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) (aprilisib). Resistant cells (H1975GR) were also less sensitive to PI3K-mTOR dual targeting inhibitor, BEZ233 compared to matched parent cells (H1975P) making them an ideal model to identify and interrogate drug resistance mechanisms. In-depth characterisation of the PI3K/mTOR mutant positive H1975 sensitive cells (H1975P) versus apoptosis 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 apoptosis resistant cells (H1975GR) versus age-matched parent cells (H1975P) to BEZ233 following 72 hour treatment was compared using a Cell Titre Blue cell viability assay (n=3).
• Alterations in 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. edeq2 was used to calculate coefficients.
• Validation of activated JAK-STAT signalling was confirmed using the IL-6/STAT3-signaling specific RT-gene profiler array (n=4). Selected genes from the array were validated by qSYBR-based qPCR, immunofluorescence (IF) and western blot analysis (n=3-4).
• 11 miRNAs (regulated by or regulators of c-Myc/PI3K) plus housekeeping control miRNAs were quantified in the H1975GR model by qPCR.
• ICSO values of a pan-PIM kinase inhibitor and novel PIM3/mTOR/PIM inhibitors IBL-301/IBL-302 inhibiting growth in H1975P versus H1975GR were compared using the Cell Titre Blue assay.
• All LncRNA experiments on ON-TARGETplus SMARTpool LncRNA, Non-TARGET control and DharmaCleav1 (Dharmacon).
• The effect of pan-PIM kinase, apoptosis 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: Apilisib resistant cells, H1975GR, are also resistant to another PI3K/mTOR inhibitor BEZ233. (A) A cell line model of acquired resistance to PI3K/mTOR inhibitors was generated over several months of chronic treatment of H1975 with Apilisib. At month 4 (D) IC50 values determined by BrdU cell proliferation assay were 2.1μM for H1975 and 1.0μM for H1975GR. At month 8 (D) similar, cell viability dose response was generated by the Cell Titre Blue assay indicating an increased resistance of H1975GR to the PI3K/mTOR inhibitor BEZ233 compared to H1975 following 72 hour treatment (ED50: 18.9μM vs 29.38μM, n=10, *p<0.05, paired student t-test, n=5).

Figure 2: Comparison of mRNA expression in Apilisib 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 Apilisib 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.

Figure 3: The gene expression profile of H1975GR versus H1975P was analysed using an IL-6/STAT3 pathway array. mRNA expression of 6-IL/6STAT3 pathway genes including SOCS1, SOCS3, SOCS5 and SOCS7 were downregulated in H1975GR and an increased expression of 12 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 4: Apilisib resistant cells, H1975GR, overexpress pro-survival and pro-inflammatory genes and under express 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) C/EBPα (2.10-fold, *p=0.001), (B) IL6R (2.93-fold, *p=0.001), (C) MMY (7.49-fold, *p=0.001), (D) MYC (2.04-fold, *p=0.001) and (E) SMAD7 (1.68-fold, *p=0.001). (F) There was a significant downregulation of (G) SOCS5 (4.63-fold, **p=0.001), (H) TNFAIP3 (1.45-fold, *p=0.05), and (I) IGFBP5 (1.34-fold, *p=0.05), **p=0.001, *p<0.01, paired student t-test, n=4.

Figure 5: Apilisib resistant cells, H1975GR, overexpress pro-survival and pro-inflammatory genes and under express regulators of cell cycle and protein synthesis. (A) mRNA and (B) c-Myc protein expression was upregulated in the H1975GR cells (both *p<0.05). (C) Expression protein of c-Myc and p-Myc was also upregulated in the H1975GR compared to H1975P (G). 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/mTOR downstream signalling molecules Akt (p-protein) (F) and p-Myc (G).

Figure 6: Protein levels of p21 are downregulated in H1975GR, while the levels of p21, c-Myc and phosphorylated mTOR are upregulated compared to age-matched sensitive H1975P.

Figure 7: Co-targeting strategies with PI3K/mTOR/PIM kinase inhibitors IBL-301/302 demonstrate greater efficacy at lower doses than pan-PIM kinase inhibitor and Apilisib sensitive/resistant cell lines.

Targeting PIM kinase alone & Co-targeting Strategies with PI3K/mTOR/PIM kinase inhibitors IBL-301/302 in H1975P/GR cells:
Results
Figure 8: PIM1 LNA knockdown assists cMyc expression in H1975GR cells. PIM expression was inhibited by treating the H1975GR cells for 72 hours with 25nM ON-TARGETplus PML1 LNA (Dharmacon), cells were also treated with 25nM of C-Myc INHIBITOR plus SMARTpool non-targeting control LNA (Dharmacon). Proteins were extracted and examined for PML and cMyc and cMyc expression by western blot analysis.

Figure 9: AD21208 & a Pan-PIM inhibitor activates MACC1 & IBL-301 & 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.

Conclusion
Our group has developed a PI3K/mTOR inhibitor resistant NSCLC cell line model that demonstrates acquired resistance to both Apolitisib and BEZ233. This indicates the utility of this model to interrogate resistance mechanisms to other PI3K/mTOR inhibitors and is not limited just to Apolitisib. This study identifies an active MACC1/PIM3/cMyC axis as well as alterations in the IL-6/STAT3 signalling pathway contributing to resistance to PI3K-mTOR inhibition and this data may provide novel effective multi-targeted therapeutic strategies for lung cancer patients. Novel PI3K/mTOR/PIM kinase inhibitors IBL-301/302 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.

This research is jointly funded by Infection Biosciences Ltd. and Enterprise Ireland.