Chapter 2: Materials and Methods

2.1 Cell culture

Cell lines were purchased from American Type Culture Collection (ATCC) or the Developmental Therapeutics Program at the National Cancer Institute. Cell lines were maintained RPMI 1640 (Invitrogen) supplemented with 10% foetal bovine serum (Invitrogen) and 1% penicillin-streptomycin-gentomycin (Invitrogen) in a humidified atmosphere at 37°C and 5% CO_2. Cell lines were subcultured twice a week at a ratio of 1:6 and 1:10 dependent on the confluency. Cell culture media was aspirated and the flask washed twice with 15ml phospho buffered saline (PBS). Cells were then detached using 10ml Trypsin-EDTA solution (SIGMA). This was then neutralised with 20ml of media and cells collected by centrifugation for 5minutes at 1500rpm.

2.2 Drug Assays

2.2.1 Cell plating

Cell lines of a known genetic background (Table 2-1) were maintained as stated above (2.1) in T-150cm^2 flasks (Costar). Prior to each experiment one 80-90% confluent flask was trypsinised and centrifuged as described above (2.1), the supernatant was aspirated and the cell pellet resuspended in 2ml of media and gently pipetted to prevent clumping of cells. 10µL of cell solution was pipetted onto a haemocytometer and cells counted. Approximately 1000-2500 cells were seeded in 6 replicates to 48-well tissue culture

plate depending on the growth rates of the cell lines in a volume of 350µl. The plates were then incubated for 24hrs at 37^{o}C.

Cell Line	Histology	LKB1	BRAF	KRAS	PIK3CA	P53	CDNKA	EGFR
		Mutation	Mutation	Mutation	Mutation	Mutation	Mutation	Mutation
SKMEL28	Malignant melanoma		c.1799T>A p.V600E			c.434_435TG>GT p.L145R		c.2257C>T p.P753S
CAL-12T	NSCLC nos	No protein[A]	c.1397G>T p.G466V			c.404G>T p.C135F	c.172C>T p.R58*	
A549	NSCLC nos	c.109C>T p.Q37*		c.34G>A p.G12S			c.1_471del471 p.M1_*157del	
NCI-H1734	NSCLC Adenocarcinoma	c.152_153insCT p.M51fs*14		c.37G>T p.G13C				
NCI-H460	NSCLC Large cell carcinoma	c.109C>T p.Q37*		c.183A>T p.Q61H	c.1633G>A p.E545K		c.1_457del457 p.?	
NCI-H2030	NSCLC Adenocarcinoma	c.949G>T p.E317*		c.34G>T p.G12C		c.785G>T p.G262V		
NCI-H1563	NSCLC Adenocarcinoma	c.816C>A p.Y272*					c.1_471del471 p.M1_*157del	
NCI-H2009	NSCLC Adenocarcinoma			c.35G>C p.G12A		c.818G>T p.R273L		
NCI-H1975	NSCLC Adenocarcinoma				c.353G>A p.G118D	c.818G>A p.R273H	c.205G>T p.E69*	c.2573T>G p.L858R
NCI-H1838	NSCLC Adenocarcinoma					c.818G>T p.R273L	c.1_471del471 p.M1_*157del	
NCI-H1792	NSCLC Adenocarcinoma			c.34G>T p.G12C		c.672+1G>A p.?		
NCI-H358	NSCLC Adenocarcinoma			c.34G>T p.G12C				
NCI-H661	NSCLC Large cell carcinoma					c.644G>T p.S215I c.473G>T p.R158L	c.457+1G>T p.?	
NCI-H226	NSCLC Squamous cell carcinoma						c.1_150del150 p.?	

Table 2-1 Genetic background and histology of the cell lines used in this study Mutation data taken from COSMIC (http://www.sanger.ac.uk/genetics/CGP/CellLines/). (A) No mutation has been found in this sample by sequencing, however immunoblot analysis revealed no protein present. NSCLC nos = NSCLC not otherwise specified.

2.2.2 Drug dilutions

CI-1040 was a kind gift from Richard Marais; 50mg was dissolved in 1ml DMSO (X) to give a stock solution of 110mM. Rapamycin was purchased from Sigma; 1mg was dissolved in 1ml DMSO to give a stock solution of 1.1mM. AMPK compound C was purchased from Merck as a 10mM solution. 6-aminonicotinamide was purchased from Sigma; 250mg were dissolved in 5ml DMSO to give a stock solution of 360mM.

2.2.3 Drug Addition

After 24hrs of incubation of the plated cells, drug dilutions were made up in fresh media. Media was then aspirated from the plated cells and replaced with 350µl of media containing the compound of interest. All drug dilutions were carried had 6 replicates. An equal volume of media was added to the untreated control cells. The plate was then incubated at 37^{o}C for 72hrs.

2.2.4 Proliferation assay

After 72hrs of incubation with the compound of interest cells were subjected to a proliferation assay. This was carried out using the CyQuant® Proliferation Assay Kit (Invitrogen). Assays were carried out according to manufacturer's instructions. Media was shaken off rather than aspirated, so as not to disturb the cells and alter the cell number. Wells washed once with PBS and PBS shaken off rather than aspirated, plates were then frozen at -70^{o}C. The CyQuant® solution was made up as needed on the day of the assay. 20x lysis buffer was diluted with nuclease-free water (Ambion) and CyQuant® fluorescent dye added, this solution was protected from the light using aluminium foil. 500µl of this solution was added to each well and plates were incubated in the dark for 5minutes at room temperature before the fluorescence was measured using Fluorescein filters.

2.2.5 Data Analysis

The 6 replicates were averaged to give a mean value and a standard deviation calculated. The rate of proliferation was then estimated relative to the untreated control. The average and standard deviation from at least 2 independent experiments was calculated for each cell line for each drug concentration.

2.3 siRNA knockdown

2.3.1 Cell plating

Cell lines of a known genetic background (Table 2-1) were maintained as stated above (2.1) in T-150cm^2 flasks. Prior to each experiment one 80-90% confluent flask was trypsinised and centrifuged as described above (2.1), the supernatant was aspirated and the cell pellet resuspended in 2ml of media and gently pipetted to prevent clumping of cells. 10µl of cell solution was pipetted onto a haemacytometer and cells counted. Approximately 500-1000 cells were seeded in quadruplicate to 96-well tissue culture plate depending on the growth rates of the cell lines in a volume of 150µl of antibiotic-free media. The plates were then incubated for 24hrs at 37^{o}C.

2.3.2 Transfection of siRNA

ON-TARGETplus single siRNAs were purchased from Dharmacon. siRNAs were resuspended in 1x siRNA buffer to a stock concentration of 20µM. Aliquots of 2µM were prepared to avoid freeze-thaw degradation. After 24hrs of incubation cells were transfected in triplicate with the siRNA using 0.25µM Lipofectamine reagent in 100µl of antibiotic-free media. Negative controls included ON-TARGETplus Non-targeting siRNAs (Dharmacon) and Lipofectamine only control. These were also carried out in triplicate. After 8hrs of incubation with the siRNA, media was aspirated gently and replaced with antibiotic–free media containing no siRNA. 48hrs later the transfection was repeated.

2.3.3 Proliferation assay

After 7 days of incubation with the compound of interest cells were subjected to a proliferation assay. This was carried out using the CyQuant® Proliferation Assay Kit (Invitrogen) as described in 2.2.4. 200µl of CyQuant® solution was added to each well and plates were incubated in the dark for 5minutes at room temperature before the fluorescence was measured using Fluorescein filters.

2.3.2 Data Analysis

Triplicates were averaged to give a mean value and a standard deviation calculated. Toxicity was estimated by comparing the non-targeting control and Lipofectamine™ 2000 control to the untreated control. The rate of proliferation for the siRNA of interest was estimated relative to the non-targeting control. The average and standard deviation from at least 2 independent experiments was calculated for each cell line.

2.4 Protein Quantification

2.4.1 Cell plating

2.4.1.1 To determine the downstream effects of CI-1040 and rapamycin.

Cells were seeded to 6-well plates and incubated at 37^{o}C for 24hrs. Cells were then treated with a range of concentrations of CI-1040 (0-10µM) or rapamycin (0-200nM) and incubated for 8 or 24hrs and protein harvested.

2.4.1.2 To estimate level of siRNA knockdown

Cells were seeded to 6-well plates and 24hrs later transfected with 10nM, 20nM or 50nM of the siRNA using 0.25µM Lipofectamine™ 2000 in antibiotic-free media. After 6hrs the media was replaced with antibiotic-free media containing 10% FCS. At 48hrs the transfection was repeated. 96hrs after the first transfection the cells were harvested for immunoblot analysis of protein levels.

2.4.2 Harvesting protein

Protein was harvested from cells seeded to 6-well plates. Cells were washed twice with ice-cold PBS and then treated with 200µl RIPA buffer (Sigma) containing protease (Sigma) and phosphatase inhibitors (Roche). The solution was than incubated with gentle shaking on ice for 30mins and then centrifuged at 13200rpm at 4^{o}C for 20minutes. The supernatant was then transferred to a new tube on ice and all samples stored at -70^{o}C.

2.4.2 Quantification of protein

Quantification of protein was carried out using the BCA assay. The working reagent was created by mixing 50 parts bicinchoninic acid with 1 part copper (II) sulphate solution. Bovine Serum Albumin (Sigma) standards were prepared in duplicate (0-1000µg/ml). Unknown samples were diluted 5-fold and 10-fold in a 96-well plate in a volume of 25µL. 200µL of BCA working reagent was added to all samples and incubated at room temperature for two hours or at 37^{o}C for 30mins. The absorbance was measured at

562nm. BSA standards were used to create a standard curve and sample concentrations estimated using this curve.

2.4.3 SDS-PAGE

Samples were diluted to 20µg/well using RIPA buffer (Sigma). 10µl of each sample was denatured and reduced by adding 3.7µL 4x NuPAGE® LDS Sample buffer (Invitrogen), 1.5µL (10x) NuPAGE® Sample Reducing Agent (Invitrogen) and boiled at 95-100^{o}C for 5-10mins. Samples were then cooled on ice and spun down.

14.5µL of samples were loaded into pre-cast NuPAGE® 4-12% Bis-Tris Gel (Invitrogen) alongside 5µL Novex® Sharp Pre-stained Protein Standards (Invitrogen). Samples were run for approximately 1 hour at 200V in 1X NuPAGE® MES SDS Running Buffer (Invitrogen) containing 1ml NuPAGE® antioxidant (Invitrogen) in an Xcell SureLock™ system.

2.4.4 Western blotting

SDS-PAGE gels were transferred to PVDF membranes (Invitrogen) using the following protocol. 1X NuPAGE® Transfer Buffer (Invitrogen) containing 10% methanol and 1ml NuPAGE® Antioxidant was prepared on the day of the transfer. Prior to transfer, blotting pads and filter paper were soaked in the transfer buffer. PVDF membranes were prepared by soaking for 1min in methanol, briefly washed with de-ionised water and finally soaked in transfer buffer. The protein samples were transferred to the PVDF membrane using the Xcell SureLock™ system at 30V for an hour. Membranes were then incubated in 1X blocking buffer (Sigma) for an hour at room temperature.

Membranes were then incubated overnight at 4°C with primary antibodies (see table 2-2). Membranes were then washed 3x for 10mins in TBST (500mM NaCl, 20mM Tris-HCl, 0.01% Tween-20, pH7.6), secondary Anti-rabbit IgG, HRP-linked Antibody (Cell Signalling Technology) was diluted 1:2500 in blocking buffer and membranes incubated at room temperature for 2hrs with the secondary antibody.

Antibody	**Supplier**	**Dilution**
cyclin D1 (C-20): sc-717	Santa Cruz Biotechnology	1:500
p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb	Cell Signalling Technology	1:1000
Phospho-p44/42 MAPK (Erk1/2)(Thr202/Tyr204) Rabbit mAb	Cell Signalling Technology	1:1000
p70 S6 Kinase (49D7) Rabbit mAb	Cell Signalling Technology	1:1000
Phospho-p70 S6 Kinase (Thr389) (108D2) Rabbit mAb	Cell Signalling	1:1000
AKR1B10 (ab62218)	Abcam	Min. 1:20000

Table 2-2 Antibodies used in this study

2.4.5 Visualisation and data analysis

Protein bands were visualised using the SuperSignal West Pico Chemiluminescent Substrate (Pierce). Equal amounts of the two solutions were mixed on the day, membranes were incubated for 5mins at room temperature, excess fluid drained, wrapped in cling film and exposed to X-ray film (Amersham) in a dark room. Relative amounts of protein were estimated using the software ImageJ.

2.5 Analysis of Affymetrix Microarray data

2.5.1 Data analysis

mRNAs were extracted from candidate cell lines and expression profiles were determined using the Affymetrix Human U133 Plus 2 GeneChip by GlaxoSmithKline. Data quality test and normalization were performed using Affy package in R (Irizarry, et al. 2003a). The quality of each array was assessed by checking the mean and standard deviation of over-all expression, percentages of present gene calls and background levels, 3': 5' ratios for spiked-in and control genes specific to the array type and the correlation among replicated samples. Background correction of each array was using robust multi-array average expression measure (Irizarry, et al. 2003; Bolstad, et al. 2003; Irizarry, et al. 2003b). The data were normalized by quantile method and expression level of each gene was log2 transformed. Genetic signature analysis was using Limma package from BioConductor (Smyth, 2004). Paired t-test was used to differences between the expression profiles of between *LKB1/KRASmut* cell lines and wild type cell lines. *P*-values were corrected by Benjamini/Hochberg (BH) paradigm with a false discovery rate (FDR) of 0.01. Unsupervised hierarchical cluster was carried out using Cluster 3.0 (Eisen et al., 1998) and presented by Java TreeView (Saldanha, 2004). The similarities between cell lines were calculated based on uncentered Pearson correlation and used average linkage for clustering. This section of analysis was carried out by Lina Chen.

2.5.2 Bioinformatic analysis of genes with significantly different expression

Genes found to be significant using the BH paradigm with an FDR of 0.01 were subjected to GO term enrichment analysis using the web-based program GoMiner (http://discover.nci.nih.gov/gominer/). Differentially expressed genes were also mapped on to metabolic pathways using the web-based program; Metacyc (http://metacyc.org/).

2.6 Expression analysis of CI-1040, rapamycin and AMPK inhibitor treated cells

2.6.1 Cell plating and drug addition

Cell lines of a known genetic background (Table 2-1) were seeded to 10cm dishes, in triplicate at time 0hrs and in duplicate for all other time points. The following day untreated controls were harvested for time point 0hrs. The remainder of the dishes were treated with 5μM CI-1040, 40nM rapamycin or 7μM AMPK inhibitor compound C. Cells were harvested and RNA extracted 8hrs and 48hrs after treatment.

2.6.2 RNA extraction

RNA extraction was carried out using the Qiagen RNeasy® kit. Approximately $1x10^6$ cells were lysed directly in the dish by addition of 600μL of RLT buffer. The lysate was collected and transferred to an RNase-free tube and vortexed to disperse clumps. Lysate was transferred to a QIAshredder® spin column in a 2ml collection tube and centrifuged for 2mins at full speed. 1 volume of 70% ethanol was added to each sample and pipette mixed. 700μL of the sample was transferred to an RNeasy spin column in a 2ml collection tube and centrifuged for 15 seconds at ≥8000g, flow-through discarded

and the same step repeated with the remainder of the sample. 700µL of RWI buffer was added to the spin column and centrifuged for 15sec at ≥8000g, flow-through discarded. 500µL of RPE buffer was added to the column and centrifuged for 15sec at ≥8000g, flow through discarded; this step was then repeated and centrifuged for 2mins at ≥8000g. The spin column was transferred to a new 2ml collection tube and spun at full speed for 1min before being transferred to a new 1.5ml collection tube and the RNA eluted by adding 50µL of RNase-free water and centrifuged for 1min at ≥8000g. Eluted samples were stored at -70^{o}C.

2.6.3 Assessing quality and quantity of RNA

RNA quality was assessed using the RNA 6000 Nano kit (Agilent). On arrival of the kit the RNA ladder was prepared by transferring to a new RNase-free vial, heat denatured at 70^{o}C for 2mins and immediately cooled on ice. Aliquots with the required amount for typical daily use were prepared in RNase-free tubes and stored at -70^{o}C. RNA 6000 Nano gel matrix was prepared by pipetting 500µL into a spin filter, centrifuging at 1500g for 10mins at room temperature. Aliquots of 65µL were prepares in 0.5ml RNase-free vials and stored at 4^{o}C for 4 weeks.

On the day of the experiment the gel-dye mix was prepared. RNA 6000 Nano dye concentrate was equilibrated at room temperature for at least 30mins, vortexed, spun down and 1µL added into a 65µL aliquot of filtered gel. The gel-dye mix was vortexed thoroughly and spun at 13000g for 10mins at room temperature.

9µL of gel dye mix was loaded into a new RNA 6000 Nano chip on the chip priming station. 1µL of RNA 6000 Nano Marker was added to all sample wells and in the well for

the RNA ladder. 1µL of the prepared RNA ladder was pipetted into the marked well and 1µL of each sample into separate wells. The loaded chip was then vortexted in an IKA vortexer for 1min at 2400rpm. The chip was then loaded straight into the Bioanalyser and gels assessed for RNA quality. RNA concentration was estimated using a Nanodrop spectrophotometer.

2.6.4 Microarray

Samples were diluted to 50ng/µL in a 96-multi well plate and the microarry performed by Peter Ellis of the Microarray team. mRNA was reverse transcribed using oligo(dT) primers for two hours at 42^{o}C which incorporates a T7 RNA polymerase binding site at the 5'-end. The RNA was then digested with RNaseH and the cDNA converted to double stranded cDNA using DNA polymerase for 2 hours at 16^{o}C. The purified cDNA was then incubated overnight at 37oC with T7 RNA polymerase and rNTPs (including biotin-tagged rUTP) to produce biotinylated single-stranded anti-sense RNA (cRNA).

Once quantified and purified 1500ng of the cRNA (per sample/array) was mixed with hybridisation buffer and applied to the array. The arrays were incubated in a humidified atmosphere at 58^{o}C for 16-20hrs followed by washing according to a standard Illumina protocol: 10min at 55^{o}C in wash buffer, 5mins at room temperature in E1BC, 10min in 100% ethanol at room temperature, 2mins at room temperature in E1BC, 10mins in blocking agent (casein in PBS) at room temperature, 10mins at room temperature in blocking agent containing 1µg/ml streptavidin-cy3 and 5mins in E1BC at room temperature. Finally slides were dried by spinning at 275 x g for 4mins. Slides were then scanned using a BeadArray reader and the output exported to BeadStudio

2.6.5 Data Analysis

Expression profiles were determined using the Illumina HumanWG-6_V3 chip. Data quality test and normalization were performed using Lumi package in R (Du, et al. 2008). The quality of each array was assessed by checking the mean and standard deviation of over-all expression, ratio of detectable probe, expression information of control probes (housekeeping genes) and the correlation among replicated samples. Background correction of each array was using variance-stabilizing transformation (Lin, et al. 2008). The data were normalized by quantile method and expression level of each gene was log transformed. Genetic signature analysis was using Limma package from BioConductor (Smyth, 2004). Paired t-test was used to access the significant level of expression change before and after drug treatment and the expression profile between *LKB1/KRAS* mutant cell lines and wild type cell lines. *P*-values were corrected by Benjamini/Hochberg paradigm with a false discovery rate of 0.01. Unsupervised hierarchical cluster has been done by using Cluster 3.0 (Eisen, et al. 1998) and presented by Java TreeView (Saldanha, 2004). The similarities between cell lines were calculated based on uncentered Pearson correlation and used average linkage for clustering. Heatmaps were produced by smcPlot from PGSEA package in BioConductor (http://www.bioconductor.org/packages/2.4/bioc/html/PGSEA.html). This section of analysis was carried out by Lina Chen.

2.6.6 Bioinformatic analysis of genes with significantly different expression

A cut-off of 1.5 for the fold change was used when comparing treated to untreated samples. Gene lists were compiled of significantly altered genes for the treated samples versus the untreated time 0hrs, for each cell line. Genes which only showed

altered expression after compound treatment in *LKB1/KRAS* mutant cell lines were subjected to further analysis. Genes found to be significant and differentially expressed in *LKB1/KRAS* mutant cell lines after compound treatment were subjected to GO term enrichment analysis using the web-based program GoMiner (http://discover.nci.nih.gov/gominer/).

2.7 NMR spectroscopy

2.6.1 Labelling of cells with ^{13}C glucose

Cells were grown in T-150cm^2 flasks until at least 50% confluent. Media was aspirated and replaced with glucose-free media containing 11.11mM ^{13}C-glucose. Cells were incubated at 37^{o}C for 24-48hrs, trypsinised as described in 2.1, ensuring that the media used for neutralising the trypsin was glucose-free.

2.6.2 Preparation of samples for NMR spectroscopy

Carried out by Jules Griffin (Department of Biochemistry, University of Cambridge). Dried aqueous extracts from the aqueous layer of the cell extract were dissolved in 600µl D_2O containing 1mM trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP) and transferred to 5mm NMR tubes for analysis. All NMR was performed on a 500MHz NMR spectrometer interfaced with a TXI 5mm probe. For 1H NMR spectroscopy a standard 1D NOESY pre-saturation pulse sequence with pre-saturation applied during the relaxation delay and mixing time was used in order to suppress the residual water signal. The sample temperature was 300K for all experiments. 256 transients were collected

into 64k data points over a spectral width of 20ppm. Spectra were collected with a relaxation delay of 2s, mixing time of 150ms and t1 of 4µs.

For all 1D spectra the 1H-NMR free induction decays (FID) were Fourier transformed after application of an exponential window function. The spectra were then phased, base line corrected, referenced to TSP at 0.00ppm and integrated using ACD labs NMR Processor (version 8, ACD, Toronto, Canada). 1H-NMR FIDs were Fourier transformed with a line broadening of 0.3Hz. The spectra were integrated in 0.01ppm buckets between 0.20 and 9.95ppm, excluding the water region (4.72-5.05ppm)

2.6.3 Pattern Recognition Methods

Carried out by Jules Griffin (Department of Biochemistry, University of Cambridge). Normalised data was imported into SIMCA-P+ version 11 (Umetrics, Umea, Sweden) for multivariate statistical analysis. The data was mean centred and scaled in SIMCA. Data were Pareto scaled to suppress the contribution from regions of the spectra containing only noise. Initially, principal components analysis (PCA) models were built to identify the major trends in datasets and identify outliers. The supervised technique partial least squares discriminate analysis (PLS-DA) was then used to look for differences between control and *LKB1/KRAS* mutant cells and identify changes responsible for the separation. Significantly changed metabolites were identified using loading column plots with error bars set at 95% confidence limit using a jack-knifing routine within the SIMCA software. R2 values were used to assess the variation explained by the models and Q2 values were used to assess the robustness of the models. Additionally, the SIMCA P+ validate function was used to ensure that the PLS-DA models were not over fitted.