PF-04691502, a dual PI3K/mTOR inhibitor has potent pre-clinical activity by inducing apoptosis and G1 cell cycle arrest in aggressive B-cell non-Hodgkin lymphomas

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Abstract. The PI3K/Akt/mTOR pathway is activated in a variety of human tumors including B-cell non-Hodgkin lymphoma (B-NHL). Targeting this pathway has been validated in solid and hematological tumors. In the present study, we demonstrated that PF-04691502, a novel PI3K/mTOR inhibitor has potent activity in a panel of aggressive B-NHL cell lines including diffuse large B-cell lymphoma (DLBCL) and mantle cell lymphoma (MCL). MTS analysis showed that PF-04691502 effectively inhibited cell proliferation with IC_{50} values ranging from 0.12 to 0.55 μM. Cells treated with PF-04691502 exhibited decreased phosphorylation of Akt and S6 ribosomal protein confirming the mechanism of action of a PI3K/mTOR inhibitor. Also, treatment of B-NHL cell lines with PF-04691502 induced apoptosis in a dose- and time-dependent manner. Moreover, PF-04691502 significantly induced G1 cell cycle arrest associated with a decrease in cyclin D1 which contributed to suppression of cell proliferation. Finally, rituximab enhanced apoptosis induced by PF-04691502. Taken together, our findings provide for the first time that PF-04691502 inhibits the constitutively activated PI3K/mTOR pathway in aggressive B-cell NHL cell lines associated with inhibition of cell cycle progression, cell proliferation and promotion of apoptosis. These findings suggest that PF-04691502 is a novel therapeutic strategy in aggressive B-cell NHL and warrants early phase clinical trial evaluation with and without rituximab.

Introduction

Phosphatidylinositol 3-kinase (PI3K) represents a family of closely related enzymes that serve to transduce downstream signaling and have a profound role in multiple critical cellular

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processes, including growth, differentiation, metabolism, survival and cellular proliferation (1,2). PI3K are divided into three distinct classes on the basis of primary structure, function and lipid substrate specificity (3). Class I PI3Ks, the most widely implicated as aberrant in cancers, are heterodimers composed of a regulatory and catalytic subunit and subdivided into 1A and 1B based on their mode of activation. Class 1A is composed of p110α, p110β and p110δ (catalytic subunits), bound by p85, p50 or p55 (regulatory subunits). Class 1B consists of a single catalytic domain, p110γ bound by the regulatory domain p101 (4). Class II PI3Ks are monomeric and three catalytic isoforms have been identified: the ubiquitously expressed PI3K-C2α and PI3K-C2β and liver-specific PI3K-C2γ. Class III PI3Ks are heterodimeric enzymes consisting of a catalytic (Vps34) and a regulatory (Vps15/p150) subunit (5).

PI3K binds to and is activated by several upstream receptors and non-receptor protein tyrosine kinases (6,7). Once activated, PI3K phosphorylates its lipid substrate phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) to PtdIns(3,4,5) P3, a critical intra-cellular lipid second messenger. This process is opposed by the tumor suppressor phosphatase and tension homolog (PTEN) which is frequently deleted or mutated in human cancers that results in constitutive PI3K activation (8). It in turn activates Akt, an important downstream effector through interacting with PtdIns(3,4,5)P3 via its pleckstrin homology (PH) domain. Activated Akt inhibits the tuberous sclerosis complexes 1 and 2 (TSC1 and TSC2), permitting activation of the mTOR complex 1 (mTORC1) and subsequent phosphorylation of proteins 70S6K1, S6 and the eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), resulting in dysregulation of protein synthesis and cell survival (9). A second complex of mTOR, known as mTORC2, appears to act as a feedback loop via Akt. Activation of mTORC2 via phosphorylated 70S6K1 induces phosphorylation of Akt at its serine 473 residue and facilitates its complete activation (10).

The oncogenic potential of the PI3K/Akt/mTOR pathway is well documented. Forced expression of PI3K is shown to induce cell line transformation (11), tumor formation (12) and angiogenesis *in vivo* (13). It has also been shown that Akt is involved in malignant transformation (14). PI3K signaling activation frequently occurs in numerous human tumors due

to multiple molecular alterations, such as mutations (PIK3CA, Akt1 and PTEN), gene amplifications (PIK3CA, Akt1 and Akt2), loss of expression of the tumor suppressors PTEN and inositol polyphosphate-4-phosphatase type II and other mechanisms (15). In addition, deregulation of the PI3K/Akt/mTOR axis is also observed when upstream oncogenes are mutated or amplified and tumor suppressor genes are deleted (16). Indeed, numerous inhibitors targeting this signaling pathway have been developed and have been shown to possess preliminary clinical activity (16).

Aggressive B-cell non-Hodgkin's lymphomas (B-NHL) such as diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma (MCL), Burkitt's lymphoma (BL) and follicular lymphoma (FL) are incurable with current chemo-immunotherapy regimens and development of novel and effective treatments based on biologically validated targets is urgently needed (17). Same as in solid tumors, activation of the PI3K/Akt/mTOR signaling pathway occurs commonly in B-NHL (18). The mechanism by which the pathway is activated differs by lymphoma subtype. In MCL, most patients have an increased copy number of PIK3CA, resulting in increased transcription and pathway activation (19). PTEN loss has been found in GCB subtype of DLBCL, which results in increased PI3K/Akt signaling and *in vitro* PI3K inhibitor sensitivity (20). Moreover, phosphorylation of Akt and p70S6K occurs in most DLBCL cell lines and patient samples (21,22). The B-cell receptor (BCR), a critical signaling pathway for B-cell survival, also induces PI3K pathway activation (23,24). Importantly, inhibition of PI3K/Akt/mTOR pathway have shown anti-lymphoma activity associated with cell cycle arrest and apoptosis both *in vivo* and *in vitro* (25-28).

PF-04691502 is a potent, selective and orally active ATP-competitive PI3K/mTOR inhibitor with Ki values of 1.6, 1.8, 1.9, 2.1 and 16 nM for human PI3K δ, α, γ, β and mTOR, respectively (29). Many *in vitro* and *in vivo* studies have shown that PF-04691502 can inhibit cell proliferation, induce apoptosis and has antitumor activity in solid tumors (30-34). However, PF-04691502 has not been tested in hematologic malignancies. In the present study, we demonstrated that PF-04691502 inhibited cell proliferation, induced cell cycle arrest and apoptosis in aggressive B-cell NHL cell lines associated with inhibition of PI3K/Akt/mTOR signaling activity.

Materials and methods

Cells and reagents. U-2932 (DLBC) and Granta-519 (MCL) cell lines were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ; Braunschweig, Germany). SUDHL-10 (DLBC) cell line was from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were maintained in RPMI-1640 medium (Mediatech, Herndon, VA, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM sodium pyruvate and 100 units/ml penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. The doubling time of each of these three cell lines is ~48 h. PF-04691502 (PI3K(α/β/δ/γ)/mTOR dual inhibitor), CAL-101 (p110δ inhibitor) and INK-128 (mTORC1/2 inhibitor) were purchased from Selleckchem (Houston, TX, USA). The compounds were dissolved at 50 mM in DMSO as a stock solution, and then further diluted

to desired concentrations for *in vitro* experiments. Rituximab was a kind donation by the affiliated hospital clinic. Anti-phospho-Akt (Ser473 and Thr308; 1:500 dilution), anti-Akt (1:500 dilution), anti-phospho-S6 (1:1,000 dilution) and anti-GAPDH (14C10; 1:1,000 dilution) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-PARP (H-250; 1:500 dilution) and anti-cyclin D1 (1:500 dilution) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Inhibition analysis of cell proliferation (MTS assay). Cells were seeded at 10,000/well in 96-well culture plates and allowed to grow for 24 h followed by the desired treatment with increasing concentrations of the indicated agents for 4 days. The studies were conducted in three separate experiments with triplicate on one plate. Viable cell densities were determined using a CellTiter 96 Cell Proliferation assay (Promega, Madison, WI, USA). Absorbance readings at 490 nm were analyzed against the control group for each drug treatment to determine cell viability. The IC_{50} values were estimated by Calcusyn software (Biosoft, Cambridge, UK).

Cell cycle analysis. Cells were treated with different concentrations of PF-04691502 for 48 h and then the cells were centrifuged at 1,500 x g for 5 min at 4°C and resuspended in PBS, fixed by drop wise addition of ice-cold ethanol (100%) to a final concentration of 70%, and incubated for 30 min on ice. Fixed cells were pelleted and treated with 100 μl of RNase A (0.2 mg/ml in PBS) for 5 min at room temperature, then suspended in 0.5 ml of ddH_2O. After staining with 4 μg/ml propidium iodide, the DNA content was determined using a BD Biosciences LSR II flow cytometer and the cell cycle profile was analyzed by ModFit software. Cell aggregates were gated out of the analysis, based on the width of the propidium iodide fluorescence signal. Each profile was compiled from 10,000 gated events.

Apoptosis assay. Using Annexin V staining to detect apoptosis, treated cells were harvested and rinsed with cold PBS once. After centrifugation for 5 min, cells were resuspended in 500 μl of 1X Annexin V binding buffer (Annexin V-FITC reagent kit, cat.#1001-1000; BioVision, Inc., Milpitas, CA, USA) and then 5 μl of Annexin V-FITC and 5 μl of propidium iodide (PI) (Annexin V-FITC reagent kit; BioVision) were added. After incubation for 5 min at room temperature in the dark, the samples were analyzed by a BD Biosciences FACSCalibur flow cytometer.

Immunoblotting. The cells were lysed in NP-40 lysis buffer containing 50 mM Tris-Cl (pH 7.4), 0.15 M NaCl, 0.5% NP-40, 1 mM DTT, 50 mM sodium fluoride, and 2 μl/ml protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and 50 μg of protein was resolved by electrophoresis on a 10% SDS-PAGE gel. The proteins were then transferred onto a nitrocellulose membrane and non-specific binding was blocked by incubating with 5% non-fat milk in TBST buffer (0.01 M Tris-Cl, 0.15 M NaCl, 0.5% Tween-20, pH 8.0) at room temperature for 1 h. The membrane was subjected to the indi-
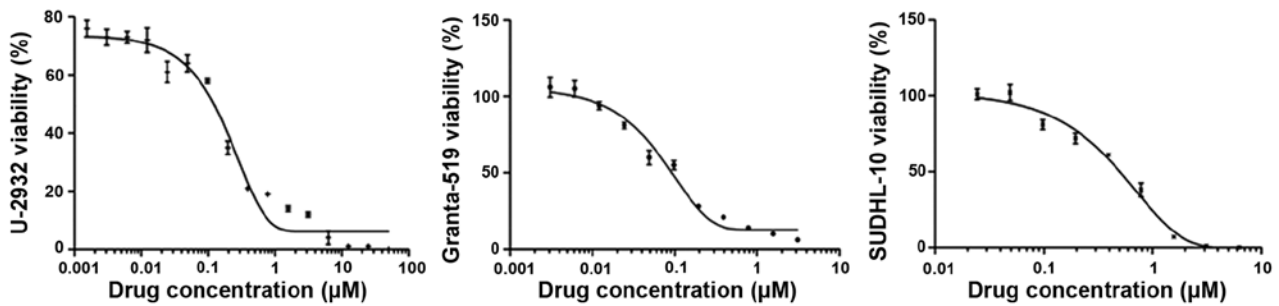
Figure 1. Antiproliferative activity of PF-04691502 in aggressive B-NHL cell lines. U-2932, Granta-519 and SUDHL-10 cells were exposed to varying concentrations of PF-04691502 for 4 days. Cell viability was assessed by MTS analysis. Points are the means of triplicate determinations ± SD.
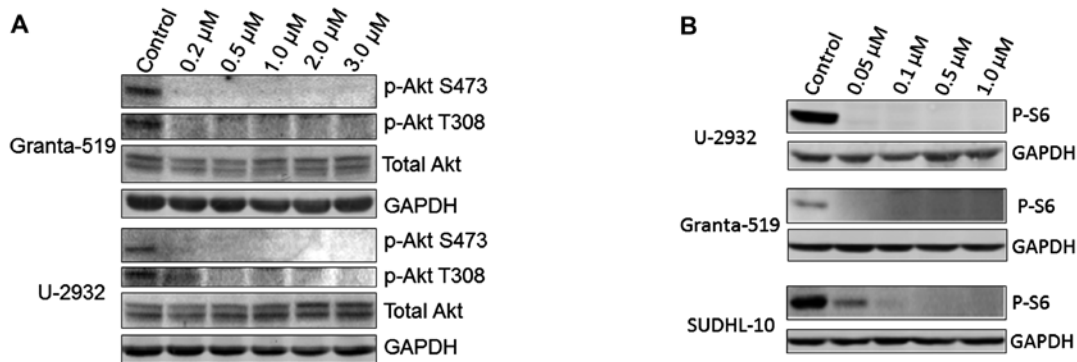

Figure 2. PF-04691502 potently inhibits phosphorylation of Akt and S6 ribosomal protein. (A) Granta-519 and U-2932 cells were untreated or treated with PF-04691502 at 0.2, 0.5, 1.0, 2.0 and 3.0 μM for 1 h. (B) U-2932, Granta-519 and SUDHL-10 cells were treated with 0.05, 0.1, 0.5 and 1.0 μM of PF-04691502 for 24 h. Cells were collected for protein isolation. Total protein (50 μg) from each lysate was resolved by SDS-PAGE and immunoblotted with antibodies against to phosphorylated Akt (Ser473 and Thr308), pan-Akt and phosphorylated S6 ribosomal protein (Ser235/236). GAPDH was used as a loading control.

cated antibodies and the proteins were detected by a LI-COR Odyssey Infrared Imaging System.

Statistical analysis. All *in vitro* experiments were performed in triplicate. The results were expressed as mean ± SD. The difference between two mean values were measured by the Student's t-test (Excel) and considered to be statistically significant at P≤0.05.

Results

Inhibition of PI3K/mTOR suppresses cell proliferation in aggressive B-NHL cells. The PI3K/mTOR pathway plays a central role in cancer cell survival and proliferation. PF-04691502, which is against all class I isoforms of PI3K and mTOR kinases, has been reported to have antitumor activity in several types of solid cancers including glioblastoma, breast, colorectal, nasopharyngeal, hepatocellular and non-small cell lung carcinoma cells (30,31,34-36). To examine whether PF-04691502 inhibits B-NHL cells, MTS assays were performed to evaluate the growth including U-2932, SUDHL-10 and Granta-519 which represents DLBCL and MCL subtypes, respectively. Consistent with previous studies in solid tumors, PF-04691502 effectively inhibited the growth of these cell lines with IC$_{50}$ values ranging from 0.121 to 0.545 μM (Fig. 1 and Table I). CAL-101, a p110δ selective PI3K inhibitor which has been shown to inhibit multiple myeloma (37), B-cell acute lymphoblastic leukemia (38) and chronic lymphocyte

leukemia (39) and INK-128, a selective mTOR inhibitor which has antitumor activity in breast, pancreatic and colorectal cancers (40-42) were also used in this experiment. Notably, the IC$_{50}$ of CAL-101 in these cells was higher than that of INK-128 (ranging from 3.15 to 15.37 μM and from 0.014 to 0.170 μM, respectively) (Table I). Together, these data demonstrate that inhibition of PI3K/mTOR signaling effectively suppresses cell proliferation in B-cell NHL cell lines.

PF-04691502 inhibits downstream signaling of PI3K in B-cell NHL cell lines. Receptor tyrosine kinase or chronic B-cell receptor signaling results in activation of the PI3K pathway, and subsequently leads to trigger the downstream targets such as Akt and S6 ribosomal protein which promote cell proliferation and survival. In the present study, we evaluated the effect of PF-04691502 on the activity of Akt and S6 in B-NHL cells. U-2932, Granta-519 and SUDHL-10 cells were treated with PF-04691502 at different concentrations for 1 and 24 h. As expected, phosphorylation of Akt at both Ser473 and Thr308 sites was completely decreased with PF-04691502. However, the total Akt protein level was unchanged upon PF-04691502 treatment, indicating that the decreased pSer473 and pThr308 were due to inhibition of phosphorylation and not Akt protein degradation or translational downregulation (Fig. 2A). Phospho-S6 ribosomal protein was also reduced markedly by PF-04691502 even at a low concentration of 0.05 μM (Fig. 2B). Hence, PF-04691502 effectively inhibits the PI3K/Akt/mTOR pathway in B-NHL cells.
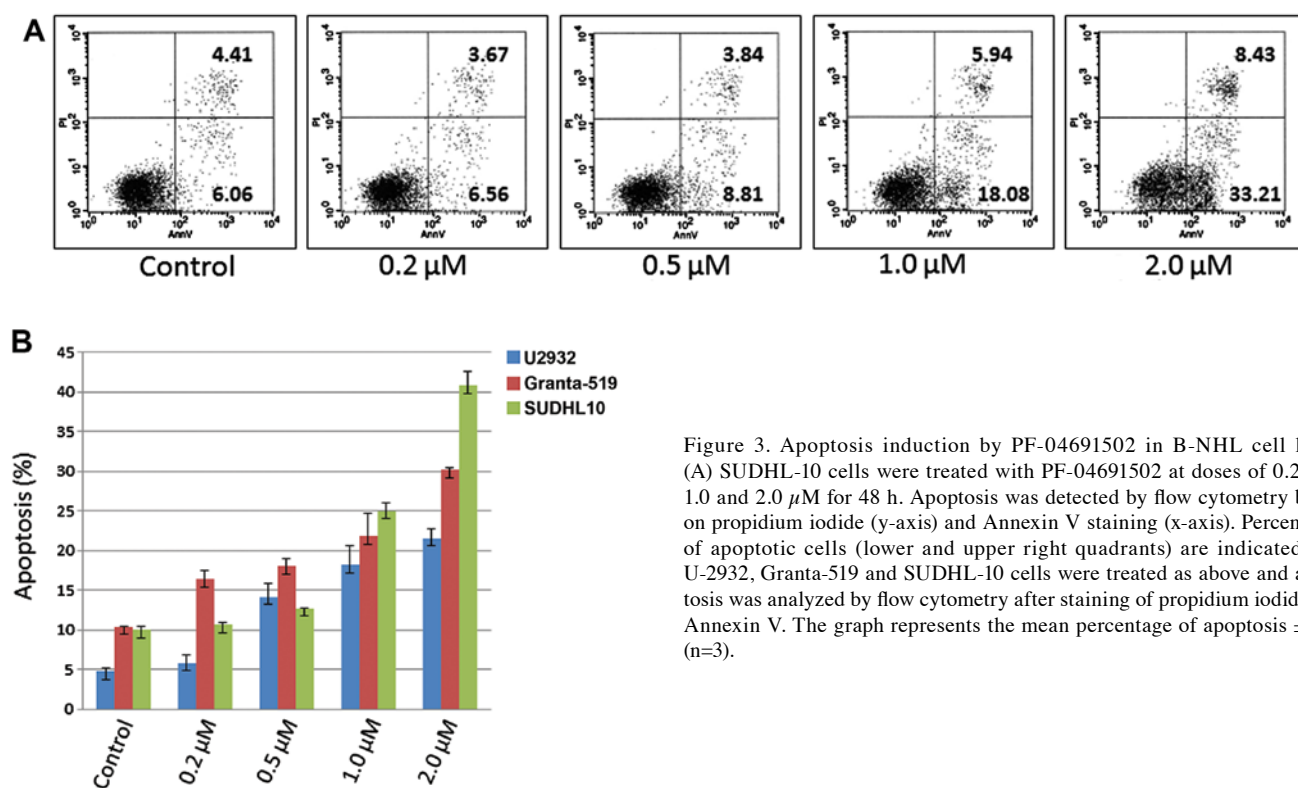
Figure 3. Apoptosis induction by PF-04691502 in B-NHL cell lines. (A) SUDHL-10 cells were treated with PF-04691502 at doses of 0.2, 0.5, 1.0 and 2.0 μM for 48 h. Apoptosis was detected by flow cytometry based on propidium iodide (y-axis) and Annexin V staining (x-axis). Percentages of apoptotic cells (lower and upper right quadrants) are indicated. (B) U-2932, Granta-519 and SUDHL-10 cells were treated as above and apoptosis was analyzed by flow cytometry after staining of propidium iodide and Annexin V. The graph represents the mean percentage of apoptosis ± SD; (n=3).

Table I. IC$_{50}$ (μM) of the different PI3K/mTOR inhibitors in B-NHL cells.

Cell lines/drugs	U-2932	Granta-519	SUDHL-10
CAL-101	12.58±0.62	3.15±0.26	15.37±0.73
PF-04691502	0.175±0.064	0.121±0.013	0.545±0.089
INK-128	0.014±0.003	0.022±0.006	0.170±0.015

PF-04691502 induces apoptosis in B-NHL cell lines. It is known that apoptosis is induced when suppressing the PI3K pathway. To examine apoptosis, U-2932, Granta-519 and SUDHL-10 cell lines were treated with PF-04691502 at varying doses of 0.2, 0.5, 1.0 and 2.0 μM for 48 h, stained with Annexin V and PI and evaluated by flow cytometry assays. As expected, PF-04691502 induced apoptosis in all three cells in a dose-dependent manner (Fig. 3A and B). These results were confirmed by demonstrating an increased level of cleaved PARP in treated B-NHL cells (Fig. 4A). In addition, apoptosis occurred as early as 6 h in Granta-519 and SUDHL10 and 24 h in U-2932 cells (Fig. 4B) indicating apoptosis induction is in a time-dependent manner in different B-NHL subtypes. Together, the data demonstrate that inhibition of PI3K/mTOR by PF-04691502 leads to apoptosis in aggressive B-NHL cells.

PF-04691502 inhibits cell cycle progression by down-regulating cyclin D1 in aggressive B-NHL cells. The PI3K/Akt/mTOR pathway plays an important role in cell cycle progression. PF-04691502 has been shown to induce G1 phase arrest in glioblastoma and hepatoma cells (30,36). To examine cell cycle progression in B-NHL, we treated U-2932,

Granta-519 and SUDHL-10 cells with PF-04691502 at 0.5, 1.0 and 2.0 μM for 48 h and DNA content was evaluated using flow cytometry (Fig. 5A and B). Treatment of B-NHL cells with PF-04691502 strongly increased G0/G1 and decreased S phase populations (Fig. 5A and B). Since cyclin D1 is a critical player in the G1/S cell cycle progression and is regulated by the PI3K pathway, we evaluated it in a representative cell line Granta-519 which highly expresses cyclin D1. Fig. 5C clearly showed that PF-04691502 inhibited cyclin D1 at the protein level which is consistent with the G1 arrest observed in B-NHL cells.

PF-04691502 in combination with rituximab enhances apoptosis in B-NHL cells. Rituximab is often used as part of the initial treatment or as part of a second-line regimen for treating non-Hodgkin lymphoma patients either by itself or along with chemotherapy. It has been reported that rituximab diminishes the constitutive activity of the PI3K/Akt signaling pathway and increases chemo-sensitization to drug-induced apoptosis (43). Here we treated U-2932, Granta-519 and SUDHL-10 cells with PF-04691502 at 0.2, 0.5 and 1.0 μM alone or in combination with 10 μg/ml of rituximab for 48 h. Apoptosis was evaluated after Annexin V and PI staining and the results clearly showed rituximab plus PF-04691502 enhanced apoptosis significantly (P<0.05) compared with rituximab or PF-04691502 alone (Fig. 6), suggesting rituximab increases the antitumor activity of PI3K/mTOR inhibitor in B-cell NHL cells.

Discussion

Most aggressive B-cell non-Hodgkin lymphomas (B-NHL) are not curable with current chemo-immunotherapy combi-
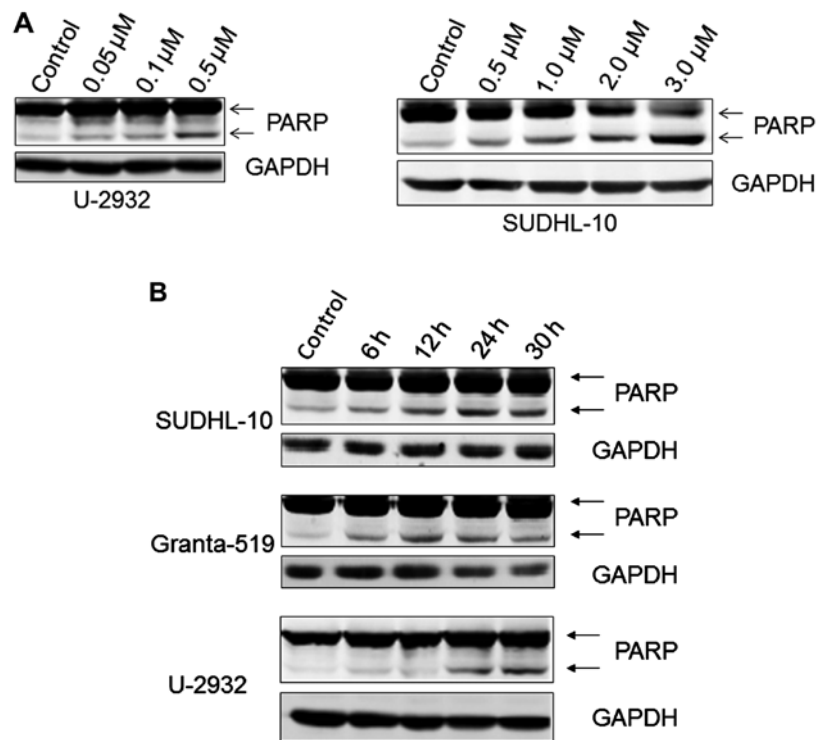
Figure 4. PF-04691502 induces PARP cleavage in B-NHL cells. (A) U-2932 and SUDHL-10 cells were not treated or treated with PF-04691502 at different concentrations as indicated for 24 h. (B) SUDHL-10, Granta-519 and U-2932 cells were treated with 0.5 μM of PF-04691502 for 6, 12, 24 and 30 h. Apoptosis was evaluated by immunoblotting to detect PARP cleavage with an anti-PARP antibody. GAPDH was used as a loading control.

nations (17). The PI3K/Akt/mTORC signaling pathway is frequently dysregulated in B-NHL (44), promoting the evaluation of novel small molecule inhibitors as an alternative treatment strategy. Indeed, disruption of PI3K and mTOR activity is now accepted as a therapeutic concept in hematologic malignancies and multiple agents are currently being investigated in various stages of clinical trials (44). In the present study, we demonstrate that PF-04691502, a novel selective dual PI3K/mTOR inhibitor, potently suppresses cell proliferation, induces G1 cell cycle arrest by inhibiting cyclin D1 protein level, and promotes apoptosis associated with suppression of Akt and S6 ribosomal protein activity in aggressive B-NHL cells including DLBCL and MCL. PF-04691502 has been shown to have potent antitumor activity in hepatocellular carcinoma cells (30), nasopharyngeal carcinoma (31) and colorectal cancer (34). A phase I trial evaluation of PF-04691502 has also been done in patients with advanced solid tumors which demonstrated that PF-04691502 was tolerable at 8 mg orally once a day, with a safety profile similar to other PI3K/mTOR inhibitors (35). However, no studies have been performed to evaluate PF-04691502 activity in aggressive B-NHL. Our results here support the inhibition of PI3K/mTOR by PF-04691502 may represent a novel therapeutic strategy that warrants clinical trial evaluation in aggressive B-NHL.

CAL-101, a potent and selective p110δ inhibitor, has been extensively studied and shown to have pre-clinical and clinical activity in lymphoid malignancies, such as CLL (44). Compared with PF-04691502, CAL-101 is less effective in suppressing cell proliferation in B-NHL (Table I), suggesting a pan-PI3K inhibitor is likely to be more active than an isoform selective inhibitor for the treatment of B-NHL. The most likely explanation is that other PI3K class I isoforms such as p110α, p110β and p110γ exist in an activated form leading to resistance. In fact, all of the PI3K class I isoforms are expressed and active in a variety of aggressive B-NHL (4,44). Additionally PF-04691502 also inhibits mTOR which is also a therapeutic target in B-NHL (44). Notably, INK-128, a potent and selective mTOR inhibitor has more activity than PF-04691502 in B-NHL cells (Table I). Further preclinical and clinical studies are needed to determine whether selective mTOR inhibitors have a therapeutic advantage over dual PI3K/mTOR or selective PI3K inhibitors.

The PI3K/Akt/mTOR signaling has been implicated to regulate both G1/S and G2/M transition. Inhibition of PI3K by LY294002 or other small molecular inhibitors and mTOR by rapamycin has been demonstrated to induce G1 cell cycle arrest in human malignances (42,45). However, recently LY294002 has been shown to block G2/M transition in retinal cells (46). The novel PI3K/mTOR inhibitors, GDC-0980, GDC-0941 and PF-04691502 have been reported to induce G1 cell cycle arrest in breast, lung, glioblastoma and hepatocellular carcinoma cells (30,36,47,48). Similarly, our data in this study show inhibition of PI3K/mTOR by PF-04691502 markedly blocked B-NHL cells from entering S phase (Fig. 5A and B). Cyclin D1, is an oncogene that regulates the G1-S cell cycle transition in B-NHL, particularly in MCL where it is overexpressed due to the t(11;14) (q13;q32) chromosomal translocation. Overexpression of constitutively active Akt extends the half-life of cyclin D1 protein whereas treatment with the PI3K inhibitor accelerated its degradation (45,49). Consistent with these findings,
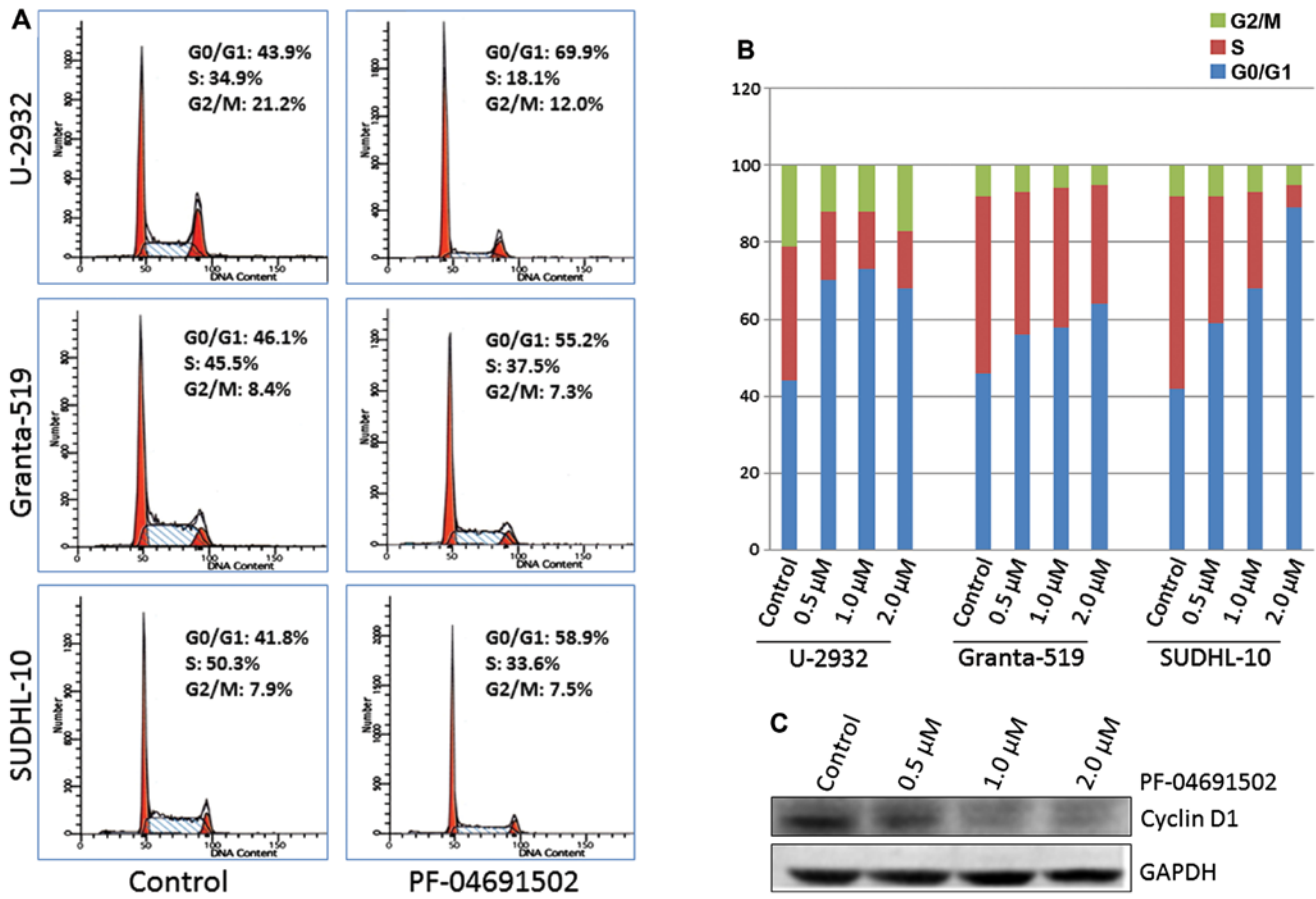
Figure 5. PF-04691502 induces G1 arrest and decreased cyclin D1 in B-NHL cell lines. (A) U-2932, Granta-519 and SUDHL-10 cells were treated with PF-04691502 at 0.5 μM for 48 h. Samples were harvested and stained with propidium iodide. DNA content was analyzed by flow cytometry. x-axis, DNA content; y-axis, cell numbers. Percentages of G0/G1, S and G2/M cell populations analyzed by ModFit software are indicated. (B) U-2932, Granta-519 and SUDHL-10 cells were treated with PF-04691502 at 0.5, 1.0 and 2.0 μM for 48 h. Cell cycle was analyzed and presented on the graph. (C) Granta-519 was treated with PF-04691502 for 48 h at 0.5, 1.0 and 2.0 μM. Cyclin D1 protein level was evaluated by immunoblotting.
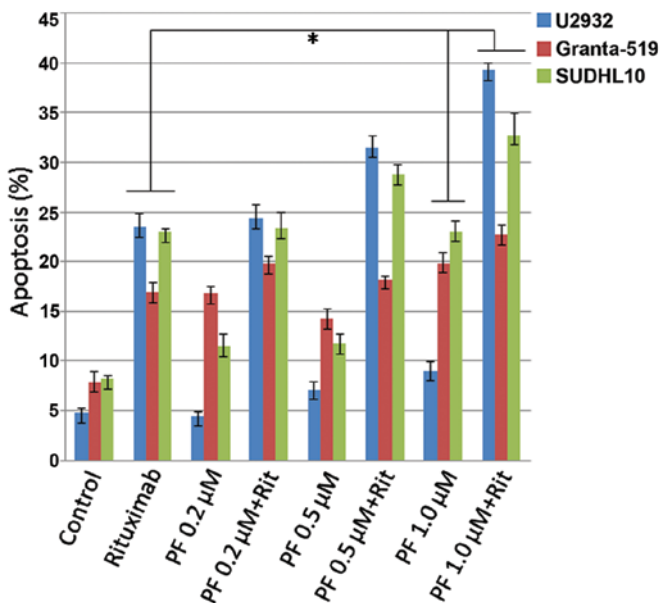

Figure 6. Rituximab enhances apoptosis induced by PF-04691502 in aggressive B-NHL. U-2932, Granta-519 and SUDHL-10 cells were not treated or treated with 10 μg/ml of rituximab, 0.2, 0.5 and 1.0 μM of PF-04691502 alone or 0.2, 0.5 and 1.0 μM of PF-04691502 combined with 10 μg/ml of rituximab, respectively for 48 h. Apoptosis was evaluated by flow cytometry after Annixin V and PI staining. The graph represents the mean percentage of apoptosis ± SD; (n=3). *P<0.05.

PF-04691502 inhibits Akt and decreases cyclin D1 protein in B-NHL (Fig. 5C).

Rituximab is a chimeric monoclonal antibody against the protein CD20 and is often used for treating leukemias and lymphomas either by itself or along with chemotherapy. Rituximab has been demonstrated to inhibit the constitutively activated Akt pathway in B-NHL cell lines, and this inhibition contributes to sensitization of drug-resistant cells to apoptosis by chemotherapeutic drugs (43). In this study, we found combination of PF-04691502 and rituximab significantly increases apoptosis in all tested B-NHL cell lines compared to a single agent treatments (Fig. 6), implying this combination may have better efficacy to treat lymphoma patients. In fact, several PI3K inhibitors including Idelalisib and XL765 (SAR245409) plus rituximab are entering clinical trials in NHL or CLL (44). Results from these trials are eagerly awaited.

In conclusion, our findings indicate that inhibition of the PI3K/mTOR pathway is an excellent therapeutic strategy for aggressive B-NHL. PF-04691502, a dual PI3K/mTOR inhibitor had strong activity in reducing tumor cell proliferation, enhancing apoptosis and induction of G1 cell cycle arrest in aggressive B-NHL. Inhibition of PI3K/mTOR pathway by PF-04691502 was confirmed by decreased phosphorylation of Akt and S6 ribosomal protein. Finally, combining PF-04691502 with rituximab increased apoptosis and may

be a synthetic lethal interaction. These results suggest that PF-04691502 with and without rituximab should be examined further as a potential therapeutic strategy for patients with aggressive B-cell NHL.

Acknowledgements

References

1. Engelman JA: Targeting PI3K signalling in cancer: Opportunities, challenges and limitations. Nat Rev Cancer 9: 550-562, 2009.
2. Yuan TL and Cantley LC: PI3K pathway alterations in cancer: Variations on a theme. Oncogene 27: 5497-5510, 2008.
3. Vanhaesebroeck B, Stephens L and Hawkins P: PI3K signalling: The path to discovery and understanding. Nat Rev Mol Cell Biol 13: 195-203, 2012.
4. Blachly JS and Baiocchi RA: Targeting PI3-kinase (PI3K), AKT and mTOR axis in lymphoma. Br J Haematol 167: 19-32, 2014.
5. Foster FM, Traer CJ, Abraham SM and Fry MJ: The phosphoinositide (PI) 3-kinase family. J Cell Sci 116: 3037-3040, 2003.
6. Chen HC and Guan JL: Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase. Proc Natl Acad Sci USA 91: 10148-10152, 1994.
7. Izuhara K, Feldman RA, Greer P and Harada N: Interleukin-4 induces association of the c-fes proto-oncogene product with phosphatidylinositol-3 kinase. Blood 88: 3910-3918, 1996.
8. Leslie NR and Downes CP: PTEN: The down side of PI 3-kinase signalling. Cell Signal 14: 285-295, 2002.
9. Engelman JA, Luo J and Cantley LC: The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev Genet 7: 606-619, 2006.
10. Sarbassov DD, Guertin DA, Ali SM and Sabatini DM: Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307: 1098-1101, 2005.
11. Kang S, Denley A, Vanhaesebroeck B and Vogt PK: Oncogenic transformation induced by the p110beta, -gamma, and -delta isoforms of class I phosphoinositide 3-kinase. Proc Natl Acad Sci USA 103: 1289-1294, 2006.
12. Chang HW, Aoki M, Fruman D, Auger KR, Bellacosa A, Tsichlis PN, Cantley LC, Roberts TM and Vogt PK: Transformation of chicken cells by the gene encoding the catalytic subunit of PI 3-kinase. Science 276: 1848-1850, 1997.
13. Jiang BH, Zheng JZ, Aoki M and Vogt PK: Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. Proc Natl Acad Sci USA 97: 1749-1753, 2000.
14. Chang F, Lee JT, Navolanic PM, Steelman LS, Shelton JG, Blalock WL, Franklin RA and McCubrey JA: Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: A target for cancer chemotherapy. Leukemia 17: 590-603, 2003.
15. Courtney KD, Corcoran RB and Engelman JA: The PI3K pathway as drug target in human cancer. J Clin Oncol 28: 1075-1083, 2010.
16. Rodon J, Dienstmann R, Serra V and Tabernero J: Development of PI3K inhibitors: Lessons learned from early clinical trials. Nat Rev Clin Oncol 10: 143-153, 2013.
17. Mahadevan D and Fisher RI: Novel therapeutics for aggressive non-Hodgkin's lymphoma. J Clin Oncol 29: 1876-1884, 2011.
18. Fang X, Zhou X and Wang X: Clinical development of phosphatidylinositol 3-kinase inhibitors for non-Hodgkin lymphoma. Biomark Res 1: 30, 2013.
19. Psyrri A, Papageorgiou S, Liakata E, Scorilas A, Rontogianni D, Kontos CK, Argyriou P, Pectasides D, Harhalakis N, Pappa V, et al: Phosphatidylinositol 3'-kinase catalytic subunit alpha gene amplification contributes to the pathogenesis of mantle cell lymphoma. Clin Cancer Res 15: 5724-5732, 2009.
20. Pfeifer M, Grau M, Lenze D, Wenzel SS, Wolf A, Wollert-Wulf B, Dietze K, Nogai H, Storek B, Madle H, et al: PTEN loss defines a PI3K/AKT pathway-dependent germinal center subtype of diffuse large B-cell lymphoma. Proc Natl Acad Sci USA 110: 12420-12425, 2013.
21. Baohua Y, Xiaoyan Z, Tiecheng Z, Tao Q and Daren S: Mutations of the PIK3CA gene in diffuse large B cell lymphoma. Diagn Mol Pathol 17: 159-165, 2008.
22. Zhao MY, Auerbach A, D'Costa AM, Rapoport AP, Burger AM, Sausville EA, Stass SA, Jiang F, Sands AM, Aguilera N, et al: Phospho-p70S6K/p85S6K and cdc2/cdk1 are novel targets for diffuse large B-cell lymphoma combination therapy. Clin Cancer Res 15: 1708-1720, 2009.
23. Choi MY and Kipps TJ: Inhibitors of B-cell receptor signaling for patients with B-cell malignancies. Cancer J 18: 404-410, 2012.
24. Inabe K and Kurosaki T: Tyrosine phosphorylation of B-cell adaptor for phosphoinositide 3-kinase is required for Akt activation in response to CD19 engagement. Blood 99: 584-589, 2002.
25. Gupta M, Hendrickson AE, Yun SS, Han JJ, Schneider PA, Koh BD, Stenson MJ, Wellik LE, Shing JC, Peterson KL, et al: Dual mTORC1/mTORC2 inhibition diminishes Akt activation and induces Puma-dependent apoptosis in lymphoid malignancies. Blood 119: 476-487, 2012.
26. Bhende PM, Park SI, Lim MS, Dittmer DP and Damania B: The dual PI3K/mTOR inhibitor, NVP-BEZ235, is efficacious against follicular lymphoma. Leukemia 24: 1781-1784, 2010.
27. Uddin S, Hussain AR, Siraj AK, Manogaran PS, Al-Jomah NA, Moorji A, Atizado V, Al-Dayel F, Belgaumi A, El-Solh H, et al: Role of phosphatidylinositol 3'-kinase/AKT pathway in diffuse large B-cell lymphoma survival. Blood 108: 4178-4186, 2006.
28. Mahadevan D, Chiorean EG, Harris WB, Von Hoff DD, Stejskal-Barnett A, Qi W, Anthony SP, Younger AE, Rensvold DM, Cordova F, et al: Phase I pharmacokinetic and pharmacodynamic study of the pan-PI3K/mTORC vascular targeted pro-drug SF1126 in patients with advanced solid tumours and B-cell malignancies. Eur J Cancer 48: 3319-3327, 2012.
29. Cheng H, Li C, Bailey S, Baxi SM, Goulet L, Guo L, Hoffman J, Jiang Y, Johnson TO, Johnson TW, et al: Discovery of the highly potent PI3K/mTOR dual inhibitor PF-04979064 through structure-based drug design. ACS Med Chem Lett 4: 91-97, 2013.
30. Wang FZ, Peng-Jiao, Yang NN, Chuang-Yuan, Zhao YL, Liu QQ, Fei HR and Zhang JG: PF-04691502 triggers cell cycle arrest, apoptosis and inhibits the angiogenesis in hepatocellular carcinoma cells. Toxicol Lett 220: 150-156, 2013.
31. Wong CH, Loong HH, Hui CW, Lau CP, Hui EP, Ma BB and Chan AT: Preclinical evaluation of the PI3K-mTOR dual inhibitor PF-04691502 as a novel therapeutic drug in nasopharyngeal carcinoma. Invest New Drugs 31: 1399-1408, 2013.
32. Devereaux K, Dall'Armi C, Alcazar-Roman A, Ogasawara Y, Zhou X, Wang F, Yamamoto A, De Camilli P and Di Paolo G: Regulation of mammalian autophagy by class II and III PI 3-kinases through PI3P synthesis. PLoS One 8: e76405, 2013.
33. Carver BS, Chapinski C, Wongvipat J, Hieronymus H, Chen Y, Chandarlapaty S, Arora VK, Le C, Koutcher J, Scher H, et al: Reciprocal feedback regulation of PI3K and androgen receptor signaling in PTEN-deficient prostate cancer. Cancer Cell 19: 575-586, 2011.
34. Fang DD, Zhang CC, Gu Y, Jani JP, Cao J, Tsaparikos K, Yuan J, Thiel M, Jackson-Fisher A, Zong Q, et al: Antitumor efficacy of the dual PI3K/mTOR inhibitor PF-04691502 in a human xenograft tumor model derived from colorectal cancer stem cells harboring a PIK3CA mutation. PLoS One 8: e67258, 2013.
35. Britten CD, Adjei AA, Millham R, Houk BE, Borzillo G, Pierce K, Wainberg ZA and LoRusso PM: Phase I study of PF-04691502, a small-molecule, oral, dual inhibitor of PI3K and mTOR, in patients with advanced cancer. Invest New Drugs 32: 510-517, 2014.
36. Yuan J, Mehta PP, Yin MJ, Sun S, Zou A, Chen J, Rafidi K, Feng Z, Nickel J, Engebretsen J, et al: PF-04691502, a potent and selective oral inhibitor of PI3K and mTOR kinases with antitumor activity. Mol Cancer Ther 10: 2189-2199, 2011.
37. Ikeda H, Hideshima T, Fulciniti M, Perrone G, Miura N, Yasui H, Okawa Y, Kiziltepe T, Santo L, Vallet S, et al: PI3K/p110{delta} is a novel therapeutic target in multiple myeloma. Blood 116: 1460-1468, 2010.
38. Lannutti BJ, Meadows SA, Herman SE, Kashishian A, Steiner B, Johnson AJ, Byrd JC, Tyner JW, Loriaux MM, Deininger M, et al: CAL-101, a p110delta selective phosphatidylinositol-3-kinase inhibitor for the treatment of B-cell malignancies, inhibits PI3K signaling and cellular viability. Blood 117: 591-594, 2011.

39. Herman SE, Gordon AL, Wagner AJ, Heerema NA, Zhao W, Flynn JM, Jones J, Andritsos L, Puri KD, Lannutti BJ, *et al*: Phosphatidylinositol 3-kinase-δ inhibitor CAL-101 shows promising preclinical activity in chronic lymphocytic leukemia by antagonizing intrinsic and extrinsic cellular survival signals. Blood 116: 2078-2088, 2010.

40. García-García C, Ibrahim YH, Serra V, Calvo MT, Guzmán M, Grueso J, Aura C, Pérez J, Jessen K, Liu Y, *et al*: Dual mTORC1/2 and HER2 blockade results in antitumor activity in preclinical models of breast cancer resistant to anti-HER2 therapy. Clin Cancer Res 18: 2603-2612, 2012.

41. Lou HZ, Weng XC, Pan HM, Pan Q, Sun P, Liu LL and Chen B: The novel mTORC1/2 dual inhibitor INK-128 suppresses survival and proliferation of primary and transformed human pancreatic cancer cells. Biochem Biophys Res Commun 450: 973-978, 2014.

42. Kampa-Schittenhelm KM, Heinrich MC, Akmut F, Rasp KH, Illing B, Döhner H, Döhner K and Schittenhelm MM: Cell cycle-dependent activity of the novel dual PI3K-MTORC1/2 inhibitor NVP-BGT226 in acute leukemia. Mol Cancer 12: 46, 2013.

43. Suzuki E, Umezawa K and Bonavida B: Rituximab inhibits the constitutively activated PI3K-Akt pathway in B-NHL cell lines: Involvement in chemosensitization to drug-induced apoptosis. Oncogene 26: 6184-6193, 2007.

44. Jabbour E, Ottmann OG, Deininger M and Hochhaus A: Targeting the phosphoinositide 3-kinase pathway in hematologic malignancies. Haematologica 99: 7-18, 2014.

45. Gao N, Zhang Z, Jiang BH and Shi X: Role of PI3K/AKT/mTOR signaling in the cell cycle progression of human prostate cancer. Biochem Biophys Res Commun 310: 1124-1132, 2003.

46. Ornelas IM, Silva TM, Fragel-Madeira L and Ventura AL: Inhibition of PI3K/Akt pathway impairs G2/M transition of cell cycle in late developing progenitors of the avian embryo retina. PLoS One 8: e53517, 2013.

47. Wallin JJ, Edgar KA, Guan J, Berry M, Prior WW, Lee L, Lesnick JD, Lewis C, Nonomiya J, Pang J, *et al*: GDC-0980 is a novel class I PI3K/mTOR kinase inhibitor with robust activity in cancer models driven by the PI3K pathway. Mol Cancer Ther 10: 2426-2436, 2011.

48. Zou ZQ, Zhang LN, Wang F, Bellenger J, Shen YZ and Zhang XH: The novel dual PI3K/mTOR inhibitor GDC-0941 synergizes with the MEK inhibitor U0126 in non-small cell lung cancer cells. Mol Med Rep 5: 503-508, 2012.

49. Diehl JA, Cheng M, Roussel MF and Sherr CJ: Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev 12: 3499-3511, 1998.