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PI3K/Akt inhibition modulates multidrug resistance and activates NF-κB in murine lymphoma cell lines

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Abstract

Upregulation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway has been described in some tumors related to multidrug resistance (MDR). The aim of this work was to analyze the relationship between PI3K/Akt, MDR and NF-κB in murine lymphoma cell lines resistant to vincristine (LBR-V160) and doxorubicin (LBR-D160) as well as in the sensitive line (LBR-). PI3K/Akt activity, analyzed by phosphatidylinositol trisphosphate production and phosphorylated Akt (p-Akt) expression, was higher in the resistant cell lines than in the sensitive one and inhibition with wortmannin or LY294002 improved apoptosis in the resistant cell lines. Vincristine but not doxorubicin increased p-Akt expression whereas co-treatment with PI3K inhibitors and vincristine increased apoptosis in the three cell lines. Wortmannin and LY294002 inhibited P-glycoprotein (Pgp) function and also increased NF-κB activity. We concluded that the PI3K/Akt pathway is involved in MDR in lymphoma cell lines and PI3K/Akt inhibition correlates down-regulation of NF-κB activity and inhibition Pgp function.

Keywords: PI3K/Akt; Apoptosis; P-glycoprotein; Vincristine; Doxorubicin; Multidrug resistance; Lymphoma; NF-κB

1. Introduction

Cancer cell resistance to different chemotherapeutic drugs, called multidrug resistance (MDR), is a major clinical obstacle in the treatment of hematological malignancies. Classic MDR is the consequence of overexpression of transporter proteins belonging to the ATP binding cassette (ABC) family such as P-glycoprotein (Pgp) and multidrug resistance related protein (MRP). Their function is to extrude antitumor agents from the cytoplasm, thus reducing intracellular drug concentrations to sublethal levels [1]. Other mechanisms involved in MDR include alterations in the apoptotic response, activation of DNA repair or stimulation of detoxifying systems [2,3]. Chemotherapeutic drugs induce a series of cellular responses that impact on tumor cell proliferation and survival. In fact, several lines of evidence have suggested a direct correlation between alteration in survival pathways and chemoresistance and some components of these pathways have been pointed as critical targets for cancer intervention [4].

The phosphatidylinositol 3-kinase (PI3K)/Akt signal transduction pathway plays a central role in intracellular processes such as cell survival, proliferation, angiogenesis and motility. Phosphatidylinositol 3-kinase is a heterodimeric lipid kinase that consists of a catalytic subunit of 110–120 kDa (p110α, p110β and p110γ) and a regulatory subunit of 55–85 kDa (p85α, p85β, p50α, p55α and p55γ). Activation of PI3K occurs by extracellular survival signals through cell surface receptors [5]. Moreover, direct binding of the catalytic subunit to Ras has also

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been shown to activate PI3K [6]. Once activated, PI3K converts phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3). This second messenger recruits the serine-threonine kinase Akt and phosphatidylinositol-dependent kinase 1 (PDK1) to the plasma membrane where Akt is phosphorylated and consequently activated by PDK. In turn, Akt phosphorylates multiple substrates including kinases, transcription factors and other regulatory molecules that collectively contribute to promote cell proliferation and malignancy by inhibiting cell death. PI3K/Akt pathway is negatively regulated by the tumor suppressor PTEN, a phosphatase that converts the second messenger PIP3 back to PIP2, resulting in inactivation of Akt. Although there are several PI3K downstream kinases, Akt is the most critical downstream kinase with regard to the regulation of tumor cell growth, survival and apoptosis [7]. The Akt signaling cascade is frequently disrupted in many human malignancies, including pancreatic, colon, ovarian and breast cancers. There are several mechanisms involved in this process: inappropriate activation of PI3K, Akt gene amplification, Akt protein overexpression and loss of PTEN [8].

The promotion of cell survival by PI3K occurs through Akt, which inhibits pro-apoptotic signals and activates anti-apoptotic genes. In fact, Akt may activate the transcription factor NF-κB through multiple mechanisms. Akt phosphorylates and activates IκB kinases (IKKs), which, in turn, phosphorylate IκB allowing NF-κB to translocate to the nucleus and activate transcription of anti-apoptotic genes such as Bcl-xL, c-IAP1 and c-IAP2. Akt can also activate members of the mitogen activated protein kinase (MAPK) family, and indirectly affect IKK and NF-κB activity [9,10].

We and others have reported that the PI3K/Akt pathway is also associated with chemoresistance in cancer cells such as leukemia, prostate and colon carcinoma and lymphoma cell lines. Inhibition of this pathway induces apoptosis and decreases growth of drug-resistant tumor cells [11–13]. A recent report in acute myelogenous leukemia has demonstrated that MRP-1 but not Pgp efflux is inhibited by the PI3K inhibitor wortmannin [14]. However, another PI3K inhibitor, LY294002, was able to block Pgp efflux in mouse leukemic cell lines [15]. Since additional studies are needed to clarify the role of PI3K in MDR, the purpose of this study was to analyze the relationship between PI3K/Akt, MDR and NF-κB in a lymphoma cell line expressing Pgp.

2. Materials and methods

2.1. Materials

Wortmannin and LY294002 were purchased from Calbiochem (La Jolla, CA, USA). Vincristine (VCR) was kindly provided by Filaxis Pharmaceuticals S.A., Argentina and doxorubicin (DOX) by Gador Pharmaceuticals, Argentina. Antibodies against PI3K-p85α, p-Akt, Akt, survivin, p-IκB-α, IκB-α, actin, anti-rabbit secondary horseradish peroxidase, anti-goat secondary horseradish peroxidase, and Western Blotting Luminol Reagent were purchased from Santa Cruz Biotechnology, Inc. and anti-PIP3 antibody was from Echelon Biosciences, Inc. Annexin V-FITC Apoptosis Detection Kit was from BioVision, Inc. RPMI 1640 was from Invitrogen and Poly-deoxy-inosinic-deoxy-cytidylic acid (Poly(dI-dC)) from GE-Amersham Biosciences. NF-κB and Oct-1 oligonucleotides were from Promega (Madison, WI, USA).

2.2. Cell culture and treatments

The vincristine resistant (LBR-V160), doxorubicin resistant (LBR-D160) and sensitive (LBR-) murine lymphoma cell lines were obtained in our laboratory and described previously [16]. Cell lines were cultured in RPMI 1640 with 10% heat-inactivated fetal calf serum at 37 °C in a 5% CO_2 atmosphere. Cells (8×10^6) were treated with either wortmannin (0.25 and 0.5 μM) or LY294002 (10 and 20 μM). DMSO was used as control, since both inhibitors were solubilized in this component. The chemotherapeutic agents VCR (0.5 μM) and DOX (0.5 μM) were used. Treatments were performed for 30 min for PIP3 production, 2 h for western blot or EMSA extracts and 24 h for apoptosis detection.

2.3. Western blot analyses

Cells were lysed with a hypotonic buffer (20 mM Tris pH 8.0, 150 mM NaCl, 100 mM NaF, 10% glycerol, 1% Nonidet P-40, 1 mM PMSF, 40 μg/ml leupeptin and 20 μg/ml aprotinin) for 30 min at 4 °C. After clarification, equal amounts of protein were separated by electrophoresis on an SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane as described previously [17]. The membrane was blocked and incubated with specific antibodies to PI3K-p85α (B-9), p-Akt (Thr 308), Akt (H-136), survivin (FL-142), p-IκB-α (B-9) and IκB-α (C-21), washed and incubated with horseradish peroxidase-conjugated secondary antibody. Actin served as an internal control and was detected with goat anti-actin antibody (C-11). After washes, the reaction was developed using a chemiluminescence detection system and visualized by autoradiography on X-ray film. Density of detected bands was quantified using Scion Image (Scion Corporation, Frederick, MD).

2.4. PIP3 production

PIP3 extraction was performed as described previously [13]. Briefly, cells were incubated with cold 0.5 M TCA for 5 min, centrifuged and resuspended in 5% TCA/1 mM EDTA. After centrifugation, neutral lipids were extracted with methanol:chloroform (2:1) and acidic lipids by adding methanol:chloroform:12 M HCl (80:40:1). The extracts were centrifuged, chloroform plus 0.1 M HCl was added to the supernatant, and centrifugation was carried out to separate organic and aqueous phases. The organic phase was dried in a vacuum dryer. Extracted lipids were spotted onto PVDF-Plus Transfer membranes and the dot membranes were blocked in PBS with 2% glycine and 3% non-fat dried milk overnight at 4 °C, and then probed with anti-PIP3 antibody (Z-P345), followed by horseradish peroxidase-labeled secondary antibody. Visualization of the immunoreactive areas was achieved using a chemiluminescent detection system and densitometric analysis was performed with Image Scion Software (Scion Corporation, USA).

2.5. Detection of apoptosis

Morphological features associated with apoptosis were analyzed by acridine orange and ethidium bromide staining [16]. A minimum number of 200 cells were counted under a fluorescence microscopy (Zeiss, Germany) and the number of cells presenting fragmented nuclei, enlarged cytoplasm and condensed chromatin were determined. The percentage of apoptotic cells was calculated as: apoptotic cells (%) = (total number of cells with apoptotic nuclei/total number of cells counted) × 100. Percentage of apoptosis for each treatment was calculated by subtraction of spontaneous apoptosis from induced apoptosis (treated apoptotic cells (%) − untreated cells (%)). For the Annexin V-staining method, cells were resuspended in binding buffer and Annexin V-FITC plus propidium iodide was added. Samples were analyzed using a FACScan flow cytometer (Becton Dickinson) and data acquired was analyzed using WinMDI 2.8 software (Scripps Institute, La Jolla, CA).

2.6. Electrophoretic mobility shift assay

Nuclear extracts were prepared as previously [18]. Briefly, cells were incubated in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.1% Nonidet P-40) and centrifuged at 11,000 × *g*. Nuclear pellets were resuspended in nuclear hypotonic buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.5 mM DTT, 0.5 mM PMSF, 0.2 mM EDTA, 25% glycerol) followed by centrifugation at 13,000 × *g*. Nuclear protein concentration was determined by the Bradford assay. Nuclear extracts (4 μg) were preincubated with (Poly(dI-dC)) in binding buffer (50 mM Tris–HCl pH 7.5, 250 mM NaCl, 2.5 mM EDTA, 5 mM $MgCl_2$, 2.5 mM DTT, 20% glycerol) and exposed to γ-32P-labeled oligonucleotide probe for the consensus binding sites of NF-κB. The DNA–protein complexes were separated on a nondenaturating 4% polyacrylamide gel and exposed to an X-ray film for 24 h at −70 °C. For cold competition experiments, proteins were preincubated with unlabeled NF-κB or Oct-1 probes in 100-fold excess.

2.7. Drug efflux pump function

Intracellular accumulation of anthracyclines was carried out as previously described [17]. Briefly, cells (1×10^6 cells) were grown in drug-free medium for 24 h prior to analysis and then stained for 40 min at 37 °C with 200 mM daunorubicin (DNR) and 8 μM cyclosporin-A (CsA) or 0.5 μM wortmannin or 10 μM LY294002. Stained cell samples were acquired and analyzed on a FACScan flow cytometer (Becton Dickinson). DNR fluorescence was collected through a 564–606 nm band-pass filter.

2.8. Statistical analysis

Statistical significance between groups was evaluated by one way-ANOVA and means were compared by the Tukey's test (apoptosis) or Dunnet's test (densitometric analysis). Differences between groups were considered significant at the level of $P < 0.05$.

3. Results

3.1. Resistant cell lines present higher PI3K/Akt activity

In order to analyze PI3K activity in the three cell lines, membrane extracts were obtained and the p85-PI3K subunit was analyzed by western blot. We observed lesser expression in LBR-D160 (45% reduction determined by densitometric analysis) than in the other two cell lines (Fig. 1A). Then, we analyzed PI3K activity by evaluating PIP3 production as well as phosphorylated Akt (p-Akt) expression and found in both cases that PI3K activity was increased in the resistant cell lines. In fact, PIP3 production was 100% higher in LBR-D160 and 73% in LBR-V160 than in LBR- (Fig. 1B) and expression of p-Akt showed an increase of 90% in LBR-D160 and 96% in LBR-V160 when compared to LBR- (Fig. 1C). These findings indicate that although resistant cell lines did

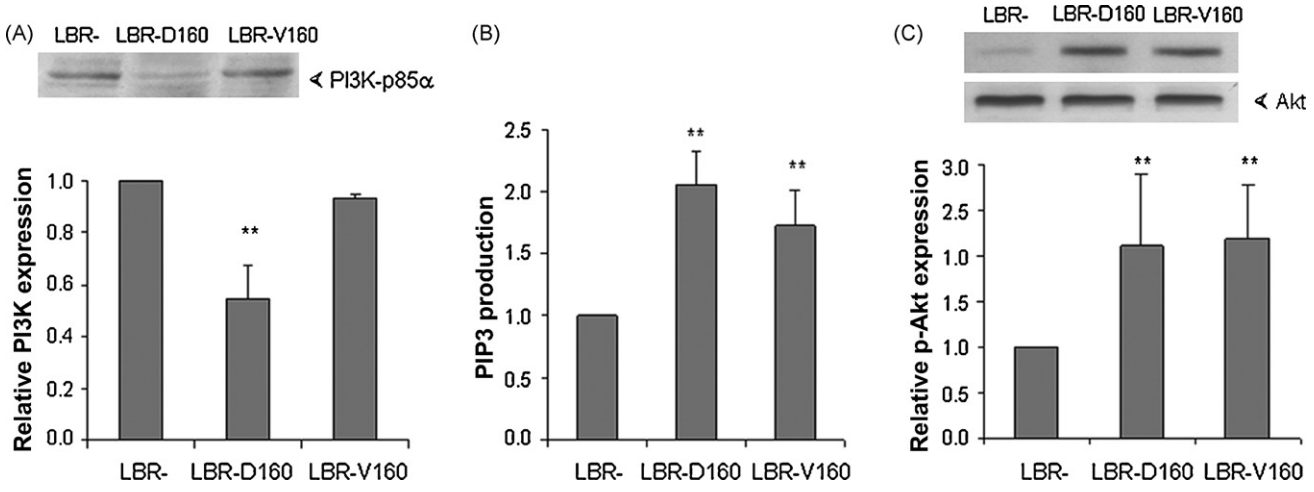

Fig. 1. Analysis of constitutive PI3K activity in the three cell lines. (A) Cellular extracts were analyzed for p85-PI3K expression by western blot. (B) Production of PIP3 was evaluated by dot blot assays. (C) Expression of p-Akt and Akt was evaluated by western blot. Density of each band was quantified using Scion Image software and the ratio of bands of LBR-D160 and LBR-V160 vs. LBR- was calculated and represented as relative expression. Each value represents the mean ± S.D. of three independent experiments. $^{**}P < 0.01$ vs. LBR-.

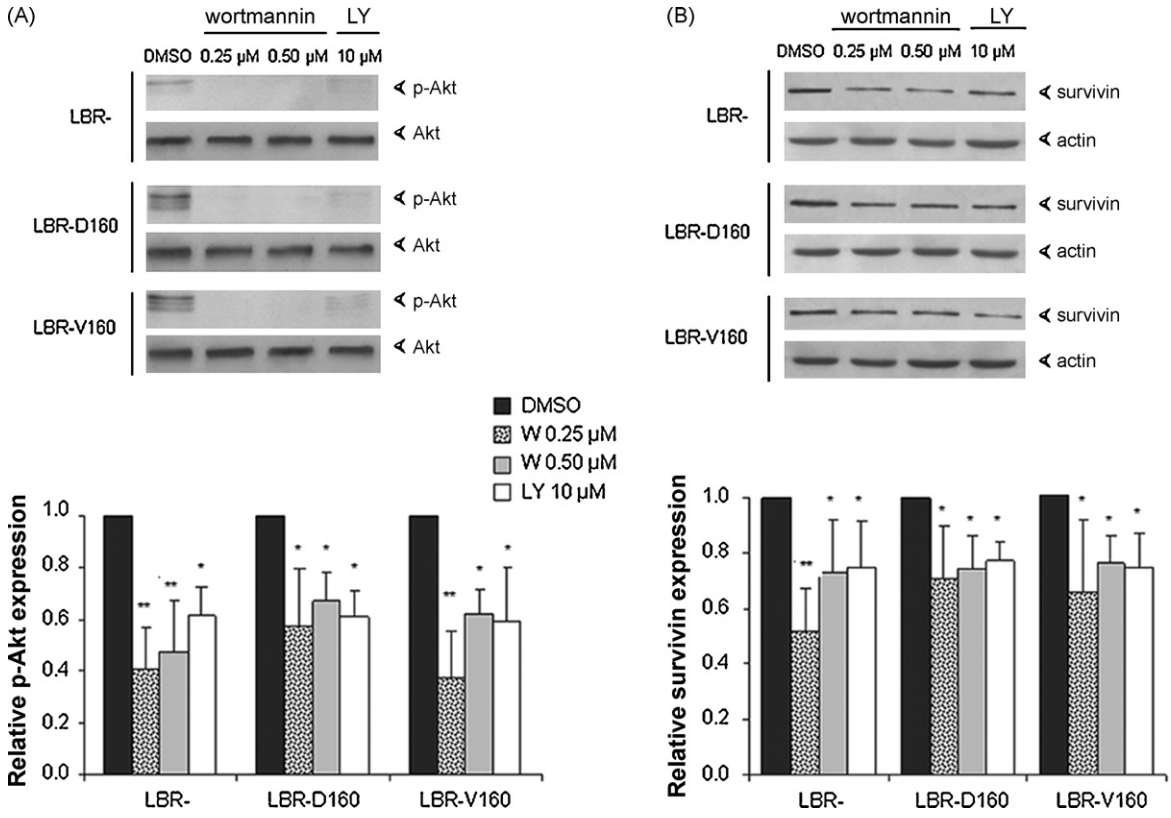

Fig. 2. Western blot analyses for p-Akt (A) and survivin (B) after wortmannin (W) or LY294002 (LY) treatment. Density of each band was quantified and the ratio of bands with each treatment vs. DMSO was calculated and shown as relative expression. Akt and actin expression are shown as loading controls. Each value represents the mean ± S.D. of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. DMSO.

not present a higher p85-PI3K expression than that of the sensitive line, PI3K activity was significantly increased in the resistant cell lines.

3.2. Wortmannin and LY294002 inhibit p-Akt and survivin expression

The principal kinase activated by PI3K is Akt, thus we decided to evaluate the influence of PI3K on p-Akt expression in these cell lines by using specific inhibitors of PI3K. Wortmannin and LY294002 treatment reduced p-Akt expression in the three cell lines without modifying Akt expression (Fig. 2A).

As previous data have indicated that the PI3K/Akt pathway can regulate survivin expression [19], we decided to evaluate this pathway in our cell lines. Survivin expression showed a significant decrease after treatment with different doses of the inhibitors of PI3K, wortmannin or LY294002 (Fig. 2B).

3.3. PI3K/Akt inhibition leads to higher apoptosis induction in the resistant cell lines

To determine the role of the PI3K/Akt pathway in the survival of cell lines, apoptosis induction after wortmannin or LY294002 treatment was analyzed by morphological features of apoptosis evidenced by acridine orange and ethidium bromide staining. As shown in Fig. 3, after 0.50 μM wortmannin treatment, LBR-D160 and LBR-V160 presented increased apoptosis when compared to LBR- (17.6 ± 7.5%, 17.4 ± 4.8% and 3.7 ± 3.4%, respectively). Additionally, 10 μM LY294002 treatment also induced higher apoptosis

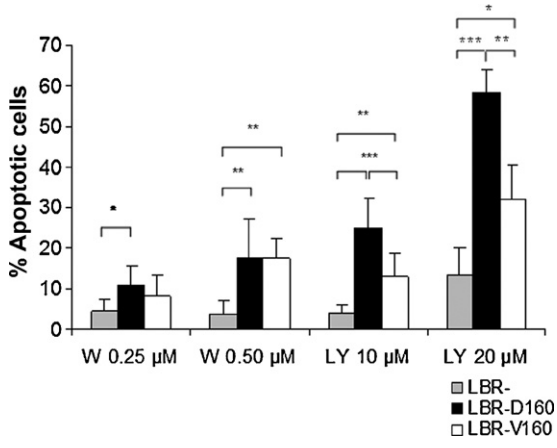

Fig. 3. Apoptosis induction after PI3K/Akt inhibition. Cells were treated with different doses of either wortmannin (W) or LY294002 (LY) and were evaluated by acridine orange and ethidium bromide staining. Values are expressed as the mean ± S.D. of % apoptotic cells of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

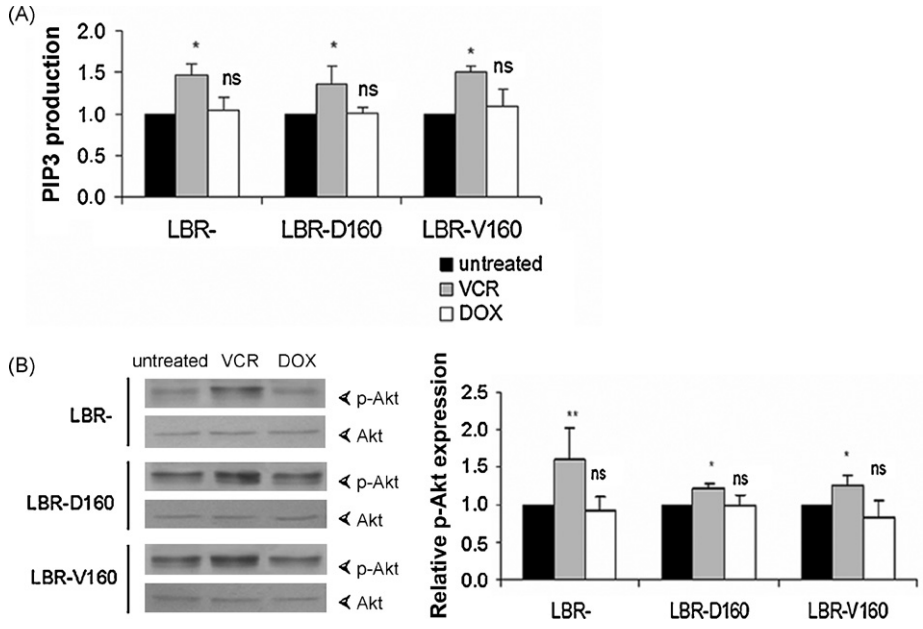

Fig. 4. Effect of VCR and DOX on PI3K/Akt pathway. (A) Production of PIP3 was evaluated by dot blot assays. Densitometric analysis of the immunoreactive areas was performed and the results are expressed as the Index (treated cells/untreated cells). (B) Cytoplasmic extracts from cells were analyzed for p-Akt or Akt expression by western blot. Densitometric analysis of the bands was performed and the results are expressed as the Index (treated cells/untreated cells), determined from three independent experiments. Bars represent means ± S.D. of three independent experiments. ns: non-significant, $*P<0.05$, $**P<0.01$ vs. untreated.

in LBR-D160 and LBR-V160 than in LBR- (25.2 ± 7.3%, 15.4 ± 5.3% and 4.0 ± 1.8%, respectively). A higher dose (20 μM LY294002) resulted in significantly different levels of apoptosis in each cell line, being LBR-D160 the cell line that showed the highest apoptosis induction (58.3 ± 5.8%). These results were confirmed by the Annexin V-staining method (data not shown). Taken together, these data suggest that the PI3K/Akt pathway is involved in the survival of lymphoma resistant cell lines and that specific inhibition of this pathway leads to apoptosis.

3.4. VCR increases the PI3K/p-Akt pathway

Since we observed higher PI3K/Akt activity in the resistant cell lines, we next decided to evaluate the effect of the chemotherapeutic agents vincristine and doxorubicin on this signaling pathway. We observed that PIP3 production was increased by about 50% after treatment with VCR in the three cell lines (Fig. 4A). Similarly, p-Akt expression was also increased after treatment with this chemotherapeutic agent. Densitometric analysis of western blot showed an increase in p-Akt expression after VCR treatment in the three cell lines: 60% in LBR-, 22% in LBR-D160 and 26% in LBR-V160. The chemotherapeutic agent DOX failed to modulate PIP3 production and p-Akt expression (Fig. 4A and B). Total Akt expression was comparable between all the treatments. Our results indicate that VCR – but not DOX – was able to increase the PI3K/Akt pathway as shown by the increased PIP3 production and p-Akt expression in the resistant cell lines.

3.5. PI3K/Akt inhibition sensitizes cell lines to VCR-induced apoptosis

Next, we evaluated the effect of co-treatment with the chemotherapeutic agents and PI3K/Akt inhibitors on apoptosis induction. We observed that in LBR- and LBR-V160 LY294002 sensitized the cells to VCR-induced apoptosis whereas in LBR-D160 both inhibitors, wortmannin and LY294002 had this effect (Fig. 5). In contrast, neither of the inhibitors significantly increased the apoptosis induced by DOX (data not shown). These results showed that co-

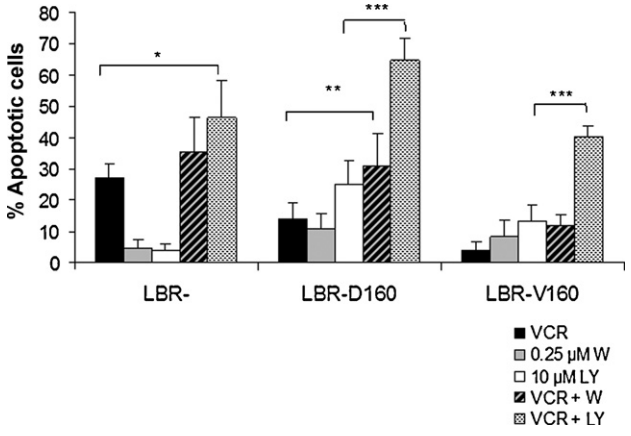

Fig. 5. Apoptosis induction after co-treatment with VCR (0.5 μM) and PI3K/Akt inhibitors, wortmannin (W) or LY294002 (LY). Values are expressed as the mean ± S.D. of apoptotic cells of three independent experiments. $*P<0.05$, $**P<0.01$ and $***P<0.001$.

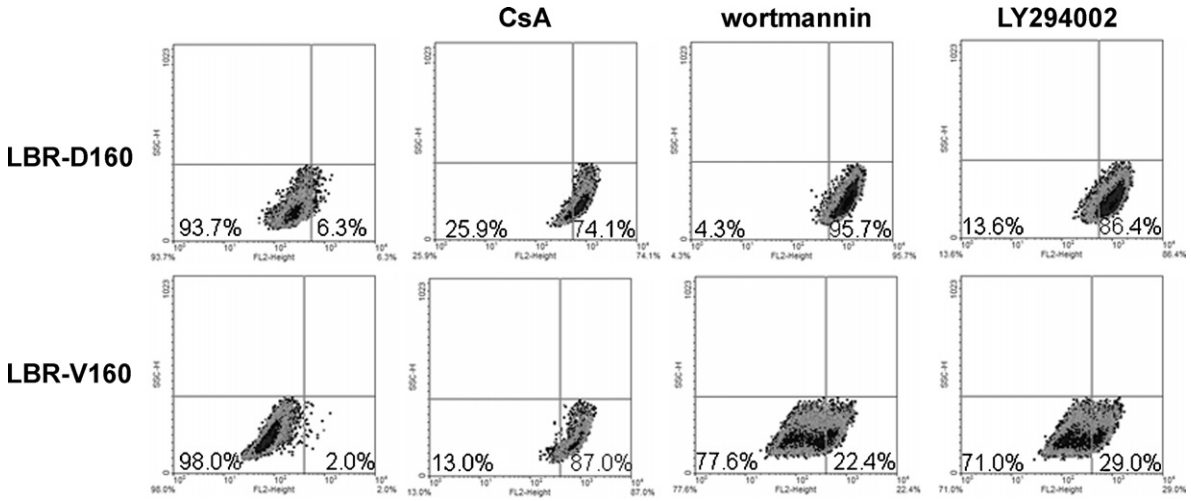

Fig. 6. Effect of wortmannin and LY294002 on Pgp modulation. Daunorubicin efflux was analyzed by flow cytometry. The resistant LBR-D160 and LBR-V160 cells were incubated with DNR alone (first column). CsA (8 μM) was used an inhibitor of drug efflux presenting enhanced intracellular fluorescence in both LBR-D160 and LBR-V160 (second column). Treatment with 0.50 μM wortmannin (third column) and 10 μM LY294002 (fourth column) at 40 min. A representative from three independent experiments is shown.

treatment with VCR and PI3K inhibitors can sensitize lymphoma resistant cell lines to this chemotherapeutic agent. However, this was not observed with DOX.

3.6. Wortmannin and LY294002 inhibit Pgp efflux

Due to previous controversial results about the effect of PI3K inhibitors on Pgp activity and our results indicating that wortmannin and LY294002 were able to sensitize resistant cells to VCR-induced apoptosis, we decided to evaluate the effect of such inhibitors on Pgp efflux. For this purpose, daunorubicin accumulation was evaluated by flow cytometry. As we have previously demonstrated [17], CsA increased intracellular fluorescence in both resistant cell lines demonstrating inhibition of Pgp efflux (Fig. 6, second column). Treatment with wortmannin and LY294002 enhanced intracellular fluorescence at 40 min in LBR-D160 and partially in LBR-V160 (Fig. 6, third and fourth column). Inhibition of Pgp efflux persisted up to 24 h only in LBR-D160 after wortmannin treatment (73.7%, data not shown). Taken together, these observations indicate that PI3K inhibitors such as wortmannin and LY294002 are able to inhibit Pgp efflux in the resistant cell lines and that Pgp blockage is almost complete in LBR-D160, whereas it is partial in LBR-V160.

3.7. PI3K/Akt inhibition activates NF-κB

Since previous data have indicated that Akt activates the transcription factor NF-κB, we decided to evaluate the NF-κB pathway through the expression and phosphorylation of its inhibitor IκB-α by western blot. As shown in Fig. 7A, treatment with wortmannin or LY294002 increased IκB-α phosphorylation leading to a decrease in the expression of IκB-α. Densitometric analysis showed a decrease in IκB-α expression after wortmannin or LY294002 treatment (20% and 23% in LBR-; 24% and 23% in LBR-D160; 29% and 35% in LBR-V160, respectively). Since increased p-IκB-α seems to lead to activation of NF-κB, we next investigated the activity of this transcription factor by EMSA assay. We observed that wortmannin enhanced NF-κB activity in a dose-dependent manner (Fig. 7B). These data show that inhibition of PI3K/Akt pathway activates NF-κB pathway.

4. Discussion

In this study we evaluated the correlation of the PI3K/Akt signaling pathway with multidrug resistance and the NF-κB survival pathway. We demonstrated that the resistant cell lines, LBR-D160 and LBR-V160, presented higher PI3K/Akt activity than the sensitive one, which is in accordance with the MDR phenotype. The production of PIP3 and the expression of p-Akt, which reveal PI3K activity, were increased in the resistant cell lines, but the expression of PI3K-p85α was decreased in LBR-D160 when compared with the other cell lines. These discrepancies could be because in these cell lines other isoforms different from the regulatory subunit p85 could be responsible for PI3K activity. In fact, mutants of the regulatory subunit of PI3K (p65-PI3K in a thymic lymphoma cell line and p76α in a human lymphoma cell line) have been described. Both proteins induce the kinase activity of PI3K and contribute to cellular transformation [20,21]. We also demonstrated that the expression of p-Akt and survivin was decreased after wortmannin or LY294002 treatment in the three cell lines without modifying Akt expression. Our results are in line with previous reports suggesting that survivin is under PI3K control [19,22]. Consequently, inhibition of the PI3K/Akt pathway with wortmannin or

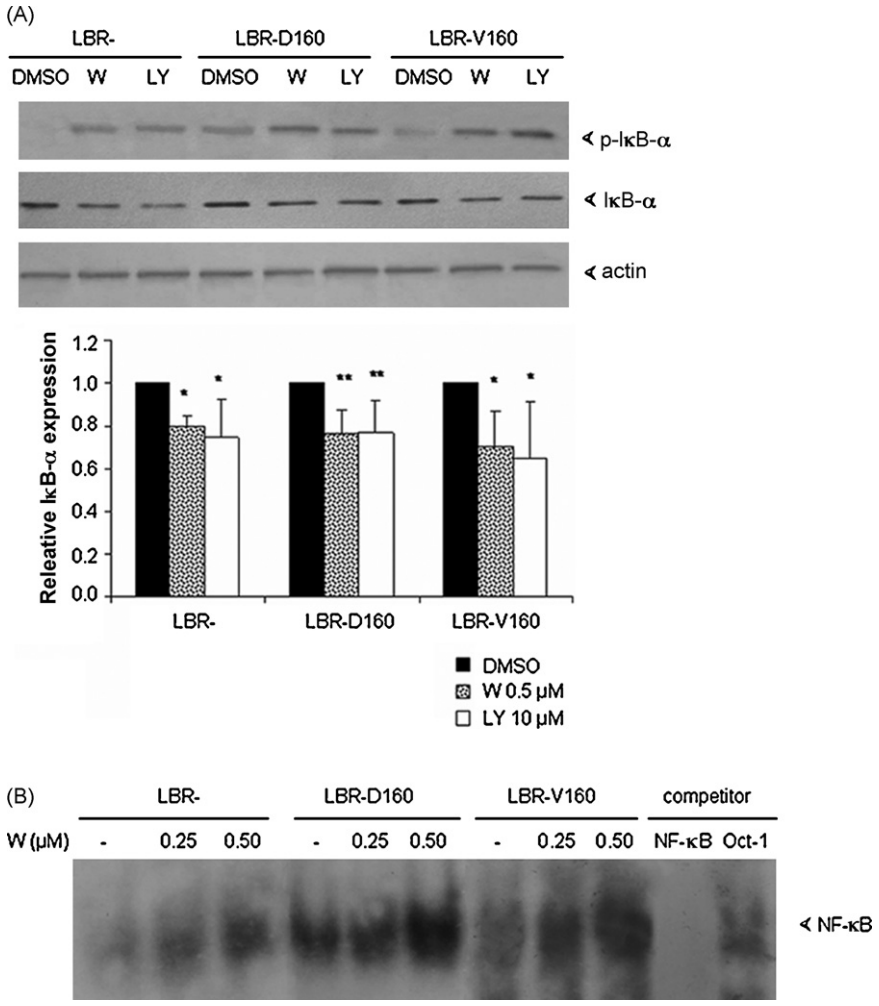

Fig. 7. Analysis of NF-κB activity after PI3K/Akt inhibition. (A) Western blot for p-IκB-α, IκB-α and actin after either 0.5 μM wortmannin (W) or 10 μM LY294002 (LY) treatment. Actin was used as loading control. A representative from four independent experiments is shown. Density of each band was quantified and the ratio of bands with each treatment vs. DMSO was calculated and shown as relative expression. Data represents the mean ± S.D. of four independent experiments. $*P < 0.05$ vs. DMSO. (B) EMSA assay for NF-κB activity after treatment with different doses of either wortmannin (W) or DMSO (−). The specificity of NF-κB activation was confirmed by competition studies with unlabeled specific (NF-κB) or nonspecific (Oct-1) binding site oligonucleotides. A representative from three independent experiments is shown.

LY294002 induced higher apoptosis levels in LBR-D160 and LBR-V160 than in LBR-, thus indicating that this pathway could be essential for the survival of MDR lymphoma cell lines.

The chemotherapeutic agent vincristine but not doxorubicin was able to increase the PI3K/Akt pathway in the three cell lines as shown by increased PIP3 production and p-Akt expression. Likely, PI3K/Akt inhibition sensitized the cell lines to VCR but not to DOX-induced apoptosis. Although some authors have reported that inhibition of PI3K chemosensitize tumor cells to DOX [11,12], others have shown that LY294002 synergistically increase the cytotoxicity induced by antimicrotubule agents like vincristine or paclitaxel [23]. Our results indicate that in these lymphoma cell lines VCR and DOX have different effects on the PI3K/Akt pathway and that inhibition of this signaling cascade chemosensitizes tumor cells only to the antimicrotubule agent. The increase in p-Akt expression was more evident in the sensitive cell line, however the apoptosis induction by co-treatment of LY294002 and VCR was more significant in the resistant cell lines than in LBR-. In addition, wortmannin synergized VCR-induced apoptosis in LBR-D160. Evidently, it is more difficult for VCR to increase p-Akt in the cell lines that already present higher p-Akt levels like the resistant cell lines. However, when the overexpressed PI3K/Akt survival pathway was inhibited in these resistant cell lines and also when the cells were co-treated with VCR, higher apoptosis induction was observed. These results also suggest that in the sensitive line LBR-, VCR has an effect on different molecular targets such as Akt but that in spite of this the cell is eliminated by apoptosis. In contrast, in LBR-D160 and LBR-V160 the presence of an active efflux pump (Pgp) diminished the intracellular concentration of VCR. Although this concentration is insufficient to induce apoptosis, it is sufficient to

activate Akt. Taken together these results suggest that in the resistant cell lines, VCR not only failed to induce apoptosis but also activated a survival pathway. For that reason, inhibition of PI3K/Akt pathway provides a molecular target for resistant cell lines to induce apoptosis in co-treatment with VCR.

We found that both PI3K inhibitors, wortmannin and LY294002, were able to block Pgp efflux in LBR-D160 and partially in LBR-V160. We have previously demonstrated that the LBR-V160 cell line has an efflux pump more active than LBR-D160 and that such difference could be a result of the coexpression of *mdr-3* and *mdr-1* genes in this cell line [13,24]. It has been recently demonstrated that LY294002 is able to block Pgp efflux in mouse leukemic cell lines [15] and that wortmannin can block the multidrug resistance-associated protein MRP1 but not Pgp in human acute myelogenous leukemia blasts [14]. Our results demonstrate that both inhibitors, wortmannin and LY294002, were able to block Pgp efflux in these lymphoma cell lines. Our data indicate that PI3K inhibitors modulate MDR by inhibiting both PI3K/Akt and Pgp functions, thus allowing the drug to accumulate in the cytoplasm and to induce apoptosis. We have recently demonstrated that treatment with oligosaccharides of hyaluronan has similar effects on the reversion of MDR [13]. In summary, our results highlight the importance of the PI3K pathway inhibition as a therapeutic approach in MDR lymphomas.

Finally we evaluated the relation between PI3K/Akt and NF-κB showing that PI3K inhibition with either wortmannin or LY294002 activates NF-κB in the cell lines. The regulatory role of the PI3K/Akt pathway in NF-κB activity appears to be cell type- and ligand-specific. Although PI3K activates NF-κB in many cell lines [25,26], a negative regulation of NF-κB by the PI3K/Akt signaling cascade has also been described [27]. In fact, LPS-induced activation of the PI3K/Akt pathway negatively regulates NF-κB and MAPK pathways. Inhibition of these signaling cascades limits the expression of inflammatory mediators thus avoiding severe tissue damage [28,29]. On the light of these findings, we suggest that in these cell lines PI3K inhibition is able to induce cell death but at the same time may activate other survival pathways, like NF-κB, acting as a possible compensatory mechanism of cell death.

In the present work, we demonstrated that PI3K/Akt pathway is involved in MDR in these lymphoma cell lines since LBR-D160 and LBR-V160 presented higher PI3K/Akt activity than the sensitive one and inhibition of this pathway resulted in higher apoptosis induction in the resistant cell lines. Besides, PI3K/Akt inhibition correlates with survivin down-regulation and NF-κB activation. PI3K inhibitors, W and LY, modulate MDR by both PI3K/Akt and Pgp function inhibition. Further investigations with other tumor models as well as *in vivo* studies will be required to better understand the role of PI3K/Akt pathway in MDR. Nevertheless, PI3K/Akt signaling cascade may be considered as an attractive target for therapeutic intervention.

Conflict of interest statement

None.

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