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ORIGINAL ARTICLE

Combination of low doses of Enzastaurin and Lenalidomide has synergistic activity in B-non-Hodgkin lymphoma cell lines

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Abstract Less toxic and more active treatments are needed for indolent lymphomas as there is no curative treatment, and patients eventually die due to complications related to their disease. The purpose of the present study was to assess the antitumour activity of the combination of low doses of Enzastaurin and Lenalidomide (Revlimid) on B-lymphoma cell lines. The combination of Enzastaurin and Lenalidomide, at doses as low as 1 μM, showed strong synergism against indolent lymphomas by reducing cell growth, producing an increase in G0–G1 phase followed by significant decrease in S phase, increasing apoptosis, and inhibiting PI3K/AKT, PKC and MAPK/ERK pathways. These preclinical findings, together with promising results obtained with Lenalidomide for the treatment of non-Hodgkin lymphoma, suggest that further evaluation of the combination of Enzastaurin and Lenalidomide for the treatment of indolent lymphomas is warranted. These compounds, with a favourable toxicity profile, are not classic chemotherapeutic agents, causing severe side effects, and could be considered an example of a new innovative attempt of an anti-cancer 'soft treatment'.

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Introduction

Indolent B-cell lymphomas represent a heterogeneous group of lymphoproliferative disorders [1, 2]. There is currently no curative treatment, and most patients eventually die from their disease. There is an urgent need to identify dysregulated signalling pathways in B-cell lymphomas and to develop target therapies. Enzastaurin, a novel targeted agent, inhibits PKC-β by interacting competitively at its ATP-binding site [3]. Several studies have shown that Enzastaurin inhibits the growth of a wide array of cultured human tumour cells [4–11]; in accordance with these studies, we found the antitumour effects of Enzastaurin are mediated through interference with the PI3K/AKT pathway [5, 9–11]. Lenalidomide (Revlimid), an oral immunomodulatory drug, has antineoplastic activity in various tumours [12–17] and is approved for the treatment of patients with multiple myeloma (MM) and myelodysplastic syndrome (MDS) bearing a 5q deletion [18, 19]. Several studies have demonstrated that Lenalidomide has a pleiotropic effect, via activity, on the microenvironment and some direct effects on the cell. This results in antiangiogenic and anti-inflammatory responses through regulation of cytokine production and T cell and natural killer cell stimulation [20–22] and antiproliferative effects on haematopoietic tumour cells [23, 24].

The rationale for combining Enzastaurin and Lenalidomide is based on the mechanisms of action of both drugs. Activation of the PI3K/AKT pathway and cell cycle progression play vital roles in cell survival and proliferation. Thus, the combination of Enzastaurin and Lenalidomide should inhibit the AKT pathway and arrest cell cycle progression, which could activate caspases cascades and trigger

apoptosis. The objective of the present study was to examine the potential effects of low doses of Enzastaurin and Lenalidomide alone, and in combination, on the signalling mechanisms responsible for inhibition of cell growth and induction of apoptosis in B-cell lymphoma cell lines. Our results showed the combination of Enzastaurin and Lenalidomide at low doses exerts strong synergistic activity leading to inhibition of AKT, PKC and MAPK/ERK signalling pathways and induction of apoptosis.

Materials and methods

Cell culture

The WSU-NHL and Karpas-422 cell lines were kindly provided by Dr. M. Introna (Ospedale Riuniti, Bergamo, Italy); the RL line was purchased from DSMZ (German Collection of Microrganisms and Cell Cultures). All three lines carry the t (14; 18), and Karpas-422 is EBV+. All cell lines were cultured in RPMI-1640 supplemented with 10 % fetal bovine serum, 2 mML-glutamine, and 100 U/ml penicillin and streptomycin. Peripheral blood mononuclear cells (PBMCs) were obtained from four patients with follicular lymphoma (FL). The leukemic phase in the four FL patients was diagnosed by microscopic analysis and confirmed by flow cytometry. After mononuclear cells were isolated with Ficoll's solution, flow cytometry showed the percentage of $CD19^{+}/CD5^{-}$ cells was more than 90 % and these cells were considered leukemic.

Drugs and reagents

Enzastaurin was a gift from Eli Lilly & Co. and Lenalidomide from Celgene. The drugs were dissolved in 100 % dimethyl sulfoxide (DMSO) at a concentration of 10^{-2} M. The final concentration of DMSO was kept at 0.1 % for all in vitro assays. All the above-mentioned reagents were purchased from Euroclone. Antibodies to cleaved caspase 3 (Asp175), caspase 9 (Asp353), PARP, AKT, phosphorylated AKT (Ser473), GSK-3β, phosphorylated GSK-3β (Ser9), p70S6, phosphorylated p70S6 (Thr421/Ser424), m-TOR, phosphorylated m-TOR (Ser2448), phosphorylated PKC (pan), BCL-2, BAD, phosphorylated BAD (Ser112) and (Ser136), p44/42 MAPK, phosphorylated p44/42 MAPK (ERK1/2) (Thr202/Tyr204), p-90RSK, and phosphorylated p-90RSK (Thr359/Ser363) were purchased from Cell Signaling. Anti-caspase-8 polyclonal antibody was purchased from BD Biosciences and anti-PKC was purchased from Sigma.

Cell viability

B-lymphoma cell lines (5×10^{4}) were seeded in triplicate in 96-well plates in the presence of Enzastaurin or Lenalidomide alone, or in combination, at a concentration of 1 μM of each compound for 24, 48, or 72 h. After treatment, cell number and viability were assessed using Trypan blue exclusion, and cell cytotoxicity was evaluated using the MTT assay (Promega) following the manufacturer's instructions. The IC_{50} values of both drugs alone were calculated from curves based on Enzastaurin concentrations ranging from 1 to 20 μM and Lenalidomide concentrations ranging from 1 to 100 μM.

Cell apoptosis

WSU-NHL cells (1×10^{6} cells/ml) were cultured for 24–72 h with Enzastaurin or Lenalidomide alone or with a combination of the two drugs at the same respective concentrations of 1 μM. After treatment, the cells were harvested in 1X binding buffer. The degree of apoptosis was assessed with an Annexin V/propidium iodide (PI)-FITC binding assay following the manufacturer's instructions (Miltenyi Biotec) and then analysed by flow cytometry (Becton Dickinson).

Cell cycle analysis

WSU-NHL cells (1×10^{6} cells/ml) were cultured with Enzastaurin or Lenalidomide alone, or in combination, at a concentration of 1 μM for 24–72 h. After treatment, the cells were incubated with 10 μM 5-bromo-2-deoxyuridine (BrdU) for 30 min. After incubation the cells were incubated with anti-BrdU mouse IgG (BD Biosciences) and then stained with the secondary antibody goat anti-mouse IgG FITC (Dako) and PI and analysed using a Coulter Epics XL MCL and EXPO 32 vers.1.2.

Western blot analysis

WSU-NHL cells (1×10^{6} cell/ml) were treated with 1 μM Enzastaurin or Lenalidomide, alone or in combination, for 48 and 72 h. Harvested cells were suspended in an extraction buffer. Samples containing equal amounts of total protein were resolved on a SDS-polyacrylamide denaturing gel and transferred to a nitrocellulose membrane. The membranes were incubated with the above mentioned primary antibodies overnight and then with horseradish peroxidase-conjugated secondary antibody for 1 h. Immunopositive bands were visualized by enhanced chemiluminescence (Pierce) according to the manufacturer's instructions. Caspase activation was also evaluated using ZVAD-fmk (Sigma).

Bone marrow stromal cell (BMSC) cultures

Bone marrow mononuclear cells were separated using the Ficoll–Hypaque technique and were cultured according to the method described by Gartner and Kaplan [25].

Co-culture of B-cell 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, cells were treated with 1 μM Enzastaurin and Lenalidomide, alone or in combination. Non-adherent cells were collected 72 h after addition of Enzastaurin and Lenalidomide and transferred to new 96-well plates, and cell proliferation was evaluated using the MTT assay.

Drug combination effects

Cell lines were cultured in triplicate in 96-well plates at a concentration of 5×10^4 cells in the absence or presence of Enzastaurin or Lenalidomide, alone or in combination at concentrations of 0.5 and 1 μM, which is lower than the IC_{50} concentrations. Cell proliferation was determined using the MTT assay.

Statistical analysis

Data are expressed as mean±standard error. Statistical differences between controls and drug-treated cells were determined by one-way analysis of variance (ANOVA). *P* values <0.05 were considered statistically significant. Data were analysed using the Stata 8.2/SE package (StataCorp LP, College Station, TX, USA). The interaction of Enzastaurin and Lenalidomide was assessed using multiple effect analysis, based on the methods described by Chou-Talalay in which a combination index (CI) of <1, =1, and >1 indicates synergism, additive effects, and antagonism, respectively [26, 27]. The mean CI was calculated from all data points with fraction affected (FA)>0.5, as values lower were not considered relevant for growth inhibition [28]. The data were processed by isobologram analysis using Stata 8.2, which calculates the CI of the combination based on the effect of the growth inhibition caused by the drugs alone relative to the effect produced by the combination.

Results

Growth inhibition studies of Enzastaurin and Lenalidomide alone and in combination

In the present study, we tested three B-NHL cell lines (WSU-NHL, Karpas-422 and RL) with Enzastaurin and Lenalidomide alone and in combination. All three cell lines have similar biological characteristics, in particular they share the same genetic features t (14;18).

To determine the effects on cell viability and the IC_{50} values of Enzastaurin and Lenalidomide alone on B-lymphoma cell lines, the cells were cultured with increasing concentrations of Enzastaurin (1–20 μM) and Lenalidomide (1–100 μM) alone for 24, 48, and 72 h.

Enzastaurin alone

Enzastaurin alone decreased the viability of all B-cell lymphoma cell lines in a time and dose-dependent manner. The IC_{50} values at 72 h were 4, 2 and 5 μM for the WSU-NHL, Karpas-422, and RL cell lines, respectively.

Lenalidomide alone

Lenalidomide alone demonstrated similar activity on all cell lines tested. The IC_{50} values at 72 h were 7, 10 and 15 μM for the WSU-NHL, Karpas-422 and RL cell lines, respectively.

Enzastaurin and Lenalidomide combined

For combination studies, we utilized Enzastaurin and Lenalidomide at concentrations of 1 μM for 24, 48 and 72 h. We observed a significant decrease in viability in all cell lines in comparison with each agent alone at the same concentration (Fig. 1, upper panel, a). For example, treatment of the WSU-NHL cell line with the combination of Enzastaurin and Lenalidomide at doses as low as 1 μM for 72 h produced a 61 % decrease in viability ($P<0.001$), whereas only minimal cell death was observed using either of these agents alone at these low concentrations.

We also evaluated the impact of combined therapy on cells from four patients with FL in leukemic phase and on normal cells from two healthy donors. We observed a considerable decrease in cell viability in patients with FL treated with the combination of Enzastaurin and Lenalidomide that did not trigger a relevant decrease in the viability of normal PBMNCs (Fig. 1b).

Finally, the combinations studies were performed using Enzastaurin and Lenalidomide at concentrations of 0.5 and 1 μM, which are lower than the IC_{50}. Based on the results of the isobologram analysis, we chose the dose of 1 μM of Enzastaurin and Lenalidomide because a this dose we reached the peak inhibition suggesting a synergistic activity of the agents with a CI of <1 in all cell lines tested (Table 1).

Apoptosis

The evaluation of the cell viability and cell cytotoxicity showed similar results with both single drugs and their combination. Therefore, based on same biological characteristics and similar response to single treatment and their combination, we decided to use WSU-NHL as model of

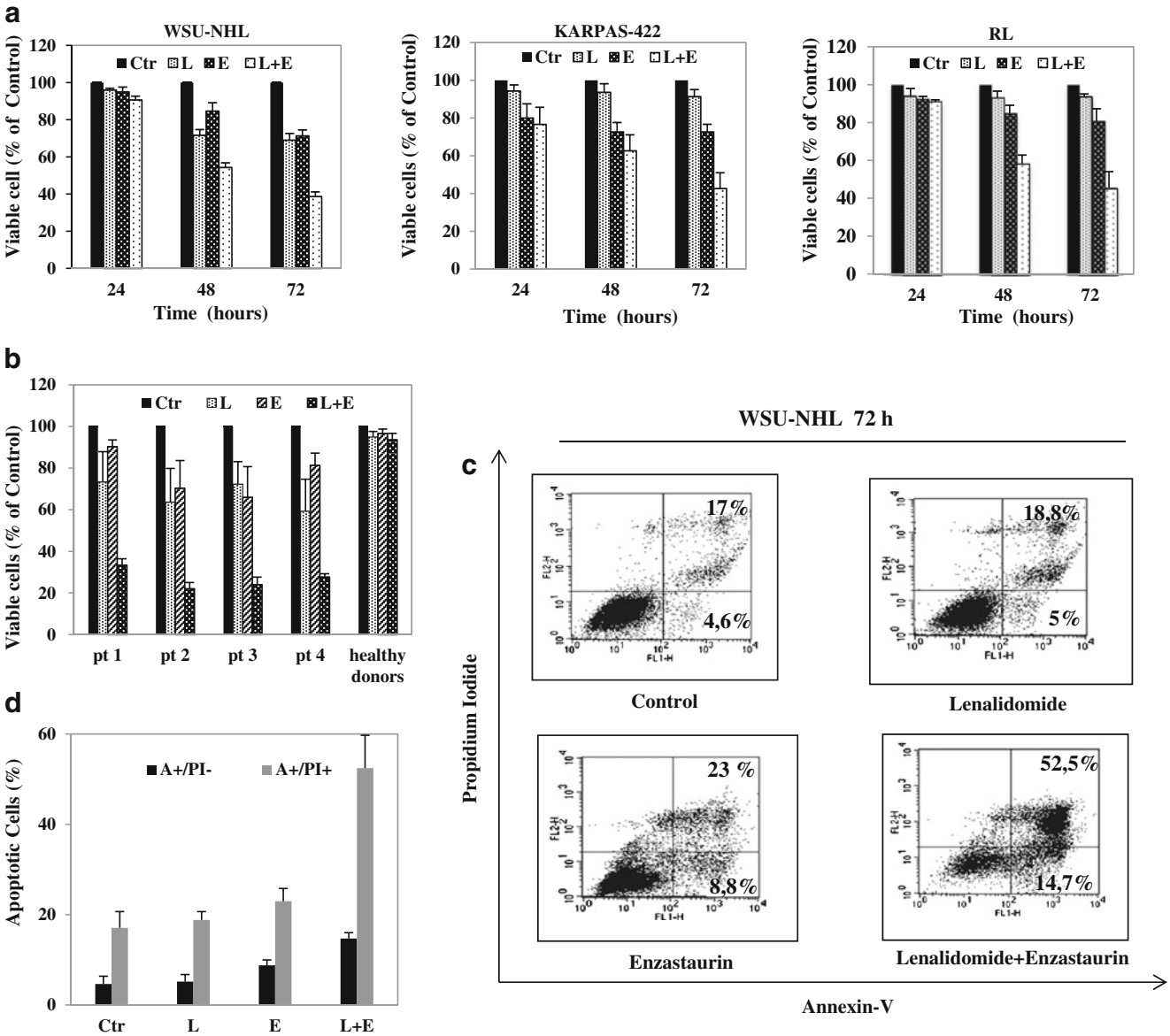

Fig. 1 *Upper panel*: **a** Antiproliferative activity of Enzastaurin or Lenalidomide alone or in combination at a concentration of 1 μM for 24, 48 and 72 h in all three B-lymphoma cell lines (WSU-NHL, Karpas-422 and RL). Data are expressed as a percentage of untreated control cells. **b** Viability of cells from patients and healthy donors after treatment. Results represent the mean±SD for the four patients with FL and the two healthy donors after 48 h. Data are expressed as percentage of untreated control cells**. c** WSU-NHL cells were cultured in the presence or absence of Enzastaurin and Lenalidomide or their combination for 72 h. Annexin V labelled (*bottom right quadrant*) represents the population undergoing apoptosis (early apoptosis). Annexin V and PI double-labelled (*top right quadrant*) represent cells that have already died by apoptosis (late apoptosis). Alive cells are represented in the bottom left quadrant. Increasing percentages of Annexin V-positive and PI-positive cells with Enzastaurin and Lenalidomide combination treatment is shown in the top right quadrants. **d** The histogram shows the increased apoptotic cells ($Annexin^{+}/PI^{-}$) in WSU-NHL cell line after 72 h of exposure to Enzastaurin and Lenalidomide in combination. Values represent the mean±SD of three independent experiments

B-lymphoma cell line to study the effects of Enzastaurin and Lenalidomide on apoptosis.

We examined whether the decrease in cell viability, induced by the combination therapy, was due to apoptosis. The apoptotic effects of Enzastaurin and Lenalidomide alone and in combination were analysed by Annexin V/PI FITC and flow cytometry in WSU-NHL cell line.

Enzastaurin alone

After treatment with doses ranging from 2.5 to 7.5 μM, Enzastaurin alone induced time- and dose-dependent increases in apoptosis, whereas the 1 μM concentration did not significantly induce apoptosis with respect to the control.

Table 1 Isobologram analysis of combination of Enzastaurin and Lenalidomide in WSU-NHL, Karpas-422 and RL cell lines

Cell lines	Lenalidomide dose (μM)	Enzastaurin dose (μM)	CI	FA	SD
WSU-NHL	0.5	0.5	0.37	74	0.23
	1	1	0.10	90	0.07
Karpas-422	0.5	0.5	0.06	77	0.05
	1	1	0.06	85	0.05
RL	0.5	0.5	0.85	68	0.23
	1	1	0.47	70	0.29

Cells were exposed to Enzastaurin and Lenalidomide at dose of 0.5 and 1 μM. Cytotoxicity was measured using MTT assay after 72 h of treatment. Results are representative of three independent experiments performed in all three B-lymphoma cell lines

Combination index (CI): CI<1, synergism; CI=1, additive effect; CI>1, antagonism. *FA* fraction of cells with growth affected in drug-treated versus untreated cells, *SD* standard deviation

Lenalidomide alone

To determine the effects of Lenalidomide alone, we utilized increasing concentrations (1–100 μM) for 24, 48, and 72 h. Even after 72 h of treatment, Lenalidomide alone did not induce cell apoptosis (data not shown).

Enzastaurin and Lenalidomide combined

The combination of 1 μM of Enzastaurin and Lenalidomide induced a significant increase in apoptosis in comparison with each single agent ($P<0.001$) (Fig. 1c, d). We examined if combining 1 μM concentrations of each agent induced extrinsic and/or intrinsic apoptotic signalling pathways. Our results showed that Enzastaurin combined with Lenalidomide in WSU-NHL cells induced activation of caspase 8 (extrinsic pathway), caspase 9 (intrinsic pathway), and caspase 3 after 72 h (Fig. 2a). Treatment of WSU-NHL cells with the combination also triggered an increase in PARP cleavage at 72 h that was abrogated by ZVAD-fmk (Fig. 2b), confirming that the apoptosis is caspase-dependent.

Cell cycle distribution

Studies with Annexin V/PI and caspases showed that apoptotic activity was induced by the combination of Enzastaurin and Lenalidomide at low concentrations in the WSU-NHL cell line. To better understand the mechanism of this effect, we analysed the cell cycle distribution determined by these drugs alone and in combination.

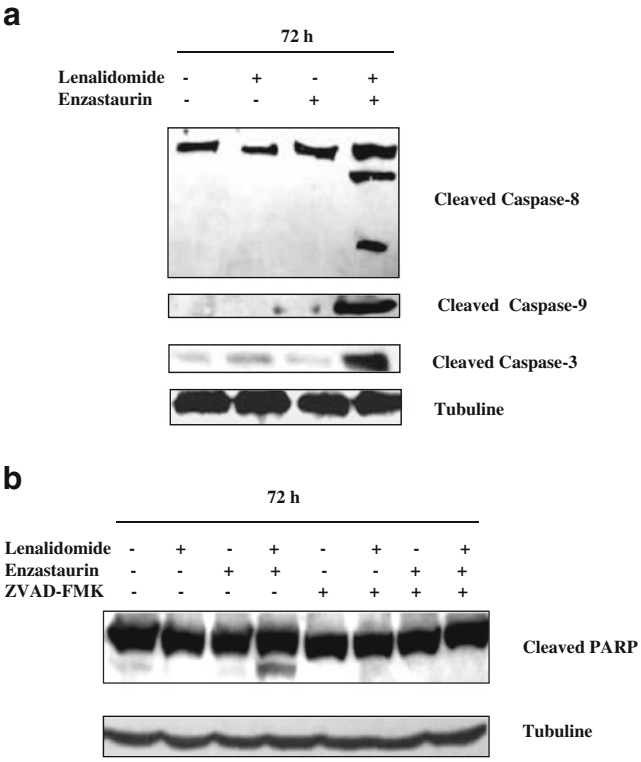

Fig. 2 Western blot of cellular extracts from WSU-NHL cells. WSU-NHL cells were cultured with Enzastaurin or Lenalidomide alone or in combination at a concentration of 1 μM for 72 h. **a** Enzastaurin and Lenalidomide in combination induced cleavage of caspase 8, caspase 9, caspase 3, and **b** PARP. Treatment of cells with ZVAD-fmk blocked PARP cleavage, confirming that apoptosis is caspase-dependent

Enzastaurin alone

Treatment with Enzastaurin (1 μM) alone did not significantly affect the cell cycle distribution with respect to control after 72 h.

Lenalidomide alone

After 72 h, treatment with Lenalidomide (1 μM) alone reduced the proportion of cells in S phase ($P<0.09$) and increased the proportion of cells in G0–G1 phase ($P<0.01$).

Enzastaurin and Lenalidomide combined

Our results indicated that the combination of Enzastaurin and Lenalidomide triggered a marked increase in cells arrested in G0–G1 phase, followed by a significant decrease in cells in S phase ($P<0.001$), in comparison with each single agent after 72 h of treatment (Fig. 3). Taken together, our results demonstrate that the combination of Enzastaurin and Lenalidomide can synergistically induce cell cycle arrest, resulting in growth inhibition.

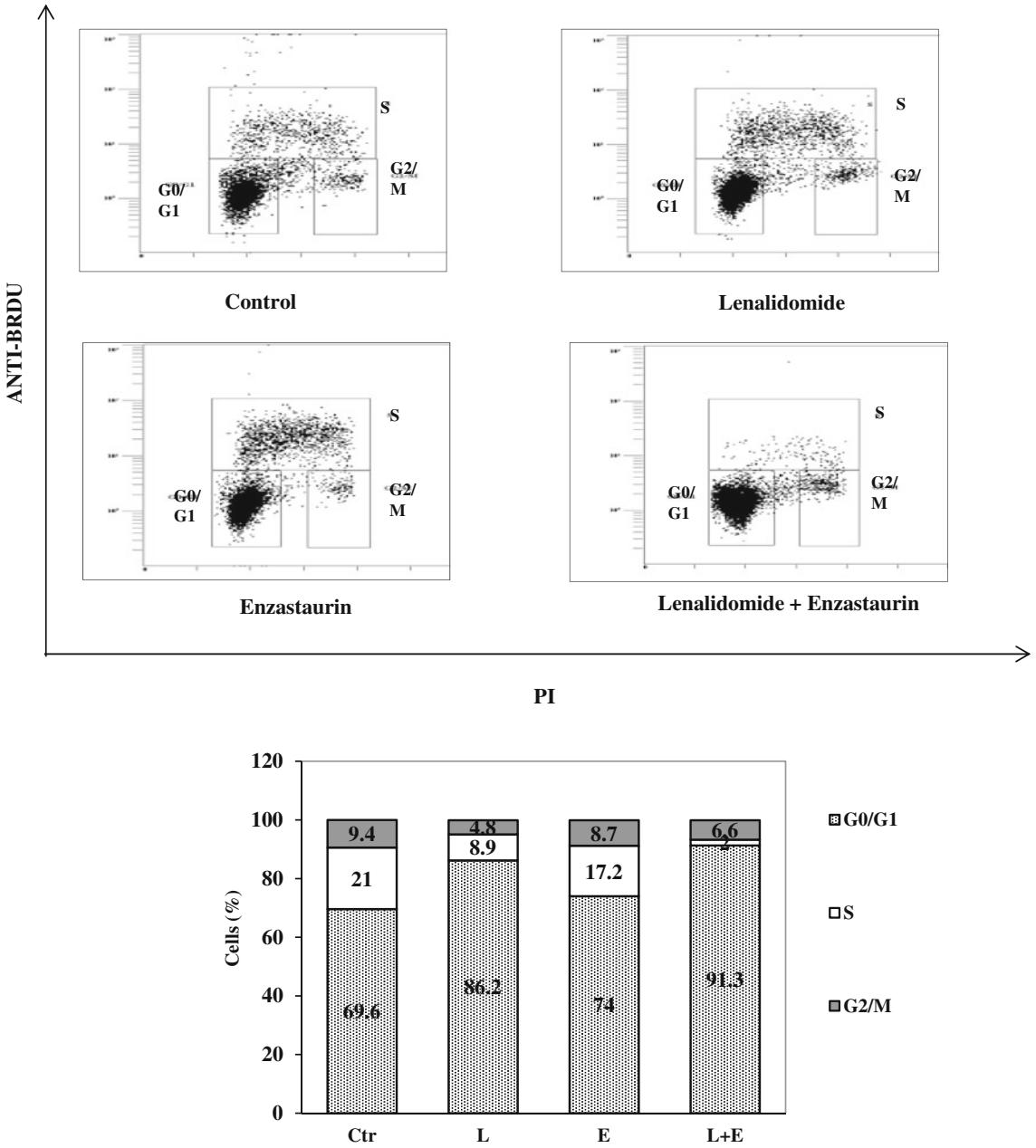

Fig. 3 Cell cycle profile (*top*) and cell cycle distribution (*bottom*) of WSU-NHL cells after 72 h of treatment with Enzastaurin and Lenalidomide alone and in combination. The cell cycle was analysed by BrdU and PI staining and flow cytometry. Results of three independent experiments are shown

Effect of Enzastaurin and Lenalidomide on signalling pathways

We examined, in the WSU-NHL cell line, the phosphorylation status of AKT and its downstream proteins, GSK-3β, m-TOR, and p70S6, using western blotting. No inhibition of the AKT pathway and its downstream targets was noted with Enzastaurin or Lenalidomide alone at this concentration (1 μM). In contrast, we observed that at 72 h the combination inhibited the AKT pathway, dephosphorylating AKT, GSK3-β, m-TOR, and p70S6 (Fig. 4a). Our studies also showed that the Enzastaurin and Lenalidomide combination decreased the expression of phosphorylated PKC (Fig. 4b); meanwhile, no inhibition of the PKC pathway was observed with Enzastaurin or Lenalidomide alone at this concentration (1 μM). The combination triggered inhibition of MAPK/ERK and its downstream effector p-90RSK; while, Enzastaurin and Lenalidomide alone at 1 μM did not affect this pathway (Fig. 5a). Furthermore, our data showed that the combination did not modify Bcl-2

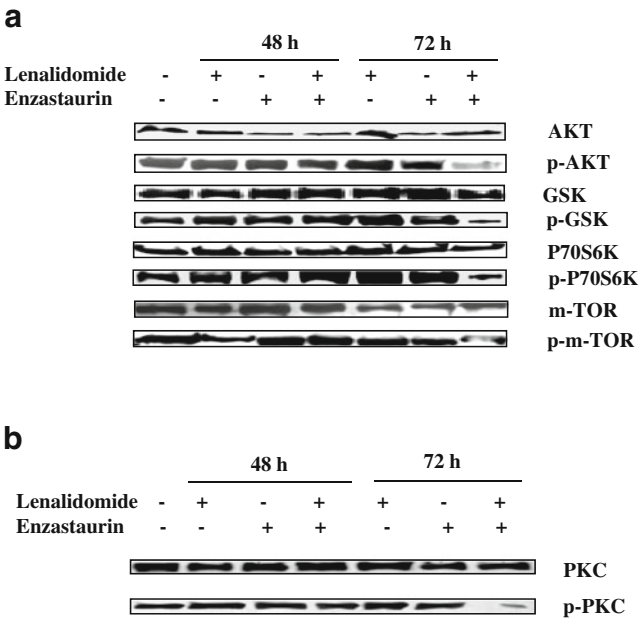

Fig. 4 Western blot of cellular extracts from WSU-NHL cells. WSU-NHL cells were treated with Enzastaurin or Lenalidomide alone or in combination for 48 and 72 h. The combination of Enzastaurin and Lenalidomide induced **a** dephosphorylation of AKT, GSK3-β, m-TOR, and p-70 and **b** PKC after 72 h

levels. We next evaluated whether combination treatment of WSU-NHL cells affected BAD, a member of the Bcl-2 family protein that has pro-apoptotic activity when dephosphorylated. After treatment with the combination, we observed a decrease in BAD phosphorylation at Ser136 and Ser112 (Fig. 5b). These findings suggest that apoptosis triggered by Enzastaurin plus Lenalidomide is mediated, at least in part, via BAD.

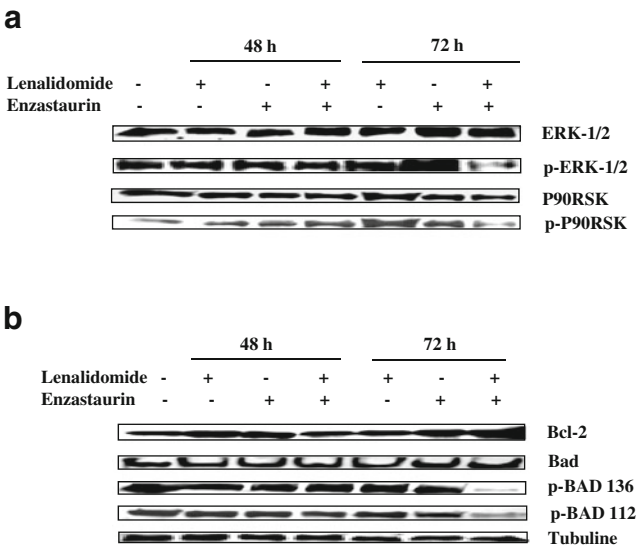

Fig. 5 Western blot of cellular extracts from WSU-NHL cells. The combination of Enzastaurin and Lenalidomide **a** induced MAPK/ERK and p90RSK dephosphorylation after 72 h and **b** did not change the expression levels of Bcl-2, but decreased phosphorylation of p-BAD (Ser112) and (Ser136)

Combination of Enzastaurin and Lenalidomide suppresses cell proliferation in B-cell lymphoma cell lines co-cultured with BMSCs

The bone marrow (BM) microenvironment could confer a growth advantage and increase drug resistance in lymphoma cells lines; therefore, we next examined whether the combination retained its ability to trigger B-NHL cell death in the BM milieu. WSU-NHL cells were cultured with or without BMSCs in the absence or presence of Enzastaurin or Lenalidomide, alone or in combination, at a dose of 1 μM. Enzastaurin and Lenalidomide alone were unable to suppress proliferation of B-cell lymphoma cell lines co-cultured with BMSCs; while, the combination inhibited cell growth in the WSU-NHL cell line as assessed by MTT (Fig. 6). These findings suggest that the combination not only directly targets WSU-NHL cells, but also overcomes the cytoprotective effects of the BM microenvironment, without toxicity of normal BMSCs.

Discussion

In recent years, there has been a rapid development of novel agents targeting tumour-specific mechanisms. Preliminary data indicate that combinations of compounds, which simultaneously target multiple tumourigenic pathways, induce

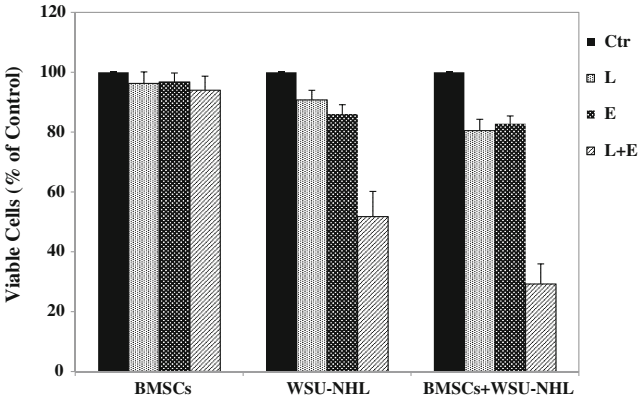

Fig. 6 Viability of WSU-NHL co-cultured with or without BMSCs. WSU-NHL cells were plated in the absence or presence of BMSC feeder layer. Enzastaurin and Lenalidomide alone or in combination were added the next day. Treatment of BMSCs with single agents and combination did not affect BMSCs cell viability, that is in favour of a specific cell type sensitivity to the drug. On the other hand the combination of Enzastaurin and Lenalidomide suppressed NHL cell proliferation alone and in presence of BMSCs confirming that the combination is effective on lymphoma cells, overcoming the protective effect of the bone marrow microenvironment. Data are expressed as a percentage of untreated control±standard deviation

more effective and lasting anti-tumour effects in different malignancies [29–33]. Although indolent non-Hodgkin's lymphomas (NHLs) usually have a relatively good prognosis, a curative treatment does not exist and most patients eventually die from their disease. Therefore, there is a need to develop optimal combinations of drugs to improve the survival outcomes of these patients. An increased understanding of signalling pathways that regulate lymphoma cell growth and survival helped identifying new molecular targets. Of particular interest are PI3K/AKT, PKC, MAPK/ERK that are involved in the regulation of cell proliferation and apoptosis and thus represent an attractive target for cancer treatment.

Enzastaurin, a PKC-β inhibitor and Lenalidomide represent two of many new target therapies with promising results. The preclinical activity of Enzastaurin led to a phase I study of the oral agent in patients with advanced cancer [34]. Based on safety data in the phase I study, Enzastaurin 525 mg was the recommended dose for additional phase II trials [34]. Of note, Enzastaurin was well tolerated at this recommended dose with no grade 3 or 4 toxicities. For these reasons, Enzastaurin is being evaluated in several clinical trials as a single agent or in combination with other compounds for the treatment of various haematologic malignancies and solid tumours [35–37]. The clinical trials with Enzastaurin have been promising and there is a strong rationale for efforts directed at identifying logical therapeutic combinations.

Lenalidomide is a second-generation immunomodulatory drug derived from Thalidomide, that has activity in a variety of B-cell malignancies. Lenalidomide has a manageable safety profile and is now being evaluated in various clinical settings, including maintenance, and in combination with other new agents in clinical trials. Further preliminary data have shown in vivo activity against aggressive and indolent lymphomas either alone or in combination with rituximab [38–40]. Its mechanism of action is complex and acts on both cancer cells and microenvironment.

In our previous study [11], we demonstrated that Enzastaurin was able to induce cell death in WSU-NHL cells, at concentrations higher than 2.5 μM, suggesting that targeting the AKT, PKC, RSK and downstream proteins may have direct antiproliferative and apoptotic effects on B-cell lymphoma cell lines. Based on our previous results and these observations, we propose a combination of two novel agents, with the goal to improve their efficacy, reducing the doses and their possible side effects. We therefore tested the combination of Enzastaurin and Lenalidomide at dose as low as 1 μM on three NHL-cell lines (WSU-NHL, Karpas-422 and RL), confirming a proapoptotic effect. Given that all three NHL-cell lines showed the same sensitivity to the drug combination, we analysed in details the mechanism of action in WSU-NHL cell line.

Several investigations have reported antiproliferative and proapoptotic effects of Lenalidomide alone on tumour cells in vitro [20, 23, 24]. Gandhi et al. [24] have shown that Lenalidomide alone inhibits Namalwa cell from Burkitt's lymphoma leading to antiproliferative effect and affecting AKT. Another report [23] showed a strong effect of Lenalidomide on cell cycle progression without affecting cell apoptosis. In this study, we demonstrated that the treatment of WSU-NHL cells with Lenalidomide alone affected the cell cycle acting both on S and G0–G1 phase but was unable to induce apoptosis. This would suggest that Lenalidomide has a more of a cytostatic than a cytotoxic effect in this cell line, justifying the combination with another drug. As was confirmed by Chou-Talalay, the combination of Enzastaurin and Lenalidomide, in fact, exerted a strong synergistic anti-lymphoma activity. Our in vitro results are partly in agreement with previous data obtained by Podar et al. [7], who reported a moderate synergistic effects of the combination of low doses of Enzastaurin and Lenalidomide in MM cells. However, the effects of combination treatment on signalling pathways have not been analysed.

The pro-apoptotic effect was also confirmed by western blot analysis that showed that Enzastaurin and Lenalidomide in combination activated both the extrinsic and intrinsic pathways of apoptosis via caspases 8 and 9. To further determine the role of upstream proteins in the drugs-induced mitochondrial apoptotic pathway, we analysed the expression of Bcl-2, antiapoptotic protein that plays an important role in the mitochondria-associated apoptotic pathway, and Bad. Bad is one of proapoptotic Bcl-2 family member proteins. Our previous study [11] showed that Enzastaurin alone induced apoptosis through Bad-mediated pathways without affecting Bcl-2 expression in B-cell lymphoma with t(14;18). Because Enzastaurin alone activated Bad through dephosphorylation at Ser^{112} and Ser^{136}, we evaluated whether Bad activity and Bcl-2 expression were affected by Enzastaurin and Lenalidomide in combination. In our experiments, Bad activation (dephosphorylation) was confirmed in Enzastaurin and Lenalidomide combination (Fig. 5b) without observing any effect on Bcl-2.

The downstream signalling cascades study also demonstrated that the combination triggered a time-dependent inhibition of AKT, PKC and MAPK/ERK phosphorylation, whereas either of these drugs alone did not affect these pathways. This may explain the increased apoptosis and the decreased cell proliferation found in WSU-NHL. The molecular mechanism whereby Enzastaurin and Lenalidomide in combination inhibit signalling pathways activity remains to be defined.

In summary, Enzastaurin and Lenalidomide are new agents with different mechanisms of action that synergize in vitro against indolent lymphomas cell lines at a very low dose. They are not classic chemotherapeutic agents causing

usual side effects and could be considered an example of new anti-cancer 'soft treatments'. Considering their strong anti-lymphoma activity, safe toxicity profiles, and the need for more active and less toxic treatments for indolent lymphomas, further study of Enzastaurin in combination with Lenalidomide is warranted.

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