NVP-BEZ235 alone and in combination in mantle cell lymphoma: an effective therapeutic strategy

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Objectives: Mantle cell lymphoma (MCL) is a distinct subtype of B-cell lymphoma; the complete response rate for standard therapies in use today is 85 – 90%. NVP-BEZ235 inhibits the PI3K/Akt/mTOR signaling axis at the level of both PI3K and mTOR. In this study, we analyzed the inhibitory effects of NVP-BEZ235 on mantle cell lines and its effects in combination with enzastaurin, everolimus and perifosine.

Methods: The effects of NVP-BEZ235 on cell proliferation and apoptosis were evaluated using MTT assay and flow cytometry analysis. The cell cycle analysis was performed applying BrdU incorporation. Western blot analysis was utilized for phosphorylation status evaluation of protein kinases. The interaction between NVP-BEZ235 and enzastaurin, everolimus and perifosine was examined by Chou-Talalay method.

Results: NVP-BEZ235 induced significant increase of apoptosis, both via intrinsic and extrinsic pathways. We found that NVP-BEZ235 inhibited mantle cells growth by induction of G1 arrest. NVP-BEZ235 exerts its antitumor activity even when mantle cells were in contact with bone marrow microenvironment. Enzastaurin, everolimus and perifosine enhanced the cytotoxicity triggered by NVP-BEZ235.

Conclusions: The above results encourage clinical development of NVP-BEZ235 in combination and the possible inclusion of patients with mantle lymphoma in Phase I/II clinical trials.

Keywords: apoptosis, combined therapy, mantle cell lymphoma, NVP-BEZ235, PI3k/Akt/m-TOR pathway

1. Introduction

Mantle cell lymphoma (MCL) is a distinct subtype of B-cell lymphoma, which is believed to originate from follicle mantle B cells [1-3]. Mantle cell lymphoma is genetically characterized by the (11;14)(q13;q32) translocation, which results in deregulated aberrant expression of cyclin D1 [4-6]. Mantle cell lymphoma is generally incurable, and patients have a poor prognosis with a median survival of 3 to 5 years [7-9]. Recent gene profiling studies suggest that in MCL many individual genes involved in signaling pathways may be either overexpressed or underexpressed [10]. Among the overexpressed genes, there are several members of phosphatidylinositol 3'-kinase (PI3K)/Akt pathway. This signaling pathway is involved in the transduction of extracellular stimuli that regulates fundamental cellular processes including cell-cycle progression, proliferation and cell growth,
apoptosis and survival [11-13]. Activated PI3K enables recruitment of serine/threonine kinase Akt to the cell membrane where it undergoes phosphorylation [14]. Phosphorylated Akt subsequently activates several other intracellular signaling proteins [15,16]. One downstream target of Akt is the mammalian target of rapamycin (mTOR) that when activated promotes mRNA translation and protein synthesis, resulting in the regulation of cell growth and proliferation, cellular metabolism and angiogenesis [17,18]. In recent years, it has been shown that PI3K/AKT signaling pathway components are frequently altered in human tumors. According to the database of Catalogue of somatic mutations in cancer, PI3K mutations appear most commonly in breast (37%), endometric (24%), urinary (17%), colon (15%) and upper digestive tract (11%) cancers. As PI3Ks play important roles in tumorigenesis, there is a great interest to develop PI3K inhibitors as potential therapeutic agents. All available PI3K inhibitors represent an optimal tool to block cancer cell proliferation, but they appear poorly cytotoxic. On these bases, recent studies have shown that the combination of PI3K inhibitors with other cytotoxic agents can increase to a great extent the cytotoxic response of different tumors [19]. The molecular events associated with activation of PI3K/AKT pathways in MCL present an important challenge for the development of a targeted therapy based on signaling pathway alterations [20]. Current treatment for NHL is not optimally effective; relapse and resistance to chemotherapy are common events and the risk of secondary malignancies an increasing concern. Monotherapy with the proteasome inhibitor, bortezomib, has shown efficacy in MCL, and combination therapy with conventional chemotherapy regimens appears promising [21-23]. Demonstration of durable complete and partial responses to monotherapy with the mTOR inhibitors (everolimus, tensirolimus and ridaforolimus) in Phase I/II monotherapy trials supports further study of this class of compounds in Phase III trials [24-26]. Despite all efforts to the contrary, current therapies are not curative and progressive disease remain the leading cause of cancer-related mortality [27]. NVP-BEZ235 is a synthetic small molecular mass compound belonging to the class of imidazoquinolines that potently and reversibly inhibits PI3K catalytic activity by competing at its ATP-binding site [28-31]. ex vivo pharmacokinetic/pharmacodynamics analysis of tumor tissue showed a time-dependent correlation between compound concentration and PI3K/AKT pathway inhibition [32]. The efficacy of the dual NVP-BEZ235 in targeting Akt and mTOR pathways has been recently proven in Waldenström Macroglobulinemia cells and in low-grade lymphoma cell lines [33,34].

We hypothesized that, on the basis of mechanisms of action of the NVP-BEZ235 and of enzastaurin, everolimus and perifosine, the agents would be more effective in combination compared with every single agent alone. Here we demonstrate a synergistic activity of NVP-BEZ235 with enzastaurin, everolimus and perifosine. In particular, the synergism of NVP-BEZ235 with enzastaurin appeared more effective than other combination in targeting some signaling pathways.

2. Material and methods

2.1 Cell culture

2.1.1 Cell lines

Granta-519 and Jeko-1 cell lines were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures; http://www.dsmz.de). Jeko-1 and Granta-519 cell lines were cultured in RPMI-1640 and DMEM, respectively, supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine and 100 U/ml penicillin and streptomycin. All the above-mentioned reagents were purchased from Euroclone.

2.1.2 Bone marrow stromal cell (BMSC) culture

Bone marrow stromal cells were separated using the method described by Gartner and Kaplan [35]. Adherent cells were long-term cultured and expanded in Iscove’s modified Dulbecco’s medium (Euroclone) supplemented with 20% FBS, 2 mM L-glutamine, and 100 U/mL penicillin and streptomycin at 37°C and 5% CO2. When an adherent cell monolayer developed, the cells were harvested in 1× phosphate buffered saline (PBS; Euroclone) containing 0.05% trypsin (Euroclone) and 0.02% ethylenediaminetetraacetic acid (Euroclone) and then washed and collected by centrifugation. The confluent adherent BMSCs showed a predominantly fibroblast morphology.

2.1.3 Patient samples

Peripheral blood mononuclear cells (PBMCs) were obtained from four patients with indolent MCL in leukemic phase and from two volunteers, affected by ITP (immune thrombocytopenia) after informed consent. The leukemic phase in the four patients was diagnosed by microscopic analysis and flow cytometry showed the percentage of CD19+/CD5+ cells was more than 80%.

2.2 Drugs

NVP-BEZ235 was a gift from Novartis Pharma. Enzastaurin, perifosine and everolimus were purchased from Selleck Chemicals. The drugs were dissolved in dimethylsulfoxide (DMSO; Euroclone) and stored at 4°C until use; every drug was diluted in culture medium immediately before use. The maximum final concentration of DMSO (< 0.1%) did not affect cell proliferation and did not induce cytotoxicity on the cell lines and primary cells tested (data not shown).

2.3 Cell proliferation assay

Mantle cell lines were treated with increasing concentrations of NVP-BEZ235 for 24, 48 and 72 h. Viability were assessed using 0.2% Trypan Blue (Euroclone) exclusion and cell proliferation was evaluated by measuring MTT (cellTiter non-radioactive cell proliferation assay; Promega) as previously
2.4 Annexin V binding assay for apoptosis

Apoptosis was quantified using annexin/propidium iodide (PI) binding assay following the manufacturer’s instructions (Miltenyi Biotec) and then analyzed by a flow cytometer Coulter Epics XL MCL and EXPO 32 vers.1.2.

Table 1. Antiproliferative activity of NVP-BEZ235 in mantle cell lines. Granta-519 and Jeko-1 cells were treated with NVP-BEZ235 (0.5 – 50 nM) for 24, 48 and 72 h. IC50 values were calculated using MTT assay.

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<td>IC95%</td>
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Values represent IC50 (mean and IC95%) obtained from three independent experiments performed in triplicate.

2.5 Cell cycle analysis

DNA synthesis in proliferating cells was determined by BrdU incorporation. Cells were spread onto 96-well plates and incubated with 10 µL 1 x 5-bromodeoxyuridine (BrdU; Sigma) for 30 min. Fixing solution was added to the cells for 30 min. The cells were washed with wash buffer and incubated for 60 min with anti-BrdU mouse IgG (BD Bioscience). After adding goat anti-mouse IgG FITC (Dako) and PI (Miltenyi Biotec), the optic density (OD) was measured at 450 nm and analyzed using a Coulter Epics XL MCL and EXPO 32 vers.1.2.

2.6 Western blot analysis

Mantle cell lines were harvested and lysed using lysis buffer (20 mM Tris-Cl, pH 7, 150 mM NaCl, 1% NP40, 10 mM EDTA, 10 mM sodium pyrophosphate, 10% glycerol) reconstituted with 5 mM NaVO3, 10 mM p-nitrophenyl phosphate (PNPP) and 5 mM phosphate inhibitor cocktail (PIC). Cell lysates (50 microg) were resolved in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. All the above-mentioned reagents were purchased from Euroclone. Western blot analysis included evaluation of PI3K, AKT, p-AKT, p-GSK-3b, p-p70S6, m-TOR, p-m-TOR, p-p90RSK, p-EBP1, cyclin D1, MAPK, p-MAPK, p-p90RSK, p-BAD (Ser112), BIM, Bcl-2, caspase 3, caspase 9, caspase 8 and PARP. PARP expression was evaluated also after 1 h of pretreatment with 40 microM of zVAD-fmk (Sigma), a broad inhibitor caspase. The treatment with zVAD-fmk did not affect cell proliferation and did not induce cytotoxicity on the cell lines tested (data not shown). All antibodies were purchased from Cell Signaling Technology.

2.7 Analysis of BCL-2 expression

Bcl-2 protein expression was determined after mantle cell line incubation with Bcl-2 Antibody Sampler Kit, purchased from Epitomics. Following incubation with fluorescently conjugated secondary antibody, analysis was performed using a flow cytometer (Coulter Epics XL MCL).

2.8 Co-culture of B-lymphoma cell lines with bone marrow stromal cells

For co-cultures, BMSCs were incubated for 48 h to reach confluence. B-cell lymphoma cell lines were then seeded in the presence or absence of BMSCs and the next day, increasing concentrations of NVP-BEZ235 were added. Non-adherent cells were collected 24, 48 and 72 h after drug addition and cell proliferation was evaluated using the MTT assay.

2.9 Drug combination effects (isobologram analysis)

Cell lines were cultured for 48 h in the presence or absence of NVP-BEZ235 (at concentrations corresponding to IC25, IC50 and IC75) in combination with enzastaurin, perifosine and everolimus (at concentrations corresponding to IC25, IC50 and IC75). Inhibitory effects of drugs combination treatment were determined using the MTT assay as described above.

described [36]. Dye absorbance was measured at 550/560 nm using a plate reader.
2.10 Statistical analysis

Statistical differences between controls and drug-treated cells were determined by one-way ANOVA (Sidak). P-values < 0.05 were considered statistically significant. Data were analyzed using the Stata 8.2/SE package (StataCorp LP).

The interaction of NVP-BEZ235 with other drugs was assessed using multiple effect analysis, based on the method described by Chou-Talalay in which a Combination Index (CI) of < 1, = 1 and > 1 indicates synergism, additive effects and antagonism, respectively [37,38]. The data were processed by isobologram analysis using Stata 8.2.

3. Results

3.1 NVP-BEZ235 suppresses proliferation of mantle lymphoma cells

We studied the effects of NVP-BEZ235 on Granta-519 and Jeko-1 cell lines proliferation. Mantle lymphoma cell lines were cultured in presence of NVP-BEZ235 (0.5 – 50 nM) for 24, 48 and 72 h; then, cell viability assessment was evaluated with trypan blue exclusion (data not shown) and IC50 values were evaluated using the MTT assay. Table 1 shows IC50 values obtained after Granta-519 and Jeko-1 cell lines treatment with NVP-BEZ235. Results were obtained from three independent experiments performed in triplicate.

We evaluated the effects of NVP-BEZ235 on PBMCs from four patients with indolent mantle lymphoma and from two donors, affected by ITP. Freshly isolated PBMCs were incubated with NVP-BEZ235 at 10 and 40 nM for 24 and 48 h and analyzed with MTT assay (Figure 1A and B). The results showed that NVP-BEZ235 decreased significantly in a dose-time-dependent manner the percentage of viable cells from patients with lymphoma but had minimal or no cytotoxic effect on PBMCs from healthy donors. Results represent the mean + standard deviation (s.d.) for the four patients and the two healthy donors. There are no appreciable changes between the four patient samples and the two donor samples.

3.2 NVP-BEZ235 induces apoptosis in mantle lymphoma cell lines

We examined the functional effects of NVP-BEZ235 on apoptosis in mantle cell lines. Jeko-1 and Granta-519 cells were treated for 24, 48 and 72 h with NVP-BEZ235 at 10 and 40 nM concentrations. NVP-BEZ235 induces significant increase (p < 0.001) of apoptosis in a dose-time-dependent manner, evidenced by annexin V/PI staining. Results represent the mean + s.d. obtained from three independent experiments. Comparable results were obtained after Jeko-1 cells treatment with NVP-BEZ235 (data not shown).

After flow cytometer analysis, we try to define mechanisms whereby NVP-BEZ235 induces apoptosis and we demonstrated that NVP-BEZ235 induced apoptosis both via intrinsic and extrinsic apoptotic pathways as demonstrated by caspase 9, caspase 8 and PARP cleavage in a dose-dependent manner. Figure 3A shows blots resulted from analysis of expression of caspase 3, caspase 8 after treatment of Granta-519 cells with increasing concentration (0 – 50 nM) of NVP-BEZ235 for 48 h. To confirm that
Effects of NVP-BEZ235 alone and in combination in mantle cell lymphoma

the apoptosis was mediated by the caspases’ activation, we cultured the Granta-519 cells in the presence of ZVAD-fmk, a broad caspase inhibitor. Notably, ZVAD-fmk decreased NVP-BEZ235-induced PARP cleavage, whereas when utilized alone it did not influence survival and cell growth after treatment (Figure 3B). Comparable western blots of cellular extracts from Jeko-1 cells were obtained (data not shown).

We studied other proteins involved in apoptotic mechanisms: BIM, p-BADser112 and Bcl-2. Induction of apoptosis in Granta-519 cells treated with NVP-BEZ235 was accompanied by upregulation of proapoptotic Bim and p-BADser112, whereas no change in Bcl-2 expression was observed.

3.3 NVP-BEZ235-induced apoptosis is associated with the down modulation of cyclin D1 and with cell cycle arrest in MCL cells

Cyclin D1 overexpression has been implicated in the pathogenesis of MCL. To determine if NVP-BEZ235-induced apoptosis was mediated through the modulation of this target, we evaluated the effects of NVP-BEZ235 on cyclin D1 levels by immunoblot analysis. Treatment of Granta-519 and Jeko-1 cells with NVP-BEZ235 at increasing concentration (0 – 50 nM) for 48 h resulted in a dose-dependent downregulation of cyclin D1 protein. Figure 5 shows blots from the expression of cyclin D1 after treatment of Granta-519 cells with increasing concentration (0 – 50 nM) of NVP-BEZ235 for 48 h. Comparable western blots were obtained from cellular extracts of Jeko-1 cells (data not shown).

To determine if the NVP-BEZ235-induced cyclin D1 downregulation resulted in defects in cell cycle progression, Jeko-1 and Granta-519 cells were treated with NVP-BEZ235 (at concentrations corresponding to IC50) for 24, 48 and 72 h and subjected to cell cycle analysis by flow cytometry. NVP-BEZ235 treatment resulted in time-dependent significantly (p < 0.05) decrease in the S-Phase entry of the cell cycle. This decrease is further confirmed by the accumulation of cells in the G1 phase of the cell cycle. Figure 6 shows the results obtained on Granta-519 (Figure 6A) and Jeko (Figure 6B) from three independent experiments. Data are expressed as mean ± s.d.

3.4 NVP-BEZ235 targets mantle cell in the context of bone marrow microenvironment in vitro

It has been widely demonstrated that bone marrow microenvironment confers growth advantages and induces drug resistance in malignant cells. We therefore thought to evaluate the antitumor activity of NVP-BEZ235 against mantle cells in the context of the bone marrow milieu. Granta-519 and Jeko-1 cells were incubated with NVP-BEZ235 at increasing concentration for 48 h in presence or absence of BMSCs (Figure 7A and B). NVP-BEZ235 showed a cytotoxic effect on cell lines even when cultured with BMSCs. NVP-BEZ235 had minimal or no cytotoxic effect on BMSCs. Results are representative of three independent experiments performed in triplicate.

3.5 NVP-BEZ235 targets the PI3K and mTOR signaling pathway

We investigated the efficacy of the NVP-BEZ235 in targeting Akt and mTOR pathways in mantle lymphoma cell lines. Cell lines were treated with increasing doses of NVP-BEZ235 (0 – 50 nM) for 48 h. Figure 8 shows blots resulted from analysis of expression of PI3K, Akt, p-Akt(ser473), p-S6R, p-GSK3β, m-TOR, p-m-TOR, p-70 and p-4EBP1 after treatment of Granta-519 cells with NVP-BEZ235 for 48 h. NVP-BEZ235 reduced expression of PI3k, Akt and GSK3β in dose-dependent manner both in the Granta that in the Jeko cell line. In parallel, NVP-BEZ235 also inhibited phosphorylation of mTOR as well as of the downstream targets p70S6 and 4EBP1 in a dose-dependent manner. Comparable western blots were obtained from cellular extracts of Jeko-1 cells (data not shown). Granta-519 and Jeko cells were cultured with NVP-BEZ235 (0 – 50 nM) for 48 h;
whole-cell lysates were subjected to western blotting using anti-MAPK, p-anti-MAPK and p-P90RSK. P90RSK is normally activated by MAPK in vitro and in vivo via phosphorylation. The phosphorylation status of p-MAPK and p-P90RSK were not affected (Figure 9).

3.6 NVP-BEZ235 in combination with enzastaurin, everolimus and perifosine

We tested the anti-lymphoma activity of NVP-BEZ235 in combination with enzastaurin, everolimus and perifosine. First we evaluated IC₅₀ values of each compound on Granta-519 and Jeko-1 cells using MTT assay, then we evaluated the cytotoxicity of the combinations using the same assay. The interaction between drugs was analyzed by isobologram analysis using the STACorp8.2 software program based upon the Chou-Talalay method to determine if the combination was additive or synergistic. We found that enzastaurin, everolimus and perifosine enhanced the cytotoxicity triggered by NVP-BEZ235; a clear synergistic interaction (CI < 1) appeared after 48 h using low concentrations of all compounds (Table 2).

3.7 NVP-BEZ235 in combination with enzastaurin, everolimus and perifosine: effects on Bcl-2 and MAPK

In the previous section we showed that NVP-BEZ235 did not have effects on Bcl-2 expression. A western blot analysis (Figure 10A) showed that enzastaurin, everolimus and perifosine did not decrease Bcl-2 expression. As isobologram analysis showed that NVP-BEZ235 combined with enzastaurin, perifosine and everolimus had synergist effects on mantle cell lines, as we decided to evaluate the effects of the combination of NVP-BEZ235 with enzastaurin, perifosine and everolimus on Bcl-2 expression. In Figure 10B and C blots showed that the combination of NVP-BEZ235 (20 nM) plus enzastaurin (5 µM) decreased Bcl-2 expression in Granta-519 and Jeko-1 cell lines, whereas the combination with perifosine and everolimus had no effect. The same Granta-519 cells treated with NVP-BEZ235 plus enzastaurin utilized for western blot analysis of Bcl-2 expression were fixed in 2% paraformaldehyde, and Bcl-2 protein expression was detected by flow cytometer. The flow cytometer analysis showed that Bcl-2-positive cells decreased after treatment with NVP-BEZ235 plus enzastaurin (74 vs 33%) (data not shown).

We studied the effects of NVP-BEZ235 in combination with enzastaurin, perifosine and everolimus on p-MAPK expression. No combinations affected phosphorylation status of MAPK (data not shown).

4. Conclusion

Improvement of our ability to control malignant lymphoma depends not only on the identification of crucial signaling pathways, but also on the ability to develop drugs that target these pathways in a synergistic manner.
and, in particular, NVP-BEZ235 has recently entered clinical trials. The mTOR kinase is the catalytic component of the complexes mTORC1 and mTORC2. Their different composition accounts for not only distinct cellular function but also for differential sensitivity to pharmacological intervention. Rapamycin allosterically inhibit only mTORC1, but not mTORC2 [14]. NVP-BEZ235 induced significant p-Akt inhibition resulting from the dual targeting of mTORC1 and mTORC2 [42]. Because NVP-BEZ235 inhibits the PI3k/Akt pathway at multiple levels, it may overcome the compensatory drug resistance mechanism that has developed with other selective inhibitors against individual targets of these pathways. NVP-BEZ235 is a first-generation PI3K inhibitor with sufficient drug-like properties to promote it as a candidate for clinical use in the treatment of cancer. Indeed, NVP-BEZ235 is being investigated in 14 Phase I/II clinical trials in advanced solid tumor patients as a single agent as well as in combination with other agents [31]. However, in the last clinical reports it is becoming evident that PI3K inhibitors as single agent entities might not hold up to their initial promise [43]. Thus, it will be important to focus on robust translational research programs to best identify key combination partners for PI3K inhibitors.

In our study, we analyzed the inhibitory effects of NVP-BEZ235 on mantle cell lines and then we evaluated its effects after combination with enzastaurin, everolimus and perifosine. We used clinically achievable concentrations of NVP-BEZ235 to identify its antitumor activity in MCL, as a result of its effect on PI3K/AKT and mTOR pathways. Our study showed that NVP-BEZ235 induces cell lymphoma growth inhibition and apoptosis even at low nanomolar concentrations and not in PBMCs from healthy individuals. Induction of apoptosis mediated by cleavage of caspases 3, 9, 8 and PARP suggests that both the intrinsic and extrinsic pathways were activated upon incubation with NVP-BEZ235. Indeed, we described that apoptotic events such as BIM and BAD [36]. NVP-BEZ235 even at high concentrations did not induce a decreased expression of the anti-apoptotic protein Bcl-2.

Treatment with NVP-BEZ235 resulted in a time-dependent down modulation of cyclin D1 and accumulation of cells in G0–G1 and G2–M phases of the cell cycle with concomitant decrease in S-phase entry. We demonstrated the selective activity of NVP-BEZ235 on PI3K, Akt and mTOR by reduced phosphorylation of their downstream targets. Our western blot experiments revealed that NVP-BEZ235 strongly inhibits phosphorylation of p-S6R, GSK3β, P70S6 and 4EBP1. It is important to highlight the role of NVP-BEZ235 in targeting mantle cells in the context of bone marrow microenvironment. By co-culturing mantle cells and primary BMSCs, we have demonstrated that NVP-BEZ235 exerts its antitumor activity even when mantle cells were in contact with bone marrow milieu. Because the bone microenvironment plays such an important role
in the resistance to conventional therapies, the ability of NVP-BEZ235 to overcome these factors is encouraging.

Enzastaurin is an ATP competitive multi-kinase inhibitor initially developed to target PKC [44]. Everolimus, an analogue of rapamycin, targets primarily mTORC1 and has demonstrated antineoplastic activity [45,46]. Perifosine is a synthetic alkylphospholipid that binds plasma membranes and inhibits Akt activation without any direct effect on related kinases such as PI3-K or PDK1 [47]. On the basis of this data, we hypothesized that the three compounds would be more effective in combination compared with every agent alone. Using the CalcuSyn software, we have shown a synergistic activity when NVP-BEZ235 was combined with all the drugs. We would underline that the combination of NVP-BEZ235 plus enzastaurin decreases Bcl-2 expression, whereas these compounds utilized as single agents did not have any effects.

In conclusion, the PI3k/Akt/mTOR axis is an important target in MCL because it regulates cell survival, protein translation and cytokine production. Here, we have demonstrated efficacy of NVP-BEZ235 in cell lines and tumor cells, highlighting its potential utility in MCL. Further, NVP-BEZ235 showed a clear synergistic effect in combination with enzastaurin, everolimus and perifosine. In particular, the combination of NVP-BEZ235 with enzastaurin reduces expression of Bcl-2 protein. The above results encourage clinical development of NVP-BEZ235 in combination and the possible inclusion of patients with mantle lymphoma in

Table 2. Analysis of drug combination effects. Granta-519 and Jeko-1 were treated with NVP-BEZ235 (B) combined with enzastaurin (E), everolimus (EV) and perifosine (P).

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CI indicates the combination index; CI < 0.9 indicated synergism. We found that enzastaurin, everolimus and perifosine enhanced the cytotoxicity triggered by NVP-BEZ235; a clear synergistic interaction (CI < 1) appeared after 48 h using low concentrations of all compounds.

Figure 10. A. Is shown a Western blot of cellular extracts from Granta-519 cells, treated with enzastaurin (2.5 µM), everolimus (1 nM) and perifosine (2.5 µM) for 48 h. B. Is shown a Western blot of cellular extracts from Granta-519 cells, treated with NVP-BEZ235 (5 nM) alone and in combination with enzastaurin (2.5 µM), everolimus (1 nM) and perifosine (2.5 µM) for 48 h. NVP-BEZ235 combined with enzastaurin reduces expression of Bcl-2 protein. B. Blot shows a clear decrease of Bcl-expression also in Jeko cells treated with NVP-BEZ235 combined with enzastaurin.
Effects of NVP-BEZ235 alone and in combination in mantle cell lymphoma

Phase I/II studies. Given the fact that compounds such as NVP-BEZ235, enzastaurin, everolimus, perifosine and MEK/ERK inhibitors are available for clinical study, future exploration of these combinations is justified.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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