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LETTER TO THE EDITOR

Dual inhibition of the PI3K/mTOR and MAPK pathways in Non-Hodgkin Lymphoma

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Short title: Dual inhibition of the PI3K and MAPK pathways

NHL subtypes are classified based on clinical and histologic characteristics, as well as lymphocyte developmental stage. NHL sub-types include diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), mantle cell lymphoma (MCL), Burkitt lymphoma (BL), primary effusion lymphoma (PEL) to name just a few. Many lymphomas such as DLBCL, MCL, and AIDS-associated lymphomas are very aggressive and rapidly fatal if unresponsive to therapy. Indolent lymphomas, such as FL are also many times not curable with conventional chemotherapy. Clearly, novel treatment paradigms are needed to improve clinical outcomes of patients with NHL.

We had previously shown that the phosphatidylinositol 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) pathway was important for NHL survival and that single and dual inhibitors of this pathway effectively inhibited

NHL proliferation *in vitro* and in mouse xenograft models of NHL including FL and PEL ^{1,2}.

Another pathway that is linked to tumorigenesis is the mitogen activated protein kinase (MAPK), and many sub-types of NHL display an activated MAPK pathway ³⁻⁵.

Although conventional chemotherapeutic drugs are useful in some cases, a significant number of NHL patients will eventually relapse and develop resistance to chemotherapy, which necessitates determining additional therapeutics. Since both PI3K and MAPK pathways are activated in NHL, we decided to investigate combination therapy involving inhibition of both pathways. It has been demonstrated that such therapy is effective against certain solid tumors ^{6,7}.

In this study we used a combination of NVP-BEZ234 and AZD6244. NVP-BEZ235 is an imidazo[4,5-c]quinoline derivative compound, shown to inhibit PI3K and mTOR kinase activity both *in vitro* and *in vivo* including PEL and FL ^{8,9}. AZD6244 is an inhibitor of MEK1 and MEK2 that does not compete with ATP binding. It is a potent and selective MEK1/2 inhibitor that demonstrates high activity in solid tumor models *in vitro* and *in vivo* ¹⁰.

Representative types of NHL cells, including SUDHL4 (DLBCL), FL-18 (FL), Raji (BL), Ramos (BL), and BC-1 (PEL), were treated with 0.1 nM, 1 nM, 10 nM, 100 nM, 1000 nM (1 μ M), and 10,000 nM (10 μ M) of the MEK inhibitor, AZD6244, either alone or in combination with 10 nM of the PI3K/mTOR inhibitor, NVP-BEZ235 for 72 hours (Figure 1a). The cells were also treated with 10 nM NVP-BEZ235 alone. An MTS assay was performed similar to what we previously described ^{1,2}. Each experiment was done in triplicate and standard deviation was calculated. The amount of NVP-BEZ235 was kept constant at 10

nM, and increasing doses of AZD6244 was tested in combination with NVP-BEZ235. In each case, we observed that AZD6244 by itself did not have any discernable effect on cell viability in cell culture, even at the highest dose of 10 μ M AZD6244 (Figure 1a). Moreover, the combination of different amounts of AZD6244 with 10 nM NVP-BEZ235 did not affect cell viability when compared to 10 nM NVP-BEZ235 alone in each case (Figure 1a). A slight decrease in cell viability was only seen at the highest dose of AZD6244 (10 μ M) with 10 nM NVP-BEZ235. This demonstrates that the combined inhibition of the PI3K and MAPK pathways was neither additive nor synergistic.

To confirm that the drug AZD6244 used in our experiments had similar activity to that recorded in previous reports ¹¹, we evaluated the effect of AZD6244 on the COLO-205 colorectal cancer cell line by culturing these cells in increasing concentrations of AZD6244 or vehicle for 72 hours. We determined an IC₅₀ of 107.18 ± 2.47 nM in COLO-205 (Figure 1a). Similar results were seen with another non-small cell lung cancer cell line Calu-6, that was previously shown to be inhibited by AZD6244 ¹¹ (data not shown). We also evaluated NHL cell viability in the presence of increasing concentrations of the chemically unrelated MEK inhibitor, PD184352 ¹². We found that PD184352 was ineffective at reducing cell viability in various NHL cell lines except at the highest dose of 10 μ M (data not shown). This data supports our observations made with the MEK inhibitor, AZD6244, and suggests that NHL cells are not addicted to the MEK/ERK pathway for survival.

To verify that each of the drugs was inhibiting their respective targets, we performed Western blot analysis on the NHL cells that were treated with the above therapies for 24 hours (Figure 1b). We used phosphorylated S6K as a downstream marker for active PI3K/mTOR as we previously described ^{1,2}, and

we used phosphorylated ERK1/2 as a downstream marker for MAPK activation¹⁰. We found that NVP-BEZ235 inhibited PI3K signaling as measured by reduced phosphorylated S6K levels, and that AZD6244 inhibited MAPK signaling as measured by reduced ERK1/2 phosphorylation (Figure 1b).

To determine whether a PI3K inhibitor only would be synergistic with AZD6244, we evaluated the combination of 25 μ M CAL-101 (a PI3K inhibitor) with increasing doses of AZD6244 on NHL cell viability. CAL-101 (5-fluoro-3-phenyl-2-[(S)-1-(9H-purin-6-ylamino)-propyl]-3H-quinazolin-4-one) is a small molecule inhibitor of the class I PI3K p110 δ isoform¹³_ENREF_11. CAL-101 has been found to have anti-tumor activity in patients with indolent NHL and hence we sought to investigate whether inhibiting PI3K p110 δ and MAPK may be synergistic against NHL¹⁴. Figure 1c depicts a representative NHL cell line (Ramos) that was tested with CAL-101 and AZD6244. We did not observe a synergistic or additive effect when different amounts of AZD6244 was combined with 25 μ M CAL-101 compared to 25 μ M CAL-101 alone (Figure 1c), although each drug appeared to be effective in inhibiting downstream effectors of the pathway. Treatment with CAL-101 inhibited phosphorylation of Akt and S6K, and AZD6244 inhibited phosphorylation of ERK1/2 (Figure 1d). We found similar results for FL-18 and Raji cells (data not shown). Thus, neither a dual inhibitor of the PI3K/mTOR pathway (NVP-BEZ235) nor a single inhibitor of PI3K (CAL-101) appear to synergize with AZD6244 in our system.

We next investigated the combination of NVP-BEZ235 and AZD6244 in a mouse xenograft model². Four-to-five-week-old athymic nude-Foxn1^{nu} female mice were injected intraperitoneally with anti-asialo GM1 antibody (Wako). The following day, 1x10⁶ FL-18 lymphoma cells were subcutaneously injected with growth-factor reduced matrigel as we previously described².

Upon development of palpable tumors, the animals were split into four groups and treated by oral gavage 6 days a week with either vehicle, 45 mg/kg NVP-BEZ235, 45 mg/kg AZD6244, or 25mg/kg each of NVP-BEZ235 and AZD6244. While tumors grew in the mice treated with vehicle, mice treated with NVP-BEZ235 showed a significant inhibition of tumor growth as we previously reported¹⁰. Interestingly, in the xenograft model, mice treated with AZD6244 alone did show a modest inhibition of tumor growth compared to vehicle treated mice. However, the combination of NVP-BEZ235 and AZD6244 did not have a synergistic or additive effect on tumor growth compared to NVP-BEZ235 or AZD6244 alone (Figure 2a).

The mice were sacrificed when maximal tumor size was reached. Tumors were excised, sectioned, and subjected to immunohistochemical analysis as we previously described¹. S6 was consistently phosphorylated in almost all cells in the vehicle control group, however we found reduced phosphorylated levels of S6, a downstream effector of the PI3K pathway in the NVP-BEZ235 treated grafts, and reduced phosphorylated ERK1/2, an effector of the MAPK pathway in the AZD6244 treated grafts (Figure 2b). Both markers were reduced in animals treated with both drugs. This suggests that both drugs were delivered to the tumors and inhibited their respective targets in the tumor cells *in vivo*. Overall, our data demonstrate that the combined inhibition of the PI3K and MAPK pathways was not synergistic or additive in preventing tumor growth of NHL cells *in vitro* or *in vivo*.

Currently, there are a number of clinical trials testing MEK and PI3K or mTOR inhibitors in patients. Our data suggest that combining inhibitors specific for these two pathways may not be an effective treatment option for NHL since there was no preclinical evidence showing any significant enhancement of cell

death *in vitro* or inhibition of tumor growth *in vivo* when these inhibitors were combined. This is in direct contrast to reports in solid tumors such as melanoma and breast cancer in which the combination of these inhibitors showed a synergy in curtailing tumor growth in similar pre-clinical models ^{6,7}. Davies et al. found that tumors with activating Ras mutations are more sensitive to AZD6244 than tumors with WT Ras ¹¹. Since activating mutations in Ras are infrequent in NHL ¹⁵, inhibition of the MEK/ERK pathway may not be as effective for NHL as for solid tumors despite the fact that ERK was highly phosphorylated in the cell lines tested.

Even though MEK/ERK and PI3K/mTOR pathways are active in NHL ^{1,4,5}, our data suggests that NHL are more reliant on the PI3K/mTOR pathway than the MEK/ERK pathway for cell survival. Normal B-lymphocyte survival is also dependent on the PI3K pathway ¹⁶ and studies have shown that B-lymphocytes lacking BCR signaling do not survive while these cells can be rescued from apoptosis by being engineered to have constitutively active PI3K ¹⁶.

Although the MEK/ERK pathway may be important in NHL pathology, we found that targeting this pathway alone is not sufficient to reduce NHL cell survival *in vitro* or *in vivo*. There are multiple arms of the MAPK pathway ¹⁷ and to see additional benefit in NHL, we may need to modulate a different and/or multiple arms of this pathway along with PI3K/mTOR inhibition to see an enhancement in the reduction of NHL cell viability. Based on these results, we propose that further preclinical studies should be performed before a combination of MEK 1/2 and PI3K inhibitors is evaluated in a clinical trial setting for patients with NHL.

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FIGURE LEGENDS

Figure 1. Simultaneous inhibition of the PI3K/mTOR and MEK/ERK pathways fails to synergistically reduce NHL cell proliferation *in vitro*. (a)

Proliferation of NHL cell lines treated with drug(s) or vehicle were evaluated by MTS Assay using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions. Cells (1×10^5 /mL) were incubated with drug(s) or vehicle for 72 hours in a 12-well plate. Twenty microliters of MTS reagent was added to 100 μ L of cell suspension in a 96-well plate and incubated at 37°C for 1 hour. Percent of viable cells after 72 hours treatment is shown on the y-axis. The percent of viable cells for untreated cells was set as 100% for each sample. Elevated absorbance values are indicative of metabolically active cells. The COLO-205 cells (5×10^3 cells/100 μ L) were added to each well of a 96-well plate. Increasing concentrations of drug(s) or vehicle were added the following day. The MTS reagent was added after 72 hours following the addition of the drug(s) or vehicle, and incubated with the cells for 4 hours at 37°C. Absorbance was measured at 490nm using a FLUOstar OPTIMA spectrophotometer. Percent viable cells after 72 hours treatment is shown on the y-axis. The percent of viable cells for vehicle treated was set as 100% for each sample. Elevated absorbance values are indicative of metabolically active cells. (b) Phosphorylation of ERK 1/2 and S6 kinase were determined by Western blot of NHL cell lines treated with AZD6244 (1 μ M) and/or NVP-BEZ235 (30nM) for 24 hours. Equal volume of DMSO (0.1%) was added for vehicle control. (c) Proliferation of cells with 25 μ M CAL-101 and increasing concentrations of AZD6244 was determined by MTS assay after 48 hours following the addition of the drug(s) or vehicle as described in panel a. (d) Phosphorylation of Akt, ERK 1/2 and S6 kinase were determined by Western

blot of NHL cell lines treated with AZD6244 (1μM) and/or CAL-101 (25μM) for 24 hours.

Anders et al. Fig. 1

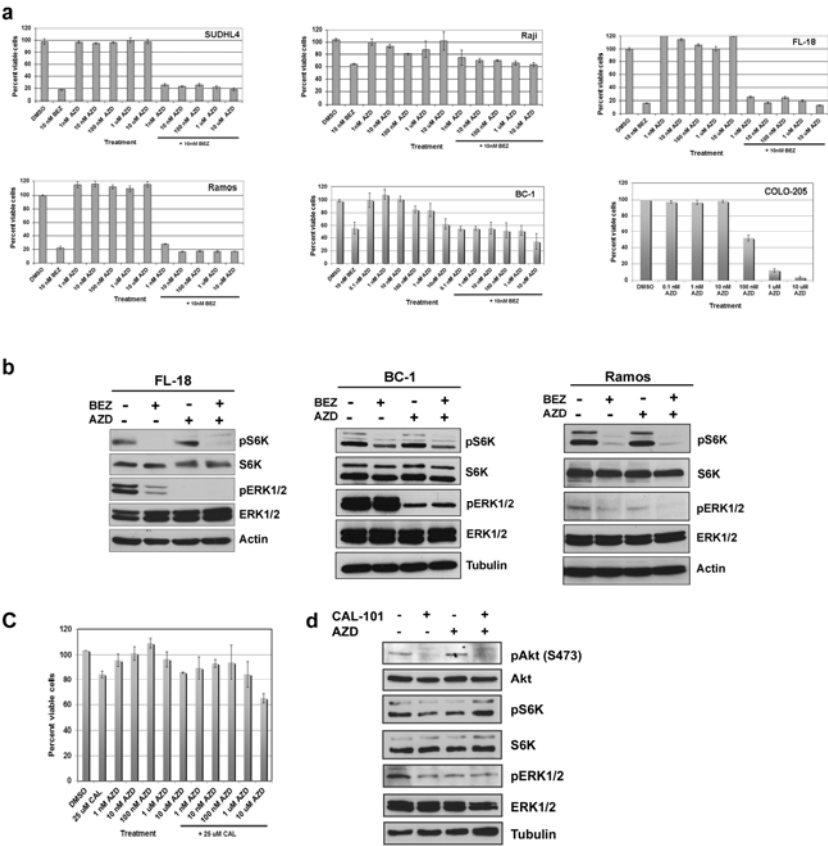


Figure 2. Combined inhibition of the MEK/ERK and PI3K/mTOR pathways is no more effective at reducing tumor burden than single blockade of the PI3K/mTOR pathway *in vivo*. **(a)** Athymic nude-Foxn1^{nu} 4-5-week-old female mice were injected subcutaneously into the right flank with 200 μ L (1×10^6) of FL-18 cells. Five mice per group were treated with equal volume of vehicle, 45 mg/kg NVP-BEZ235, 45 mg/kg AZD6244, or 25 mg/kg each of NVP-BEZ235 and AZD6244 (combination therapy). Treatment involved oral gavage 6 times per week. Tumor volume was determined daily (L x W x D) and plotted over time. Shown for each time point is a box and whisker plot of the results per group. The horizontal bar indicates the median, the box indicates 25% and 75% of the data and the whiskers represent 1.5 x the interquartile range, which encompasses 50% of the data. **(b)** Phosphorylation of ERK 1/2 or S6 protein from each treatment group was evaluated on paraffin-embedded tumors by immunohistochemical staining. Sections were counterstained with hematoxylin, and visualized at magnifications of 200X using a Leica DMLS microscope. Images were acquired using the Leica DFC480 camera and associated Leica Firecam software. Stored TIFF images were evaluated using Adobe Photoshop CS5.1.

