

ORIGINAL ARTICLE: RESEARCH

The oral protein-kinase C β inhibitor enzastaurin (LY317615) suppresses signalling through the AKT pathway, inhibits proliferation and induces apoptosis in multiple myeloma cell lines

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Abstract

Deregulation of the protein kinase C (PKC) signalling pathway has been implicated in tumor progression. Here we investigated the PKC inhibitor enzastaurin for its activity against multiple myeloma (MM) cells. Enzastaurin suppresses cell proliferation in a large panel of human myeloma cell lines (HMCLs), with IC₅₀ values ranging from 1.3 to 12.5 μ M and induces apoptosis, which is prevented by the ZVAD-fmk broad caspase inhibitor. These results are consistent with decreased phosphorylation of AKT and GSK3- β , a downstream target of the AKT pathway and a pharmacodynamic marker for enzastaurin. Furthermore, enzastaurin cytotoxicity is retained when HMCLs were cocultured with multipotent mesenchymal stromal cells. Enzastaurin has additive or synergistic cytotoxic effects with bortezomib or thalidomide. Considering the strong anti-myeloma activity of enzastaurin *in vitro* and in animal models and its safe toxicity profile, phase II studies in MM patients of enzastaurin alone or in combination with other drugs are warranted.

Keywords: Signal transduction pathways, multiple myeloma, enzastaurin, AKT, apoptosis, caspases

Introduction

Multiple myeloma (MM) is a malignant proliferation of bone marrow (BM) plasma cells (PCs) characterised by marked biological and clinical heterogeneity and high genomic instability [1]. Despite the recent introduction of new drugs and treatment strategies, MM remains associated with a poor prognosis. Therefore, new treatment approaches are needed to improve patient outcome. In recent years, important insights into the pathogenesis of MM have emerged, including the contribution to myeloma cell survival made by growth factors [e.g., interleukin (IL)-6 and insulin-like growth factor (IGF)-1], stromal cells, and

dysregulation of various signal transduction pathways [2–6]. Interactions between malignant cells and the BM microenvironment induce the transcription and secretion of cytokines (TNF α , IL-6, IGF-1, SDF1 α , VEGF) by both the PCs and the BM stromal cells, triggering signalling pathways that promote cell proliferation and prevent apoptosis [7]. These insights have stimulated the search for new therapeutic interventions that specifically target pathways required for myeloma cell survival.

The protein kinase C (PKC) family of serine/threonine protein kinases has been implicated in multiple processes that control cell growth and differentiation, apoptosis, cell invasiveness,

tumor-induced angiogenesis and drug efflux [8]. Members of the PKC family play important roles in the regulation of cell growth and survival in several hematological malignancies, notably B cell lymphomas [9,10]. Recently, a link between PKC activity and the activity of the phosphatidylinositol-3-kinase (PI3-K)/Akt pathway, a prominent regulatory pathway governing the apoptotic response, has been shown [11–14]. Constitutive activation of PI3K/AKT signalling axis has recently been found in acute myeloid leukemia (AML) [15,16]. Further, AKT activation might be one of the factors contributing to the decreased apoptosis rate observed in patients with high risk myelodysplastic syndromes [17]. Some AKT inhibitors might be promising therapeutic agents for the treatment of those AML cases characterised by up regulations of PI3K/AKT pathway [18]. Deregulation of this signalling pathway may play an important oncogenic role in MM. Constitutive activation of the PI3-K/Akt pathway occurs frequently in MM *in vivo* and also has been observed in growth factor-independent MM cell lines [19,20]. Moreover, PI3K-dependent, constitutive GSK3- α/β phosphorylation has been detected in the OPM2 cell line [21]. The biological mechanisms that lead to deregulated PI3-K activation in MM are still unknown.

Enzastaurin (LY17615), an acyclic bisindolylmaleimide, was initially considered as a selective PKC- β inhibitor to be administered orally. The well-established role of PKC- β in angiogenic signalling prompted the initial evaluation of the anti-angiogenic activity of enzastaurin in tumor models [22,23]. On the basis of its striking ability to suppress angiogenesis, enzastaurin was advanced for clinical development. In addition to its anti-angiogenic effects, enzastaurin has been shown to suppress proliferation and induce apoptosis of tumor cells in culture and in human colon and glioblastoma xenografts by suppressing the phosphorylation of AKT and its downstream effectors glycogen synthetase 3-beta (GSK3- β) and ribosomal protein S6 [11]. Inhibition of cell growth and induction of apoptosis via specific inhibition of the AKT signalling pathway was also demonstrated in two cutaneous T-cell lymphoma cell lines [24].

These observations and recent results on the anti-myeloma effects of enzastaurin in myeloma cells and cell lines [25–27] prompted us to investigate the effect of enzastaurin on the proliferation and survival of a large panel of human myeloma cell lines (HMCLs). The results show that enzastaurin inhibits cell proliferation, induces apoptosis, and decreases AKT and GSK3- β phosphorylation. Further, enzastaurin cytotoxicity is retained when HMCLs are co-cultured on a layer of BM-derived multipotent mesenchymal stromal cells (MSCs), a system that mimics the BM microenvironment.

Materials and methods

Cell culture and reagents

The HMCLs NCI-H929, OPM2, J2N3, U266 and KMS-12-PE were obtained from the DMSZ-German collection of Microorganism and Cell Culture, Germany. RPMI 8226 was obtained from LGC Promochem-UK, the European distributor for ATCC cultures. The KMS-28-BM, KMS-34, KMS-18, KMS-11, KMS-26, KMM-1 and KMS-20 cell lines were kindly provided by Dr. T. Otsuki, Kawasaki Medical School, Okayama, Japan; KM4 and AMO1 by Dr. S. Iida, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; and SKMM1 by Dr. F. Malavasi, Department of Genetics, University of Torino, Italy. The MM1.S cell line was kindly provided by Dr. Steven Rosen, Feinberg School of Medicine, Northwestern University, Chicago, IL. The CMA-01, CMA-02 and CMA-03 cell lines were established in our laboratory [28]. All other cell lines are well characterised and well described in Drexler et al. [29] and Inoue et al. [30]. All cell lines were cultured at 37°C in a 5% CO₂ humidified atmosphere in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% foetal calf serum (FCS) at concentrations ranging from 4×10^5 to 8×10^5 cells/mL. CMA-01, CMA-02 and CMA-03 were cultured in the presence of 20 U/mL recombinant human IL-6 (R&D Systems, Minneapolis, MN).

MSC cell lines were kindly provided by Dr. M. Introna, Division of Hematology, Ospedali Riuniti, Bergamo, Italy. Enzastaurin was a gift from Eli Lilly & Co. (Indianapolis, IN). A 10 mM stock solution of enzastaurin was prepared in 100% DMSO, and aliquots were stored at 20°C. Dexamethasone and thalidomide were purchased from Sigma (St. Louis, MO). Bortezomib was obtained from Jansen-Cilag.

Monoclonal antibodies against total PKC- β I and PKC- β II and against PARP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against total PDK-1 was purchased from Upstate/Millipore (Billerica, MA); the antibody against phosphorylated PDK-1 was obtained from Cell Signalling Technologies (Beverly, MA). Antibodies against the total and phosphorylated forms of GSK3- β and AKT and against the cleaved/activated forms of caspase-3 and caspase-9 (all polyclonal except for monoclonal anti-AKT) were obtained from Cell Signalling Technologies (Beverly, MA). Anti-caspase-8 polyclonal antibody was purchased from BD Biosciences (San José, CA).

Cell proliferation assay

2×10^4 cells were seeded in triplicate in 96-well plates in a total volume of 200 μ L IMDM with 10%

FCS. After 24 h, the culture medium was changed with fresh medium with or without enzastaurin. After 24, 48 and 72 h of treatment, cell number and viability were assessed by Trypan blue exclusion, and cell proliferation was evaluated with the Cell Proliferation Reagent WST-1 kit (Roche, Indianapolis, IN), which measures the metabolic activity of viable cells, according to the manufacturer's instructions. The data are expressed as percentage of untreated control cells. Each data point represents the mean value (percentage) \pm SD. IC₅₀ values were calculated from curves based on enzastaurin concentrations ranging from 2.5 to 12.5 μ M.

Cell proliferation was also determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium Cell Titer AQueous assay (Promega, Madison, WI), which measures 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 is the average of four independent determinations. The data are expressed as the percentage of formazan produced by untreated cells in the same assay.

Preparation of cell extracts and Western blot analysis

Cells were seeded in a 25-cm² cell culture flask at a density of 5×10^6 cells/flask in IMDM medium supplemented with 10% FCS. For the analysis of PKC- β I and PKC- β II expression by Western blotting, cells were harvested 24 h after seeding. To study the effects of enzastaurin on caspase activation and AKT and GSK3- β phosphorylation, IC₅₀ concentrations of enzastaurin were added 24 h after seeding, and cells were collected after 1–5, 24 and 48 h of treatment. 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), a broad caspase inhibitor, which was added 1 h before enzastaurin at a concentration of 40 μ M.

Harvested cells were washed twice with phosphate-buffered saline (PBS) and lysed on ice in PCLB buffer containing protease inhibitors, as described previously [31]. One hundred micrograms of total protein for each cell line was separated by SDS-PAGE and transferred to a nitrocellulose filter. The membranes were incubated with primary antibodies overnight and then with horseradish peroxidase-conjugated secondary antibodies for 1 h. Immuno-positive bands were visualised by enhanced chemiluminescence (Super-Signal, PIERCE, Rockford, IL) according to the manufacturer's instructions.

Cell cycle analysis

Cell lines were plated in 24-well plates at a concentration of 5×10^5 cells/mL, and on day 1 cells were treated or not with enzastaurin concentrations corresponding to the respective IC₅₀ values. Cells were collected after 12, 24, 48 and 72 h, fixed in 70% ethanol, and stored at 4°C until analysis. Before cytofluorimetric analysis, 10^6 cells were washed twice in PBS and incubated in staining solution (10 μ g/mL propidium iodide, 100 μ g/mL RNase, 0.05% Nonidet P-40) at room temperature in the dark for 5 h. Cell cycle profiles were determined using MODFIT software (Verity Software House, Topshem, ME) on a FACScan flow cytometer (Becton Dickinson, San José, CA).

Analysis of apoptosis versus necrosis by flow cytometry

Cell lines were cultured in 24-well plates at a concentration of 5×10^5 cells/mL in the presence or absence of IC₅₀ concentrations of enzastaurin added on day 1. Cells were harvested after 24, 48 and 72 h of treatment, washed twice in PBS, resuspended in a solution of 40 μ g/mL propidium iodide (PI) in PBS, and left at room temperature in the dark for 30 min. Cells were then analysed by flow cytometry, producing a dot plot of forward scatter (FSC) vs. PI fluorescence on a log scale.

Coculture of MM cell lines with mesenchymal stromal cells

For cocultures, 5×10^3 MSCs were seeded in triplicate in 96-well plates and incubated for 48 h to reach confluence. HMCLs in IMDM medium were then seeded at 2×10^4 cells/well in the presence or absence of MSCs. The next day, increasing concentrations of enzastaurin were added. Non-adherent cells were collected 72 h after enzastaurin addition and transferred to new 96-well plates, and cell proliferation was evaluated by WST-1 assay. Collected cells were also stained with anti-CD138 PE (BD Biosciences, San José, CA) and analysed on the FACScan flow cytometer to determine cell identity.

Combination of enzastaurin with other drugs

Cell lines were cultured in 24-well plates at a concentration of 5×10^5 cells/mL in the presence or absence of enzastaurin (at concentrations corresponding to IC₂₅ values) alone or in combination with bortezomib and thalidomide (at concentrations corresponding to the IC₂₅ and IC₇₅). Cells were harvested 24 h after treatment and washed twice with

PBS. Cell proliferation was determined using an MTT assay as described above.

The interaction between enzastaurin and bortezomib or thalidomide, was examined by isobologram analysis using the StaCorp 8.2 software program based upon the Chou-Talalay method. A combination index (CI) of < 1, = 1, and > 1 indicates synergism, additive effects and antagonism, respectively [32,33].

Results

PKC-β expression in MM cell lines

Because enzastaurin was initially considered as an ATP-competitive, PKC-β selective inhibitor, we analysed the expression of the PKC-β isoforms I and II at the protein level in our panel of 20 HMCLs. Analysis of PKC-βI isoform expression by Western blotting showed that three cell lines (AMO1, JJN3 and KMS26) expressed both PKC-βI and PKC-βII, eleven cell lines expressed only one of the two isoforms, and six cell lines lacked detectable PKC-β expression (Table I).

Enzastaurin treatment suppresses tumor cell proliferation in MM cell lines

To evaluate the ability of enzastaurin to suppress tumor cell proliferation in culture, the 20 HMCLs were incubated in the presence of increasing concentrations of enzastaurin (range 2.5–12.5 μM) for 72 h. IC₅₀ values were calculated by evaluation of cell proliferation with the WST-1 assay and by cell viability assessment with Trypan blue exclusion. Enzastaurin showed a growth inhibitory effect in all HMCLs. The seven most sensitive cell lines had IC₅₀ values ranging from 1.3 to 6.5 μM. Thirteen cell lines were less sensitive (IC₅₀ range, 8.0–12.0 μM) (Table II and Figure 1). Because enzastaurin has a high protein-binding affinity, clinically relevant concentrations are best achieved in the presence of a low FCS percentage. Therefore, MM1.S, CMA-01, AMO1, OPM2, KMS-28 and U266 cell lines were also cultured in IMDM containing 1% FCS 24 h before treatment with enzastaurin. As expected, we

observed a strong reduction in IC₅₀ values, with values ranging from 1.2 to 5.0 μM (Table II). However, we decided to conduct all the experiments under the optimal culture conditions with 10% FCS to avoid subjecting the cells to further stress stimuli.

Enzastaurin induces apoptosis in sensitive cell lines

Next, the molecular mechanism by which enzastaurin induces cytotoxicity was investigated. Three cell lines were selected for further studies on the basis of

Table II. HMCLs IC₅₀ values (μM) for Enzastaurin.

IC ₅₀ ranges	1.3–6.5	8–12	
	MM.1S	KM4	U266
	CMA-01	CMA-02	KMS-20
	AMO1	CMA-03	NCI-H929
	KMS-26	KMS-18	SKMM-1
	KMS-11	KMS-28	OPM2
	KMS-34	JJN3	KMS-12
	RPMI 8226	KMM-1	

	IC ₅₀ in medium with 10% FCS	IC ₅₀ in medium with 1% FCS
MM.1S	5.2	1.2
CMA-01	2.8	1.6
AMO1	3.6	2.0
OPM2	11.7	2.2
KMS-28	9.0	1.7
U266	11.0	5.0

IC₅₀ values were evaluated in all HMCLs cultured in presence of 10% FCS and, in six cell lines, also in presence of 1% FCS.

Table I. PKCβ I and PKCβ II expression in HMCLs.

PKCβ I/II pos.	PKCβ I pos.	PKCβ II pos.	PKCβ neg.
AMO1	KMS-18	CMA-03	CMA-01
JJN3	KMS-20	KMS-11	CMA-02
KMS-26	KMS-28	NCI-H929	KM-4
	MM1.S	U266	KMM-1
	OPM2		KMS-12
	RPMI 8226		KMS-34
	SKMM-1		

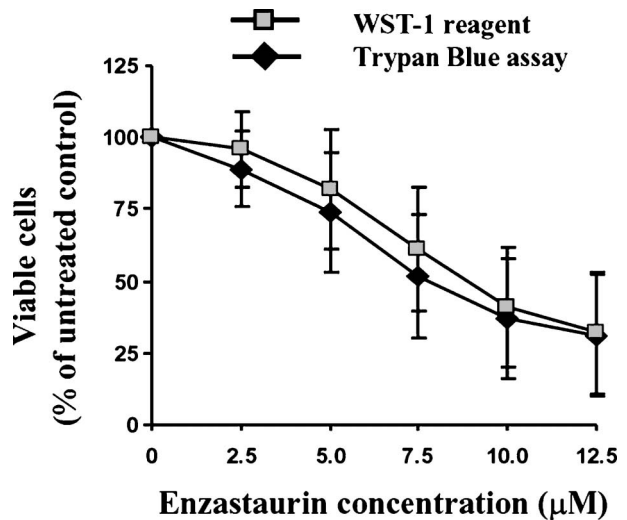


Figure 1. Anti-proliferative activity of enzastaurin in HMCLs. The 20 HMCLs were incubated with increasing concentrations of enzastaurin for 72 h in medium supplemented with 10% FCS. Cell number and viability were assessed by Trypan blue exclusion, and cell proliferation was evaluated with the WST-1 reagent. Graphic represent mean ± standard deviation of the 20 HMCLs. Data are expressed as percentage of untreated control cell.

their sensitivity to enzastaurin and PKC- β expression: MM1.S and RPMI 8226 (among the most sensitive cell lines) and KMS-18 (a less sensitive cell line), all of which express PKC- β I.

To determine whether enzastaurin induces apoptosis, as suggested by an increased proportion of cells with a sub- G_0/G_1 DNA content, two cell lines (MM1.S and KMS-18) were treated with enzastaurin concentrations corresponding to the respective IC_{50} for 72 h. At 12, 24, 48 and 72 h of culture, cell cycle was analysed by flow cytometry. Starting at 24 h of culture, enzastaurin induced a significant increase in the percentage of cells in the sub- G_0/G_1 fraction and a concomitant decrease in cells in the G_0/G_1 phase of the cell cycle compared with untreated controls [Figure 2(A)].

In parallel to the cell cycle analysis, apoptosis induction by enzastaurin was evaluated by flow cytometry analysis of membrane permeability. Due to extensive membrane damage, necrotic and late apoptotic cells are quickly stained by a short incubation with PI, whereas apoptotic cells show much lower uptake of dye. Moreover, when cells die or become apoptotic, their morphology changes and a reduction in FSC signal is observed. Combining analysis of PI fluorescence with FSC, it is therefore possible to distinguish healthy (PI-negative), apoptotic (reduced FSC and dim PI fluorescence) and necrotic (bright PI fluorescence) cells [Figure 2(B)]. In MM1.S and KMS-18 cell lines, enzastaurin induced an increase in the percentage of apoptotic

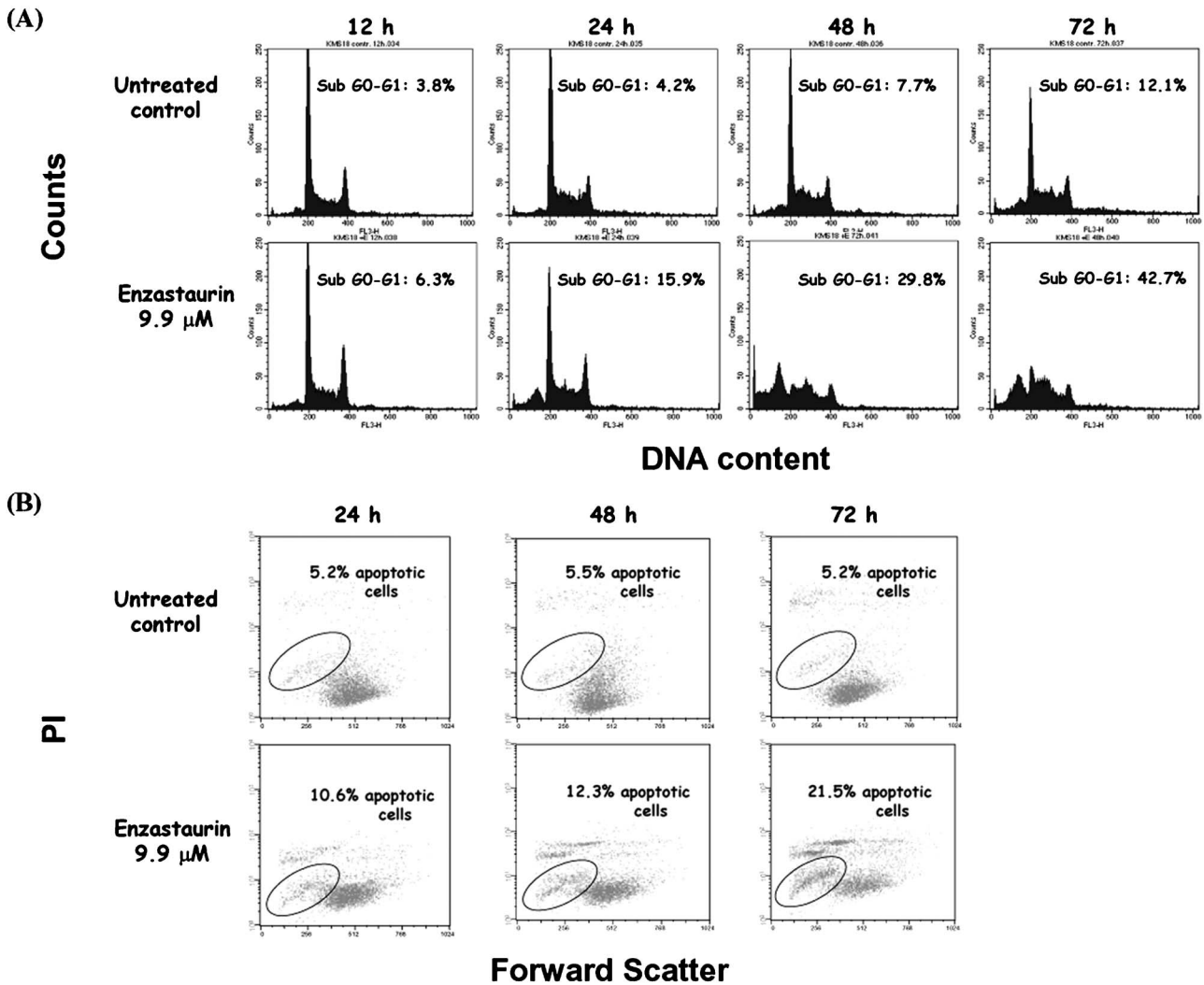


Figure 2. Proapoptotic activity of enzastaurin in HMCLs. MM1.S and KMS-18 cell lines were treated with enzastaurin concentrations corresponding to the respective IC_{50} for 72 h. At 12, 24, 48 and 72 h of culture, the cell cycle stage (PI staining) and membrane permeability (PI versus FSC) were analysed by flow cytometry. (A) Representative histograms for cell cycle analysis of KMS-18 cell line. (B) Representative flow cytograms (PI versus FSC) for membrane permeability of KMS-18 cell line. Early apoptotic cells (highlighted by circles) were identified as a population showing low PI fluorescence and reduced FSC, whereas necrotic and late apoptotic cells showed bright PI fluorescence and reduced FSC.

cells compared with untreated controls in a time-dependent fashion.

Effect of enzastaurin on caspase activation

To determine whether enzastaurin-induced apoptosis is a caspase-dependent event, we studied caspase activation in MM1.S, KMS-18 and RPMI 8226 cell lines after exposure to the respective IC₅₀ concentration of the drug. Figure 3(A) shows that enzastaurin induced the appearance of the cleaved caspase-3 fragment in all cell lines. Involvement of the mitochondrial apoptotic pathway was also indicated by the increase in cleaved caspase-9 fragment in all three cell lines. No caspase-8 activation was induced in MM1.S and KMS-18 cell lines within 48 h of enzastaurin treatment, while a weak activation was observed at 48 h in RPMI 8226. These findings suggest that enzastaurin mainly induced activation of intrinsic pathway of apoptosis.

Activation of the apoptotic pathway was confirmed by cleavage of the PARP enzyme in MM1.S and RPMI 8226 cell lines [Figure 3(B) and data not shown]. To examine whether a caspase inhibitor could block enzastaurin-induced cytotoxicity, the cells were cultured with enzastaurin in the presence of ZVAD-fmk, a broad caspase inhibitor. Importantly, ZVAD-fmk decreased enzastaurin-induced

PARP cleavage. Taken together, these results suggest that enzastaurin triggers caspase-dependent apoptosis in HMCLs and that the induced apoptosis is at least partly mediated by activation of the intrinsic apoptotic pathway.

Enzastaurin decreases phosphorylation of AKT and GSK3-β

Enzastaurin was initially considered as an ATP-competitive, PKC-β selective, small molecule inhibitor. Therefore, we examined whether pathways known to be influenced by PKC-β activity might be affected by enzastaurin treatment of human myeloma cells. Because PKC-β activity has been linked to many intracellular signalling cascades, including the PI3K/Akt pathway, the phosphorylation status of AKT and of GSK3-β, a downstream AKT substrate, were examined by Western blotting. Following enzastaurin treatment of MM1.S and RPMI 8226 cell lines with drug concentrations corresponding to the respective IC₅₀ or higher concentrations, a decrease in AKT^{ser473} phosphorylation was observed in RPMI 8226 (2 h after treatment) and in MM1.S (48 h after treatment) cell lines [Figure 4(A)]. Enzastaurin treatment markedly decreased the phosphorylation of GSK3-β in both cell lines within 1 h of treatment [Figure 4(B)]. Phosphorylation of PDK1, a

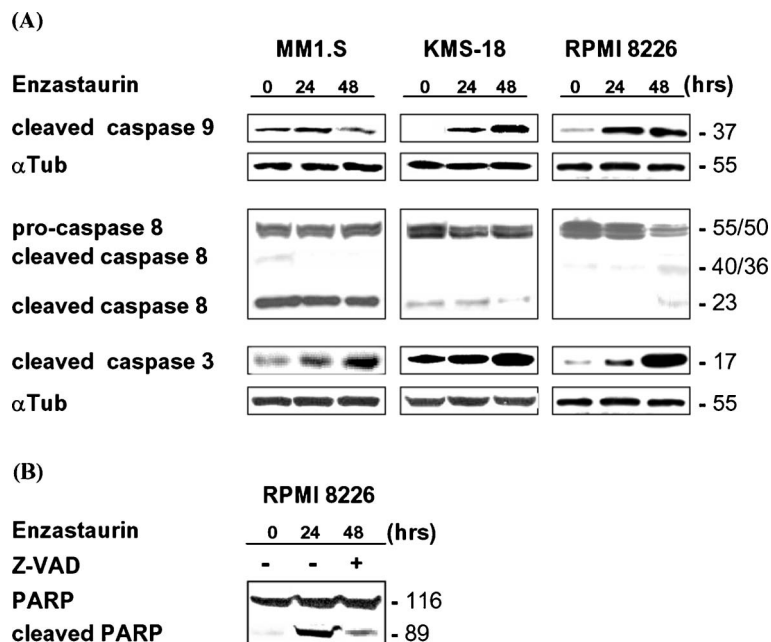


Figure 3. Enzastaurin induces cleavage of caspases and PARP. At 0, 24 and 48 h of treatment with enzastaurin, cellular extracts of MM1.S, KMS-18 and RPMI 8226 were analysed by Western blotting with anti-caspase and anti-PARP antibodies. (A) Western blots of cellular extracts probed with antibodies against the cleaved forms of caspase-3 and caspase-9 and against caspase-8. Cleavage of caspase 3 and 9 is observed in all the three cell lines, while a weak cleavage of caspase 8 is observed at 48 h only in RPMI 8226. (B) Western blots of cellular extracts of RPMI 8226, untreated or treated with ZVAD-fmk, probed with anti-PARP antibody.

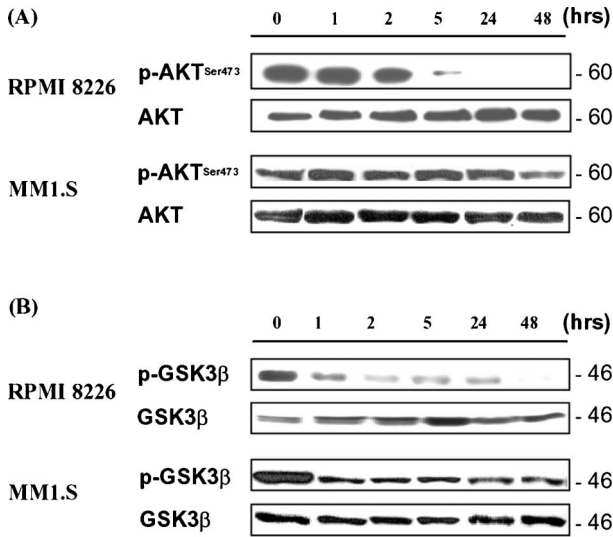


Figure 4. Enzastaurin decreases phosphorylation of AKT and GSK3- β . To determine whether enzastaurin affects AKT^{Ser473} and GSK3- β ^{Ser9} phosphorylation, MM1.S and RPMI 8226 were treated with a drug concentration corresponding to the respective IC₅₀. (A) Enzastaurin decreased AKT^{Ser473} phosphorylation in both cell lines. (B) Enzastaurin decreased GSK3 β ^{Ser9} phosphorylation in both cells lines.

molecule upstream of AKT in the PI3K/Akt pathway, was not affected by enzastaurin treatment in MM1.S and RPMI 8826 cell lines (data not shown).

Enzastaurin suppresses cell proliferation in MM cell lines cocultured with MSCs

MM cells are dependent upon the BM microenvironment for growth and survival. Thus, the investigation of new anti-cancer drugs for MM is best performed in a system that, at least in part, reconstitutes this microenvironment. We first investigated whether enzastaurin is cytotoxic for stromal cells. BM multipotent MSCs, grown in culture from normal BM adherent cells, were treated with increasing doses of enzastaurin. Cell proliferation was measured 72 h after enzastaurin treatment by the WST-1 reagent. As shown in Figure 5, enzastaurin at concentrations up to 12.5 μ M was not cytotoxic to MSCs. The highest drug concentration tested was 20 μ M, at which the cytotoxicity was 15% (data not shown).

Next, the cytotoxic activity of enzastaurin on HMCLs cocultured with MSCs was measured. MM1.S and KMS-18 cell lines were cultured on a MSC feeder layer in the presence or absence of increasing concentrations of enzastaurin. After 72 h, non-adherent cells were collected, and cell proliferation was evaluated by means of a WST-1 assay. Cells were also stained with anti-CD138 PE and analysed on the flow cytometer to confirm that >97% of the

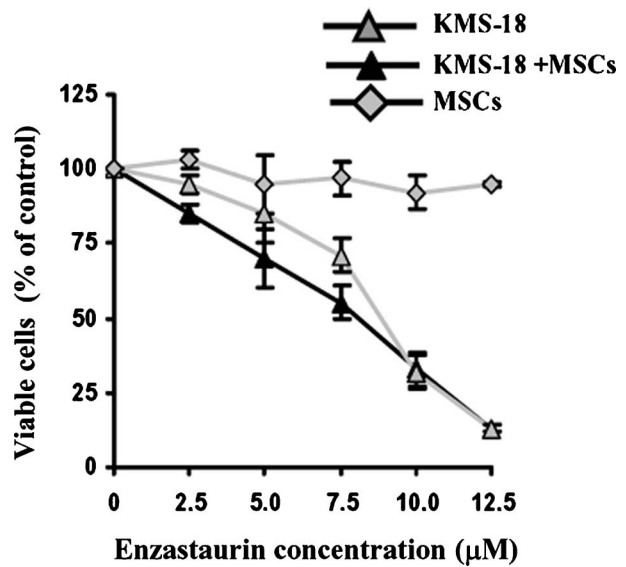


Figure 5. Anti-proliferative activity of enzastaurin on HMCLs cultured on MSCs. MSCs were plated at 5000 cells/well and incubated in the absence or presence of increasing concentrations of enzastaurin. After 72 h of treatment, cellular proliferation was evaluated by WST-1 reagent. MM1.S and KMS-18 cells were plated on a MSC feeder layer in the absence or presence of increasing concentrations of enzastaurin. After 72 h, non-adherent cells were collected, and cell proliferation was measured by WST-1 reagent. Representative results for MSCs and the KMS18 cell line alone or in the presence of MSCs are shown. The data are expressed as percentage of untreated control wells and represent the mean \pm standard deviation of triplicate wells.

non-adherent cells were MM cells (CD138-positive). Enzastaurin was cytotoxic for MM1.S and KMS-18 cell lines even when cultured in a system mimicking the BM microenvironment (Figure 5).

Enzastaurin has additive effects with other drugs

MM1.S and RPMI 8226 cell lines were cultured for 24 h with bortezomib and thalidomide in the presence of enzastaurin. Cytotoxicity was assessed by MTT assay. These agents enhanced the cytotoxicity triggered by enzastaurin in a dose-dependent fashion. Isobologram analysis demonstrated that enzastaurin plus bortezomib or thalidomide had an additive or synergistic effect ($IC \leq 1$) (Table III).

Discussion

In recent years, remarkable progress has been made in the treatment of MM patients, resulting in improved periods of survival. However, MM still remains an incurable disease, and its course is characterised by frequent relapses and the development of resistance to chemotherapeutic agents. Thus, new agents able to overcome MM cell resistance are needed. An increased understanding of signalling pathways that

Table III. Isobologram analysis of combination of enzastaurin with bortezomib and thalidomide.

Enzastaurin	Bortezomib	CI	CI lower	CI upper
0.7	0.15	1.000	0.942	1.060
4	0.8	0.734	0.620	0.848
Enzastaurin	Thalidomide			
0.7	0.1	1.000	0.974	1.046
4	1	0.983	0.904	1.026

Enzastaurin has additive or synergistic effects with bortezomib and thalidomide. MM1.S cells were cultured in presence of enzastaurin (0.7 μM = IC_{25} and 4 μM = IC_{75}) in combination with bortezomib (0.15 μM = IC_{25} and 0.8 μM = IC_{75}) and thalidomide (0.1 M = IC_{25} and 1 M = IC_{75}). Proliferation was measured using MTT assay after 24h of treatment. CI indicates the combination index. $\text{CI} < 1$: synergism, $\text{CI} = 1$: additive effects and $\text{CI} > 1$: antagonism. The results are representative of 3 independent experiments.

regulate MM cell growth and survival can help to identify new molecular targets.

Members of the PKC family have been implicated in the pathogenesis of neoplastic diseases; thus, PKC represents a promising therapeutic target. The PKC family consists of at least 12 isoforms involved in signal transduction pathways that regulate growth factor response, proliferation and apoptosis. Increased levels of PKC have been observed in solid tumors [11,12], and members of the PKC family have also been implicated in hematological malignancies [9,10].

Enzastaurin is an orally administrated PKC inhibitor that binds to the ATP-binding site of PKC- β , competing with ATP binding and preventing enzyme activation. In preliminary studies, enzastaurin demonstrated a significant anti-tumor activity against cell lines of solid tumors and hematological malignancies, including cutaneous T-cell lymphoma and leukemia [11,20–24]. Recently, *in vitro* studies showed that enzastaurin inhibits proliferation, survival and migration of MM and Waldenström macroglobulinemia cell lines [25–27,34]. Furthermore, tumor growth is reduced by enzastaurin in a xenograft mouse model [26]. In the present study, we evaluated the effects of enzastaurin on a large panel of HMCLs and investigated possible mechanisms by which it exerts its cytotoxic effects. Our results demonstrated that PKC- β isoforms are often expressed in HMCLs, although sometimes at low levels: 14 of the 20 analysed HMCLs express either one or two isoforms of PKC- β .

Enzastaurin suppressed cell proliferation in the large panel of HMCLs, with IC_{50} values ranging from 1.3 to 12.5 μM or from 1.2 to 5.0 μM when cells were cultured in medium with 10% or 1% FCS, respectively. These effects are also evident in cell lines lacking PKC- β expression. It is known that enzastaurin can

inhibit other PKC isozymes; thus, inhibition of proliferation in cell lines lacking PKC- β expression may be mediated by enzastaurin-induced effects on other PKC isozymes. By cell cycle analysis and detection of caspase and PARP cleavage, we showed that enzastaurin induces apoptosis by activation of the intrinsic pathway. The apoptosis is partially prevented by the ZVAD-fmk broad caspase inhibitor. These results are consistent with the observed decreased phosphorylation of AKT and GSK3- β , a downstream target of the AKT pathway and a pharmacodynamic marker for enzastaurin. Regarding the role of enzastaurin in inducing caspase activation, published results are often conflicting. Some researchers have found that apoptosis induced by enzastaurin in MM1.S cell line may be caspase independent. This different result could be due to different methods utilised, flow cytometry versus immunoblotting [25]. Other researchers have found activation of either intrinsic or extrinsic apoptosis pathways, as if enzastaurin inhibited check points shared by both apoptotic pathways [34]. However this observation was made in different cell lines. Further other authors, utilizing lower enzastaurin doses have observed activation of caspase 8 and 3, but not of caspase 9 [26].

The mechanism by which enzastaurin suppresses signalling through the ATK pathway is unclear. In *in vitro* kinase assays, enzastaurin did not inhibit AKT, suggesting that the kinases responsible for phosphorylation of GSK3- β and AKT may not be directly inhibited by enzastaurin [11]. Thus, it is conceivable that interference with the AKT signalling pathway arises from the effects of enzastaurin on multiple PKC family members. Enzastaurin did not inhibit PDK1, a signalling molecule upstream of PKC. In addition, our studies demonstrated that enzastaurin cytotoxicity is retained when HMCLs are co-cultured with MSCs in a system that mimics the BM microenvironment. Moreover, additive or synergistic effects of enzastaurin with bortezomib or thalidomide were observed.

Preclinical toxicology studies showed that enzastaurin is well tolerated in rats and dogs. The dose of 525 mg once a day is well tolerated in patients enrolled in a dose-finding Phase I trial [35]. This dose is recommended for a phase II trial. Several clinical trials examining the effects of enzastaurin on solid tumors and malignant lymphomas are ongoing. Considering the strong anti-myeloma activity of enzastaurin *in vitro* and in animal models and the safe toxicity profile, phase II studies in MM patients of enzastaurin alone or in combination with other drugs are warranted. Furthermore, enzastaurin could be utilised in combination studies with agents having different toxicity profiles and molecular targets to examine additive effects in enhancing tumor shrinkage and preventing regrowth.

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