medicine and [2]Robert H. Lurie Comprehensive Cancer Center of Northwestern University, Chicago, Illinois

Abstract

Enzastaurin (LY317615), an acyclic bisindolylmaleimide, is an oral inhibitor of the protein kinase Cβ isozyme. The objective of this study was to assess the efficacy of enzastaurin in inducing apoptosis in multiple myeloma (MM) cell lines and to investigate possible mechanisms of apoptosis. Cell proliferation assays were done on a variety of MM cell lines with unique characteristics (dexamethasone sensitive, dexamethasone resistant, chemotherapy sensitive, and melphalan resistant). The dexamethasone-sensitive MM.1S cell line was used to further assess the effect of enzastaurin in the presence of dexamethasone, insulin-like growth factor-I (IGF-I), interleukin-6, and the pan-specific caspase inhibitor ZVAD-fmk. Enzastaurin increased cell death in all cell lines at clinically significant low micromolar concentrations (1 – 3 μmol/L) after 72 hours of treatment. Dexamethasone and enzastaurin were shown to have an additive effect on MM.1S cell death. Although IGF-I blocked the effect of 1 μmol/L enzastaurin, IGF-I did not abrogate cell death induced with 3 μmol/L enzastaurin. Moreover, enzastaurin-induced cell death was not affected by interleukin-6 or ZVAD-fmk. GSK3β phosphorylation, a reliable pharmacodynamic marker for enzastaurin activity, and AKT phosphorylation were both decreased with enzastaurin treatment. These data indicate that enzastaurin induces apoptosis in MM cell lines in a caspase-independent manner and that enzastaurin exerts its antimyeloma effect by inhibiting signaling through the AKT pathway. [Mol Cancer Ther 2006;5(7):1783 – 9]

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Requests for reprints: Mujahid A. Rizvi, Division of Hematology/Oncology, Department of Medicine, Northwestern University Feinberg School of Medicine, Lurie Building 3-250, 303 East Superior Street, Chicago, IL 60611. Phone: 312-695-6180; Fax: 312-695-6189. E-mail: m-rizvi@md.northwestern.edu

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Introduction

Multiple myeloma (MM) is characterized by malignant transformation and proliferation of a single clone of plasma cells that produce a monoclonal immunoglobulin. MM accounts for 10% of all hematologic malignancies and 1% of all malignant disease in the United States (1). The annual incidence of MM is 4 per 100,000 (1). In the last decade, the treatment of MM has evolved rapidly. The use of high-dose chemotherapy followed by autologous stem cell transplant has increased remission rates and progression-free survival, and overall survival compared with conventional chemotherapy (2). Unfortunately, a large proportion of patients relapse, and the search for new, more effective salvage therapies is needed.

The protein kinase C (PKC) family consists of 11 serine/threonine protein kinase isoforms that are involved in cell proliferation and differentiation, gene transcription, and tumor-induced angiogenesis. The PKC pathway has been shown to play a role in the regulation of cell growth in hematologic malignancies. In diffuse large B-cell lymphoma, oligonucleotide microarray gene expression profiles were used to predict outcomes and PKCβ was found to be overexpressed in refractory/fatal cases of diffuse large B-cell lymphoma (3, 4). In MM, the role of the PKC pathway has not been extensively studied; however, there is some evidence that MM cell migration may in fact be modulated by the vascular endothelial growth factor via a PKC-dependent pathway (5). Ni and colleagues (6) showed that the U266, RPMI8226S, and K620 MM cell lines expressed PKCδ, PKCι, PKCμ, and PKCζ. PKCβ was expressed in the RPMI8226S and K620 cell lines, but not in the U266 cell line. In addition, the K620 cell line expressed PKCα, PKCγ, PKCε, and PKCθ. The U266 cell line was treated with safingol, a general PKC inhibitor, and rottlerin, a PKCδ specific inhibitor, and both induced apoptosis in MM cells (6). A phase II trial using bryostatin 1, a PKC modulator, was conducted in MM patients; although the treatment was well tolerated, none of the nine patients responded (7).

Enzastaurin (LY317615), an acyclic bisindolylmaleimide, is an oral inhibitor of the PKCβ isozyme. In a phase I clinical trial, patients treated with 525 mg/d enzastaurin achieved steady-state plasma concentrations of 8 μmol/L, with a mean plasma exposure of 2 μmol/L (8). Enzastaurin selectively inhibits PKCβ at low concentrations, but also inhibits the other PKC isozymes at concentrations achieved physiologically in clinical trials. It does not inhibit other cellular serine/threonine kinases and tyrosine kinases, such as, IκB kinase α, IκB kinase β, c-Jun-NH_2-kinase, mitogen-activated protein kinase kinase, stress-activated protein kinase, mitogen-activated protein kinase, AMP-activated

protein kinase, PRK2, PKBα, PKBβ, PDK1, epidermal growth factor receptor, platelet-derived growth factor receptor, fibroblast growth factor receptor, Bruton's tyrosine kinase, SRC, and Abl (9). PKCβ is a part of the signal cascade of vascular endothelial growth factor; therefore, initial data has focused on the effects of enzastaurin on angiogenesis. Because there is data to suggest that gliomas are dependent on vascular endothelial growth factor–mediated angiogenesis, a phase II trial of enzastaurin in high-grade gliomas is currently under way. Eighty-five patients have been treated with enzastaurin and 79 patients are evaluable for response. Seventy-two percent of the patients had glioblastoma multiforme. Treatment with enzastaurin was well tolerated with minimal drug-related toxicity: three patients had grade 3 to 4 hematologic toxicity and one patient had grade 2 hepatotoxicity. Thirty-six patients received more than one cycle of enzastaurin and 13 of these patients had stable disease for >3 months. Objective radiographic responses have been seen in 14 patients (10 glioblastoma multiforme patients), one of whom had a complete response and is still receiving enzastaurin 13 months after enrollment (10).

In addition to the anti–vascular endothelial growth factor effects, enzastaurin in the low-μmol/L range has been shown to suppress proliferation in a wide variety of cancer cell lines: leukemia, non–small cell lung cancer, colon cancer, melanoma, ovarian cancer, renal cancer, prostate cancer, and breast cancer cell lines (9). Graff et al. (9) have shown that enzastaurin (1–4 μmol/L) induces apoptosis in the HCT116 colon carcinoma cell line and U87MG glioblastoma cells. In addition, they have shown a time-dependent loss of GSK3β phosphorylation after exposure to enzastaurin (1–3 μmol/L) in the colon cancer cell line, U87MG glioblastoma cells, and in xenograft-bearing mice. A similar loss of GSK3β phosphorylation was seen in the mouse peripheral blood mononuclear cells and xenograft tissues. GSK3β phosphorylation is associated with PKCβ activity directly and is also a downstream target of the AKT pathway. They concluded that enzastaurin may exert its antineoplastic effects by inhibiting the AKT pathway and further that GSK3β phosphorylation is a reliable pharmacodynamic marker for enzastaurin activity. We were able to corroborate these findings with enzastaurin in cutaneous T-cell lymphoma cell lines (HuT-78 and CRL-2105; ref. 11).

Here, we show that enzastaurin induces apoptosis in MM cell lines at concentrations achieved in phase I clinical trials. We also show the loss of GSK3β and AKT phosphorylation when myeloma cells are exposed to enzastaurin.

Materials and Methods

Chemicals

Enzastaurin was obtained from Eli Lilly and Co. (Indianapolis, IN). Interleukin-6 (IL-6), insulin-like growth factor-I (IGF-I), and ZVAD-fmk was purchased from R&D Systems (Minneapolis, MN). Dexamethasone was obtained from Sigma (St. Louis, MO).

Cell Culture

A variety of MM cell lines with unique characteristics were used. MM.1S is a glucocorticoid-sensitive cell line established from the peripheral blood of a MM patient (12). $MM.1R_e$ (1.414) and $MM.1R_L$ are subclones of MM.1S with partial to complete resistance to glucocorticoids. The U266 cell line is also resistant to glucocorticoids. RPMI8226S is a chemotherapy-sensitive cell line, whereas RPMI8226-LR 5 (selected with melphalan) is a chemotherapy-resistant cell line. The chemotherapy-sensitive and chemotherapy-resistant cell lines were provided by Dr. William Dalton (H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL).

MM cell lines were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone, Inc., Logan, UT), 2 mmol/L glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL plasmocin, and 2.5 μg/mL fungizone. Cells were maintained at 37°C in an incubator with 5% CO_2. These cell lines have a doubling time of ~72 hours.

Enzastaurin Treatment

Twenty-four hours before treatment with enzastaurin, the myeloma cell lines were transferred to RPMI 1640 with 1% fetal bovine serum due to the high protein binding affinity of enzastaurin (concentration of glutamine, penicillin, streptomycin, plasmocin, and fungizone were kept constant; ref. 9). The MM cells were seeded into culture at concentrations that would ensure logarithmic growth over the duration of the experiment. The concentration and time of treatment with enzastaurin is detailed in the individual experiments. The cells were maintained in standard culture conditions over the course of the experiment. To determine whether enzastaurin-induced apoptosis was caspase-dependent, ZVAD-fmk, a pan-specific caspase inhibitor, was used at 40 μmol/L. ZVAD-fmk was always added 1 hour before enzastaurin treatment.

Cell Proliferation Assay

MM cells were cultured into 96-well dishes at a concentration of 25,000 per well and incubated with the indicated drugs for 72 hours. Cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium Cell Titer Aq_{ueous} assay (Promega, Madison, WI), which measured the conversion of a tetrazolium compound into formazan by a mitochondrial dehydrogenase enzyme in live cells. The amount of formazan is proportional to the number of living cells present in the assay mixture. Each data point was the average of four independent determinations. The data were expressed as the percentage of formazan produced by the untreated cells in the same assay.

Immunoblotting Analysis

MM cells were cultured and grown at a concentration of 1×10^6/mL. Cells were treated and harvested at the desired time points. Cell pellets were homogenized in lysis buffer [10 mmol/L KPO_4 (pH 7.0), 1 mmol/L EDTA, 5 mmol/L EGTA, 10 mmol/L $MgCl_2$, 50 mmol/L B-glycerol phosphate, 1 mmol/L sodium orthovanadate, 2 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 0.5% NP40, and 0.1% deoxycholate]. Homogenates were centrifuged at 4°C

for 10 minutes at 16,100 × *g*, the supernatants were collected, and protein concentrations were determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Thirty micrograms of protein were loaded with sample buffer [125 mmol/L Tris (pH 6.8), 4% SDS, 20% glycerol, 100 mmol/L DTT, and 0.05% bromphenol blue] onto a precast 8% to 16% Tris-glycine gels (Invitrogen/Novex, Carlsbad, CA). Proteins were then transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). After protein transfer, membranes were blocked with 5% bovine serum albumin in TBS for at least 1 hour and subsequently incubated with the appropriate primary antibody at a 1:1,000 dilution overnight at 4°C. Phospho-GSK3β$^{(serine9)}$, GSK3β, and AKT antibody were purchased from Cell Signaling Technologies (Beverly, MA). To detect poly(ADP-ribose)polymerase (PARP), a mouse monoclonal antibody was used (BD PharMingen, San Diego, CA). Phospho-AKT$^{(serine473)}$ antibody was purchased from R&D Systems (Minneapolis, MN). The R&D Systems antibody required a different blocking solution that contained 5% nonfat dry milk, 25 mmol/L Tris, 0.15 mol/L NaCl, and 0.1% Tween 20 (pH 7.4). A primary antibody concentration of 0.5 μg/mL was used for the phospho-AKT$^{(serine473)}$ antibody. The following day, blots were washed with TBS-T (TBS with 0.1% Tween 20) and incubated for 1 hour at room temperature with a 1:5,000 dilution of horseradish peroxidase–linked secondary antibody (Amersham, Arlington Heights, IL). After washing the blots with TBS-T, they were developed using enhanced chemiluminesence (ECL Plus; Amersham), and the signal was visualized with X-ray film (Hyperfilm; Amersham).

Cell Cycle Analysis

Cells (2×10^6) were treated with enzastaurin at the concentration indicated in the figure legends. After incubation for 72 hours, cells were collected, centrifuged at 500 × *g*, washed with cold PBS, and fixed overnight with 40% ethanol at 4°C. Cells were washed with cold PBS, and the pellet was resuspended in 50 μg/mL RNase A (diluted in PBS), and incubated for 30 minutes at 37°C. The samples were then resuspended in 25 μg/mL propidium iodide in 38 mmol/L sodium citrate buffer. Flow cytometry was done on a Coulter EPICs XL instrument and data were analyzed using the System II software package.

Annexin V Staining

Cells (1×10^6/mL) were treated with 3 μmol/L enzastaurin and harvested after 72 hours. As cells undergo apoptosis, the integrity of the cell membrane is disrupted and phosphatidylserine is exposed. Detection of phosphatidylserine on the outer leaflet of apoptotic cells was done by using Annexin V binding according to the instructions of the manufacturer (BD PharMingen). Briefly, cells were incubated with 5 μg/mL FITC-conjugated Annexin V in the presence of 5 μg/mL propidium iodide and screened by flow cytometry (Coulter EPICs XL). Annexin V–FITC positive, PI-negative cells were scored as early apoptotic. Annexin V–FITC positive, PI-positive cells were scored as late apoptotic.

Statistical Analysis

Data were analyzed using GraphPad InStat statistical software (GraphPad Software, Inc., San Diego, CA). Unpaired *t* tests were done and two-tailed *P* values are reported.

Results

Enzastaurin Inhibits Cell Proliferation in MM Cell Lines

MM.1S, MM.1R$_e$, MM.1R$_L$, RPMI8226S, RPMI8226-LR5, and U266 MM cell lines were treated with increasing concentrations of enzastaurin. The cell proliferation assay, as described in Materials and Methods, was measured after 24 and 72 hours of treatment with enzastaurin. There was no evidence of inhibition of cell growth at clinically relevant concentrations at 24 hours (data not shown). At 72 hours, there was evidence of decreased cell proliferation at low μmol/L concentrations (1–3 μmol/L; Fig. 1). The mean percentage of viable cells present after 72 hours of treatment with 3 μmol/L enzastaurin was 40% (range 18–81%). The RPMI8226S (chemotherapy sensitive) and the RPMI8226-LR5 (chemotherapy resistant) cell lines were more resistant to enzastaurin, with 61% and 81% viable cells present, respectively, after 72 hours of exposure to 3 μmol/L enzastaurin.

Enzastaurin and Dexamethasone Show Additive, Not Synergistic, Effects

MM.1S cells were treated with increasing concentrations of enzastaurin. Using cell cycle analysis, we determined the fraction of cells in the sub-G$_1$ phase, indicating cells undergoing apoptosis (Fig. 2A). The percentage of cells undergoing apoptosis increased with increasing concentrations of enzastaurin. After 72 hours of exposure to 3 μmol/L enzastaurin, there was a 2.7-fold (52% of cells in the sub-G$_1$ phase) increase in the proportion of cells in the sub-G$_1$ phase compared with untreated controls (18.7% of

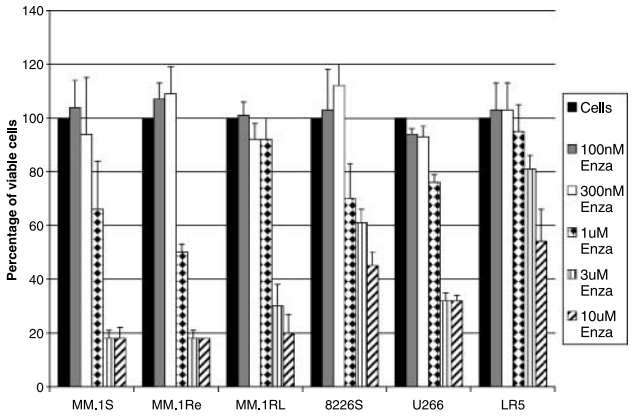
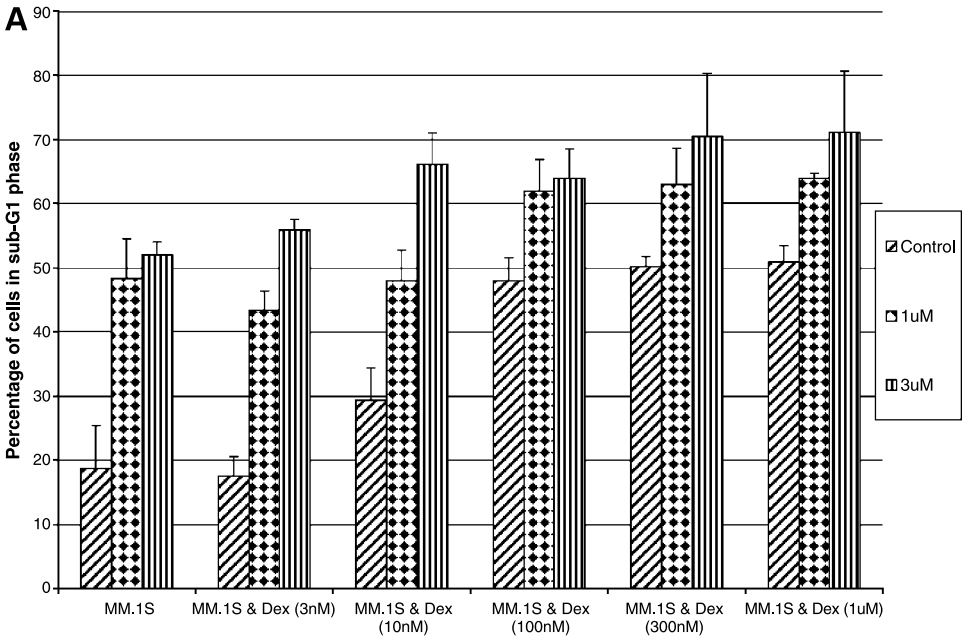


Figure 1. Enzastaurin treatment decreases the number of viable cells. MM cell lines were treated with increasing concentrations of enzastaurin (*Enza*) for 72 h. The cell proliferation assay measured the amount of formazan, which is directly proportional to the number of living cells present compared with the control.



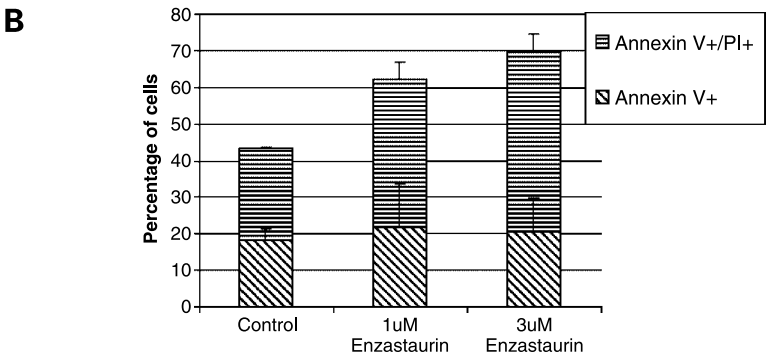


Figure 2. Enzastaurin treatment induces apoptosis in MM cells. **A,** MM.1S cells were treated with increasing concentrations of enzastaurin for 72 h. The same set of experiments was repeated with increasing concentrations of dexamethasone (*Dex*) simultaneously with enzastaurin. DNA content was measured by staining with propidium iodide followed by flow cytometry as described in Materials and Methods. **B,** MM.1S cells were treated with increasing concentrations of enzastaurin for 72 h. Annexin V staining was determined by flow cytometry as described in Materials and Methods. *PI,* propidium iodide.

cells in the sub-G_1 phase). The same set of experiments was repeated with the addition of increasing concentrations of dexamethasone simultaneously with enzastaurin (Fig. 2A). There was no evidence that low doses of dexamethasone (3 nmol/L) had any effect on MM.1S cells (percentage of cells in the sub-G_1 phase was 17.6% compared with 18.7% in the control). We were unable to show synergistic effects between suboptimal doses of dexamethasone and clinically relevant doses (1–3 μmol/L) of enzastaurin (percentage of cells in the sub-G_1 phase was 55.9% with 3 nmol/L dexamethasone and 3 μmol/L enzastaurin and 52% with 3 μmol/L enzastaurin). There was, however, evidence of an additive effect at higher concentrations of dexamethasone and enzastaurin (1–3 μmol/L).

We used a parallel assay to determine whether cell cycle analysis was accurate in determining the percentage of cells undergoing apoptosis. We used Annexin V–FITC binding as a confirmatory assay. The cell cycle analysis was a more conservative assay in determining the percentage of cells undergoing apoptosis (Fig. 2B).

IGF-I Partially Inhibits the Effects of Enzastaurin on MM.1S Cells whereas IL-6 Has No Effect

IL-6 has pleiotropic effects on hematopoietic and non-hematopoietic cells and is an important growth factor for MM cells (13, 14). IL-6 facilitates B-cell differentiation into immunoglobulin-secreting plasma cells and has been shown to prevent drug-induced apoptosis (15). IGF-I is a crucial growth and survival factor for MM (16) and has been shown to inhibit dexamethasone-induced apoptosis (17). Cell cycle analysis was used to determine whether either IL-6 or IGF-I could affect enzastaurin-induced apoptosis. Cells were treated with 1 and 3 μmol/L enzastaurin for 72 hours with or without IL-6 (1 ng/mL). In a similar fashion, MM.1S cells were treated with enzastaurin in the presence or absence of IGF-I (100 ng/mL). Enzastaurin-induced apoptosis was not affected by the addition of IL-6 (Fig. 3A and B). There was a 3.4-fold increase in the percentage of cells in the sub-G_1 phase with 3 μmol/L enzastaurin irrespective of whether IL-6 was present or not. Experiments were done using higher concentrations of IL-6 (5 and 50 ng/mL) with similar

results (data not shown). In contrast, IGF-I inhibits enzastaurin-induced apoptosis at lower concentrations of enzastaurin (1 μmol/L). Forty-eight percent of cells were in the sub-G_1 phase after a 72-hour exposure to 1 μmol/L enzastaurin compared with 22% of cells with 1 μmol/L enzastaurin and 100 ng/mL IGF-I ($P = 0.048$). However, a higher concentration of enzastaurin (3 μmol/L) was able to overcome the inhibitory effects of IGF-I ($P = 0.24$; Fig. 3A and B).

Enzastaurin Induces Apoptosis in a Caspase-Independent Manner

PARP is a 116 kDa protein that is a substrate for activated caspase-3. The cleaved form of PARP is detected at 85 kDa by immunoblotting. Cells were treated with enzastaurin (2 μmol/L) for the indicated times, harvested, and whole cell extracts were prepared, fractionated, and immunoblotted as described. As shown in Fig. 4A, immunoblots of whole cell lysates of MM.1S cells treated with enzastaurin for 2, 4, 24, and 72 hours showed PARP cleavage as early as 24 hours of incubation. Dexamethasone was used as a positive control because it is known to induce apoptosis in MM.1S cells, and there was evidence of PARP cleavage after a 24-hour exposure to dexamethasone (1 μmol/L).

Cell cycle analysis was used to further elucidate the role of caspase activation in enzastaurin-induced apoptosis in MM cells. MM.1S cells were treated with enzastaurin (1 and 3 μmol/L) for 72 hours as described in the above experiments in the presence and absence of ZVAD-fmk (40 μmol/L), a pan-specific caspase inhibitor added 1 hour before enzastaurin. The percentage of cells in the sub-G_1 phase in the untreated control and the ZVAD-fmk-treated control was 27% and 18%, respectively (Fig. 5). This indicated that there was caspase-dependent apoptosis present in MM.1S cells at baseline. After treatment with enzastaurin (3 μmol/L for 72 hours), the percentage of cells undergoing apoptosis in the ZVAD-fmk–untreated (57%) and ZVAD-fmk–treated cells (51%) was similar ($P = 0.28$). Dexamethasone-induced apoptosis is caspase dependent. We treated MM.1S cells with 1 μmol/L dexamethasone in the presence and absence of ZVAD-fmk (40 μmol/L). There was a 40% decrease in the relative number of cells undergoing apoptosis in the ZVAD-fmk–treated samples ($P < 0.01$). Our data suggest that enzastaurin-induced apoptosis may be caspase independent.

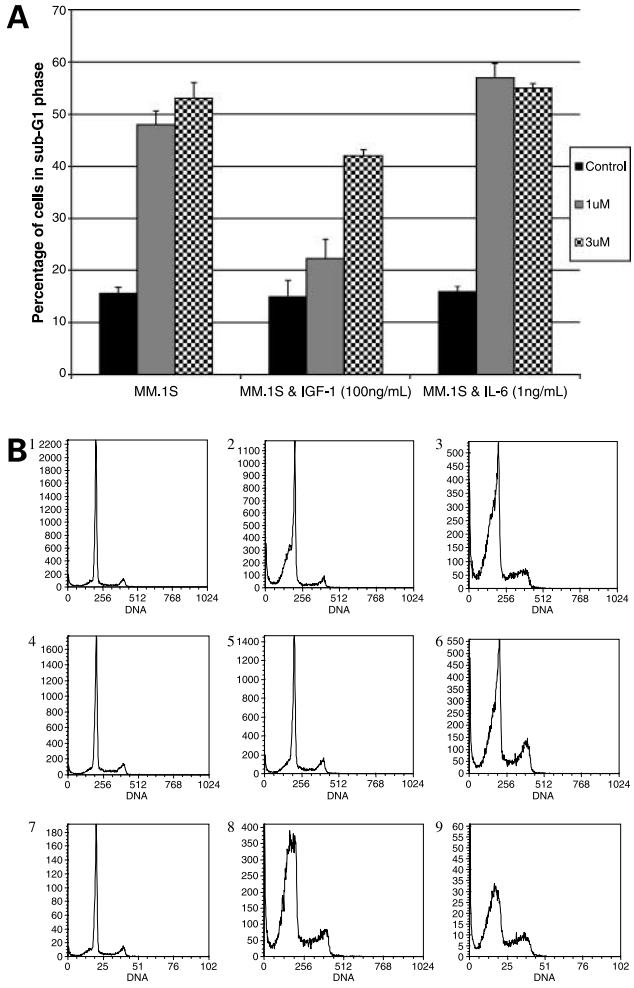


Figure 3. Effect of MM growth factors on enzastaurin-induced apoptosis. **A,** enzastaurin treatment induces apoptosis in MM cells by virtue of increased sub-G_1 phase cell population. MM.1S cells were treated with increasing concentrations of enzastaurin for 72 h. The same set of experiments was repeated separately with the addition of IL-6 (1 ng/mL) and IGF-I (100 ng/mL). DNA content was measured by staining with propidium iodide followed by flow cytometry as described in Materials and Methods. **B,** representative flow cytograms. *1,* control; *2,* 1 μmol/L enzastaurin; *3,* 3 μmol/L enzastaurin; *4,* 100 ng/mL IGF-I; *5,* 100 ng/mL IGF-I and 1 μmol/L enzastaurin; *6,* 100 ng/mL IGF-I and 3 μmol/L enzastaurin; *7,* 1 ng/mL IL-6; *8,* 1 ng/mL IL-6 and 1 μmol/L enzastaurin; *9,* 1 ng/mL IL-6 and 3 μmol/L enzastaurin.

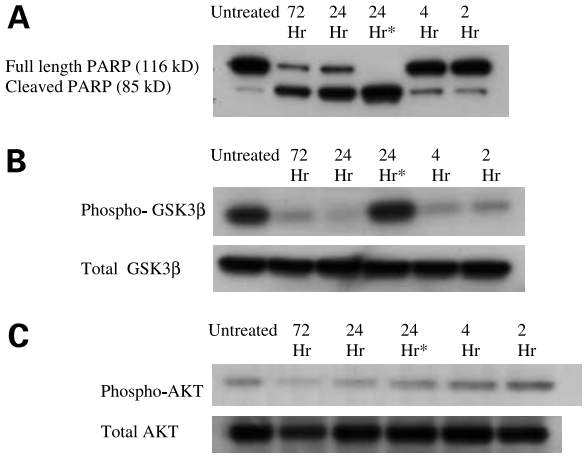


Figure 4. Effect of enzastaurin on PARP cleavage and kinase phosphorylation. MM.1S cells were treated with enzastaurin (2 μmol/L) for the indicated times. **A,** cleavage of PARP in enzastaurin-treated cells. *, 1 μmol/L dexamethasone. **B,** decrease in phosphorylation of GSK3β. *, 1 μmol/L dexamethasone. **C,** decrease in phosphorylation of AKT. *, 1 μmol/L dexamethasone. Whole cell lysates were prepared and immunoblotted for PARP, phospho-GSK3β and total GSK3β, and phospho and total AKT as described in Materials and Methods. Immunoblots are representative of two similar experiments.

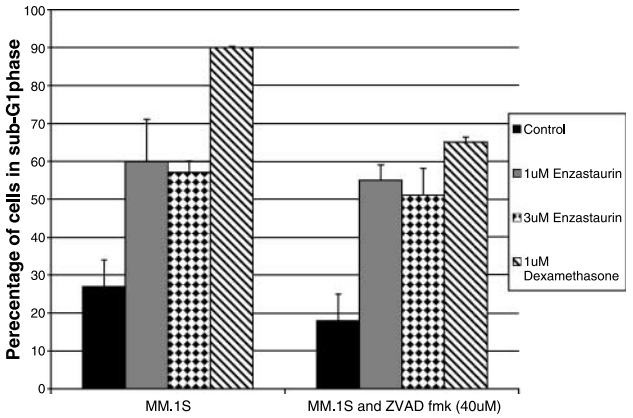


Figure 5. Enzastaurin induces caspase-independent apoptosis. Enzastaurin treatment induces apoptosis in MM cells by virtue of increased sub-G_1-phase cell population. MM.1S cells were treated with increasing concentrations of enzastaurin. ZVAD-fmk was added 1 h before the addition of enzastaurin. DNA content was measured by staining with propidium iodide followed by flow cytometry as described in Materials and Methods.

Enzastaurin Decreases Phosphorylation of GSK3β and AKT

GSK3β is a pharmacodynamic marker for enzastaurin and has been linked to PKCβ directly and is also a downstream target of the AKT pathway. MM.1S cells were treated with 2 μmol/L enzastaurin for 2, 4, 24, and 72 hours. The cells were harvested, and whole cell extracts were prepared, fractionated, and immunoblotted as described in Materials and Methods. Dexamethasone was used as a negative control and there was no evidence of loss of phosphorylation of GSK3β$^{(serine9)}$ or AKT$^{(serine473)}$ after a 24-hour exposure to dexamethasone (1 μmol/L). Enzastaurin treatment showed a distinct, time-dependent reduction of GSK3β$^{(serine9)}$ and AKT$^{(serine473)}$ phosphorylation, whereas total protein levels remain unchanged (Fig. 4B and C). We conclude that enzastaurin may exert its antimyeloma activity by inhibiting the PKCβ and AKT pathway.

Discussion

There are ~13,700 new patients diagnosed with MM and almost 11,000 patients succumb to this illness every year in the United States (1). It is only recently that progress has been made in terms of survival in patients with MM. Unfortunately, the course of the disease is characterized by frequent relapses and development of resistance to chemotherapy and other agents. Therefore, it is imperative that newer agents with novel mechanisms of action be tested in MM. Here, we have evaluated the effects of enzastaurin in MM cell lines and investigated possible mechanisms by which it exerts its effects.

PKC act by regulating cell growth and differentiation in hematopoietic cells by modulating signal transduction (18–20). The data on PKC effects in MM cells, however, is limited. Recent reports in the literature describe a role for PKC in AKT activity. PKCβII can phosphorylate AKT at serine 473 and activate AKT (21). Graff et al. (9) present evidence that enzastaurin may affect human tumor cells, directly inducing a loss of phosphorylation of GSK3β, and AKT in a colon cancer cell line (HCT 116). We have shown a time-dependent loss of phosphorylation of GSK3β and AKT in the MM.1S cell line. A decrease in AKT phosphorylation was seen at 24 hours, a time at which cleavage of the PARP protein is detected. This also correlates with our findings and those of Graff et al. (9) who found evidence of significant apoptosis with enzastaurin after 72 hours of exposure. The loss of GSK3β at an earlier time point may have been due to PKC inhibition alone.

Enzastaurin is an oral agent given once daily. In phase I clinical trials, it was well tolerated with minimal adverse events (8). The efficacy of enzastaurin in MM cell lines, the MM.1S cell line in particular, makes it an attractive agent for further evaluation. We have shown enzastaurin-induced apoptosis in MM cells at clinically significant concentrations (1–3 μmol/L). We have also shown an additive effect with enzastaurin and dexamethasone, a commonly used drug in MM. Our investigation revealed that the effects of enzastaurin are not suppressed in the presence of IL-6 *in vitro*. This held true in the face of higher concentrations of IL-6. IGF-I, however, inhibits enzastaurin-induced apoptosis at lower concentrations (1 μmol/L). The inhibitory effects of IGF-I were reversed at higher concentrations of enzastaurin (3 μmol/L). Mitsiades et al. (22) have previously shown that IGF-I promotes MM cell growth by stimulating the activation of NF-κB and AKT in MM cell lines. In contrast, IL-6 does not activate NF-κB and induces less AKT activity compared with IGF-I (22). Our data is consistent with their findings because only IGF-I was able to inhibit enzastaurin-induced apoptosis. This has therapeutic implications and may prove beneficial in patients who have relapsed or refractory disease by targeting MM cells through different mechanisms.

The results of this study provide a platform for further evaluation of the role of PKCs in the pathogenesis of MM and the role of enzastaurin in the treatment of MM. It would be interesting to evaluate microarray gene expression profiles and chromosomal analyses of patients with MM to see whether the expression of PKCβ was associated with poor prognostic factors and sensitivity to antimyeloma agents. We are currently in the process of initiating a phase II trial with enzastaurin in MM patients. In addition to studying the effects of enzastaurin in patients, further studies to elucidate the role of PKCs in the pathogenesis of MM will be done.

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