# A Systems Biology Approach; Proteomic Study on Rapamycin Treated Human Cancer Cell Line

ASMS 2010

Sudha Rajagopalan, Nilanjan Guha, Yugandhar Reddy, Siji Joseph and Syed Lateef, Agilent Technologies India Pvt. Ltd, Bangalore India

# A Systems Biology Approach; Proteomic Study on Rapamycin Treated Human Cancer Cell Line

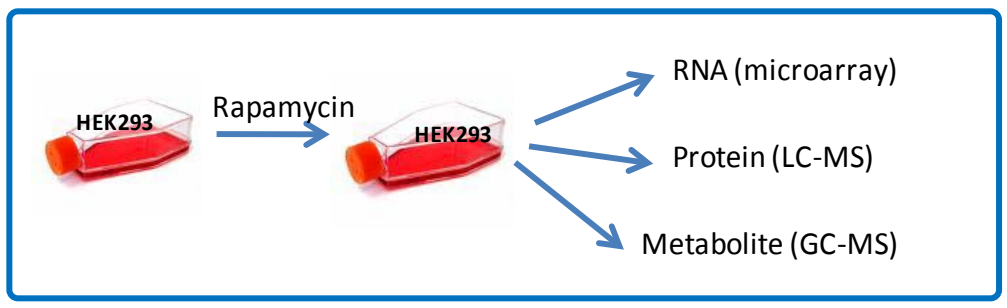
## Introduction

*Rapamycin is an immunosuppressant drug which is recently shown to inhibit cancer growth. Rapamycin specifically inhibits mTOR activity and cellular hyper-proliferation in many cells types resulting in G1 growth arrest. A comprehensive study at genome, proteome and metabolome level will enable better understanding of the biological processes affected by this drug. Multiple instrument platforms and advanced software are required for such an analysis. In the current study, we have used systems approach to understand the effect of rapamycin treatment on various biological pathways using state of the art platforms from Agilent. Effect of rapamycin treatment was studied on human cancer cell line HEK293.*

## Study Design

*Human cell line HEK293 was treated with rapamycin and cells were harvested at 15min, 45min, 2hrs and 16hrs after rapamycin treatment. Control samples treated with vehicle alone were also harvested at each time point. RNA, Protein and metabolites extracted from treated and control samples were compared. Five biological replicates were performed on five different days.*

Figure1 Study Design




## Experimental

### Cell Culture and harvesting cells

*HEK293 cells (ATCC: CRL-1573) were cultured in DMEM medium containing 10% fetal bovine serum, 10 U of penicillin/ml, and 10 µg of streptomycin/ml. Cells were grown to an approximate density of $10^5$/ml, 40 nM rapamycin or DMSO vehicle was added, Cells were harvested at 15, 45, 120 min and 16 h and aliquoted so that each tube contains 10^6 cells.*

### RNA Analysis

*RNA was extracted from the harvested cells using Stratagene Absolutely RNA kit (Cat# 400814). Both miRNA and mRNA species are quantitatively recovered from the Stratagene kit, allowing gene expression studies of both miRNA and mRNA from the same sample.*

*The RNA integration number (RIN) values were determined for the extracted RNA using RNA 6000 Nano Lab Chip kit on the Agilent 2100 Bioanalyzer and the quality of RNA was found to be good.*

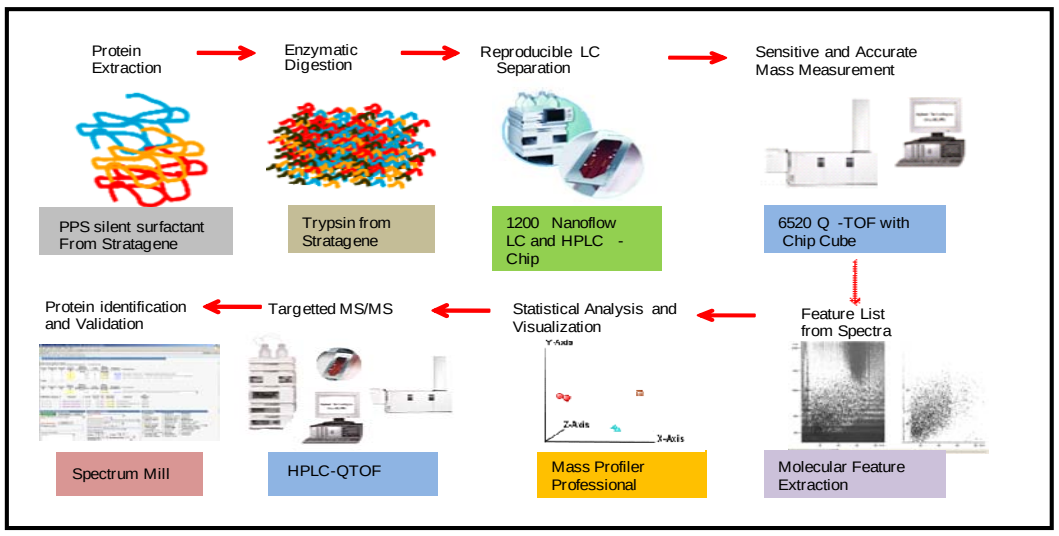
*25 ng of RNA samples for each control and treated samples were labeled with Cy3 using Agilent's Low Input Quick Amp kit) and gene expression microarray analysis was performed using Agilent Human GE 4X44K V2 Microarray kit (G4845A). The data was analyzed using GeneSpring 11.0.2. (Agilent) Entities having a p-value≤0.1 and fold change≥1.4 were considered differentially expressed.*

## Experimental

### Protein Analysis

*Protein was extracted using PPS silent surfactant (Stratagene), reduced, alkylated and digested. Samples were analysed in five replicates on HPLC chip-QTOF system. Differential features obtained in the comparison of control and treated samples were then identified using targetted MS/MS analysis. Workflow used for the proteomics analysis is shown below.*

Figure2 Workflow used for proteomics Analysis




*All LC/MS experiments were performed on an Agilent 1200 Series HPLC-Chip/MS interfaced to an Agilent 6520 Accurate-Mass Q-TOF LC/MS. The LC system consisted of a capillary pump for sample loading, a nanoflow pump and a thermostated microwell-plate autosampler. The HPLC-Chip configuration consisted of a 160 nL enrichment column and a 150 mm x 75 µm analytical column (Zorbax 300SB-C18). Mobile phases employed were: A). 0.1% formic acid in water and B). 90% acetonitrile with 0.1% formic acid. LC and MS conditions used were as reported earlier.[1]*

*Mass profiler professional (MPP), Agilent version B02.00 was used for statistical comparison of the LC-MS data and SpectrumMill version B03.00 (Agilent) was used for data base searching of the MS/MS data.*

### Metabolome Analysis

*Metabolites were extracted from one million cells with 2:1:2 methanol-water/chloroform mixture and derivatized in two steps before GC-MS analysis. First, using methoxyamine hydrochloride in pyridine to derivatize carbonyl groups, then using N-methyl- N trimethylsilyltrifluoroacetamide containing 1% trimethylchlorosilane to derivatize carboxyl, hydroxyl, amino, imino or sulphonyl groups. d27-myristic acid, was used as internal standard for the GC-MS analysis.*

*Agilent 7890 GC coupled with Agilent 5975C inert MSD was used for the data collection. A 30m long Agilent DB-5MS column with 10m Duragard precolumn (Part Number: 122-5532G) with 0.25 µm film thickness and 250µm diameter was used for the separation.. All GC-MS experiments were performed as described in the user manual (G1676-80000)[2].*

*Statistical comparison between control and treated samples were performed using MPP (Agilent) Differentially expressed metabolites were identified using Agilent Fiehn GC/MS Metabolomics RTL Library (version June 2008).*

# A Systems Biology Approach; Proteomic Study on Rapamycin Treated Human Cancer Cell Line

## Results and Discussion

### *Protein Analysis*

*Approximately Forty proteins were significantly (P value less than 0.05 and fold change greater than 10) differentially expressed between the treated and control samples at 16h of rapamycin treatment.*

Table 1 A partial list of Differentially expressed peptides and the proteins from which they are derived

| Peptide Sequence | Regulation | RT | m/z | Mass | Prot.Accession No. | Protein Name |
|---|---|---|---|---|---|---|
| (K)VDFPQDQLTALTGR(I) | up | 31.79 | 780.9076 | 1560.802 | 6648067 | Malate dehydrogenase, mitochondrial precursor |
| (K)AFYPEEISSMVLTK(L) | up | 37.1 | 807.9243 | 1614.808 | 4529894 | HSP70-HOM |
| (K)AFYPEEISSMVLTK(L) | up | 37.1 | 538.9521 | 1614.808 | 4529894 | HSP70-HOM |
| (K)LQMEAPHIIVGTPGR(V) | down | 23.83 | 540.2879 | 1618.873 | 4503529 | eukaryotic translation initiation factor 4A isoform 1 |
| (K)DPVQEAWAEDVDLR(V) | down | 30.14 | 821.8985 | 1642.771 | 33286418 | pyruvate kinase |
| (K)DLADELALVDVIEDK(L) | up | 48.23 | 829.4246 | 1657.853 | 5031857 | lactate dehydrogenase A |
| (K)NQVALNPQNTVFDAK(R) | up | 19.45 | 829.9158 | 1658.85 | 167466173 | heat shock 70kDa protein 1B |
| (K)ILLANFLAQTEALMR(G) | down | 51.94 | 852.4857 | 1703.951 | 18201905 | glucose phosphate isomerase |
| (K)ILLANFLAQTEALMR(G) | down | 51.94 | 568.6612 | 1703.951 | 18201905 | glucose phosphate isomerase |
| (K)VAVLGASGGIGQPLSLLLK(N) | up | 48.08 | 897.0407 | 1793.09 | 6648067 | Malate dehydrogenase, mitochondrial precursor |
| (R)AMADPEVQQIMSDPAMR(L) | up | 28.4 | 945.4072 | 1889.855 | 5803181 | stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing protein) |
| (R)IFANTPDSGCVLGMRKR(A) | down | 32.95 | 641.329 | 1921.974 | 4505749 | phosphofructokinase, muscle |
| (R)LTLYDIAHTPGVAADLSHIETK(A) | down | 38.47 | 592.0709 | 2365.24 | 6648067 | Malate dehydrogenase, mitochondrial precursor |
| (R)AEGSDVANAVLDGADCIMLSGETAK(G) | down | 42.8 | 1247.544 | 2494.144 | 33286418 | pyruvate kinase |
| (R)AEGSDVANAVLDGADCIMLSGETAK(G) | down | 42.83 | 832.0589 | 2494.144 | 33286418 | pyruvate kinase |
| (R)VETGVLKPGMVVTFAPVNVTTEVK(S) | down | 38 | 629.5953 | 2515.384 | 4503471 | eukaryotic translation elongation factor 1 alpha 1 |
| (K)SGDAAIVDMVPGKPMCVESFSDYPPLGR(F) | down | 41.35 | 999.131 | 2995.4 | 4503471 | eukaryotic translation elongation factor 1 alpha 1 |
| (K)EIAEAYLGYPVTNAVITVPAYFNDSQR(Q) | up | 48.17 | 1001.162 | 3001.494 | 167466173 | heat shock 70kDa protein 1B |

Figure 3 PCA plot on replicate LC-MS control and rapamycin treated (16h) samples, reveals that the variance in the data can be explained by which class it came from

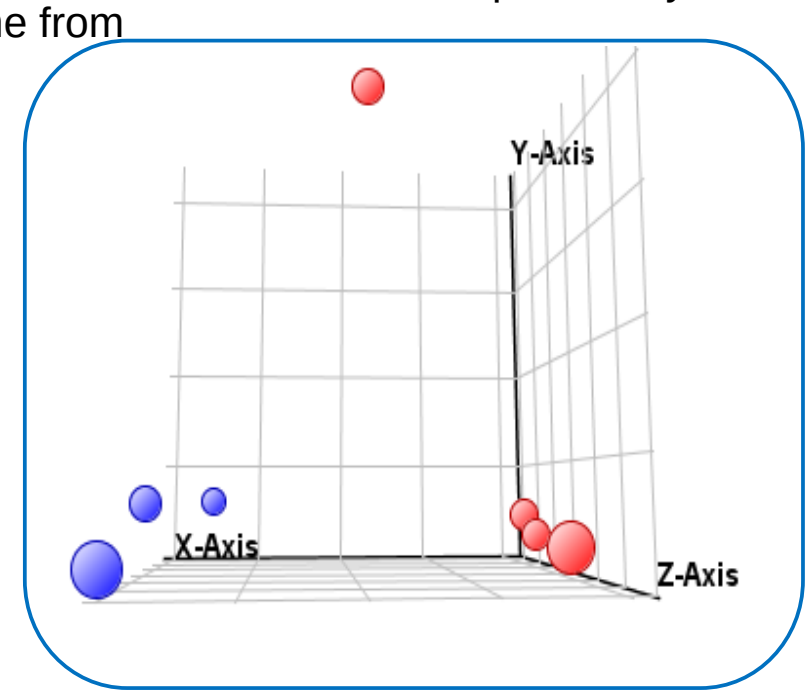



Figure 4 Mass spectrum between 29.2-29.4 minutes showing one of the differentially expressed peptides DPVQEAWAEDVDLR derived from the protein pyruvate kinase

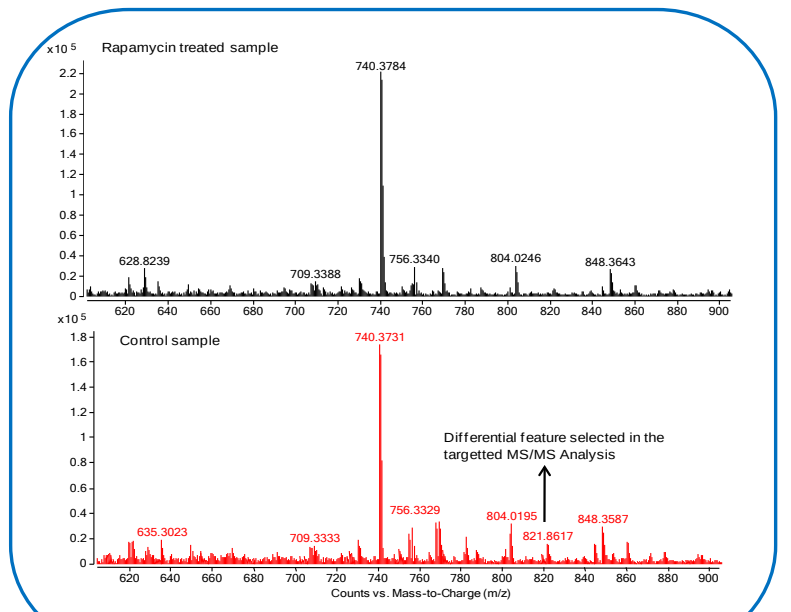
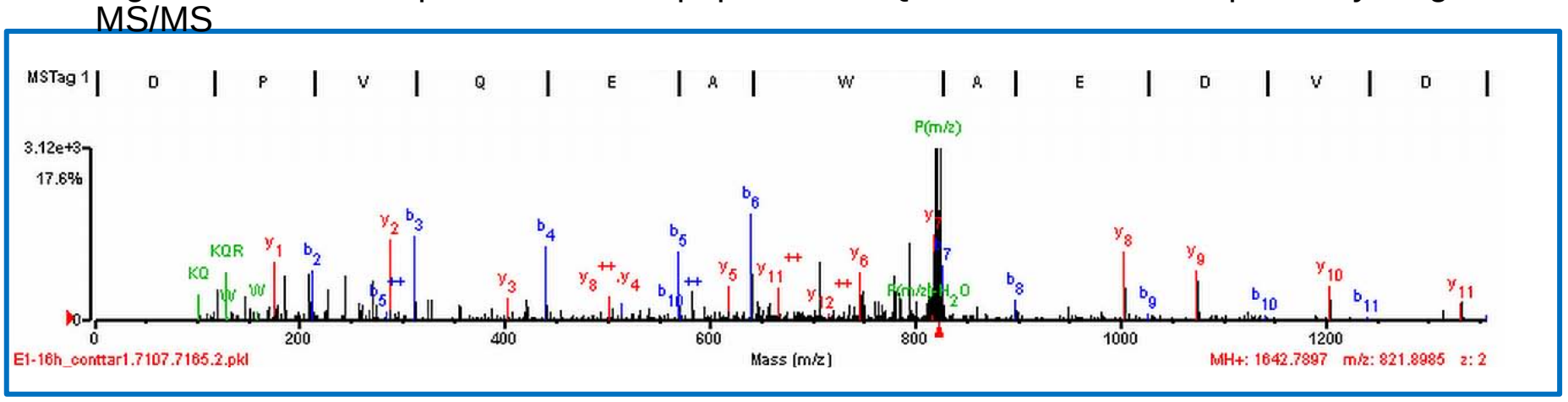



Figure 5 MS/MS spectrum of the peptide DPVQEAWAEDVDLR acquired by targeted MS/MS




The peptide DPVQEAWAEDVDLR is co-eluting with several other high abundant peptides. This peptide may not get selected in the data dependant MS/MS analysis.

# A Systems Biology Approach; Proteomic Study on Rapamycin Treated Human Cancer Cell Line

## Results and Discussion

### *Genome Analysis*

*Approximately 72 genes were differentially expressed between the control and rapamycin treated samples at 16h. Gene expression analysis suggests that transcription, translation, cell growth, and the stress response are some of the biological processes affected by rapamycin treatment.*

Table 2 A Partial list of differentially expressed genes

| Fold change | Regulation | Gene Symbol | Protein anme |
|---|---|---|---|
| 1.6216595 | up | ATF3 | activating transcription factor 3 |
| 1.4631783 | down | C11orf52 | chromosome 11 open reading frame 52 |
| 1.6093587 | down | TMEM86A | transmembrane protein 86A |
| 1.5000856 | up | HSPA9 | heat shock 70kDa protein |
| 1.434173 | up | ZFP36L1 | zinc finger protein 36, C3H type-like 1 |
| 1.5229359 | up | HSPH1 | heat shock 105kDa/110kDa protein 1 |
| 1.5675275 | up | INPP5F | inositol polyphosphate-5-phosphatase F |
| 1.5505759 | up | ETNK1 | ethanolamine kinase 1 |
| 1.4378525 | up | TBCE | tubulin folding cofactor E |
| 1.5906347 | up | GNPDA2 | glucosamine-6-phosphate deaminase 2 |
| 1.4255726 | up | MRPL19 | mitochondrial ribosomal protein L19 |

### *Metabolome Analysis*

*Approximately 30 metabolites were found to be differentially expressed between the control and rapamycin samples at 16h. The metabolite list included up-regulation of several sugars and amino acids.*

Table 3 A Partial list of differentially expressed metabolites

| Compound name | [r] Vs [c] regulation | Fold change | Mass | Retention Time |
|---|---|---|---|---|
| gluconic acid lactone 1 | down | 16 | 147 | 17.66 |
| malonic acid 1 | down | 16 | 73 | 8.45 |
| D-glucose 1 | up | 16 | 186 | 17.63 |
| talose 1 | up | 16 | 205 | 17.66 |
| N-acetyl-D-glucosamine 1 | up | 16 | 147 | 18.743 |
| 10-hydroxydecanoic acid | up | 16 | 227 | 15.71 |
| glycerol 1-phosphate | up | 16 | 299 | 16.04 |
| Creatinine | up | 16 | 115 | 13.68 |
| fructose 1 | up | 16 | 133 | 17.17 |
| D (+)altrose 1 | up | 4.92 | 189 | 17.7 |
| L-threonine 2 | up | 3.98 | 73 | 11.54 |
| L-pyroglutamic acid | up | 3.96 | 156 | 13.24 |
| glycine | up | 3.79 | 174 | 10.46 |
| 2-amino-1-phenylethanol | up | 3.27 | 174 | 9.42 |
| L-(+) lactic acid | up | 3.27 | 73 | 6.8 |

**Figure 6: Total ion chromatogram from 17.6 - 19.5 minutes showing differential expression of one of the glucose derivatives**

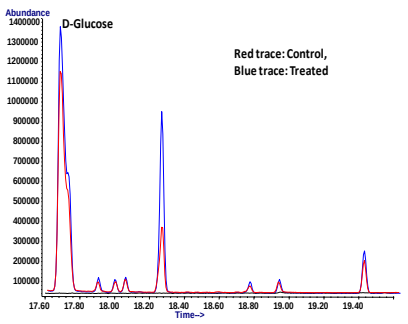



Figure 7 Fragmentation pattern observed in the experiment matches well with the library, confirming glucose identification.

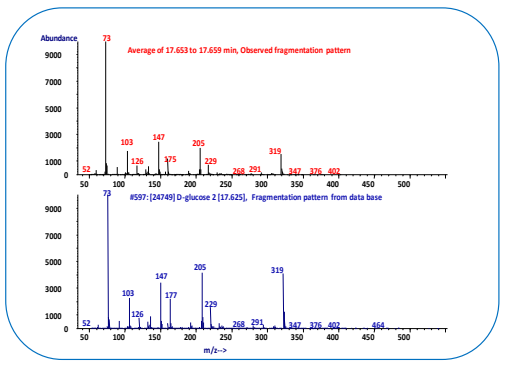



Table 4 Biological processes affected by rapamycin

| *Biological Process* | *RNA (microarray)* | *Protein (LC-MS)* | *Metabolite (GC-MS)* |
|---|---|---|---|
| *Glycolysis/ gluconeogenes* | •Glucosamine-6-phosphate deaminase 2 | • Pyruvate kinase<br>• Glucose phosphate isomerase<br>• Malate dehydrogenase, | • *Glucose*<br>• *Fructose* |
| *Stress Response* | Heat shock 70kDa protein | Heat shock 70kDa protein | ---- |
| *Protein Synthesis* | ---- | Eukaryotic translation initiation factor 4A | Glycine, Cysteine Threonine,,Valine *Leucin, Alanine* |

Green : Elevated  Red: Suppressed

## Conclusions

- *Glycolysis/gluconeogenesis pathway is reported to be affected by rapamycin treatment[3]. In this study, down regulation of pyruvate kinase, glucose phosphate isomerase and up-regulation of malate dehydrogenase are observed, indicating glycolysis is reduced and gluconeogenesis is enhanced due to rapamycin.*
- *Up-regulation of the gene for glucosamine-6-phosphate deaminase 2 and elevation of glucose in metabolite analysis supports this observation.*
- *Up-regulation of Heat shock proteins, which is known to be a stress response is observed in both proteomics and gene expression analysis.*
- *Rapamycin treatment is reported to result in decreased protein levels[4]. Observation of down regulation of eukaryotic translation initiation factor 4A in proteomic analysis and up-regulation of amino acids in the metabolite analysis indicates down regulation in protein synthesis.*
- *Proteomic, genomic and metabolomic analysis using various platforms enables better understanding of biological process.*

### *Future Direction*

*Preliminary results obtained in this study is encouraging and further experiments on more biological replicates are currently in progress. Integration of data from genome, proteome and metabolome analysis to elucidate biological processes will be available in future versions of Agilent GeneSpring software.*

### *References*


1. *Agilent Publication 5990-4404EN Profiling Approach for Biomarker Discovery using an Agilent HPLC-Chip Coupled with an Accurate-Mass Q-TOF LC/MS.*
2. *Agilent Publication G1676-90000 Agilent G1676AA Fiehn GC/MS metabolomics RTL library user guide.*
3. *Houde VP, Brûlé S, Festuccia WT, Blanchard PG, Bellmann K, Deshaies Y, Marette A. Diabetes. 2010 Mar 18.*
4. *Tao Peng,. Golub, TR and. Sabatini DM Mol.Cell.Biol. 2002, p. 5575–5584.*




Agilent Technologies