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Effects of enzastaurin, alone or in combination, on signaling pathway controlling growth and survival of B-cell lymphoma cell lines

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Abstract
Immunochemotherapies have improved outcomes in indolent lymphoma. However, response durations progressively shorten following each treatment, and the majority of patients eventually die from the disease. Thus, new, less toxic, and more active treatments are needed. Protein kinase C (PKC), which has been repeatedly implicated in B-cell lymphoma progression, may be a new target for lymphoma cell growth inhibition. Enzastaurin, a PKC-β inhibitor, has toxic effects on a variety of cancer cells. The purpose of the present study was to assess the antitumor activity of enzastaurin on B-cell lymphoma cell lines and to investigate the underlying antitumor mechanisms. Enzastaurin induced apoptosis and inhibited phosphorylation of PKC, RSK, AKT, and downstream proteins. Moreover, our results reveal a new mechanism for enzastaurin-induced apoptosis via BAD activation. Finally, enzastaurin synergizes in its effects with chlorambucil and fludarabine, respectively. Taken together, our results strongly support clinical evaluation of enzastaurin in patients with B-cell lymphoma.

Keywords: *PKC, PI3K/AKT, signaling pathways, apoptosis, B-cell lines, indolent lymphoma*

Introduction

Indolent non-follicular lymphomas (INFLs) include a rather heterogeneous subgroup of lymphomas such as small lymphocytic lymphomas, lymphoplasmacytic lymphomas, and marginal-zone lymphomas [1]. Although several studies have shown that outcomes of follicular lymphoma changed with the introduction of rituximab [2,3], therapies for INFLs have been poorly investigated. Typical initial treatment strategies vary from the use of a single agent to high-dose therapies [4], but alkylating single-agent chemotherapy can still be considered the standard treatment [5]. INFLs in an advanced stage have a relatively poor prognosis, with low complete response to conventional chemotherapy and short median time to treatment failure [4]. The outcome for refractory/relapsed patients is even worse. Recently, a fludarabine and cyclophosphamide combination as a frontline therapy, with or without rituximab, improved outcome. However, the toxicity profile is of concern, and more data are needed to confirm results [6,7]. Follicular lymphoma (FL) is an indolent lymphoma associated with follicular center B cells, and typically contains the Bcl-2 chromosomal translocation t(14;18), which leads to overexpression of the anti-apoptotic intracellular protein, Bcl-2 [8]. FLs are sensitive to chemotherapy; however, patient relapses occur, and response duration becomes progressively shorter, and the majority of patients eventually die from the disease. Thus, there is a need for new, less toxic, and more active treatments.

A family of at least 12 distinct isoenzymes of protein kinase C (PKC) exists, functioning through serine/threonine kinase activity. The PKC signaling

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pathway plays a key role in tumor cell proliferation, survival, differentiation, cytokine secretion, and migration, and in tumor-induced angiogenesis [9]. PKC has been implicated repeatedly in the malignant progression of B-cell lymphoma [10], and PKC-β is a key component of the B-cell receptor signaling pathway and clearly involved in the normal function of B cells. PKC-β knockout mice have impaired humoral and B-cell proliferation response [11,12]. Considering its proposed role in lymphomagenesis, PKC-β may be a new target for lymphoma cell growth inhibition.

Enzastaurin (LY317615.HCl), an acyclic bisindolylmaleimide, was initially developed as an adenosine triphosphate (ATP)-competitive selective inhibitor of PKC [13]. In addition to its major target PKC-β, enzastaurin also potently inhibits other PKC isoforms. Although enzastaurin was initially developed for anti-angiogenic cancer therapy, recent preclinical studies have shown its direct effect on a variety of human cancer cells, inducing apoptosis [14]. Enzastaurin was also shown to target the phosphoinositide 3-kinase (PI3K)/AKT pathway and to inactivate BAD, a pro-apoptotic member of the Bcl-2 family of proteins. The purpose of the present study was to assess the antitumor activity of enzastaurin on B-cell lymphoma cell lines and to investigate the underlying antitumor mechanisms. We found that enzastaurin inhibits phosphorylation of some signaling pathways and induces caspase activation, leading to apoptosis. In addition, enzastaurin synergizes with chlorambucil and fludarabine.

Materials and methods

Cell culture

Cell lines. The WSU-NHL and Karpas 422 lines were kindly provided by Dr. M. Introna (Division of Hematology, Ospedali Riuniti, Bergamo, Italy); the RL line was purchased from DSMZ. All three lines carry the t(14;18), and Karpas 422 is Epstein–Barr virus positive (EBV+). All cell lines were cultured at 37°C in a 5% CO_2 humidified atmosphere in RPMI-1640 (Euroclone Ltd., UK) supplemented with 10% fetal bovine serum (FBS; Euroclone Ltd.), 2 mM L-glutamine, and 100 U/mL penicillin and streptomycin (Euroclone Ltd).

Bone marrow stromal cell cultures. Bone marrow (BM) mononuclear cells were separated using the Ficoll–Hypaque technique according to the method described by Gartner and Kaplan [15]. Adherent cells were long-term cultured and expanded in Iscove's modified Dulbecco's medium (IMDM; Euroclone Ltd.) supplemented with 20% FBS, 2 mM L-glutamine, and 100 U/mL penicillin and streptomycin at 37°C and 5% CO_2. When an adherent cell monolayer developed, the cells were harvested in 1 × PBS (Euroclone Ltd.) containing 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) and then washed and collected by centrifugation. The confluent adherent bone marrow stromal cells (BMSCs) showed a predominantly fibroblast morphology.

Patient samples. Peripheral blood mononuclear cells (PBMCs) were obtained from four patients with FL in leukemic phase and from two healthy volunteers after informed consent. After isolation by centrifugation in Ficoll's solution, mononuclear cells were more than 90%.

Drugs and reagents

Enzastaurin was a gift from Eli Lilly & Co. (Indianapolis, IN, USA). It was initially dissolved in 100% dimethylsulfoxide (DMSO; Sigma Chemical Co.) at a concentration of 10^{-2} mol/L and stored in small aliquots at −20°C. Chlorambucil and fludarabine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Monoclonal antibodies against total PKC-βI and PKC-βII were purchased from Sigma Chemical Co. Antibodies to cleaved caspase 3 (Asp175), PARP, p-PKC (pan), AKT, phosphorylated AKT(Ser473), GSK-3β, phosphorylated GSK-3β(Ser9), p70S6, phosphorylated p70S6(Thr421/Ser424), m-TOR, phosphorylated m-TOR (Ser2448), p90RSK, p-p90RSK, BAD, p-BAD (Ser112), and p-BAD(Ser136) were purchased from Cell Signaling Technologies (Beverly, MA, USA). Anti-caspase 8 polyclonal antibody was purchased from BD Biosciences (San Jose, CA, USA).

Cell proliferation assay

A total of 5×10^4 cells were seeded in triplicate in 96-well plates and treated with increasing concentrations of enzastaurin or DMSO (solvent). After 24, 48, and 72 h of treatment, cell number and viability were assessed using trypan blue exclusion, and cell proliferation was evaluated using MTT (CellTiter Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI, USA), which measures the conversion of a tetrazolium compound into formazan by a mitochondrial dehydrogenase enzyme in live cells. Briefly, 15 μL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well for 4 h at 37°C. A solubilization solution was added for 1 h, and the absorbance was then measured at 550/630 nm using a plate reader (DAS, Italy). Each data point is the average of four

independent determinations. Inhibitory concentration values IC_{25}, IC_{50}, and IC_{75} were calculated from curves based on enzastaurin concentrations ranging from 1 to 20 μM.

Annexin V binding assay for apoptosis

Cell lines were cultured in six-well plates at a concentration of 1×10^6 cells/mL in the presence or absence of enzastaurin at different concentrations (2.5–20 μM) added on day 1. Cells were harvested after 48 and 72 h of treatment in 1 × binding buffer (Miltenyi Biotec, Germany). The degree of apoptosis was assessed with an annexin–fluorescein isothiocyanate (FITC) binding assay following the manufacturer's instructions (Miltenyi Biotec). The harvested cell suspension was incubated with annexin V for 15 min at room temperature in the dark, washed in 1 × binding buffer, incubated with propidium iodide (PI), and then analyzed by flow cytometry (Becton, Dickinson and Company).

Preparation of cell extracts and Western blot analysis

Cells were seeded in a 75 cm^2 cell culture flask at a density of 1×10^6 cells/mL in RPMI medium supplemented with 10% FBS and treated with enzastaurin for 48 and 72 h, using half-maximal inhibitory concentrations (IC_{50}). Harvested cells were washed with ice-cold PBS and suspended in an extraction buffer (20 mM Tris-Cl, pH 7, 150 mM NaCl, 1% NP40, 10 mM/L EDTA, 20 mM NaF, 5 mM sodium pyrophosphate, 10% glycerol) reconstituted with 5 mM $NaVO_3$, 10 mM *p*-nitrophenyl phosphate (PNPP), and 5 mM phosphate inhibitor cocktail (PIC) (Pierce, Rockford, IL, USA). Samples containing equal amounts of total protein were resolved in a sodium dodecyl sulfate (SDS)-polyacrylamide denaturing gel and transferred to a nitrocellulose membrane. The membranes were incubated with primary antibodies overnight and then with horseradish peroxidase-conjugated secondary antibody for 1 h. Immunopositive bands were visualized by enhanced chemiluminescence (SuperSignal; Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

The antibodies used for immunoblotting included anti-PKC-βI, anti-PKC-βII, anti-p-PKC, anti-AKT, anti-p-AKT, anti-GSK-3β, anti-p-GSK-3β, anti-p70S6, anti-p-p70S6, anti-m-TOR, anti-p-m-TOR, anti-p90RSK, anti-p-p90RSK, anti-BAD, anti-p-BAD(Ser112), anti-p-BAD(Ser136), anti-caspase 3, anti-caspase 8, and anti-PARP. To better understand the role of caspase activation in enzastaurin-induced apoptosis, cell lines were treated with enzastaurin as described in the above experiments, in the presence or absence of ZVAD-fmk (Sigma, St. Louis, MO, USA), a broad caspase inhibitor, which was added 1 h before enzastaurin at a concentration of 40 μM.

Co-culture of B-lymphoma cell lines with BMSCs

For co-cultures, 5×10^3 BMSCs were seeded in triplicate in 96-well plates and incubated for 48 h to reach confluence. B-cell lymphoma cell lines were then seeded at 2×10^4 cells/well in the presence or absence of BMSCs. The next day, increasing concentrations of enzastaurin (2–10 μM) were added. Non-adherent cells were collected 72 h after enzastaurin addition and transferred to new 96-well plates, and cell proliferation was evaluated using the MTT assay.

Detection of t(14;18) with fluorescence in situ hybridization

Interphase fluorescence *in situ* hybridization (FISH) was performed on nuclei isolated from B-lymphoma cell lines. The cells were probed using a mixture of chromosome 14q32 sequence (IGH Spectrum Green) and chromosome 18q21 sequence (BCL2 Spectrum Orange) (Vysis, Woodcreek, IL, USA), as described previously [16]. Slides were analyzed on an epifluorescence microscope (DMRAZ, Leica), and hybridization signals were evaluated based on 100–200 nuclei on each slide.

Analysis of Bcl-2 expression

Cell lines were seeded onto a 75 cm^2 cell culture flask at a density of 1×10^6/mL in complete RPMI medium and treated with enzastaurin for 24, 48, and 72 h, using the IC_{50}.

Suitable aliquots of untreated and treated cells were used for DNA extraction with the DNeasy Blood & Tissue Kit (Qiagen, Milan, Italy). Quantification of the Bcl-2/JH fusion gene was performed using the Quant Bcl-2/JH kit (Diachem, Napoli, Italy), as recommended by the manufacturer. This kit includes FAM-Tamra-labeled Taqman probes and primers for the real-time quantification of samples, reference plasmid, and albumin (used as a reference gene), allowing detection of between 1 and 1×10^{-5} rearranged copies by amplification of the major breakpoint region of the Bcl-2 gene and JH region of immunoglobulins. In addition, a qualitative polymerase chain reaction (PCR) analysis of Bcl-2 was performed, as previously described [17].

The same treated cells were fixed in 2% paraformaldehyde, and Bcl-2 protein expression was determined by flow cytometry. The cells were permeabilized with 1 × fluorescence-activated sell

sorting (FACS) permeabilizing solution (BD Biosciences, San Jose, CA, USA) and incubated with blocking buffer (0.5% bovine serum albumin in $1 \times$ PBS) for 30 min. Then, cells were incubated with appropriate components of the Bcl-2 Antibody Sampler Kit, purchased from Epitomics (Burlingame, CA, USA), for 30 min at room temperature (RT), followed by incubation with fluorescently conjugated secondary antibody for 30 min at RT. Analysis was performed using a BD FACScan flow cytometer equipped with CellQuest Software (BD, Franklin Lake, NJ, USA).

Analysis of drug combination effects (isobologram analysis)

Cell lines were cultured in triplicate in 96-well plates at a concentration of 5×10^4 cells in a total volume of 100 μL RPMI with 10% FBS in the presence or absence of enzastaurin (at concentrations corresponding to IC_{25} and IC_{50}) alone or in combination with chlorambucil and fludarabine (at concentrations corresponding to IC_{25} and IC_{50}). Cell proliferation was determined using the MTT assay as described above.

Statistical analysis

The interaction between enzastaurin and chlorambucil or fludarabine was examined by isobologram analysis using the Stata 8.2 software program based upon the Chou–Talalay method. A combination index of <1, $=1$, and >1 indicates synergism, additive effects, and antagonism, respectively [18,19].

Results

Enzastaurin suppresses in vitro proliferation of cells and cell lines of B-cell lymphoma

We evaluated the ability of enzastaurin to suppress B-cell lymphoma cell proliferation in culture. B-cell lymphoma cell lines were cultured for 24, 48, and 72 h in the presence of enzastaurin (0.5–20 μM). Cell viability assessment was evaluated with trypan blue exclusion, and IC_{50} values were calculated using the MTT assay (Figure 1). Enzastaurin decreased proliferation of cell lines at 48 and 72 h, with IC_{50} values ranging between 1.5 and 10 μM. Because enzastaurin has a high protein-binding affinity, clinically relevant concentrations are best achieved in the presence of a low fetal calf serum (FCS) percentage. However, we decided to conduct the entire experiment under optimal culture conditions with 10% FCS to avoid subjecting the cells to further stress. We also evaluated the effects of enzastaurin on cells from four patients with FL and from two healthy donors. Freshly isolated PBMCs were incubated with increasing doses of enzastaurin (1–10 μM) for 48 h. The results showed that enzastaurin decreased survival of lymphoma cells from patients with FL but had minimal or no cytotoxic effect on PBMCs from healthy donors (Figure 2).

Enzastaurin induces apoptosis in B-cell lymphoma cell lines

Using flow cytometry analysis, we showed that enzastaurin induces time- and dose-dependent

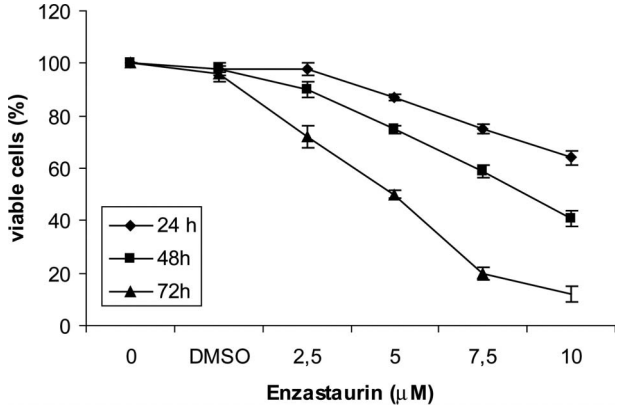

Figure 1. Antiproliferative activity of enzastaurin in B-lymphoma cell lines. The three B-lymphoma cell lines were incubated with increasing concentrations of enzastaurin for 24, 48, and 72 h in medium supplemented with 10% FCS. Cell proliferation was assessed by trypan blue exclusion. Results represent the mean ± standard deviation for the three cell lines. Data are expressed as a percentage of untreated control cells.

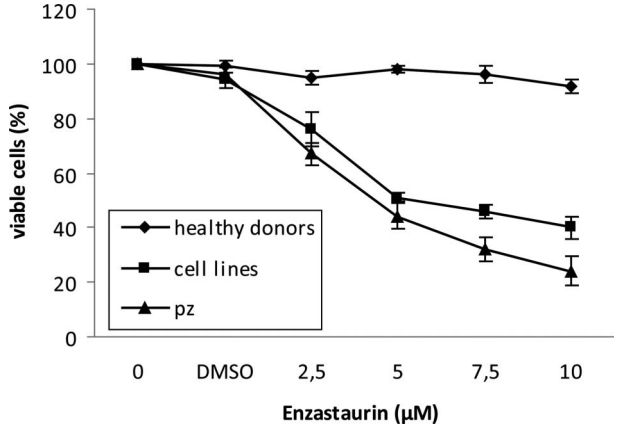

Figure 2. Antiproliferative activity of enzastaurin on B-cell lymphoma cells. The three cell lines, and PBMCs isolated from four patients with follicular lymphoma and from two healthy donors, were incubated with increasing concentrations (0.5 − 10 μM) of enzastaurin for 48 h. Results represent the mean ± standard deviation for the four patients, the three cell lines, and the two healthy donors. Data are expressed as a percentage of untreated control cells.

increases in apoptosis. After 72 h of treatment with doses ranging from 2.5 to 7.5 μM, the percentage of apoptotic cells increased, from 11% to 59% (Figure 3). To determine the mechanisms of enzastaurin-induced apoptosis, we investigated the effect of enzastaurin on the RL, WSU-NHL, and Karpas cell lines using immunoblotting. Enzastaurin induced activation of the intrinsic pathway, as suggested by caspase 3 and poly(ADP-ribose) polymerase (PARP) cleavage [Figure 4(a)]. To examine whether a caspase inhibitor could block enzastaurin-induced apoptosis, we cultured the cells in the presence of ZVAD-fmk, a broad caspase inhibitor. Notably, ZVAD-fmk decreased enzastaurin-induced PARP cleavage [Figure 4(b)], while utilized alone did not influence survival and cell growth at 72 h. Further, enzastaurin induced caspase 8 cleavage, which suggests activation of the extrinsic pathway [Figure 4(b)].

Enzastaurin suppresses phosphorylation of PKC-βI/II

We studied the expression of PKC-βI/II using Western blot analysis, and the results showed that the two proteins were abundantly expressed in all three cell lines [Figure 5(a)]. RL cell lysates, after 72 h of treatment with enzastaurin at concentrations corresponding to IC_{25} (1 μM), IC_{50} (5 μM), and IC_{75} (10 μM), were subjected to Western blotting using anti-p-PKC antibody. The blot showed a dose-dependent decrease in p-PKC expression [Figure 5(b)].

Enzastaurin suppresses phosphorylation of AKT and its downstream targets

We sought to examine whether enzastaurin treatment might affect pathways in B-cell lymphoma cells known to be influenced by PKC activity. Because PKC-β activity has been linked to many intracellular signaling cascades, including the PI3K/AKT pathway, we examined the phosphorylation status of AKT, GSK-3β, m-TOR, p70, and RSK using Western blotting. Following cell line treatment with enzastaurin at concentrations corresponding to the respective IC_{50} for 48 and 72 h, we observed a decrease in AKT (Ser473), GSK-3β, m-TOR, p70, and RSK phosphorylation in all cell lines; utilizing the same concentration we observed a decrease in phosphorylation of p-PKC after 24 and 48 h [Figure 6(a)].

Because enzastaurin suppressed phosphorylation of AKT and RSK, we next evaluated whether enzastaurin treatment of B-lymphoma cell lines affected BAD, a pro-apoptotic member of the Bcl-2 family of proteins. This protein has pro-apoptotic activity when dephosphorylated. As Figure 6(b) shows, Ser112 and Ser136 phosphorylation of BAD was detectable and decreased in a time-dependent manner after enzastaurin treatment.

Effect of enzastaurin on Bcl-2 expression

Interphase FISH performed on our three cell lines detected and confirmed the presence of t(14;18) in these B-lymphoma cell lines. Thus, we evaluated the effects of enzastaurin on Bcl-2 expression. Cell lines were cultured for 24, 48, and 72 h in the presence of enzastaurin at the IC_{50} concentration, and DNA was isolated from treated samples. Qualitative PCR confirmed the persistence of the Bcl-2/JH fusion gene (Figure 7), and quantitative analysis identified no decrease in the fusion gene after enzastaurin treatment (data not shown).

Flow cytometry analysis performed on the same treated cells showed no changes in Bcl-2 expression versus control, confirming the results obtained with PCR.

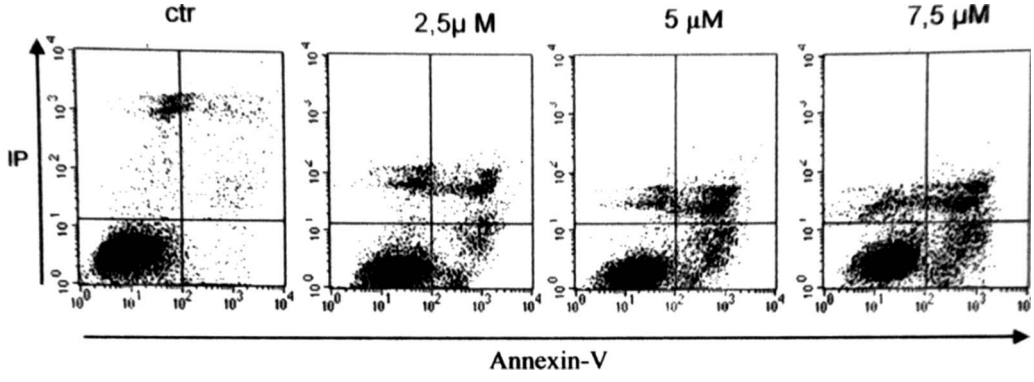

Figure 3. Flow cytometry analysis of enzastaurin apoptotic effects on B-lymphoma cell lines. All three cell lines were cultured with enzastaurin for 72 h at doses ranging from 2.5 to 7.5 μM. The percentage of cells undergoing apoptosis was determined by annexin V and PI staining. Annexin V- and PI-positive cells were considered as apoptotic. The figure shows results for the WSU-NHL cell line. After treatment, the percentage of apoptotic cells increased, from 11% to 59%.

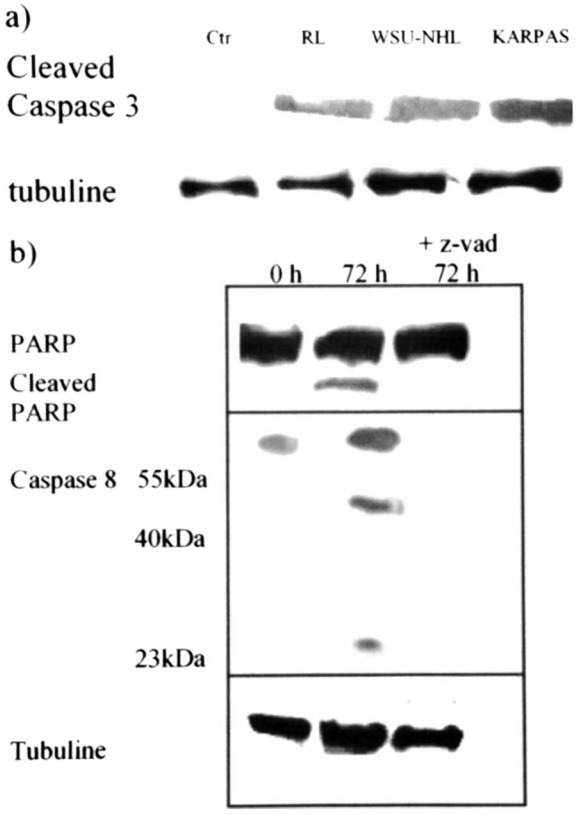

Figure 4. Western blot of cellular extracts from RL, WSU-NHL, and Karpas 422 cells, treated with enzastaurin at concentrations corresponding to IC_{50} values for 72 h. Cellular extracts were probed with antibodies against the cleaved form of caspase 3, PARP, and caspase 8. (a) Cleavage of caspase 3 was observed in all three cell lines. (b) Enzastaurin treatment for 72 h induced cleavage of PARP, inhibited by ZVAD-fmk, and cleavage of caspase 8. The figures show results obtained with the RL cell line.

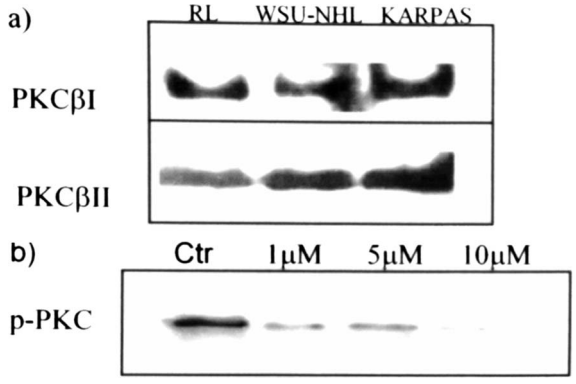

Figure 5. Expression of PKC-βI/II in B-lymphoma cell lines. (a) Cell lysates from RL, WSU-NHL, and Karpas 422 were subjected to Western blotting using anti-PKC-β antibody. All three cell lines expressed PKC-βI and PKC-βII. (b) Cell lysates from RL after treatment for 72 h with enzastaurin at concentrations corresponding to IC_{25}, IC_{50}, and IC_{75} were subjected to Western blotting using anti-p-PKC antibody. The blot showed a dose-dependent decrease of expression of p-PKC.

Enzastaurin suppresses cell proliferation in B-cell lymphoma cell lines co-cultured with BMSCs

Because the BM microenvironment could confer a growth advantage and increase drug resistance, we next studied the possible protective effect conferred by BMSCs on B-cell lymphoma cell lines. All three cell lines were cultured with enzastaurin (2.5, 5, 7.5, and 10 μM) in the presence and absence of BMSCs. After 72 h, cytotoxicity was assessed using the MTT assay. The results demonstrated that enzastaurin was cytotoxic for cell lines even when cultured in a system mimicking the BM microenvironment, without toxicity for normal BMSCs (Figure 8).

Analysis of drug combination effects

The three B-cell lymphoma cell lines were cultured for 48 and 72 h with chlorambucil or fludarabine in the presence of enzastaurin. Cytotoxicity was assessed using the MTT assay. Both drugs enhanced enzastaurin cytotoxicity. Isobologram analysis demonstrated that enzastaurin in combination with either chlorambucil or fludarabine had a significant synergistic effect (combination index < 1) (Table I). The effects of the combination were also evaluated using flow cytometry analysis after annexin V/PI staining and showed that each of the combinations had apoptotic effects (data not shown).

Finally, we studied the effects of the combination on caspase 3 of cellular extracts of RL cells. After 72 h of treatment, cell lysates were subjected to Western blotting using anti-caspase 3. The Western blot results showed a clear cleavage of caspase 3 in all samples (Figure 9).

Discussion

In recent years, remarkable progress has been made in the treatment of FL, resulting in improved patient survival. Less progress has been observed in other subsets of indolent lymphoma. However, all of these lymphomas remain incurable diseases, and their course is characterized by frequent relapses and the development of resistance to chemotherapeutic agents. Thus, new agents that can overcome lymphoma cell resistance are needed. An increased understanding of signaling pathways that regulate lymphoma cell growth and survival can help in identifying new molecular targets. PKC is involved in signal transduction pathways that regulate cell proliferation and apoptosis and thus represents an attractive target for cancer treatment.

Enzastaurin, initially developed as an ATP-competitive selective inhibitor of PKC-β [14] and used for anti-angiogenic cancer therapy, has shown activity against a variety of human cancer cells by

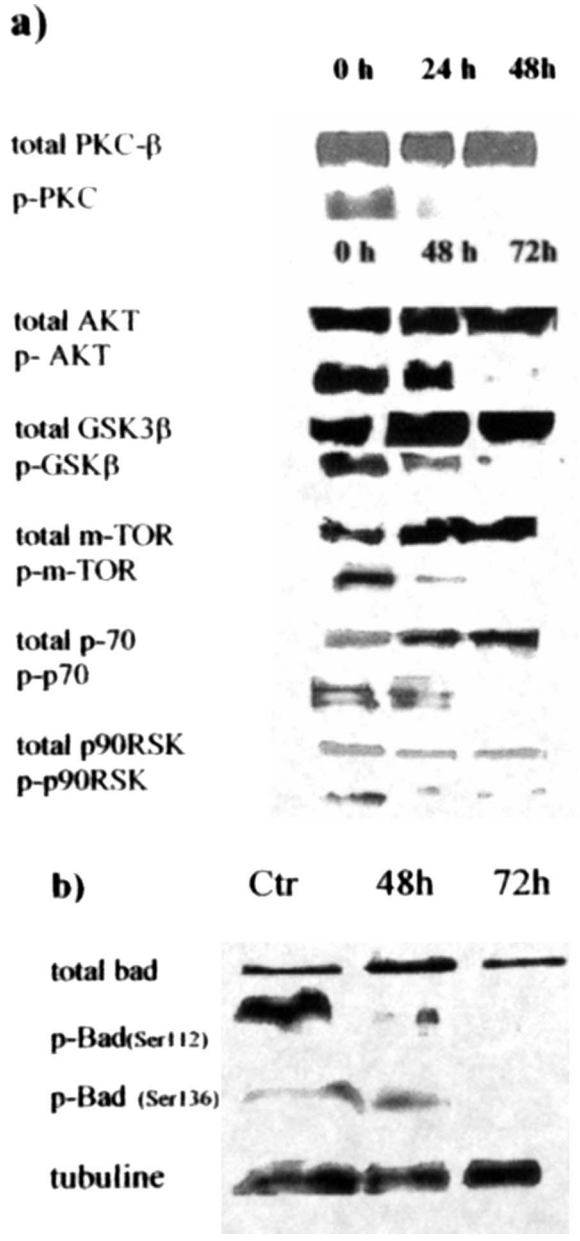

Figure 6. Enzastaurin decreases phosphorylation of PKC, AKT, GSK-3β, m-TOR, p70, and RSK. RL, WSU-NHL, and Karpas 422 cell lines were cultured with enzastaurin at IC_{50} doses for 48 and 72 h. The figures show the blot obtained for the RL cell line. (a) Whole cell lysates were subjected to Western blotting using anti-PKC-β, p-PKC, AKT, p-AKT, GSK-3β, p-GSK-3β, m-TOR, p-m-TOR, p70, p-p70, p90RSK, and p-p90RSK. (b) Enzastaurin decreased phosphorylation of p-BAD(Ser112) and p-BAD(Ser136).

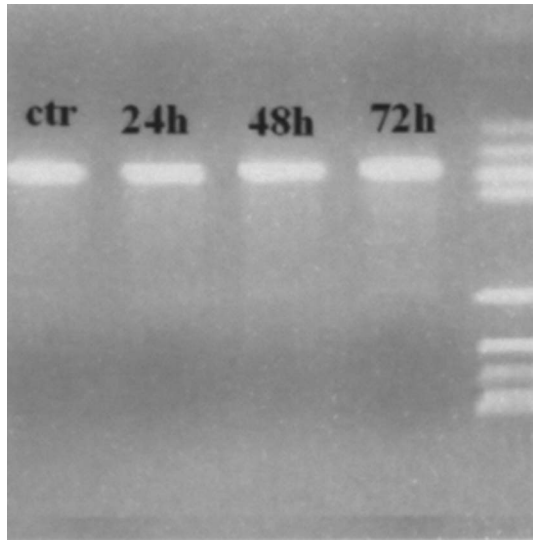

Figure 7. Ethidium bromide-stained agarose gel of PCR products of samples from the RL cell line treated with enzastaurin at IC_{50} dose for 24, 48, and 72 h. The expression of Bcl-2/JH is not modified after enzastaurin treatment.

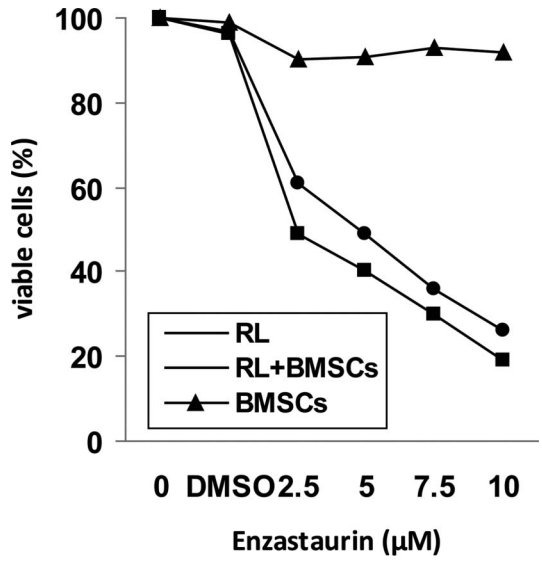

Figure 8. Antiproliferative activity of enzastaurin in RL, WSU-NHL, and Karpas. BMSCs were plated at 5000 cells/well and incubated for 48 h. B-lymphoma cell lines were plated in the absence or presence of a BMSC feeder layer. Increasing concentrations of enzastaurin were added the next day. After 72 h, non-adherent cells were collected, and cell proliferation was measured by MTT assay. Enzastaurin was cytotoxic for cell lines even when cultured with BMSCs. Data are expressed as a percentage of untreated control cells ± standard deviation.

inducing apoptosis [20–24] Enzastaurin also targets the PI3K/AKT pathway and inhibits GSK-3β phosphorylation [20]. In the present study, we evaluated the cytotoxicity and antitumor effects of enzastaurin on B-cell lymphoma cell lines bearing t(14;18) and positive for PKC-βI/II expression.

Enzastaurin inhibited proliferation of cell lines and of cells from patients with a range of IC_{50} values varying from 1.5 to 15 μM. In addition, our results demonstrated that enzastaurin cytotoxicity is retained when B-cell lymphoma cell lines are co-cultured with BMSCs in a system that mimics the BM microenvironment. The apoptotic effects of enzastaurin were mediated through the activation of caspases 3 and 8 and PARP, revealing involvement of intrinsic and extrinsic apoptotic pathways

Table I. Isobologram analysis of combination of enzastaurin with chlorambucil and fludarabine.

Enzastaurin dose (μM)	Chlorambucil/ fludarabine dose (mM)	FA	CI
With chlorambucil			
1 (IC_{25})	5 (IC_{25})	0.59	0.87
5 (IC_{50})	5	0.37	0.76
1	25 (IC_{50})	0.51	0.55
5	25	0.25	0.54
With fludarabine			
1	20 (IC_{25})	0.62	0.78
5	20	0.34	0.7
1	100 (IC_{50})	0.45	0.75
5	100	0.25	0.9

Enzastaurin-treated RL, WSU-NHL, and Karpas 422 cells were exposed to chlorambucil or fludarabine. Proliferation was measured using the MTT assay after 72 h of treatment. Results are representative of three independent experiments performed in quadruplicate on the RL cell line.
CI, combination index: CI < 1, synergism, CI = 1, additive effect, and CI > 1, antagonism; FA, fraction of cells with growth affected in drug-treated versus untreated cells.

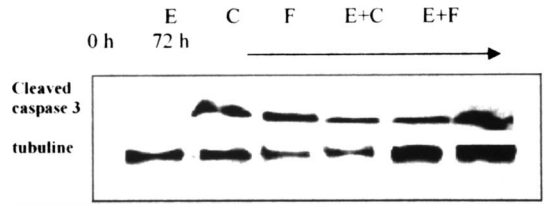

Figure 9. Analysis of drug combination effects. Cellular extracts of RL were treated with enzastaurin, chlorambucil, or fludarabine alone and in combination at low concentrations (IC_{25}). After 72 h of treatment, cell lysates were subjected to Western blotting using anti-caspase 3. Western blot clearly shows cleavage of caspase 3.

in enzastaurin-induced cell death. Our studies showed that enzastaurin treatment decreased the expression of phosphorylated PKC-β, RSK, AKT, and some AKT downstream targets such as GSK-3β, m-TOR, and p70S6. The mechanisms associated with enzastaurin-induced apoptosis in cancer cells are not clear. Recent studies have noted the importance of the PI3K/AKT signaling pathway in enzastaurin-induced apoptosis in human cancer cell lines [21,25].

AKT activation maintains a survival signal that protects cells from apoptosis [23]. However, experiments with *in vitro* kinase assays have shown that enzastaurin causes virtually no inhibition of AKT. Thus, it was suggested that enzastaurin may indirectly suppress the AKT signaling pathway through its inhibitory effect on PKC proteins [20]. However, the mechanisms underlying enzastaurin activity and linking the PKC and AKT pathways are still unknown. In mammalian cells, two apoptotic pathways, extrinsic, via activation of cell death receptors, and intrinsic, via disruption of the mitochondria, have been well characterized. Some Bcl-2 family proteins, like BAX, are important for intrinsic cell death, while others, like Bcl-2, can prevent cell death. Our results have shown that enzastaurin did not suppress Bcl-2. Our cell lines present t(14;18), and we thought that the induction of apoptosis was related to the reduction of Bcl-2 expression. Surprisingly, we did not observe modification of this protein. Thus we evaluated other mechanisms. Our results showed a new mechanism involved in enzastaurin-induced apoptosis in B-cell lymphoma cell lines, involving BAD activation. BAD is a pro-apoptotic Bcl-2 family member protein and is inactivated by phosphorylation at two critical sites, Ser112 and Ser136. When phosphorylated at these sites, BAD fails to interact with the anti-apoptotic Bcl-XL protein, thus supporting cell survival [26]. In our experiments, BAD activation (dephosphorylation) was observed in enzastaurin-treated B-cell lymphoma cell lines. Dephosphorylation of BAD at Ser112 is thought to be mediated by RSK inactivation in enzastaurin-treated cancer cells, while the AKT pathway mediates dephosphorylation of BAD at Ser136 [27,28]. After enzastaurin treatment, we indeed observed dephosphorylation of BAD at Ser112 and Ser136 in accordance with RSK and AKT inactivation. BAD is a pro-apoptotic Bcl-2 family member protein, and recently, it was demonstrated that enzastaurin affects the expression of the anti-apoptotic Bcl-2 family proteins in some human gastric cancer cell lines [29]. Our data showed that enzastaurin induces apoptosis by a different mechanism, through p90RSK- and BAD-mediated pathways, in addition to inhibiting the AKT signaling cascades. After careful investigation, we also demonstrated that enzastaurin induces apoptosis without affecting Bcl-2 expression.

Finally, our results showed a strong synergistic effect of enzastaurin in combination with chlorambucil or fludarabine, drugs usually used in the treatment of indolent lymphoma. Preclinical toxicology studies have shown that enzastaurin is well tolerated in rats and dogs. In addition, a dose of 525 mg once a day was well tolerated in patients enrolled in a dose-finding phase I trial [30]. Considering its strong anti-lymphoma activity, safe toxicity profile, and the need for more active and less toxic treatment for indolent lymphoma, a phase II study of enzastaurin alone or in combination is warranted.

In summary, enzastaurin induced cell death in B-cell lymphoma cell lines by activation of a caspase-mediated pathway. These results are consistent with the observed decreased phosphorylation of RSK and AKT that induces dephosphorylation of BAD at

Ser136 and Ser112, respectively. Further, enzastaurin showed a strong synergistic effect in combination with chlorambucil or fludarabine.

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