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ORIGINAL ARTICLE

L744,832 and Everolimus Induce Cytotoxic and Cytostatic Effects in Non-Hodgkin Lymphoma Cells

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Abstract Non-Hodgkin Lymphoma (NHL) constitutes a very heterogeneous group of diseases with different aggressiveness. Diffuse large B-cell lymphoma (DLBCL) and Burkitt's lymphoma (BL) are two clinically aggressive lymphomas from the germinal center, very heterogeneous and with different genetic signatures. Several intracellular pathways are involved in lymphomagenesis, being BCR/PI3K/AKT/mTOR and RAS/RAF pathways the most frequently ones. In this context the therapeutic potential of a mTOR inhibitor – everolimus – and a RAS/RAF pathway inhibitor – L744,832 – was evaluated in two NHL cell lines. Farage and Raji cells were cultured in the absence and presence of several concentrations of everolimus and L744,832 in monotherapy and in combination with each other, as well as in association with the conventional chemotherapy drug vincristine. Our results show that everolimus and L744,832 induce antiproliferative and cytotoxic effect in a time-, dose-, and cell line-dependent manner, inducing cell death mainly by apoptosis. A potentiation effect was observed when the drugs were used in combination. In conclusion, the results suggest that everolimus and L744, 832, alone or in combination, could provide therapeutic benefits in these subtypes of NHL.

Keywords Diffuse Large B-cell Lymphoma · Burkitt's lymphoma · Everolimus · L744,832 · Apoptosis

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Introduction

Lymphomas are lymphoproliferative malignancies arising at multiple stages of normal lymphoid cell development. In 90 % of the cases they are classify as non-Hodgkin lymphoma (NHL), being the other 10 % Hodgkin lymphomas. NHL constitute a very heterogeneous groups of diseases that can affected various cells types, B and T lymphocytes or NK cells, with different stages of maturation (immature and mature) and aggressiveness (indolent as follicular lymphoma and aggressive forms as Diffuse large B cell and Burkitt's lymphoma) [1]. The Diffuse large B cell lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma (NHL), accounting for approximately 25 to 30 % of these hematological neoplasias in Western countries [2] and for 30 to 40 % of newly diagnosed lymphomas [3]. The DLBCL is more frequently a B-cell neoplasia, in particular of germinal center centroblasts. It is a clinically aggressive lymphoma with a high rate of cellular proliferation, being able to progressively infiltrate the lymph nodes, the bone marrow, and other organs [2]. Additionally, this type of lymphoma consists in a heterogeneous entity in terms of morphological, biological, and clinical features [4]. Another lymphoma from the germinal center is Burkitt's lymphoma (BL), a very aggressive form of NHL with high proliferation index that spreads to blood and bone marrow [5]. There are three different variants of BL: endemic, sporadic, and immunodeficient. Endemic BL occurs in some parts of Africa and almost all the cases are EBV positive,

while sporadic forms are less associated with EBV and accounts for 1–2 % of all adult lymphomas in Europe and the United States. One of the molecular hallmarks of this disease is the deregulation of c-MYC [6]. NHL conventional treatment consists of chemotherapy protocols, like CHOP (cyclophosphamide, vincristine, and prednisolone) that could be followed by radiotherapy, depending on disease characteristics, such as localized or advanced disease [3, 6–11]. The introduction of anti-CD20 monoclonal antibodies in the clinical practice improves the outcome of patients with mature B cell lymphomas, especially when combine with CHOP regimens [1]. However, the side effects and acquisition of resistance to the treatment, additionally to the possibility of relapse justify the improvement of new therapeutic approaches.

Exploring particular molecular aspects of these diseases could contribute to the development of some target therapy. Several intracellular pathways are related to lymphomagenesis, such as the RAS/MAPK pathway and PI3K/AKT/mTOR pathway [11–14]. The starting point of RAS/MAPK pathway consists in activation of RAS by addition of a farnesyl group at the carboxylic terminal, process mediated by farnesyltransferase. This particularly pathway is involved in the regulation of proliferation and differentiation [15]. One indirect way of targeting this pathway is by the inhibition of farnesyltransferase, which will block the activation of RAS. L744,832 is one farnesyltransferase inhibitors (FTI) that have been tested as anticancer agents. PI3K/AKT/mTOR signaling pathway is essential in survival, resistance to apoptosis, and cell cycle progression, and is other altered pathway in NHL. This pathway could be inhibited in multiple points and several molecules are on clinical trials or already approved to some cancers. Everolimus is an inhibitor of mTOR (mTORi) approved by FDA to metastatic renal cell carcinoma and for advanced $ER^+/HER2^-$ breast cancer that induce cell death and reduce cell proliferation [16, 17]. In this context, we investigate the anticancer activity of L744,832 and everolimus, in monotherapy as well as in combination with vincristine (conventional drug), in two NHL cell lines.

Materials and Methods

Cell Culture Conditions

We used two NHL cell lines: Farage cells a model of DLBCL and Raji cells as a Burkitt Lymphoma model, both obtain from American Type Culture Collection (ATCC). The cell lines were routinely grown in Roswell Park Memorial Institute 1640 medium (RPMI 1640), containing 2 mM L-glutamine, 25 mM HEPES-Na, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco, Invitrogen), supplemented with heat-inactivated fetal bovine serum (FBS) (Gibco, Invitrogen), and kept in culture at 37 °C in a humidified atmosphere with 5 % CO_2. Farage cells were maintained with 20 % of FBS and seeded at a density of 0.5×10^6 cells/ml in all performed assays, while Raji cells were supplemented with 10 % and seeded at 0.3×10^6 cells/ml. In order to evaluate the antiproliferative and cytotoxic effect of L744,832 and everolimus (EVE), the cells were cultured for 72 h in the absence and presence of L744,832 (Enzo Life Science) in concentrations ranging from 1 to 100 μM, and EVE (Sigma-Aldrich) in concentrations ranging from 5 to 100 μM. Lower doses of these compounds were also tested in double combination with 0.1 nM of vincristine (VCR; Sigma-Aldrich) and in a triple combination scheme.

Metabolic Activity Evaluation

The resazurin assay, methodology based on mitochondria enzymes activity, was used to assess the metabolic activity of the cells in absence and presence of L744,832, EVE, and/or VCR. Resazurin was prepared as a stock solution of 100 μg/ml in phosphate-buffered saline (PBS). After treatment, a final concentration of 10 μg/ml of resazurin solution was added to the cells, which were then incubated at 37 °C for 4 h. Following incubation, the absorbance at 570 nm and 600 nm was measured using a Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments), and metabolic activity was calculated as a percentage of control cells.

Cell Death Analysis

Cell death was examined through morphological analysis using optic microscopy and by flow cytometry (FC) through annexin and propidium iodide double staining. After an incubation period of 48 h, cells untreated and treated with the different drugs were collected and seeded in glass slides. Then, cell smears were stained with May-Grünwald solution (0.3 %*v/v* in methanol; Sigma-Aldrich) for 3 min, diluted in a 1:1 ratio with distilled water, and then staining with Giemsa solution (0.75 % p/v in glycerol/methanol 1:1; Sigma-Aldrich) diluted 1:8 in distilled water for 15 min. After rinsing with distilled water, smears were left to dry at room temperature and cell morphology was analyzed by light microscopy using a Nikon Eclipse 80i microscope equipped with a Nikon Digital Camera DXm 1200 F. For flow cytometry assays, cells were collected and washed with PBS by centrifuged at 400 xg for 5 min. Then, cells were resuspended in 100 μl of binding buffer and incubated with 5 μl of annexin V-FITC (AV) and 2 μl of propidium iodide (PI) staining solution (ImmunoStep) for 15 min at room temperature in the dark. Cells were then analyzed in a FACS Calibur (Becton Dickinson) flow cytometer equipped with an argon laser. CellQuest software (Becton Dickinson) was used for the acquisition of data and results were analyzed with the Paint-a-Gate software. Results were expressed in percentage of viable cells (AV^-/PI^-), early apoptotic (AV^+/PI^-), late apoptotic/necrotic (AV^+/PI^+), and necrotic cells (AV^-/PI^+).

Evaluation of Proteins Expression by Flow Cytometry

The expression levels of activated Caspase 3, Lamin A/C and Ki-67 were assessed by FC using monoclonal antibodies labeled with fluorescent probes. Cells of each condition were incubated with a monoclonal antibody anti-activated caspase 3-phycoerythrin (PE; BD Pharmingen, Becton Dickinson), monoclonal anti-Lamin A/C PE or anti-Ki-67 PE (Santa Cruz Biotechnology) antibodies according to manufacturer's protocol. Briefly, cells were fixed with 100 μl of fix solution (IntraCell, Immunostep) for 15 min and then washed by centrifugation at 300 xg for 5 min. Cells were then permeabilized by incubated for 15 min with 100 μl of permeabilization solution (IntraCell, Immunostep) and 1 μg of the respective antibody. After washing, cells were analyzed by FC. The results are presented as mean intensity fluorescence (MIF) arbitrary units and represent MIF detected in the cells, which is proportional to the protein concentration in each cell.

Cell Cycle Analysis

For cell cycle analysis, the cells incubated with different treatments were harvested in order to obtain a density of $1x10^6$ cells/ml. Cells were then washed by centrifuged for 5 min at 300 xg, and fixed by incubation at 4 °C for 30 min with 200 μl of 70 % ethanol. After a wash step by centrifugation, 500 μl of propidium iodide solution with RNase (PI/RNase, Immunostep) was added to the cell pellet and incubated for 15 min at room temperature. Cell cycle distribution was analyzed using the ModFit LT software (Verity Software House). Results were expressed in percentage of cells in the different cell cycle phases (G_0/G_1, S, and G_2/M) according with the PI intensity. A sub-G_1 population was also identify, when present, corresponding to apoptotic cells.

Statistical Analysis

Data were expressed as mean±SEM of the number of independent experiments indicated in the figure legends. Student's *t* test, ANOVA, and Dunnett's post-test were used to determine the statistical significance, considering a p value of< 0.05.

Results

L744,832 and Everolimus Decrease Metabolic Activity of NHL Cells

The effect of L744,832 and EVE in metabolic activity of Farage and Raji cells were represent in Fig. 1. Our results show that all conditions tested induce a decrease in metabolic activity, in a time-, dose- and cell type dependent manner. As represented in Fig. 1a, the FTI induces a reduction on metabolic activity of Farage cells with an IC_{50} after 24 h between 50 and 75 μM. Revealing the importance of incubation time, the IC_{50} reduces for a value between 25 and 50 μM after 48 h. For some L744,832 concentrations, a slightly reversion of effect was also observed after 72 h of incubation. In Raji cells (Fig. 1d), FTI also decreased the metabolic activity, however these cells required a higher dose in comparison with DLBCL cells, being the IC_{50} after 48 h between 50 and 75 μM. EVE reduces significantly the metabolic activity of Farage and Raji cells also in a dose- and time-dependent manner. The IC_{50} was achieved when the Farage cells were exposed to 75 μM EVE for 24 h and 25 μM for 48 h (Fig. 1b). In case of Burkitt cells, the dose of Eve necessary to achieve the IC_{50} was lower, with values between 15 and 25 μM for 24 h, and less than 15 μM after 48 h of incubation (Fig. 1e).

Drug combination is one common strategy used to minimize the toxicity of therapeutic. Based on results in monotherapy, we tested a double combination of each inhibitor with VCR and a triple combination, represented in Fig. 1c and f. Our results show a significant decrease of metabolic activity when cells are treated with drugs combination, compared with untreated and/or monotherapy treated cells. The double combination between L744,832 and VCR showed an additive effect during the 72 h, decreasing the metabolic activity from 75 % achieved in monotherapy to approximately 55 % at the end of study. The same pattern was observed in the combination of EVE with VCR. A very pronounce effect was noted in the triple combination in Farage cells, at the end of 24 h of incubation the metabolic activity reduced from 75 % in monotherapy to less than 25 % of activity. At the same time of incubation, metabolic activity of Raji cells decreased from 65 to 30 %. The use of three inhibitors at the same time was the most effective combination scheme used in the study, specially pointing out the potentiation synergistic effect between each drug in DLBCL cell line. However, in Burkitt cells the triple combination did not revealed statistical differences comparing with the double combination of Everolimus and Vincristine.

L744,832 and Everolimus Induce Cell Death by Caspases-Dependent Apoptosis

We analyzed cell death induced by L744,832 and everolimus using AnnexinV/PI double staining as well as by morphological analysis. As illustrated in Fig. 2a, both inhibitors in monotherapy and in combination scheme decreased the percentage of viable cells (V) with a significant increase of cells in apoptosis (in Farage cells mainly early apoptosis and in Raji cells late apoptosis/necrosis), being the effects dose dependent. Supporting these results, the morphological analysis revealed

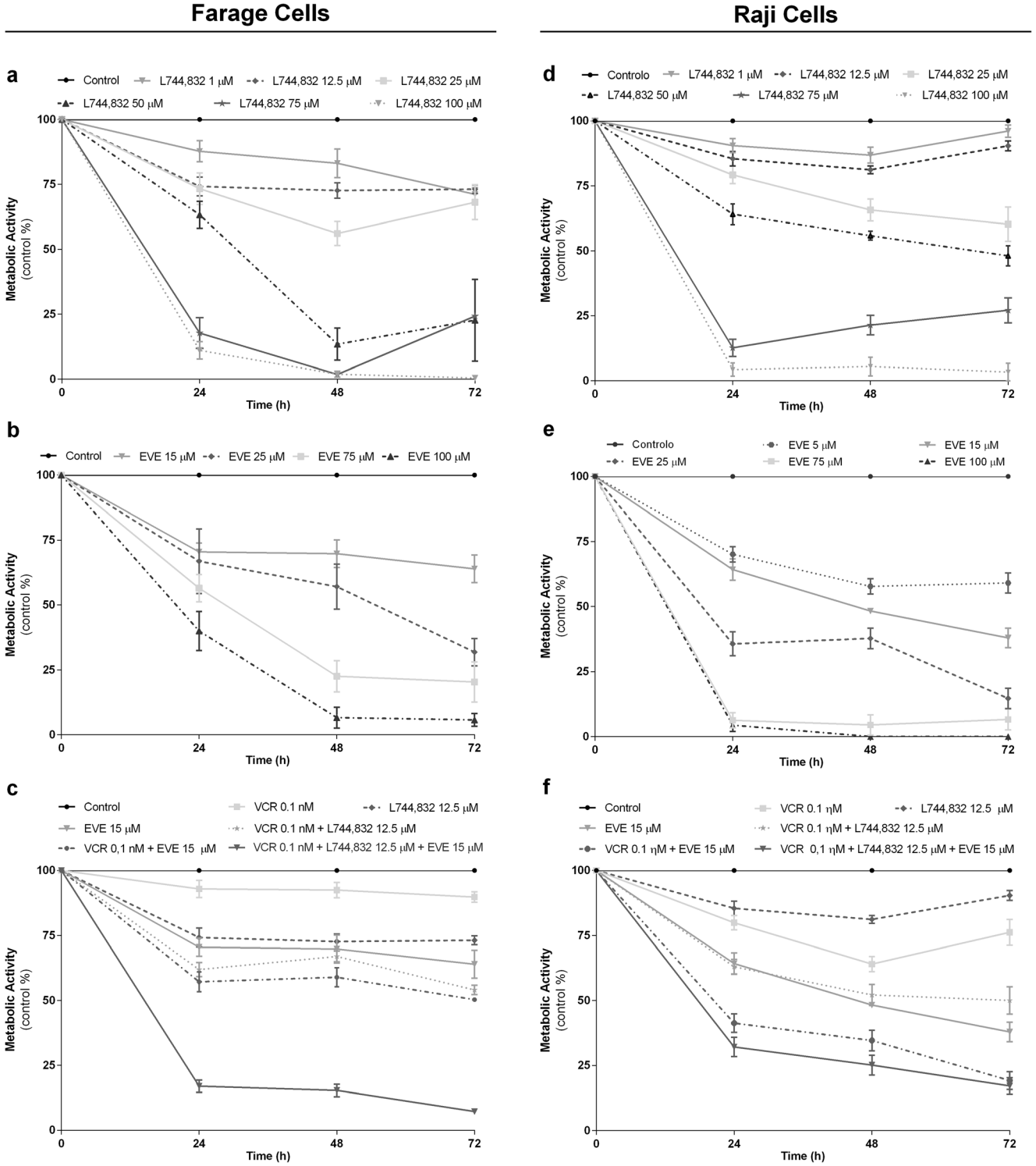

Fig. 1 Dose response curves. Farage and Raji cells were incubated during 72 h, in the absence or in the presence of increasing concentrations of L744,832 (**a**/**d**) and everolimus (**b**/**e**) in monotherapy or in combination scheme with vincristine (**c**/**f**), as indicated in figure. Dose response curves were established by resazurin reduction each 24 h, as described in Methods and Materials. Results are expressed in percentage (%) normalized to control. Data are expressed as mean±SE obtained from at least 5 independent experiments. Everolimus, EVE; vincristine, VCR

typical characteristic of cell death mediated by apoptosis. In Farage cells treated with L744,832 and EVE was observed cellular contraction, nuclear fragmentation, and blebbing (Fig. 2b). The same pattern was noted in Raji cells, with particular contribution of necrotic process in cells exposed to L744,832. We also analyzed the expression levels of activated

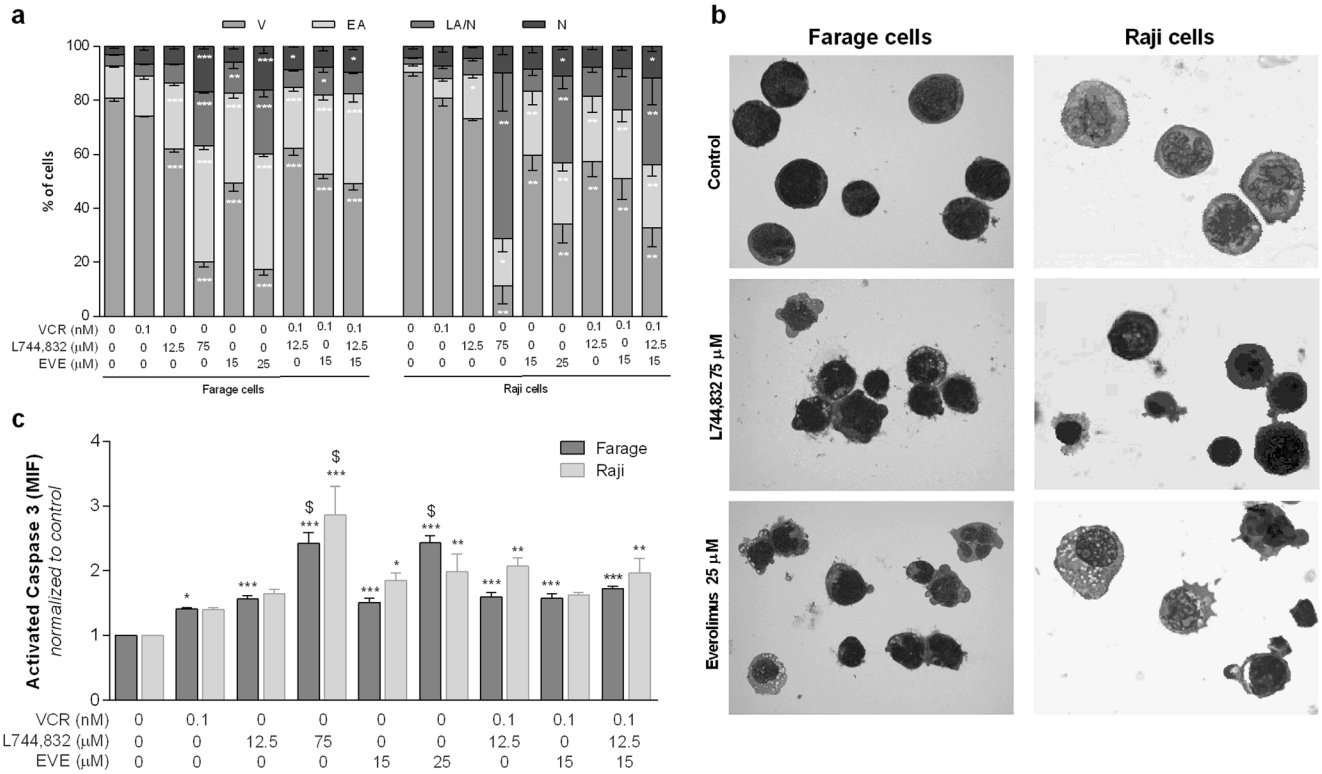

Fig. 2 Analysis of cell death induced by L744,832 and everolimus in NHL cell lines. In **a** cell death was detected by annexin V and propidium iodide staining and analysed by flow cytometry; data are expressed as percentage (%) of viable cells (V), early apoptotic cells (EA), late apoptotic/necrotic cells (LA/N) and necrosis (N). In **b** cell smears were stained with May-Grünwald-Giemsa (amplification: 500×). In **c** the activated caspase 3 expression levels were analysed by flow cytometry. Results were obtained after 48 h of incubation and represent mean±SE of at last 4 independent experiments. Everolimus, EVE; vincristine, VCR; * $p<0.05$; ** $p<0.01$; *** $p<0.001$ (comparison with control); $ $p<0.05$ (comparison with lower dose of correspondent inhibitor)

caspase 3, a frequently activated apoptotic cell death protease, to confirm the previous results. As shown in Fig. 2c, L744,832 and EVE induced significant increase the expression levels of activated Caspase 3, compared with control cells. Caspase 3 activation was dose dependent, being the differences statistical significant when comparing lower and high doses. In combination schemes, the expression levels of activated caspases 3 were similar to those induce by the equivalent doses in monotherapy.

L744,832 and Everolimus Promotes Cell Cycle Arrest in G_0/G_1 Phase

The compounds in study could not only induce cell death but also affect the cell cycle progression, and to confirm that we analysis the distribution of cells through cell cycle phases. In Farage cells, EVE induced a significant cell cycle arrest in G_0/G_1 phase, when compared with untreated cells, and this effect was dependent on the dose (Table 1). In the same way, L744,832 also induced G_0/G_1 phase arrest. In all tested combination schemes were detected an arrest in G_0/G_1 phase, not only with statistical significance when comparing to control cells but also when compared with equivalent doses in monotherapy (Table 1). Particularly in the case of L744,832, the association with VCR potentiated the ability to affect the cell cycle progression achieving the differences statistical meaning. In Burkitt cells, the higher dose of L744,832 induced significant G_0/G_1 phase arrest (Table 1). Everolimus exhibits the same ability to interfere with cell cycle in Raji cells, however the lower dose presents a higher effect. In the association of Everolimus with VCR or in triple combination a significant increase in the percentage of cells in G_0/G_1 was detected (Table 1).

Additionally, by the same technique the presence of a sub-G_1 peak was detected, corresponding to DNA fragmentation typical of apoptotic cells. The percentage of cells in sub-G_1 peak was dose- and therapeutic scheme-dependent, being higher in monotherapy conditions for both cell lines. In Farage cells, the percentage of sub-G_1 population significantly increased in cells treated with everolimus (15 μM and 25 μM), and in cells treated with the higher concentration of L-744,832 (75 μM). Similarly, in Raji cells the same pattern was observed, however the differences were only significant in the higher dose of both compounds. Furthermore, the major differences in the sub-G_1 peak were observed in the triple therapeutic scheme (Table 1).

Table 1 Effects of L-744,832 and Everolimus in cell cycle of Farage and Raji cells

		Sub-G_1 (%)	G_0/G_1 (%)	S (%)	G_2/M (%)
Farage Cells	Control	4.5±0.5	46.0±4.0	36.0±3.0	18.0±1.0
	Monotherapy				
	VCR 0.1 nM	5.0±0.0	39.5±3.5	55.5±2.5 *	5.0±1.0 *
	L-744,832 12.5 μM	12.0±1.0	57.0±2.0	32.5±2.5	10.5±4.5
	L-744,832 75 μM	22.5±3.5 *	59.0±1.0	34.5±3.5	6.5±2.5 *
	Eve 15 μM	20.0±1.0 *	68.5±6.5 *	24.0±6.0	7.5±0.5 *
	Eve 25 μM	32.5±1.5 *	91.0±2.0 *	4.0±2.0 *	5.0±0.0 *
	Combination				
	VCR 0.1 nM+L-744,832 12.5 μM	11.0±0.0	73.5±1.5 *$	22.5±1.5	4.0±0.0 *
	VCR 0.1 nM+Eve 15 μM	12.5±0.5	93.5±0.5 *#	1.5±0.5 *#	5.0±0.0 *
	VCR 0.1 nM+L-744,832 12.5 μM+Eve 15 μM	18.5±3.5 *	89.5±0.5 *$#	6.0±1.0 *$#	4.5±0.5 *
Raji Cells	Control	0.8±0.6	39.2±4.0	41.6±1.6	19.1±2.0
	Monotherapy				
	VCR 0.1 nM	0.5±0.3	39.0±1.3	42.9±2.9	18.1±3.3
	L-744,832 12.5 μM	1.3±0.5	41.5±1.9	38.8±1.3	19.7±1.3
	L-744,832 75 μM	45.0±2.0 *	84.0±3.5 *	4.3±0.7 *	11.7±2.8
	Eve 15 μM	0.5±0.5	87.0±1.1 *	7.7±1.0 *	5.3±0.5 *
	Eve 25 μM	7.0±1.5 *	75.3±5.3 *	13.5±2.0 *	11.2±2.3
	Combination				
	VCR 0.1 nM+L-744,832 12.5 μM	1.8±0.6	50.3±5.8	29.7±2.3 *$	20.0±2.0
	VCR 0.1 nM+Eve 15 μM	1.8±1.1	64.4±4.1 *#	8.1±4.0 *	27.3±1.3 #
	VCR 0.1 nM+L-744,832 12.5 μM+Eve 15 μM	7.3±3.6 *$#	64.0±1.8 *$#	19.3±0.9 *$#	16.7±1.7 #

* $p<0.05$ comparing with Control, $ $p<0.05$ comparing with L-744,832 12.5 μM, # $p<0.05$ comparing with Eve 15 μM

L744,832 Modulate Farnesylation and Everolimus Decreases Ki-67 Expression in DLBCL and Burkitt Cells

We used Lamin A/C and Ki-67 expression levels to confirm that L744,832 and everolimus were acting in the proper targets, as describe in literature. Pre-Lamin A/C needs a farnesyl group to be transformed in Lamin A/C, and this action is mediated by farnesyltransferase. L744,832 treated cells showed a significant decrease of Lamin A/C levels (Fig. 3a), and this effect was dose dependent. In Farage cells, the association of lower dose of L744,832 with VCR induced an equivalent decrease in Lamina A/C levels, as observed with higher doses of L744,832 in monotherapy. Triple combination induced a lower farnesyltransferase inhibition comparing with double combination (higher Lamin A/C levels than double combination), however farnesyltransferase inhibition in triple combination was significantly different from control cells. This suggests that EVE reverse the potentiation effect of farnesyltransferase inhibitor induced by L744,832, since the levels were similar to those detected with 12.5 μM of L744, 832 alone. In Raji cells, the combination schemes also show a farnesyltransferase inhibition but without potentiation of the effect. The results in these schemes were similar to those obtain in monotherapy. Ki-67 is an essential protein to cell progress into different phases of cell cycle, being useful to confirm antiproliferative effects of some compounds. EVE induced a significant decrease in expression levels of Ki-67, being the effect dose dependent in both cell lines (Fig. 3b). A reduction of Ki-67 expression was also detected in combination schemes. The triple association showed higher levels of Ki-67 than double combination, suggesting that L744,832 impairs the antiproliferative effects of Everolimus.

Discussion

Several studies explored the role of multiple signaling pathways in lymphomagenesis, trying to explain the heterogeneity between different subtypes. Particularly in ABC subtype of DLBCL, Lenz et al. showed that chronic activation of B-cell receptor signaling could be interrupted by inhibition of downstream kinases, such as SRC, PKC, and PI3K/mTOR [4]. The same pathway plays a very important role in Burkitt lymphomogenesis. In this particular disease were observed a tonic BCR signaling via PI3K pathway for contra balance the proapoptotic signals associated with c-MYC deregulation [18, 19]. These evidence stimulated the search of new molecules

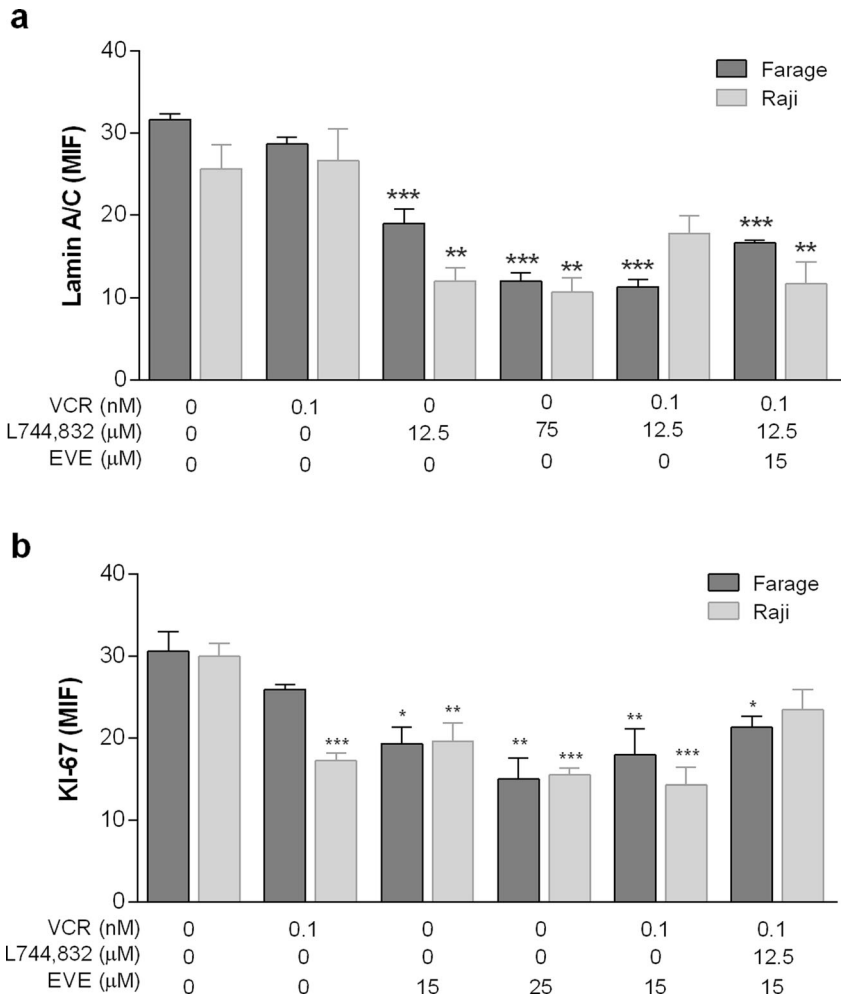

Fig. 3 Evaluation of Lamin A/C and Ki-67 protein expression levels in Farage and Raji cells by flow cytometry. Lamin A/C (**a**) and Ki-67 (**b**) expression levels were analysed by flow cytometry. Both cell lines were incubated during 48 h, in the absence or in the presence of L744,832, everolimus (EVE) and/or vincristine (VCR) in the concentrations indicated in the figure. Results were expressed as mean intensity fluorescence (MIF) normalized to control and represented as mean±SE of at least 4 independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$

that could interfere with deregulated pathways in NHL and other lymphoproliferative disease.

In our study, we evaluated the therapeutic potential of L744,832 (FTI an indirect inhibitor of RAS/MAPK) and everolimus (a mTOR inhibitor), in monotherapy and in combination with conventional drug vincristine. We demonstrated that L744,832 has a cytotoxic effect in a DLBCL and Burkitt cell lines in monotherapy and in combination scheme, in a time and dose-dependent manner. However, Farage cells seems to be more sensitive to FT inhibition then Raji cells, since requires smaller doses of FTIs to achieve the IC_{50}. Similar results were observed in CEM cells, an acute lymphoblastic leukemia cell line, when treated with a FTI [20]. The cell death induced by this drug was mediated by apoptosis, confirmed by expression of caspases 3, changes in morphologic aspects, and by phospholipids distribution at cell membrane. The identification of a sub-G_1 peak in the cell cycle analysis confirmed the DNA fragmentation after FTI treatment, a typical process in cells dyeing by apoptosis. In fact, the different techniques used to, direct or indirect, assess the cell death were in agreement and reveal statistical significant. The activation of this mechanism of death by FTI was in concordance with described by others authors that tested similar molecules [20]. All the results emphasize the relevance of this pathway in lymphoproliferative disorders. Additionally, our study showed antiproliferative effect of L744,832 sustained by the increase of cells in G_0/G_1 phase. Song and collaborators [21] showed that the L744,832 leads to an increase in the number of tetraploid cells in pancreatic adenocarcinoma cells, by stopping progression of the cell cycle through G_2/M. However, other studies using hematological cell lines show a decrease in the expression of cyclin D1 [20], a protein involved in cell cycle progression from G_1 to S phases. These observations suggest that the effect of this FTI on cell cycle is dependent on the cell type. To confirm the molecular action of L744,832, we used Lamin A/C levels as a marker of FTI efficacy, as describe in literature [22]. Lamins are nuclear membrane structural molecules, important for certain cellular functions, such as cell cycle control, DNA replication, and chromatin organization [23]. These molecules constitute a marker for enzyme farnesyltransferase activity, since the activation of Lamins from pre-Lamin is dependent

of FT [24]. In accordance with literature, we observed a lower expression of Lamin A/C in cells exposed to FTI, even in cases of combination with other drugs [25, 26].

In phase I and II trials, the mTOR inhibitors demonstrated clinical activity against a variety of solid tumors [27, 28]. Everolimus is one mTOR inhibitors already approved for some specific cases of solid tumors; however not used in practice for hematological cases. Preclinical studies in human multiple myeloma cells using a xenograft model confirmed the activity of an mTOR inhibitor in myeloma cells through inhibition of proliferation, angiogenesis, and induction of apoptosis [29]. In a B-cell lymphoma mouse model, Wendel and colleagues [30] demonstrated that activated AKT promoted lymphomagenesis by disabling apoptosis. Taken in account the importance of PI3K/AKT/mTOR pathway in hematological cancers, we confirmed the ability of everolimus to induce not only a markedly cell cycle arrest but also cell death in DLBCL and Burkitt cells. This compound has been mainly described by the ability to block the cell cycle progression [31]. In both cell lines tested, everolimus induced a significant arrest in G_0/G_1 phase in monotherapy and in combination scheme. To support this finding, we evaluated the expression levels of a protein essential for cell cycle progression, the Ki-67 [32]. Ki-67 is expressed in all proliferating cells that are in the active phases of the cell cycle but absent in resting cells. In accordance with cell cycle analysis, Ki-67 expression levels were lower with increasing doses of everolimus. Not so describe in literature, we observed that this mTORi was also able to induced cytotoxic effects. Activation of apoptotic process by everolimus was demonstrated by the positivity for annexin-V, the presence of blebbing (morphologic analysis) and DNA fragmentation (sub-G_1 population), as well as by the increased expression of activated caspases 3. The changes induced by everolimus treatment were statistically significant in the different methods used. Some authors justify the induction of apoptosis by mTORi with down-regulation of anti-apoptotic proteins (MCL-1, BCL-2, BCL-XL, cIAP, STAT3) [33] and with the negative regulation of the NF-κB pathway [34]. Everolimus inhibit FKBP51 immunophillin, a cofactor of the IKK complex that positively regulates NF-κB activity, promoting apoptosis [27]. Shortly, everolimus acts by down-regulation of cell cycle proteins regulators, altering the expression levels of anti-apoptotic proteins, and also have anti-angiogenic effects [33–35]. Particular in our study, Burkitt cells revealed to be more sensitive than DLBCL cells to everolimus, reinforcing the importance of tonic BCR activation in this disease. Blocking of PI3K/AKT/mTOR pathway promotes the increase of apoptotic signals, may be correlated with c-MYC alterations, justifying the higher sensibility of Burkitt cells to mTOR inhibitors [18, 19, 36].

Knowing the cross-talk between the different signaling pathways, many authors defend the combination of multiple drugs, by that targeting multiple points in a single shoot [21, 31]. With double and triple combination, we achieve a significant cytotoxic and cytostatic effect with lower doses of each compound. These results supports the possibility of increase effects and at same time minimizes the secondary toxicity associated with combination of multiple drugs, offering more therapeutic benefits with potentially less side-effects.

In summary, although be an in vitro study, our results suggest that L744,832 and everolimus induce cell cycle arrest in G_0/G_1 phase and apoptosis in NHL cells, in monotherapy and in therapeutic combination.

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Compliance of Ethical Standards

Conflict of Interest The authors declare no conflict of interests.

References

1. Shankland KR, Armitage JO, Hancock BW (2012) Non-Hodgkin lymphoma. Lancet 380(9844):848–57
2. Stein H, Warnke RA, Chan WC, Jaffe ES, Chan JKC, Gatter KC, Campo E (2008) Diffuse large B-cell lymphoma, not otherwise specified. In: Swerdlow S, Campo E, Harris N, Jaffe E, Pileri S, Stein H et al (eds) WHO classification of tumours of haematopoietic and lymphoid tissues. IARC, Lyon, pp 233–237
3. Schneider C, Pasqualucci L, Dalla-Favera R (2011) Molecular pathogenesis of diffuse large B-cell lymphoma. Semin Diagn Pathol 28(2):167–177
4. Lenz G, Staudt LM (2010) Aggressive lymphomas. N Engl J Med 362(15):1417–1429
5. Kenkre VP, Stock W (2009) Burkitt lymphoma/leukemia: improving prognosis. Clin Lymphoma Myeloma 9(Suppl 3):S231–8
6. Bornkamm GW (2009) Epstein-Barr virus and its role in the pathogenesis of Burkitt's lymphoma: an unresolved issue. Semin Cancer Biol 19(6):351–65
7. Vose JM, Chiu BC, Cheson BD, Dancey J, Wright J (2002) Update on epidemiology and therapeutics for non-Hodgkin's lymphoma. Hematol Am Soc Hematol Educ Prog 2002:241–262
8. Hennessy BT, Hanrahan EO, Daly PA (2004) Non-Hodgkin lymphoma: an update. Lancet Oncol 5(6):341–353
9. Castagna L, Magagnoli M, Demarco M, Santoro A (2007) Lymphomas. Updat Cancer Ther 2(2):101–110
10. Michallet AS, Coiffier B (2009) Recent developments in the treatment of aggressive non-Hodgkin lymphoma. Blood Rev 23(1):11–23
11. Murawski N, Pfreundschuh M (2010) New drugs for aggressive B-cell and T-cell lymphomas. Lancet Oncol 11(11):1074–85

12. Parcells BW, Ikeda AK, Simms-Waldrip T, Moore TB, Sakamoto KM (2006) FMS-like tyrosine kinase 3 in normal hematopoiesis and acute myeloid leukemia. Stem Cells 24(5):1174–1184
13. Small D (2006) FLT3 mutations: biology and treatment. Hematol Am Soc Hematol Educ Prog 2006:178–184
14. Morschhauser F, Bezombes C, Jardin F (2010) Targeting molecular pathways. Education Program for the 15th Congress of European Hematology Association 4(1):118–123
15. Hachem A, Gartenhaus RB (2005) Oncogenes as molecular targets in lymphoma. Blood 106(6):1911–1923
16. Easton JB, Houghton PJ (2006) MTOR and cancer therapy. Oncogene 25(48):6436–6446
17. Polivka J Jr, Janku F (2014) Molecular targets for cancer therapy in the PI3K/AKT/mTOR pathway. Pharmacol Ther 142(2):164–175
18. Niemann CU, Wiestner A (2013) B-cell receptor signaling as a driver of lymphoma development and evolution. Semin Cancer Biol 23P:410–421
19. Bojarczuk K, Bobrowicz M, Dwojak M, Miazek N, Zapala P, Bunes A, Siernicka M, Rozanska M, Winiarska M (2015) B-cell receptor signaling in the pathogenesis of lymphoid malignancies. Blood Cells Mol Dis 55(3):255–65. doi:10.1016/j.bcmd.2015.06.016
20. Costa CB, Casalta-Lopes J, Andrade C, Moreira D, Oliveira A, Gonçalves AC, Alves V, Silva T, Dourado M, Nascimento-Costa JM, Sarmento-Ribeiro AB (2012) Farnesyltransferase inhibitors: molecular evidence of therapeutic efficacy in acute lymphoblastic leukemia through cyclin D1 inhibition. Anticancer Res 32(3):831–838
21. Song SY, Meszoely IM, Coffey RJ, Pietenpol JA, Leach SD (2000) K-Ras-independent effects of the farnesyl transferase inhibitor L744,832 on cyclin B1/Cdc2 kinase activity, G2/M cell cycle progression and apoptosis in human pancreatic ductal adenocarcinoma cells. Neoplasia 2(3):261–272
22. Adjei AA, Davis JN, Erlichman C, Svingen PA, Kaufmann SH (2000) Comparison of potential markers of farnesyltransferase inhibition. Clin Cancer Res 6(6):2318–2325
23. Beck LA, Hosick TJ, Sinensky M (1990) Isoprenylation is required for the processing of the lamin a precursor. J Cell Biol 110(5):1489–1499
24. Kilic F, Salas-Marco J, Garland J, Sinensky M (1997) Regulation of prelamin A endoprotease activity by prelamin A. FEBS Lett 414(1):65–68
25. Brunner TB, Hahn SM, Gupta AK, Muschel RJ, McKenna WG, Bernhard EJ (2003) Farnesyltransferase inhibitors: an overview of the results of preclinical and clinical investigations. Cancer Res 63(18):5656–5668
26. Appels NM, Beijnen JH, Schellens JH (2005) Development of farnesyl transferase inhibitors: a review. Oncologist 10(8):565–578
27. Chan S (2004) Targeting the mammalian target of rapamycin (mTOR): a new approach to treating cancer. Br J Cancer 91(8):1420–1424
28. Wolpin BM, Hezel AF, Abrams T, Blaszkowsky LS, Meyerhardt JA, Chan JA, Enzinger PC, Allen B, Clark JW, Ryan DP, Fuchs CS (2009) Oral mTOR inhibitor everolimus in patients with gemcitabine-refractory metastatic pancreatic cancer. J Clin Oncol 27(2):193–198
29. Frost P, Moatamed F, Hoang B, Shi Y, Gera J, Yan H, Frost F, Gibbons J, Lichtenstein A (2004) In vivo antitumor effects of the mTOR inhibitor CCI-779 against human multiple myeloma cells in a xenograft model. Blood 104(13):4181–4187
30. Wendel HG, De Stanchina E, Fridman JS, Malina A, Ray S, Kogan S, Cordon-Cardo C, Pelletier J, Lowe SW (2004) Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. Nature 428(6980):332–337
31. Houghton PJ (2010) Everolimus. Clin Cancer Res 16(5):1368–1372
32. Scholzen T, Gerdes J (2000) The Ki-67 protein: from the known and the unknown. J Cell Physiol 182(3):311–322
33. Yang Q, Guan KL (2007) Expanding mTOR signaling. Cell Res 17(8):666–681
34. Kelly KR, Rowe JH, Padmanabhan S, Nawrocki ST, Carew JS (2011) Mammalian target of rapamycin as a target in hematological malignancies. Target Oncol 6(1):53–61
35. Recher C, Dos Santos C, Demur C, Payrastre B (2005) MTOR, a new therapeutic target in acute myeloid leukemia. Cell Cycle 4(11):1540–1549
36. Sander S, Calado DP, Srinivasan L, Kochert K, Zhang B, Rosolowski M, Rodig SJ, Holzmann K, Stilgenbauer S, Siebert R, Bullinger L, Rajewsky K (2012) Synergy between PI3K signaling and MYC in Burkitt lymphomagenesis. Cancer Cell 22:167–179