Activation of the MACC1/PIM/cMyc axis confers resistance to PI3K-mTOR inhibition in PIK3CA mutant NSCLC

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Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer mortality globally, having a 5 year survival rate of less than 15%. PI3K-mTOR signalling has been implicated in various hallmarks of cancer and is frequently dysregulated in NSCLC making it an ideal therapeutic target. Pan- and isoform-specific inhibitors of the PI3K pathway are currently being evaluated in clinical trials. However all patients treated thus far develop progressive disease due to the emergence of bypass signalling mechanisms. We developed a panel of lung cancer preclinical models to elucidate potential mechanisms of resistance to PI3K-mTOR inhibitor GDC-0980 (apitolisib). Resistant cells (H1975GR) were also less sensitive to PI3K-mTOR dual targeting inhibitor, BEZ235 compared to matched parent cells (H1975P) making them an ideal model to identify and interrogate drug resistance mechanisms. In-depth characterisation of the PIK3CA mutant positive H1975 sensitive cells (H1975P) versus apitolisib resistant cells (H1975GR) identified activation of MACC1 as an early marker of drug resistance and the subsequent activation of all three PIM kinases.

Methods

- The sensitivity of apitolisib resistant cells (H1975GR) versus age-matched parent cells (H1975P) to BEZ235 following a 72 hour treatment was compared using a Cell Titre Blue cell viability assay (n=3).
- Alterations to the mRNA expression profile of H1975GR versus H1975P were initially screened using the Human LncRNA Expression Array V4.0 (Arraystar Inc.) which profiles 20,730 mRNAs and 40,173 LncRNAs. edgeR was used to calculate counts per million (CPM), fold-change and p-values.
- Validation of activated JAK-STAT signalling was confirmed using the IL-6/STAT3 signalling-specific RT^2 gene profiler array (n=1). Selected genes from the array were validated by SYBR-based qPCR, immunofluorescence (IF) and western blot analysis (n=3-4).
- 11 miRNAs (regulated by or regulators of c-Myc/PIM) plus housekeeping control miRNAs were quantified in the H1975P/GR model by QPCR.
- IC50 values of a pan-PIM kinase inhibitor and novel PI3K/mTOR/PIM inhibitors IBL-301/IBL-302 inhibiting growth in H1975P versus H1975GR were compared using the Cell Titre Blue assay.
- All siRNA experiments were carried out using 25nM ON-TARGET *plus* SMARTpool siRNAs, Non-TARGET control and Dharmafect1 (Dharmacon).
- The effect of pan-PIM kinase, apitolisib and PI3K/mTOR/PIM inhibitors on MACC1 and c-Myc expression were examined in H1975P/GR cell lines by western blot analysis.

Results

Development of a PI3K/mTOR inhibitor-resistant cell line model:

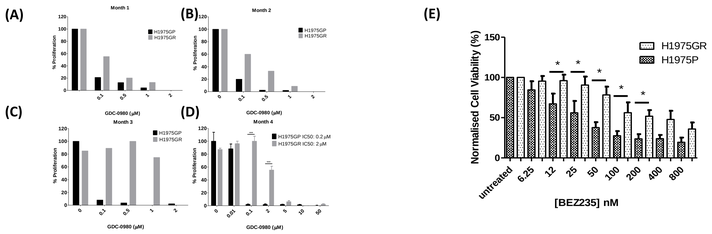

Figure 1: Apitolisib resistant cells, H1975GR, are also resistant to another PI3K-mTOR inhibitor BEZ235.
(A-D) A cell line model of acquired drug resistance to PI3K-mTOR inhibition was generated following several months of chronic treatment of H1975 with Apitolisib. At month 4 (D) IC50 values determined by BrdU cell proliferation assay were 2.2μM and 0.08μM for H1975GR and age-matched H1975P, respectively. (E) Similarly, cell viability dose response curves generated by the Cell Titre Blue assay indicated an increased resistance of H1975GR to the PI3K-mTOR inhibitor BEZ235 compared to H1975P following 72 hour treatment (IC50= 188.49nM vs. 25.38nM, n=3)(*p<0.05, paired student t-test, n=3).

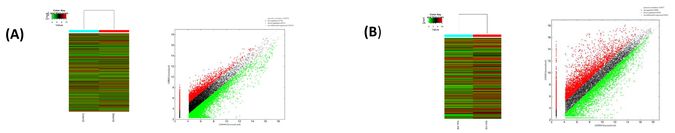

Figure 2: Comparison of mRNA expression in Apitolisib resistant cells, H1975GR versus sensitive cells H1975P
Heatmap and scatter-plot data of cell line models at month 1 (A) and month 4 (B) of acquired drug resistance to Apitolisib screened for differential mRNA and lncRNA using the Human LncRNA Expression Array V4.0 (Arraystar Inc.) which profiles 20,730 mRNAs and 40,173 LncRNAs.

Activation of MACC1/PIM/cMyc axis in Apitolisib resistant cells:

Gene Name	H1975GR vs H1975P (Month 1) Fold Change	H1975GR vs H1975P (Month 4) Fold Change
MACC1	77.89	44.89
PIM1	-	19.8
PIM2	1.69	3.3
PIM3	2.08	31.98
MYC	2.1	25.65
MET	1.65	6.06
HGF	10.06	2.2

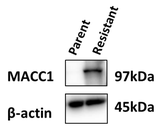

Table 1.0 Fold change of mRNA expression of MACC1 and its target genes in H1975GR versus H1975P at Month 1 & 4 of Apitolisib treatment.
Differential mRNA expression was quantified using the Human LncRNA Expression Array V4.0 (Arraystar Inc.). Validation of MACC1 expression in month 4 parent and apitolisib resistant cell lines by western blot analysis

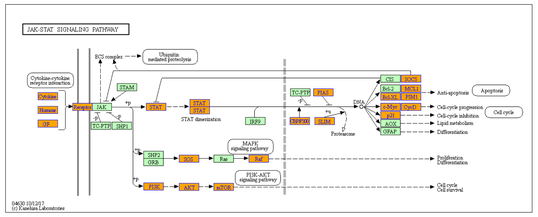

Figure 3.0 Pathway analysis of JAK-STAT signalling in H1975GR versus H1975P at Month 4 of Apitolisib treatment.
Pathway analysis of the JAK-STAT signalling (Kegg pathway) highlighting genes that are overexpressed in H1975GR cells versus H1975P were developed using Pathview (cut-off values used FC ≥ 1.5).

Validation of activated IL-6/STAT3 signaling in Apitolisib resistant cells:

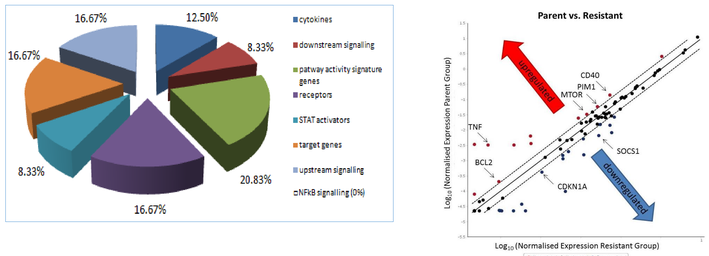

Figure 4: The gene expression profile of H1975GR versus H1975P was analysed using an IL-6/STAT3 pathway array.
mRNA expression of IL-6/STAT3 pathway-related genes was compared between H1975GR and age-matched H1975P. 22 genes involved in molecular functions of IL-6/STAT3 signalling were found to be differentially expressed between the two cell lines. As indicated on scatter plot, a number of genes altered by ≥2-fold in the array were chosen for further validation by qPCR .

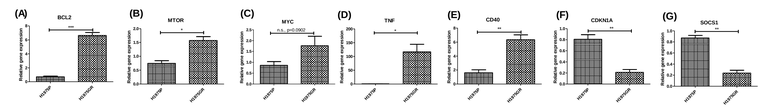

Figure 5: Apitolisib resistant cells, H1975GR, overexpress pro-survival and pro-inflammatory genes and under express negative regulators of cell cycle and protein synthesis.
Seven genes were chosen from the array for further validation by SYBR-based qPCR. There was a significant upregulation of (A) anti-apoptotic BCL2 (10.6-fold, p<0.001), (B) mTOR (2.5-fold, p<0.05), (C) MYC (2.04-fold, p=0.09) (D) TNF (>100-fold, p<0.05) and (E) the TNF receptor co-stimulatory molecule CD40 (3.6-fold, p<0.01), In contrast there was a downregulation of (F) CDKN1A (3.7-fold, P<0.01) and (G) SOCS1 (3.8-fold, P<0.01). *p<0.05, **p<0.01, ***p<0.001, paired student t-test, n=4.

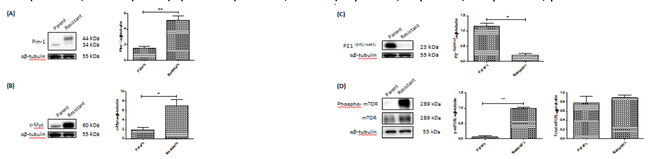

Figure 6: Protein levels of p21 are downregulated in H1975GR, while the levels of PIM1, c-Myc and phosphorylated mTOR are upregulated compared to age-matched H1975P.
(A) PIM1 and (B) c-MYC protein expression was upregulated in the H1975GR cells (both p<0.05) (C) Protein expression of p21$^{CIP1/WAF1}$ (i.e. CDKN1A) was largely suppressed in H1975GR compared to H1975P (D) Total mTOR protein was not significantly altered in H1975GR however an overexpression of phospho-mTOR was found (p<0.05). Additionally acquired resistance to PI3K-mTOR blockade resulted in increased phosphorylation of the PI3K/Akt/mTOR downstream signalling molecule S6 riboprotein (p<0.05). *p<0.05, paired student t-test, n=3.

Targeting PIM kinase alone & Co-targeting Strategies with PI3K/mTOR/PIM kinase inhibitors IBL-301/302 in H1975P/GR cells:

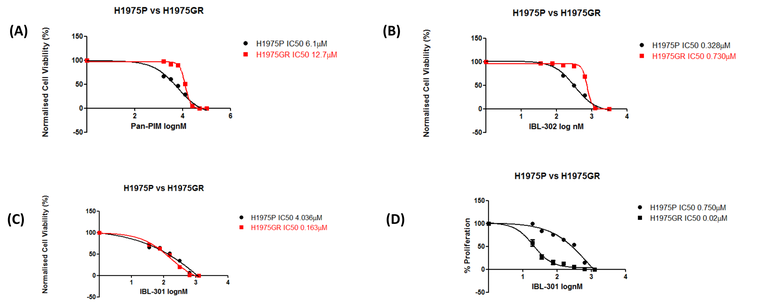

Figure 7: Co-targeting strategies with PI3K/mTOR/PIM inhibitors IBL-301/IBL-302 demonstrate greater efficacy at lower doses than a pan-PIM kinase inhibitor in Apitolisib sensitive/resistant cell lines
The cell viability of H1975P versus H1975GR cells were measured by cell titre blue assay following 72 hour treatment with a pan-PIM inhibitor (A), H1975P cells had a lower IC50 6.1μM than H1975GR cells IC50 12.7μM or IBL-302 (B) H1975P had an IC50 of 0.328μM and H1975GR had an IC50 of 0.730μM respectively. Finally the cells were treated with IBL-301 and measured by a cell titre blue assay (C) and BrdU assay (D) both demonstrating H1975GR cells are more sensitive to IBL-301 than H1975P cells at low doses of the triple inhibitor. Cell viability IC50 doses were determined by non-linear regression.

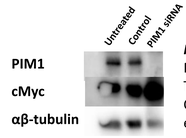

Figure 8: PIM1 siRNA knockdown activates cMyc expression in H1975GR cells.
PIM1 expression was inhibited by treating the H1975GR cells for 72 hours with 25nM ON-TARGET*plus* SMARTpool PIM1 siRNA (Dharmacon), cells were also treated with 25nM of ON-TARGET *plus* SMARTpool non-targeting control siRNA (Dharmacon). Proteins were extracted and examined for PIM1 and cMyc expression by western blot analysis.

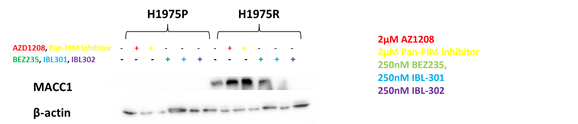

Figure 9: AZD1208 & a Pan-PIM inhibitor activates MACC1 & IBL-301 and IBL-302 inhibit MACC1 expression in H1975GR cells.
MACC1 expression was not detected in H1975P cells. Both pan-PIM inhibitors activated MACC1 expression while IBL-301 and IBL-302 inhibited MACC1 expression.

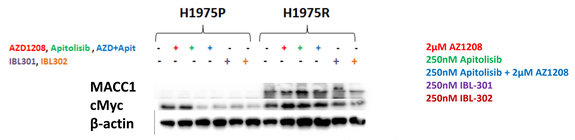

Figure 10: Pan-PIM inhibition activates cMyc & MACC1 expression & PI3K/mTOR/PIM kinase inhibition decreases cMyc and MACC1 expression.
MACC1 expression was not detected in H1975P cells. AZD1208 activates MACC1 & cMyc while Apitolisib decreases cMyc but not MACC1 expression. IBL-301 and IBL-302 inhibit both MACC1 and cMyc expression in H1975P and H1975GR cells.

Conclusion

Our group has developed a PI3K-mTOR inhibitor resistant NSCLC cell line model that demonstrates acquired resistance to both Apitolisib and BEZ235. This indicates the utility of this model to interrograte resistance mechanisms to other PI3K-mTOR inhbitors and is not limited to just Apitolisib. This study identifies an activated MACC1/PIM/cMyc axis as well as alterations in the IL-6/STAT3 signalling pathway contributing to resistance to PI3K-mTOR inhibition and these data may provide novel effective multi-targeted therapeutic strategies for lung cancer patients. Novel PI3K/mTOR/PIM inhibitors IBL-301/IBL-302 have shown promising in vitro data and may overcome resistance driven by MACC1 and PIM kinase and provide a more durable response in patients. These triple targeted therapies warrant further investigation as a therapeutic strategy for NSCLC.

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