CMI Clinical Management Issues

Synergy of NVP-BEZ235 and enzastaurin in mantle cell lymphoma

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Abstract

Mantle cell lymphoma (MCL) is a neoplasm classified as a B-cell malignancy, that accounts for approximately 3 to 8% of Non-Hodgkin's lymphoma (NHL) cases diagnosed annually. MCL is difficult to treat and seldom considered cured. The pathobiology of MCL is complex and includes alteration in the cell cycle, abnormalities in the DNA damage response, and constitutive activation of key antiapoptotic pathways including phosphatidylinositol 3-kinase (PI3K)/Akt and nuclear factor-kB. This has promoted the identification of new targeted treatments and new agents that have shown promising efficacy for future MCL therapies. The phosphatidylinositol 3-kinase (PI3K) mammalian target of rapamicin (mTOR) pathway mediates proliferation, survival, and drug resistance in lymphoma cells. NVP-BEZ235 (BEZ235) is a new, orally bioavailable inhibitor of PI3K and mTOR and a representative of a new class of anti-tumour agents.
In this study, we analysed the in vitro inhibitory effects of NVP-BEZ235 on mantle lymphoma cell lines (GRANTA-519 and JeKo-1) and its effects in combination with enzastaurin, everolimus, and perifosine. Our data suggest that in mantle lymphoma cell lines, BEZ235 in combination with enzastaurin elicits its antitumour effect better than combined with perifosine and everolimus. Our data reveal that the drug combination targets phosphorylation of PI3K/Akt/mTOR pathways and induces both intrinsic and extrinsic apoptosis pathways. Furthermore, inhibition of Bcl-2 anti-apoptosis family members may, in part, explain the efficacy of signalling blockade in lymphoma cells and suggests an additional therapeutic targeting strategy. Therefore, these preclinical data support the potential use of BEZ235 in patients with mantle lymphoma, and in particular provide rationale for combination with enzastaurin.

Keywords: Lymphoma; PI3K signalling pathway; Innovative therapy
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INTRODUCTION

In recent years, advances in cancer have produced information critical to our understanding of cell growth, proliferation, and cell death in malignant cells. The intracellular machinery and signalling cascades that are active in lymphomas have been dissected and reveal multiple potential targets for new agents [1].

Mantle cell lymphoma (MCL) is a distinct subtype of B-cell lymphoma which is believed to originate from follicle mantle B cells [2-4]. MCL is genetically characte-

Key points

***Unmet need**. Mantle cell lymphomas generally have a poor prognosis, and PI3K inhibitors appear poorly cytotoxic*
***Drugs studied**. NVP-BEZ235 (a PI3K/Akt pathway inhibitor) combined with enzastaurin, everolimus, and perifosine and tested in vitro in two mantle cell lines*
***Innovative aspects**. Research has focused on a drug to be combined with NVP-BEZ235 in order to strengthen its cytotoxic activity*

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Disclosure
The authors declare that there are no conflicts of interest of any kind and that they have not received any payment for the preparation of the manuscript

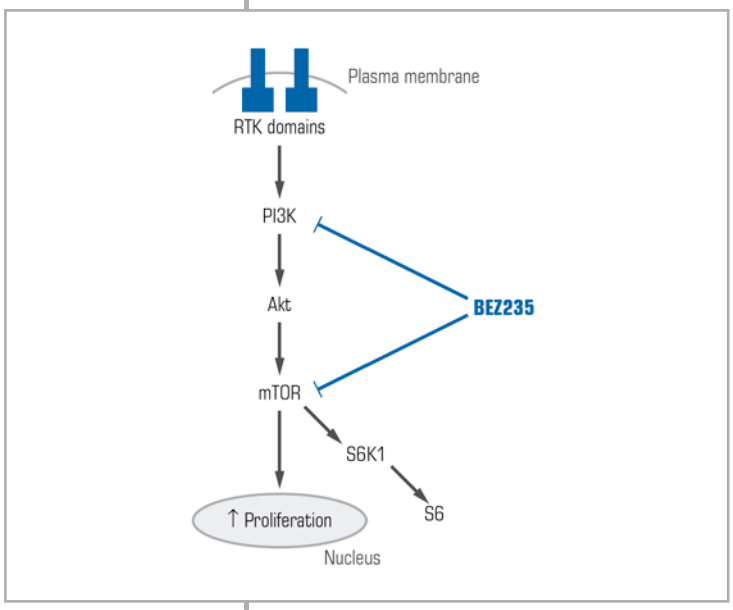

Figure 1. *Metabolic pathway affected by BEZ235 action*

mTOR = mammalian target of rapamicin; PI3K = phosphoinositide 3-kinase; RTK = receptor tyrosine kinase

rised by the t(11;14)(q13;q32) translocation which results in deregulated aberrant expression of cyclin D1 [5-7]. MCL is generally incurable and patients have a poor prognosis with a median survival of 3 to 5 years [8-10]. Recent gene profiling studies suggest that in MCL many individual genes involved in signalling pathways may be either overexpressed or underexpressed [11].

Monoclonal antibodies (mAbs) targeting surface proteins and tumour cell survival pathways have become widely adopted in the treatment of patients with lymphoma. These include improvement of patients' outcomes when combined with chemotherapy and limited toxicity profiles, making mAbs ideal alternative options for heavily pretreated patients with relapsed/refractory disease [12,13].

Monotherapy with the proteasome inhibitor, bortezomib, has shown efficacy in MCL, and combination therapy with conventional chemotherapy regimens appears promising. Bortezomib triggers oxidative stress that converges on the upregulation of the pro-apoptotic protein NOXA to induce cell death. Inhibition of the proteasoma impacts many other pathways and this may be particularly important when considering combination therapies [14,15].

The family of lipid kinases termed "phosphoinositide 3-kinases" (PI3Ks) has been found to have key regulatory roles in many cellular processes, including cell survival, proliferation and differentiation [16-18]. The PI3Ks are grouped into three classes, I, II, and III, on the basis of their structural characteristics and substrate specificity [19,20]. Of these, the most commonly studied are the class I enzymes, which are activated directly by cell surface receptors. Class I PI3Ks are further divided into class IA enzymes that are activated by receptor tyrosine kinases (RTKs), G-protein-coupled receptors (GPCRs) and oncoproteins, and class IB enzymes that are regulated exclusively by GPCRs. To date, only class IA enzymes have been clearly implicated in human cancers [20].

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 signalling pathway alterations [21].

The PI3Kdelta selective inhibitor idelalisib, formerly called "CAL-101", has been shown to exert potent antitumour effects across a range of B-cell malignancies [22]. Demonstration of durable complete and partial responses to monotherapy with the mTOR inhibitors (everolimus, temsirolimus, and ridaforolimus) in phase I/II monotherapy trials supports further study of this class of compounds in phase III trials [23,24]. Despite all efforts to the contrary, current therapies are not curative and progressive disease remains the leading cause of cancer-related mortality [25]. 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 [26-30] (Figure 1).

Ex vivo pharmacokinetic/pharmacodynamic analysis of tumour tissue showed a time-dependent correlation between compound concentration and PI3K/Akt pathway inhibition [31].

The efficacy of the dual NVP-BEZ235 in targeting Akt and mTOR pathways has been recently proven in Waldenström macroglobulinaemia cells and in low grade lymphoma cell lines [32,33].

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 tumours [34]. Four different classes of PI3K pathway inhibitors are interesting: dual PI3K-mTOR inhibitors, PI3K inhibitors (that do not inhibit mTOR), Akt inhibitors, and mTOR catalytic site inhibitors (Table I).

Target	Drug	Pharmaceutical company	Clinical Trial, phase	Reference
PI3K/mTOR	SF-1126	Semafore Pharm.	I	35
	NVP-BEZ235	Novartis	I/II	ClinicalTrial.gov
	NVP-BGT226	Novartis	I/II	ClinicalTrial.gov
	XL765	Exelixis	I	36
PI3K	PX-866	Oncothyreon	I	37
	XL147	Exelixis	I	38
	NVP-BKM120	Novartis	I/II	39
	GDC-0941	Genentech/Piramed	I	40
	Idelalisib	Calistoga Pharm.	II	41
Akt	MK-2206	Merck	I	42
	Enzastaurin	Lilly	I/II/III	ClinicalTrial.gov
	Perifosine	Æterna Zentaris	I/II	ClinicalTrial.gov
mTOR	Rapamycin and analogues	Pfizer	I/II/III	ClinicalTrial.gov

Table I. *Selection of PI3K pathway inhibitors*

For several years my research group studies the effects of some innovative drugs inhibitors of signalling pathways on lymphoma cell lines. In particular, we analysed the inhibitory effects of NVP-BEZ235 on mantle cell lines and its effects in combination with enzastaurin, everolimus, and perifosine.

Enzastaurin, an oral serine/treonine kinase inhibitor which suppressed signalling through the PI3K/Akt pathway, in relapsed and refractory MCL resulted in modest clinical activity [43]. Perifosine targets the pleckstrin homology domain of Akt, thereby preventing its translocation to the plasma membrane. It thus inhibits Akt without affecting the activity of PI3K [44].

IN VITRO STUDY

We hypothesised 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. We demonstrated 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 combinations in targeting some signalling pathways. Results using MTT assay were expressed as fraction of cells killed by the individual drug or the combination in the drug-treated versus untreated cells.

GRANTA-519			JeKo-1		
NVP-BEZ235 (nM)	E (μM)	CI	NVP-BEZ235 (nM)	E (μM)	CI
5	2.5	0.402	5	2.5	0.53
30	10	0.292	30	10	0.278
55	25	0.101	55	25	0.084
NVP-BEZ235 (nM)	EV (nM)	CI	NVP-BEZ235 (nM)	EV (nM)	CI
5	1	0.76	5	1	0.389
30	4	0.691	30	4	0.587
55	10	0.262	55	10	0.096
NVP-BEZ235 (nM)	P (μM)	CI	NVP-BEZ235 (nM)	P (μM)	CI
5	2.5	0.548	5	2.5	0.542
30	10	0.269	30	10	0.258
55	25	0.041	55	25	0.056

Table II. *Analysis of drug combination effects. GRANTA-519 and JeKo-1 were treated with NVP-BEZ235 combined with enzastaurin (E), everolimus (EV), and perifosine (P). 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 hours using low concentrations of all compounds*

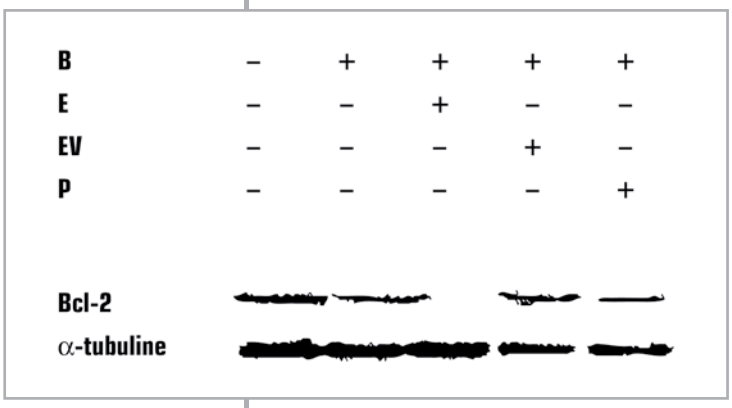

Figure 2. *Western blot of cellular extracts from GRANTA-519 cells, treated with NVP-BEZ235 (B; 5 nM) alone and in combination with enzastaurin (E; 2.5 μM), everolimus (EV; 1 nM) and perifosine (P; 2.5 μM) for 48 hours. NVP-BEZ235 combined with enzastaurin reduces expression of Bcl-2 protein*

The interaction between drugs was analysed by isobologram analysis using the STACorp8.2 software program based upon the Chou-Talalay method to determine if the combination were additive or synergistic [45]. We found that enzastaurin, everolimus, and perifosine enhanced the cytotoxicity triggered by BEZ235; a clear synergistic interaction (CI<1) appeared after 48 hours using low concentrations of the all compounds (Table II).

We examined the functional effects of BEZ235 alone and in combination on apoptosis in lymphoma cells. We demonstrated that BEZ235 (20 nM) alone after 24 hours induces an increase of 8-10% of apoptotic cells versus untreated, instead BEZ235 (20 nM) in combination with enzastaurin (5 mM) after 24 hours induces a 25% increase. We next defined mechanisms whereby BEZ235 alone and in combination induces apoptosis in lymphoid cells. In particular, BEZ235 combined with enzastaurin induces both intrinsic and extrinsic apoptosis pathways with caspase 3, caspase 9, caspase 8 cleavage. We also showed that the combination of BEZ235 and enzastaurin decreases viability and induces apoptosis in lymphoma cell lines and peripheral blood mononuclear cells (PBMCs) from lymphoma patients. The combination has no effect on normal PBMCs and suppresses cell proliferation of lymphoma cell lines when co-cultured with bone marrow stromal cells in a system that mimics the bone marrow microenvironment. BEZ235, enzastaurin, everolimus, and perifosine are inhibitors of intracellular pathways, though we investigated effects of BEZ235 alone and in combinations with the other compounds in targeting p-AKT, p-mTOR, p-GSK3beta, p-p70, p-p90, p-MAPK, p-4EBP1 and cyclin D1 pathways by Western Blot.

In addition, we demonstrated that BEZ235 plus enzastaurin resulted in increased expression of pro-apoptotic Bim, and in decreased expression of anti-apoptotic Bcl-2, which could not be abrogated by BEZ235 alone (Figure 2).

In conclusion, our data suggest that in B cell lymphoma cell lines, BEZ235 in combination with enzastaurin elicits its antitumour effect better that combined with perifosine and everolimus. Our data reveal that the drug combination targets phosphorylation of PI3K/Akt/mTOR pathways and induces both intrinsic and extrinsic apoptosis pathways. Furthermore, inhibition of Bcl-2 anti-apoptosis family members may, in part, explain the efficacy of signalling blockade in lymphoma cells and suggests an additional therapeutic targeting strategy.

DISCUSSION

Improvement of our ability to control malignant lymphoma depends not only on the identification of crucial signalling pathways activated in tumour cells, but also on the definition of how the different kinases work and interact with each other to convey signals promoting cell growth and survival. Abnormal activation of the PI3K/Akt/mTOR pathway has been validated as

In vitro tests: an overview

Isobologram analysis
Analysis on a plot containing isoboles, i.e. lines joining points of equal activity of drugs. It is generally used to predict the effect of a combination of drugs, thus establishing if it is going to be additive, synergistic, or antagonistic.

Western Blot
Test aimed at the identification of a specific protein: firstly, it is separated through electrophoresis, then transferred on a membrane, and finally identified by the use of antibodies directed against its epitopes.

an important step towards the initiation and maintenance of human tumours by preclinical studies [46-48]. The newly developed series of ATP-competitive PI3K/mTOR inhibitors fit these criteria and in particular NVP-BEZ235 has recently entered clinical trials. NVP-BEZ235 induced significant p-Akt inhibition resulting from the dual targeting of mTORC1 and mTORC2 [49]. Because NVP-BEZ235 inhibits the PI3K/Akt pathway at multiple levels, it may overcome the compensatory drug resistance mechanism that have developed with other selective inhibitors against individual targets of this 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 22 phase I/II clinical trials in advanced solid tumour patients as a single agent as well as in combination with other agents [50]. 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 [51]. Thus, it will be important to focus on robust translational research programs the best to identify key combination partners for PI3K inhibitors.

In the first part of our study we analysed the effect of NVP-BEZ235 alone on mantle cell lymphoma. 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 exerted its antitumour activity even when mantle cells were in contact with bone marrow microenvironment.

Inhibition of oncogenic signalling with targeted small molecule inhibitors is powerful therapeutic approach to treat molecularly-driven tumours. Such inhibitors can be efficacious as single agents, but improved anti-tumour activity can often be achieved by combining with other cancer therapeutics. In the second part of our study, we analysed the inhibitory effects of NVP-BEZ235 on mantle cell lines, and then we evaluated its effects after combination with enzastaurin, everolimus, and perifosine. Enzastaurin (LY317615) is a drug used to inhibit PKCβ in clinical and preclinical studies. Enzastaurin was found to be fairly specific for PKCβ by competing with ATP at the enzyme's nucleotide triphosphate binding site, thereby blocking its activation [52]. Preclinical studies have shown that enzastaurin induces apoptosis and suppresses proliferation in many cancer cell lines in the micromolar range, comparable to the concentration range that can be achieved in the plasma of clinical trial subjects [53]. Everolimus, a derivate of rapamycin, functions along with its intracellular receptor FKBP12 as highly selective allosteric inhibitor of mTORC1. Everolimus inhibits proliferation in a wide variety of tumour cell lines both *in vitro* and *in vivo* and has received FDA-approval for the treatment of a subset of cancer types [54,55]. Perifosine is a synthetic alkylphospholipid that binds plasma membranes and inhibits Akt activation without any direct effect on related kinases such as PI3K or PDK1. Hideshima et al. have recently reported that perifosine is able to completely inhibit the constitutive phosphorylation of Akt in multiple myeloma (MM) cells *in vitro* [56]. At concentrations in which peripheral blood mononuclear cells from normal volunteers are unaffected, perifosine kills plasma cells from myeloma patients. Further studies have demonstrated that perifosine induces typical apoptotic biochemical changes in myeloma cell lines *in vitro*. Perifosine is also able to block the proliferative response typically observed in myeloma cells after adherence to stroma *in vitro* and reduce tumour growth [57].

On the basis of this data, we hypothesised 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 these drugs. We would underline that the combination of NVP-BEZ235 plus enzastaurin decreases Bcl-2 expression, while these compounds utilised as single agents did not have any effect. The above results encourage clinical development of NVP-BEZ235 in combination and the possible inclusion of patients with mantle lymphoma in phase I/II studies. It will be interesting to see if this synergy can be translated into clinical practice and if the interaction of allosteric and ATP-competitive inhibitors is a phenomenon which can be applied more broadly to other targeted therapies.

We cannot say that concentrations used in the experiments are clinically relevant or achievable, because a cell culture does not reproduce the complex metabolic system of a living organism. We hope to be able to continue our study in mice, so to assess the doses of the drugs are effective against lymphomas.

Based on the identification of several new therapeutic agents affecting different regulatory pathways in lymphomas, we think that the new challenge is to identify rational pharmacological combinations to enhance the potency of single agents and improve patients outcome.

With the plethora of signal pathways and the host of pharmaceutical agents becoming available in the near future, it is important to demonstrate the preclinical rationale to conduct the phase I studies. In addition, the observation of single-agent anti-tumour activity in humans with relapsed disease and an understanding of the toxicity profile are critical to designing studies in combination with standard agents or where the agent is used as consolidation or maintenance. It is truly an exciting time for both investigators and patients.

The ability to offer hope to a relapsed B-cell lymphoma patient makes the current practice of haematology/oncology stimulating and rewarding.

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