5-2015

MEASURING SINGLE CELL RESPONSES TO LAPATINIB IN A HETEROGENEOUS POPULATION

Preety Priya

MEASURING SINGLE CELL RESPONSES TO LAPATINIB IN A HETEROGENEOUS POPULATION

Preety Priya, MS

APPROVED:

Prahlad Ram, Ph.D., Supervisory Professor

Joya Chandra, Ph.D.

Ju-Seog Lee, Ph.D.

Luay K. Nakhleh, Ph.D.

William Mattox, Ph.D.

APPROVED:

Dean, The University of Texas Graduate School of Biomedical Sciences at Houston

MEASURING SINGLE CELL RESPONSES TO LAPATINIB IN A HETEROGENEOUS POPULATION

A THESIS

Presented to the Faculty of The University of Texas Health Science Center at Houston

and

The University of Texas MD Anderson Cancer Center Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements for the Degree of MASTER OF SCIENCE

by

Preety Priya, MS

February 2015

Acknowledgement

I would like to thank all my committee members, Dr. Prahlad Ram, Dr. Joya Chandra, Dr. Ju-Seog Lee, Dr. Luay Nakhleh, Dr. William Mattox, for their advise, support and insights. I am especially grateful to Dr. Ram, who spent extra time with me guiding me smoothing through the development of my thesis project.

To my family and friends, I would be where I am now without all of you. I am extremely blessed to have all of you in my life. Lastly, I would like to thank the UT Graduate School of Biomedical Sciences for giving me such a wonderful and unique opportunity in this program.

Abstract

Measuring single cell responses to Lapatinib in a heterogeneous population

Preety Priya

Advisory Professor: Dr. Prahlad Ram, Ph.D.

Cancer is not one disease but a saga of diseases and is the outcome of disturbed homeostasis in the normal cells due to the deregulation of its genetic makeup. With advent of technologies that allow detailed molecular characterization of tumors, targeted therapies have emerged as a more promising and specific mode of treatment. However, a major challenge with targeted therapy is the acquired resistance in the cancer cells to these therapies, quite often very rapidly in the course of a few months. One of the major targets in cancer has been the EGFR/ErbB2 network in breast and other cancer types. Prior work from our lab and others have shown alterations in the cellular network whereby compensatory upregulation of alternative pathways such as glucose uptake and metabolism can lead to acquired resistance to anti- EGFR/ErbB2 therapy in breast cancer to Lapatinib [1]. However, one the of the very important unanswered questions at the cellular and molecular level is the mechanisms that leads to selection of cells that are resistant to Lapatinib whereby there exists two possibilities: 1. Cells are intrinsically resistant and are less likely to respond to the drug and get selected for 2. Cells switch response phenotype over time leading to increased metabolism and resistance. In this proposal I will develop a predictive computational model that can be used to dynamically model the response of cells to lapatinib and determine what underlying response mechanisms can lead to adaptive resistance cell populations based on single cell dynamics. Models to predict the internal environment of the cell by the

phenotype and vice versa will be a very novel approach to understand the adaptive resistance mechanism and to overcome it. Here, I propose to utilize an Agent-based cellular automata model to represent the cellular microenvironment, which can track the cellular response to drugs by tracking the metabolite or signaling levels which can then be experimentally constrained and tested using live cell FRET reporter constructs.

Table of Contents

List of Figures

List of Tables

1 Introduction

Human epidermal growth factor receptor 2 (Her2) is overexpressed in almost 20-30% of breast cancers (A.C. Wolf M.E. Hammond 2007). Trastuzumab targets Her2 and has been an effective mode of treatment for women with Her2-positive breast cancer and administered with the adjuvant therapy. Lapatinib is given to patients who don't respond to Trastuzumab. Lapatinib is a small-molecule kinase inhibitor, which interrupts both Her2 and epidermal growth factor receptor (EGFR) pathways. Lapainib is given orally alone or in combination with chemotherapy and with Trastuzumab. But patients acquire resistance to Lapatinib within a year. Multiple studies have been focused to understand the underlying mechanism of development of the resistance to Lapatinib. This study is focused on developing the computational model to study the development of resistance to Lapatinib in a heterogeneous population of cells. Previous studies have indicated the perturbation in the activity levels of AMP-activated protein kinase (AMPK) and Mitogen activated protein kinases (MAPK) in the resistant cells. So, in this study the computational model is built to investigate the cross talk between AMPK and MAPK and to examine if their correlation can attribute to the development of Lapatinib resistance in cells. The agent based modeling system has been utilized to build the computational model. The model is constrained by the variables such as different media conditions (with or without Lapatinib), activity levels of AMPK & MAPK, a phenotypic switch in the heterogeneous cell population and a cell death rate.

1.1 HER2-positive-EGFR/ErbB2

Breast carcinoma is among the most common reasons for cancer morbidity in women across the world based on the breast cancer statistics (Susan G. Komen Statistics, 2014). Present research focuses on targeting molecular factors, which can improve the diagnosis and treatment of the disease. Her2 oncogene is recognized as one of the important molecular markers for characterizing breast cancer subtypes. Tumors that express Her2 are called Her2 positive breast cancer and targeted therapies to Her2 are utilized clinically. Normally one copy of Her2 gene (ErbB2 gene family) is present in chromosome 17 of the cell; however, the Her2 positive tumors overexpress the Her2 gene. [2]

Signal transduction pathways in breast epithelial cells include activation of the transmembrane tyrosine kinase growth factor receptors (EGFR/HER1, HER2, HER3, and HER4). This signal transduction pathway regulates cellular growth, survival and differentiation involving PI3K/Akt and the Ras/Raf/MEK/MAPK pathways.

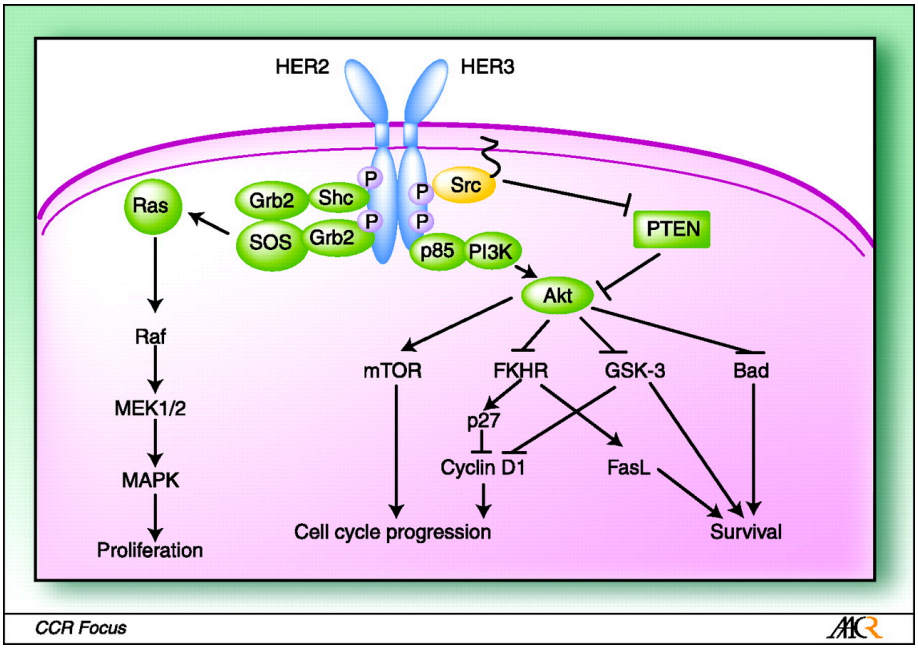


Figure 1: The ErbB signaling pathway [3]

ErbB family receptors dimerize upon ligand binding. This dimerization leads to the activation of intracellular tyrosine kinase. Ligand binding leads the heterodimerization of Her2 -Her3. FKHR, forkhead in rhabdomyosarcoma; Grb2, growth factor receptor-bound protein 2; GSK-3, glycogen kinase synthase-3; MAPK, mitogen-activated protein kinase; mTOR, molecular target of rapamycin; PI3K, phospatidyl-inositol 3-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SOS, son-of-sevenless guanine nucleotide exchange factor.

Her2 (also known as Neu or ErbB2), a receptor tyrosine kinase belonging to the human epidermal growth factor receptor (EGFR) family that also includes EGFR/Her1, Her3, and Her4, is an important component of cell-signaling networks, and is implicated in the growth of a variety of cancers [4-7]. The receptors of the EGFR family activate through dimerization (Figure 1), which is promoted by the binding of ligands from the EGF family to the receptors' extracellular regions ('ectodomains'). This activation process relies on a number of allosteric interactions in the extracellular, transmembrane, and intracellular portions of the receptor [8, 9], which lead to the formation of a specific asymmetric active dimer of the intracellular kinase domains [10]. Her2 is unique in the family in that it does not homodimerize under normal conditions, and its ectodomain does not bind ligands. Instead, it activates through heterodimerization with other members of the family (EGFR and Her3, in particular) when they are bound to ligands [6, 11]. This change in structural conformation leads to activation of tyrosine kinase domain intrinsic to the cell followed by subsequent phosphorylation of specific tyrosine kinase residues. These tyrosine kinase residues serve as docking site for signaling molecules, which leads to the activation of many downstream signaling pathways like MAPK pathway, PI3K pathway. Approximately 20-30% [12] of breast cancer cases have an amplification of the Her2 gene (~2-20 folds). This amplification of the Her2 gene leads to excessive cell signaling and cell division and ultimately tumor formation. This makes Her2 positive breast cancer one of the aggressive subtypes and thus, Her2 is a valuable treatment target.

For therapies to be effective various diagnostic techniques have been introduced to confirm the amplification of the Her2 gene and also predict the sensitivity to cytotoxic drugs.

Currently four testing techniques are being used: the IHC test (ImmunoHistoChemistry) diagnoses the excess of Her2 protein in cancer cells and results are given as 0 (negative), 1+ (negative), 2+ (borderline) and 3+ (positive, i.e., overexpression of Her2 protein), FISH (Florescence In Situ Hybridization) detects the excess copies of Her2 gene in cancer cells and the results show positive for amplification and negative for no Her2 amplification, SPoT-Light Her2 CISH Test (Subtraction Probe Technology Chromogenic In Situ Hybridization) and Inform Her2 Dual ISH test also detects the extra copies of Her2 like FISH and delivers results as positive for amplification and negative for no amplification.

1.2 Targeted Therapy-Trastuzumab

The aggressiveness of Her2 positive breast cancer correlates with the poor survival rate. Her2 positivity and its high level of expression in tumor tissues make it an ideal target for the therapies. Presently there are two approved Her2-targeted therapies: monoclonal antibodies (Trastuzumab) and small molecule inhibitors (Lapatinib).

Trastuzumab (Herceptin) is humanized anti-HER2 monoclonal antibody, which specifically targets Her2 in cancerous cells. Trastuzumab was approved in 1998 by FDA as a first line treatment for the metastatic disease in combination with paclitaxel. Trastuzumab is used for patients who have already received chemotherapy. The antigen specific sites of Trastuzumab bind to the extracellular domain of Her2 receptor and prevent the binding of ligand and in turn impede the activation of intracellular tyrosine kinase. Trastuzumab therapy has significantly increased the response rates and overall survival in patients with Her2+ metastatic breast cancer.

Mice model studies have shown the inhibition of angiogenesis by Trastuzumab in Her2 positive breast cancer. Preclinical studies have suggested that Trastuzumab recruits immune effector cells responsible for antibody-dependent cytotoxicity. In clinical studies, the joint analysis of National Surgical Adjuvant Breast and Bowel Project (NSABP) B-31 and North Central Cancer Treatment Group (NCCTG) N9831 trials showed that addition of trastuzumab to adjuvant chemotherapy significantly improves disease free survival by 52% compared to chemotherapy only. This study also led to the approval of Trastuzumab for the adjuvant treatment of Her2+ and node-positive cancer in 2006.

But there are multiple mechanisms through which patients develop resistance to Trastuzumab. Nagata et al. suggested the role of PTEN loss (decreased expression) attributing to the resistance [13]. PTEN inhibits AKT inducing growth arrest in cells. Low levels of PTEN insufficiently inhibit AKT and cells continue to grow not responding to Trastuzumab. Activation of alternate pathways like IGF-IR could also contribute to the resistance [14]. IGF-1R is a tyrosine kinase receptor, which, when overexpressed, leads to cell proliferation and metastatisation. Expression of other Her receptors like Her3 or EGFR can also be another reason. Expression of TGF-a plays a potentially important role in development of resistance. Upregulation of src activity (pernelle lavaud, fabric andre 2014) or MUC4, increased VEGF expression (pernelle lavaud, fabric andre 2014) and overexpression of c-MET (pernelle lavaud, fabric andre 2014) are also studied as the mechanisms of development of resistance to Lapatinib. (pernelle lavaud, fabric andre 2014).

ErbB targeting drug	Targeted therapy	Type of Cancer
Trastuzumab	Monoclonal Antibody (domain IV of Her2)	Her2 positive breast
Afinitor	Tyrosine kinase inhibitor	Her2 negative breast
Lapatinib	Tyrosine kinase inhibitor	Her2 positive breast
Pertuzumab	Monoclonal Antibody (domain II of Her2) (2011 gloria Fuentes, Maurizio scaltriti)	Her2 positive breast
Afatinib	Tyrosine kinase inhibitor	NSC-Lung
Cetuximab	Monoclonal Antibody (domain III of Her2)	NSC-Lung, Colorectal
Erlotinib	Tyrosine kinase inhibitor	NSC-Lung, Pancreas, Renal
Everolimus	Tyrosine kinase inhibitor	Kidney, Her2 negative breast
Panitumumab	Monoclonal Antibody (domain III of Her2)	Her2 positive breast

Table 1: Approved Her2 targeting drugs

The table lists clinically approved monoclonal antibodies and small molecule kinase inhibitors, which target Her2 in various types of cancer. The monoclonal antibodies and their target epitope is mentioned.

Various strategies are considered for overcoming the trastuzumab resistance in patients. Small-molecule kinase inhibitors are considered as effective anticancer therapeutics. These kinase inhibitors target the ATP binding site of the intracellular domain of kinases and inhibits the downstream signaling. Lapatinib is one of the clinically approved small tyrosine kinase inhibitors. Contrary to Trastuzumab, it targets and inhibits the intracellular tyrosine kinase domain of Her2. Lapatinib interrupts Her2 signaling and also enhances Trastuzumab dependent antibody-dependent cell-mediated cytotoxicity (ADCC) (pernelle lavaud, fabric andre 2014). Some of the small molecule inhibitors studied under combinatorial therapy are: Lapatinib, Gefitinib, Erlotinib.

1.3 Targeted Therapy-Lapatinib (Tykerb)

Lapatinib is a small molecule dual inhibitor of tyrosine kinase. Preclinical trials have shown its efficacy in blocking EGFR and Her2 mediated signal transduction. Lapatinib competitively binds to the ATP binding pocket of EGFR/Her2, which prevents the ATP molecule to bind to the protein kinase domain, shown in the figure. This inhibits the auto-phosphorylation and activation of downstream signal transduction.

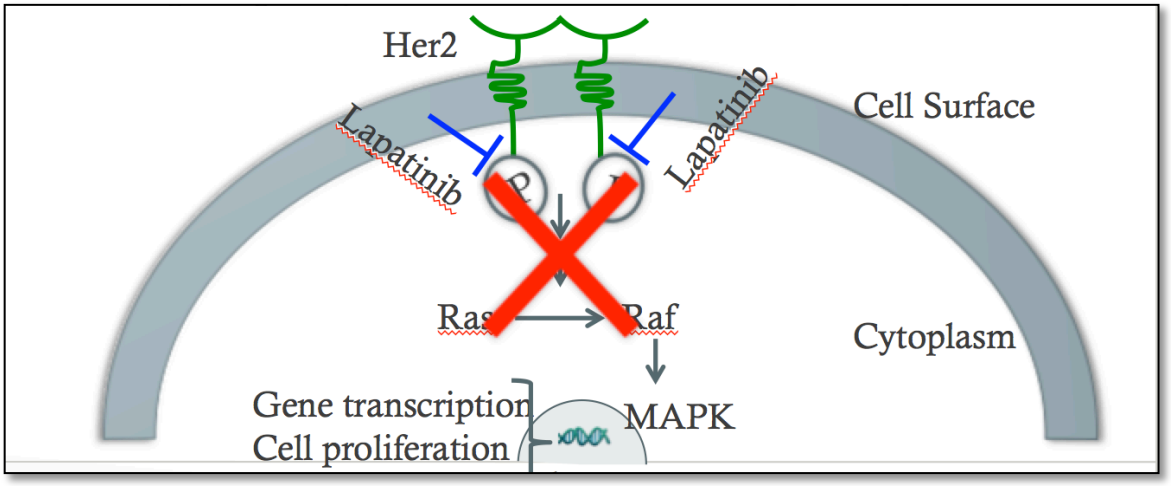


Figure 2: Dual tyrosine kinase inhibition by Lapatinib

The dimerization leads to the activation of intracellular kinase domain. Lapatinib bonds to the ATP binding site of the tyrosine kinase. This blocks the transmission of signal to downstream molecules and the growth signaling is interrupted.

Phase II clinical trial of Lapatinib in combination with Paclitaxel has shown an overall response rate of 33% in Her2 positive metastatic breast cancer [16]. Lapatinib is administered orally and it is speculated that it may cross the blood-brain barrier and reduce the risk of CNS metastasis [16]. In the combination with Capacitabine, Lapatinib has shown efficacy in improving progression-free survival (). The combination of Lapatinib with Trastuzumab has been investigated in phase III study and was found to improve progression free survival and overall survival ().

Primary and acquired resistance in patients has restricted treatment-involving Lapatinib. Phase II trials have shown the response rate of 20-30% to Lapatinib in Her2-positive metatstatic breast cancer [17]. Patients responding to the treatment have also acquired resistance to the treatment within a year. The mechanism of drug sensitivity and acquired resistance has not been fully understood. Studies have shown that the resistance can be attributed to ER (estrogen receptor) and PR (progesterone receptor) signaling upregulation due to increased activity levels of FOXO3a and Bcl-2 [18]. Another study has shown the activation of AXL [19]. Overexpression of AXL leads to invasiveness of breast cancer. Src overactivation has also been considered as a potential mechanism to Lapatinib resistance in various studies [20]. In addition studies have also shown an upregulation of metabolic functions can lead to decreased lapatinib sensitivity and adaptive resistance [20].

1.4 MAPK & AMPK

Overexpression of Her2 leads to increased downstream Ras-Raf-mitogen-activated protein kinase signaling. Mitogen activated protein kinases belong to CMGC kinase (CDK/MAPK/GSK3/CLK) group and are involved in cell signaling for proliferation, differentiation, gene expression, cell survival and apoptosis [21]. They undergo phosphorylation in their activation loop to become active. The MAPK cascade involves multiple layers of pathways. The ERK1/2 pathway is a known MAPK pathway. ERK pathway is involved in cell proliferation, which makes it a target pathway for cancer studies where cells show elevated proliferation rate. Lapatinib inhibits MAPK-Erk1/2 activity in Her2 positive breast cancer cell lines; tumor xenografts; and in clinical tumor biopsies. Work from the Ram Lab has shown the complete inhibition of Her family in presence of Lapatinib but the MAPK activity was partially inhibited. [20]

From previous studies conducted in the Ram lab, SKBr3 cells were made resistant to Lapatinib by treating them with Lapatinib over a year. Western blot analysis had shown that Lapatinib could inhibit Her2 family phosphorylation but could only partially inhibit the activity of MAPK. Along with the partial inhibition of MAPK from western blot, gene profiling indicated the up regulation of glucose uptake and metabolism pathways. Western blot analysis also indicated the upregulation of AMPK.

AMPK is involved in maintaining cellular energy homeostasis. The heterotrimeric subunit of AMPK is responsible for its enzyme activity. It is the metabolic regulator in glucose uptake, fatty acid oxidation and biogenesis of glucose transporters [22]. AMPK activation positively

regulates signaling pathways, which replenishes the ATP supply in fatty acid oxidation and autophagy. AMPK also negatively regulates ATP-consuming process like gluconeogenesis, lipid and protein synthesis.

High activity levels of AMPK were also seen in Lapatinib resistant cells. Since tumors are dependent on aerobic glycolysis (Warburg effect) for energy (ATP), metabolic proteins, fatty acid and nucleic acid biosynthesis [23], the activation of metabolic stress response via AMPK might contribute to Lapatinib resistance.

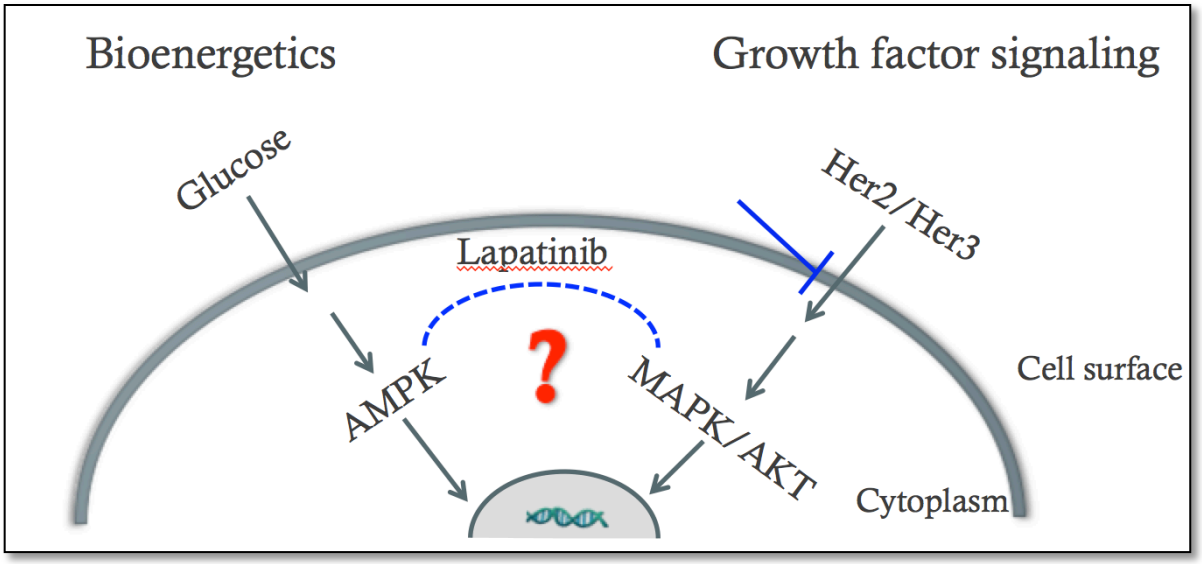


Figure 3: Cross talk between AMPK and MAPK to develop resistance against Lapatinib

Lapatinib inhibits intracellular Her2 signaling. The potential crosstalk between AMPK at the downstream of glucose pathway and MAPK at the downstream of growth signaling pathway can lead to the cellular resistance to Lapatinib.

The figure shows that AMPK, playing a crucial role in maintaining cellular energy homeostasis, is at the downstream of glucose pathways and MAPK is at the downstream of growth factor signaling pathway. The activity levels of MAPK and AMPK can be correlated to study the Lapatinib resistance. A computational model can represent this biological scenario and can give the flexibility to study the speculated mechanism of resistance if there is a cross talk between AMPK and MAPK.

With the intent of designing the computational model to study the resistance in cells, various factors have to be considered, some of which are: heterogeneity in the MAPK and AMPK activity levels across the cellular population, doubling time of cells, growth and division rates, death rate of cell, cellular interactions with neighboring cells, phenotypic switch leading to enhanced or reduced activity levels of MAPK and AMPK between cells, different media conditions. Designing and constraining the computational model requires tracking the activity levels of AMPK and MAPK. A FRET (Florescence resonance energy transfer) based analysis in microfluidic system can be efficient to track the activity levels in a heterogeneous population of cells. Microfluidic system involves study of cellular population in a fluid media including the manipulation and analysis of cultured cells. Microfluidics give the advantage of controlling the media and environmental conditions (like oxygen, glucose, CO_2) and performing time point studies. So, in my study, cells can be monitored under different media conditions and activity levels of AMPK and MAPK can be tracked over time. Fluorescence studies involve the tracking of energy emitted by the fluorophore associated with the protein. FRET can be employed to track the activity levels of AMPK and MAPK in cells under the microfluidic system.

1.5 FRET: Florescence resonance energy transfer

We know that there is heterogeneity in the population of cells, which can be critical to their chance of survival and proliferation in different environmental conditions. So, it is important to track variability among single cells and their behavior across the cellular population. Highly sensitive detection and analysis of a single cell has been a challenge for researchers. In my study I have used florescence resonance energy transfer to track individual cells. FRET generates fluorescence signals sensitive to molecular conformation with a separation of approx. 10 nm. Florescence microscopy produces contrast by exploiting the display of light emission based on ground and excited states of electrons. [24-26]

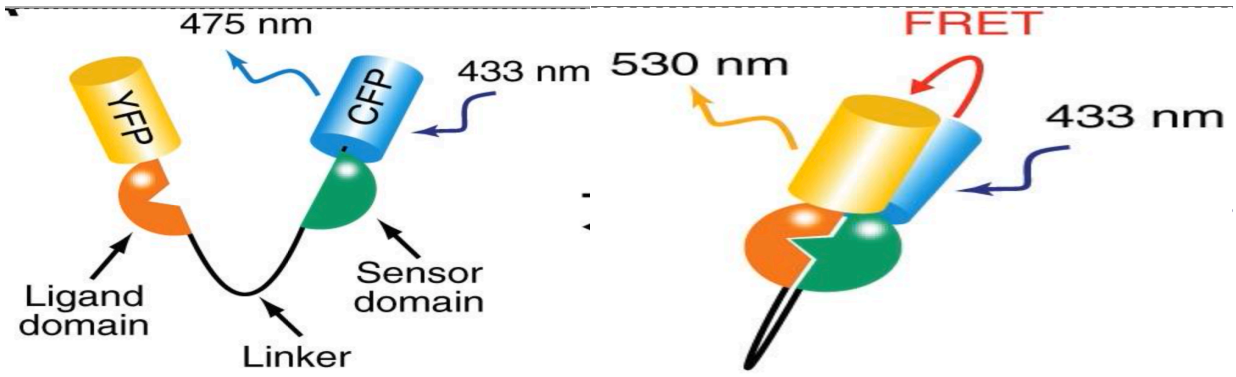


Figure 4: Florescence Resonance Energy Transfer [27]

The FRET system consists of Ligand domain, sensor domain connected by a linker protein. Fluorophores are attached to both domains. Activation of protein of interest leads to the conformational change bringing the two domains together. This brings the two fluorophores next to each other and energy is transmitted and emitted in the form of fluorescence.

FRET is unique in providing signals sensitive to intra and an intermolecular interaction resolving molecular intersection and a conformation with a spatial resolution far exceeding the inherent diffraction limit of conventional optical microscopy and also is compatible with super resolution techniques. The FRET technique involves transfer of energy from a fluorophore in excited state being a donor to another chromophore which acts as acceptor [28].

Single cell FRET imaging has been applied in determination of the pathway of tumor cell apoptosis study [24]. In this study the spatio-temporal dynamics of caspase-3 and caspase-8 activation in living cells real time with FRET technique on SCAT3 and Bid-CFP. To acquire the temporal profile of caspase, the SCAT3 expressing cells were treated with PDT. The fluorescence microscope collected the cellular emission of fluorescence. Recently, scientists have implemented genetically encoded FRET sensors for measurement of pyruvate transport, production and mitochondrial pyruvate consumption [29].

A previous study in the Ram lab had established that there is a heterogeneous population of cells, which behave differently in the presence of Lapatinib in the media. The phosphorylation was tracked using FRET system on microfluidic plate. In the experiment EKAR (ERK activity reporter) FRET sensors were used to track ERK phophorylation. The EKAR sensor consists of a fluorescent protein FRET pair, a substrate phosphorylation peptide from Cdc25C containing the consensus MAPK target sequence and proline directed phosphor-binding domain. ERK activation leads to the phosphorylation of the substrate sequence followed by subsequent binding by the phosphor-binding domain. This leads to the conformational rearrangement and brings the

receptor and donor domain of FRET close enough for the interaction to take place between the flurophores during the ERK activation. [30]

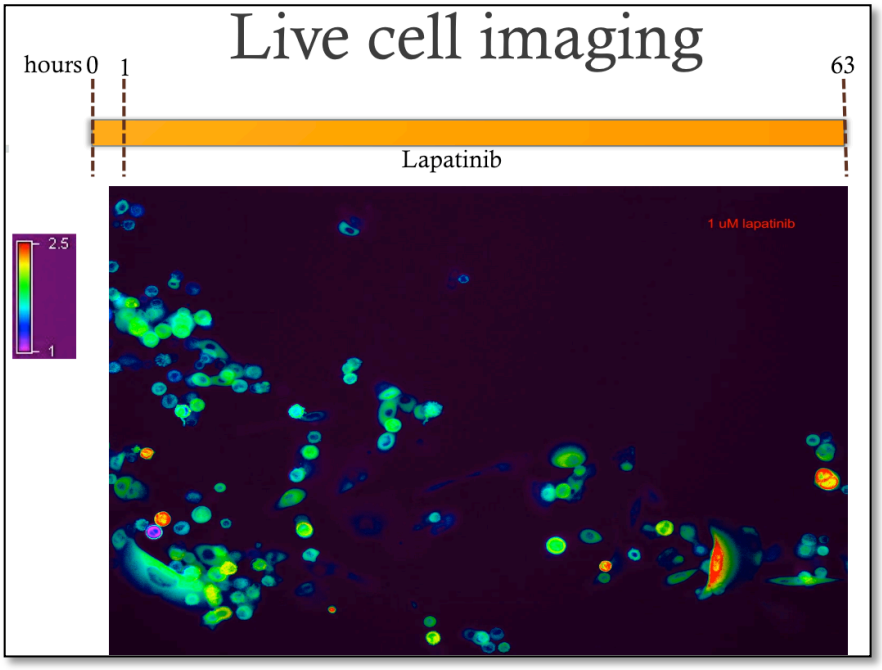


Figure 5: FRET imaging

MCF10A cells in complete media in a microfluidic system. After 1hour Lapatinib was added to the media and cells were tracked for 63hours. FRET images were taken every 15 minutes. The fluorescence shows the heterogeneity in the levels of MAPK in cellular population. In the presence of Lapatinib the cells become inactive towards the end of experiment emitting no fluorescence.

The figure shows the population of cells with heterogeneous MAPK activity levels. The cells with high levels of MAPK are shown in yellow and red colors and cells with lower MAPK levels have green bluish colorization. The cells were tracked for 63h and the cells with lower MAPK levels were observed to die earlier than the cells with high MAPK activity levels. At the end of 63h all the cells had died. This experiment indicated that high levels of MAPK could contribute to the differential sensitivity to Lapatinib.

1.6 Agent Based Model

In the study of complex diseases like cancer, scientific illation of any vision involves theoretical, experimental and computational approaches. Computational models (mathematical modeling and simulation) are especially important as they come from a thorough understanding of theoretical exploration. Agent based modeling is a computational approach, which demonstrates the evolution of a dynamic system by exploring and simulating its individual constituents. It is an unbiased approach describing live systems evolving over time by analyzing the interconnection of its individual agents.

Agent based modeling is a suitable representation of biological systems due to variety of reasons: [31]

1. The individual agents of the system set the rules and guidelines of the model. These rules are flexible and can be modified as the model evolves.

2. The model evolves by the interaction of the individual agents with each other and with the system. This results in tracking intricate details by modeling the system for more accuracy.

3. Agent-based model can be constructed even without the complete knowledge of the system. So, in the initial phase the model can still be built based on the ideology representing the simplest level of the vision.

4. Considering the random exhibit of behavior of biological systems, the agent-based models can determine the rule based on the proportionality of the behavior in consideration to the entire system and then convert it to the rules between individual agents.

5. It excludes the difficulties during the isolation of human systems and ethical problems related to the experimentation due to its existence on virtual system.

6. Also, in cases where the biological systems are very complex with multiple interactions and range of inputs, the agent-based model proves more appropriate to obtain results and multiple runs.

Many modeling software are available to run agent based models. Software like Swarm, NetLogo and AnyLogic are widely utilized to apply ABM. This algorithm has been largely used for representing biomedical applications including population dynamics [32] and human immune system (Association for Computing Machinery. 2006.). Zhihui Wang ET. Al. used ABM simulation for non-small cell lung cancer studies to study the spatio- temporal expansion of virtual glioma cells in 2D [33].

These studies have shown the usefulness of ABM simulation from molecular scale up to cellular level and also the applicability and flexibility of this platform in cancer studies. In my study ABM has been used as predictive models to study the behavior of single cells in a heterogeneous population.

2 Hypothesis

Intracellular MAPK and AMPK activity in a heterogeneous cellular population contributes to the development of resistance to Lapatinib.

To test the hypothesis, the cells were treated with Lapatinib and the activity levels of individual cells were tracked using live cell reporters (FRET) and agent based model was used to build predictive models to predict molecular activities of AMPK and MAPK and correlate with phenotypes like cell division and cell death.

3 Specific Aims

Aim 1: Development of computational model

Rationale: To computationally model the cellular activity to mimic the biological behavior in computational simulations

Aim 2: Simulations of single cell and the population dynamics in response to perturbation by EGF and Lapatinib

Rationale: To computationally investigate the intracellular activity with cells in different media and stress conditions and the corresponding changes in cell proliferation.

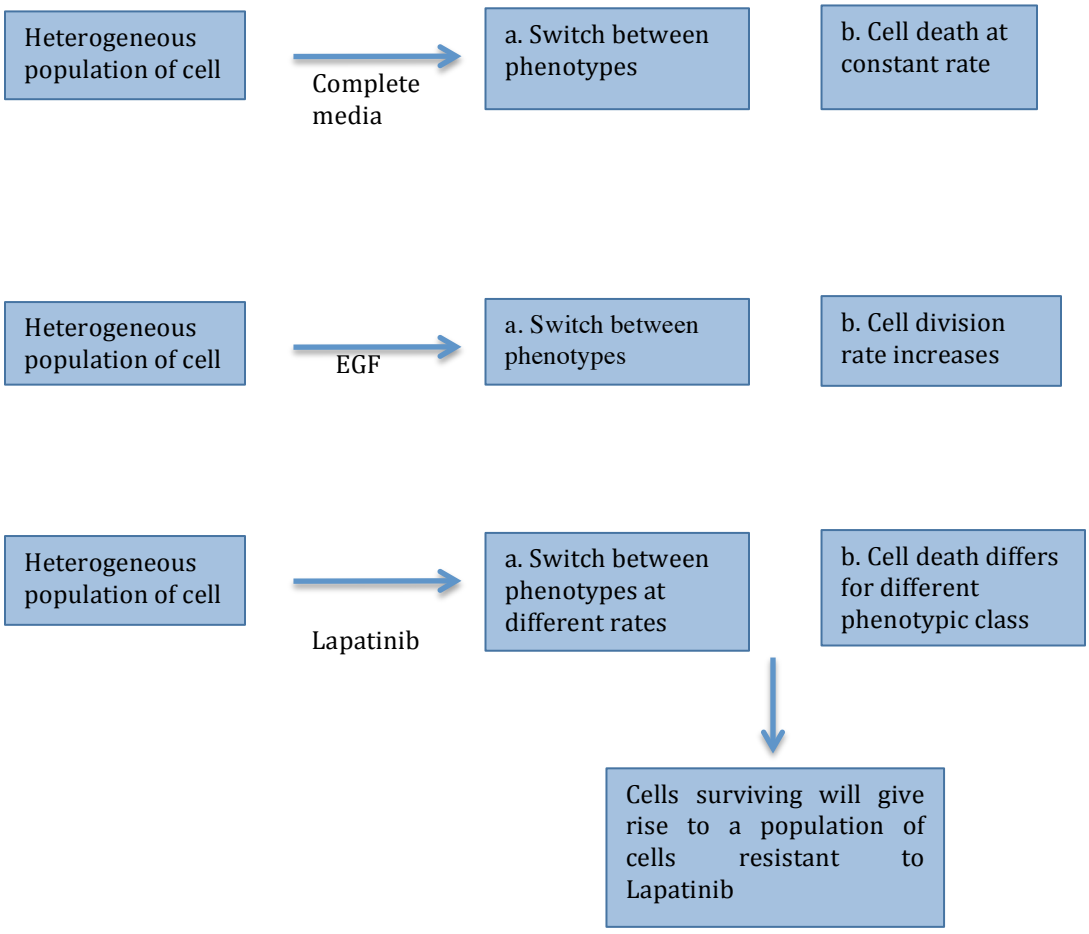
Aim 3: Computationally predict cellular behavior that leads to adaptive resistance

Rationale: Correlate phenotypic switch with cell death rate to study the development of resistance under different conditions of media and rates of cell death and cell phenotype switching.

4 Methodology

To study Lapatinib resistance based on the correlation between MAPK and AMPK levels, the computational models were based on the hypotheses that intrinsic population of cell gives rise to resistant cells. The alternate hypothesis to this is that cells switch phenotype under stress of Lapatinib. The hypothesis is tested based on the computational agent based modeling system. To simulate the agent-based model, the Netlogo framework was used. The model built uses default grids (also called patches), which are referred to here as culture dishes. The patches provide the area for the agents to move and perform actions. The model holds 4 kinds of agents (called turtles in NetLogo): high MAPK high AMPK, high MAPK low AMPK, low MAPK high AMPK and low MAPK low AMPK. The functions associated to agents are to move, divide, switch between different kinds of agents . The concept involved is that the cells (agents) divide more quickly in the presence of growth stimulator and only certain class of agents survive and grow in the presence of Lapatinib (growth inhibitor). These agents are regarded as lapatinib resistant cells, which further give rise to a population of cells completely resistant to lapatinib. The environmental conditions are invoked using stochastic impulses to gain more realistic understanding of effect of environment perturbations on the agents.

4.1 Conceptual Model and Logic implementation in the prototype



The heterogeneous population of cells switch between different phenotypic classes and die at a constant rate (3%) in the presence of complete media. When EGF is stochastically added, the division rate of cells is enhanced. When Lapatinib is added to the media, it acts as growth inhibitor and increases the cell death rate in the cellular population, which also varies between the different phenotypic classes. The model tracks the number of surviving cells in each phenotypic class with different environmental conditions.

4.2 States

All the agent sets in this agent-based model have 3 states: movement, division and death. Apart from that the agents also switch between four phenotypic classes and attain their characteristics.

Movement: At any time step all turtles can move in a patch in their neighborhood chosen at random.

Division: At any time step the turtles divide to two turtles of same phenotypic class.

Death: At any time step the life counter of agent is decremented by the user input value. If the life counter of an agent reaches 0 then the agent is removed from the simulation.

Switch: turtles switch between different phenotypic class. The rate of switching is user defined.

4.3 Transition and Simulation

To stimulate the process, the model is initiated with a user-defined number of agents in each phenotypic class. In the program the ‘set’ button resets the code and ‘go’ button starts running the code till the code terminates.

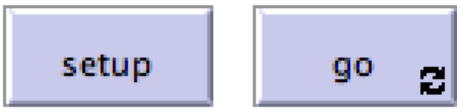


Figure 6: Start button to initiate the process

The model consists of 4 phenotypic groups of cells representing heterogeneous population of cells, which are high MAPK high AMPK, high MAPK low AMPK, low MAPK high AMPK and low MAPK low AMPK. The constructor class represents the interface look of cells:

```
set-default-shape highhigh "circle"
 create-highhigh initial-highhigh
 [
   set color green
   set size 1.5
   ;set energy random (high-gain)
   setxy random-xcor random-ycor
 ]
```

The initial number of cells in each class is set by the slider (11 in this case), which can be set to any number in between 0 to 100.

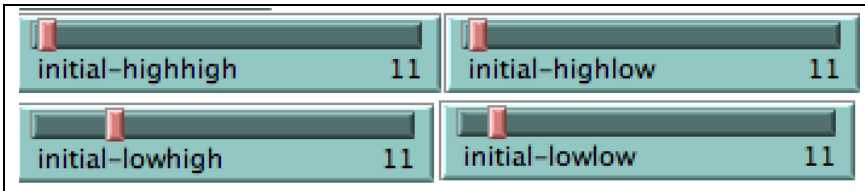


Figure 7: Slider bar for user-input of initial cell count

The basic functionality of the cells represented by the program is the cell movement, cell division and cell death.

The move function makes the cells to move in the program:

```
to move
  rt random step-size
  avoid-walls
  lt random step-size
  fd 1
end
```

The function here indicates the cells to move stepwise and forward one step at a time.

The cell division is indicated by the below code:

```
to reproduce-highlow

    hatch 1 [random-float 360 fd 1]

end
```

The hatch command is the primitive command of the Netlogo, which creates new cells identical to their parents and asks the daughter cells to run the commands directed for it.

The cell death is shown by the death function:

```
to death-lap-low
  [ die ]
end
```

The die command is also primitive Netlogo command, which makes the cells to die.

The cells with different MAPK and AMPK levels differ in their cell division rate, which is set by the slider at the user interface.

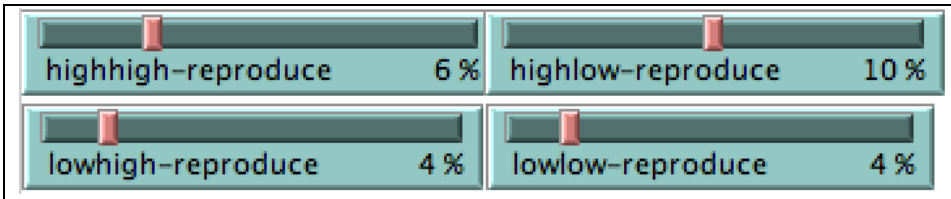


Figure 8: Slider bar at user interface for cell division rate

The initial cell death is set to 10% for all classes of cells. The cells with different phenotypic composition behave differently under different stress conditions and in the presence of growth stimulator. Also, these cells switch phenotype, which is shown by the code:

```
switch [set breed one-of ( list highlow lowhigh lowlow ) set color pink]
```

The code here indicates the cells switch phenotype at random to other phenotypic classes and the cells with switched phenotype are pink in color.

In the presence of EGF the growth of the cells are increased which is directed and controlled by the slider in the interface. When Lapatinib is switched on cells with different phenotype is measured for their different cell death rate. The sliders in the user interface of the program control these different conditions.

Figure 9: Slider bar for cell division in presence of EGF

The counter function keeps a track of cell numbers from all the four classes and as the cells switch the classes.

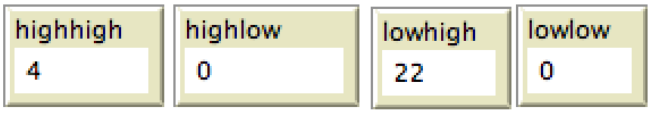


Figure 10: Counter to keep track of the number of cells

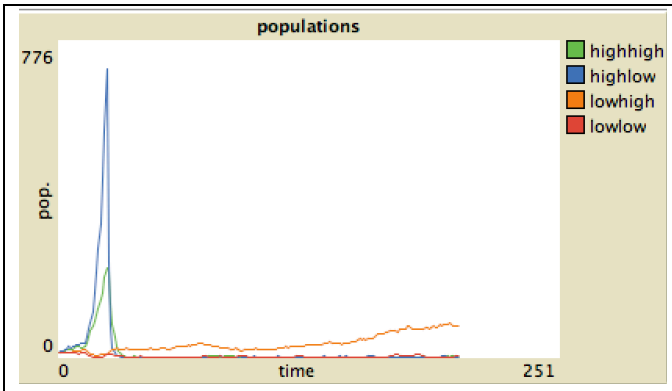


Figure 11: Histogram indicating the number of cells in different classes with time

5 Results and Discussion

5.1 Development of Computational Agent based model

Agent based modeling is a computational method used to study complex systems. It comprises of agents and their interactions with each other and with their environments in a computer-simulated environment. The code (in SCALA) for the model is written from the point of view of each of the agent types represented in the model and their environments. Behavior can be described in terms of activities or conditional statements, which constrains the model to mimics the biological scenario. After identifying the agents and its environment the model is constrained with a set of rules for the behavior.

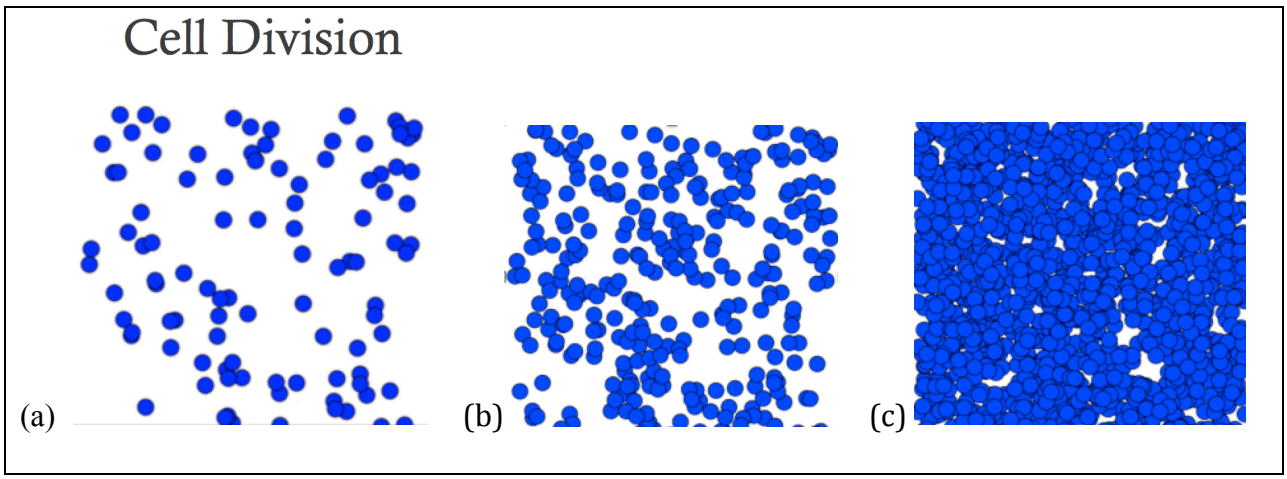


Figure 12: Model Version 0: Simulation of cell division replicated in computational model

(a) Culture dish seeded with initial number of cells
(b) Cells dividing to two daughter cells during the simulation
(c) The culture dish becoming confluent with the cells and the simulation ends

The computational model in the figure represents computational simulation of cell division. The agents in the model are the cells and their environment is the culture dish. The model consists of a cell culture dish with initial number of cells. The variable in the model are the number of cells and doubling time of the cells. The model is constrain by the following rules:

- Culture dish is seeded with initial number of cells
- Cells divide at their doubling time
- The process (simulation) ends when the culture dish is confluent

In the simulation when the cell reaches its doubling time, it divides to two daughter cells. The simulation runs till the culture dish becomes confluent with the cells.

5.2 Simulations of single cell and the population dynamics in response to perturbation by EGF and Lapatinib

After the designing of the agent-based model for the cell division, the next model designs cells (agents) treated under different media conditions (environments).

Cells in Media

Figure 13: Cells in different phenotypic group under complete media.

Cells in red have low MAPK and low AMPK. Cells in orange have low MAPK and high AMPK Cells in blue have high MAPK and low AMPK. Cells in green have high MAPK and high AMPK. Cells change to pink during transitioning to different phenotype. The graph is cellular population in each group tracked over various time points.

The figure shows a heterogeneous population of cells in a complete media. During the simulation the cells divide to populate the culture dish. They also, switch from one group to other group, shown in pink. The simulation indicates the uninterrupted cell growth in the complete media. The graph shows the number of cells in each class over the period of time. From the graph it is evident that the number of cells increases in each phenotypic group with time. In this simulation cells are not in any kind of environmental stress allowing them to divide, grow, switch and die at their own pace without any pressure.

Cells in EGF

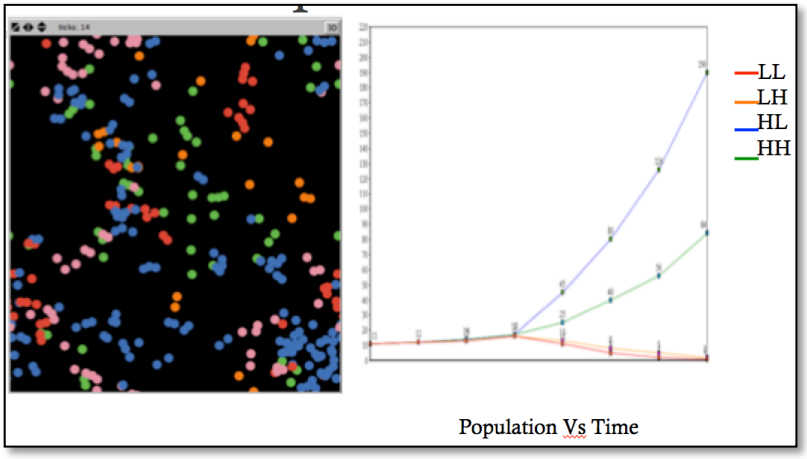


Figure 14: Cells in various phenotypic groups in presence of EGF

The graph indicates the number of cells in each group over time. The cell count increases overtime in the presence of growth stimulator, EGF

The figure shows a heterogeneous population of cells in media and EGF is spiked in the media after 10 ticks (number maintained throughout the simulation for all the media conditions). The graph shows the rapid growth in number of cells with High MAPK and low AMPK since AMPK is the growth suppressor and comparatively low cell division and growth with cell having high MAPK and high AMPK. The model also shows the phenotypic switch in cells from high MAPK to low MAPK and high AMPK to low AMPK. After switching the phenotype the cells behave in their newly switched phenotype. The simulation indicates the rapid cell growth in media with growth stimulator EGF. The graph shows the rapid increase in groups of cells with high MAPK. Also, the cells with low MAPK may switch to high MAPK increase the cell count of high MAPK group.

Lapatinib in media

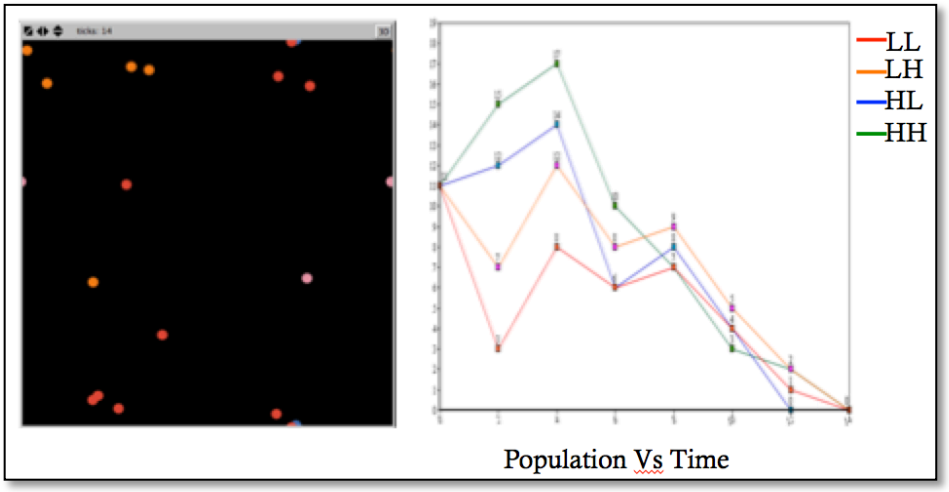


Figure 15: Cells in various phenotypic groups in presence of Lapatinib

The graph indicates the number of cells in each group over time. The cell count in each group decreases over time in presence of Lapatinib

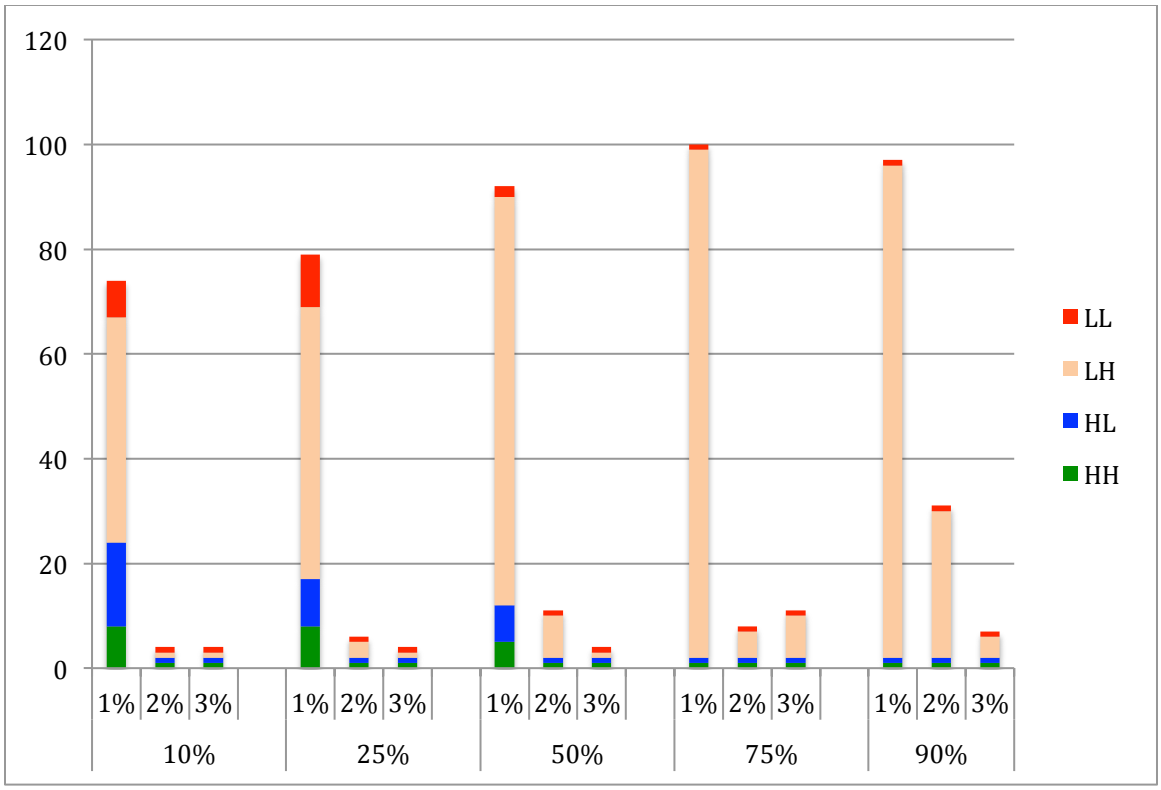
The figure shows a heterogeneous population of cells in media and Lapatinib is added to the media. The graph shows the rapid decline in the cell division in the cells. The simulation also involves the cells switching their phenotype from low to high MAPK and AMPK and vice versa. The graph indicates the time point growth of cells resistant to Lapatinib and decline in the number of Lapatinib sensitive cells. At the end of the simulation all the cells in the culture dish die.

The aim here was to understand cellular behavior in different media conditions. The growth stimulator EGF enhances the cell division while Lapatinib restricts the cell division ultimately letting the cells to die. So, considering the phenotypic switch and regulating the cellular death rate can give more information about the correlation of the levels of MAPK and AMPK and cellular response to drug Lapatinib.

5.3 Computationally predict cellular behavior that leads to adaptive resistance

To predict the cellular behavior, cells were put under different stress conditions in presence of Lapatinib:

1. Death rate
2. Phenotype switch

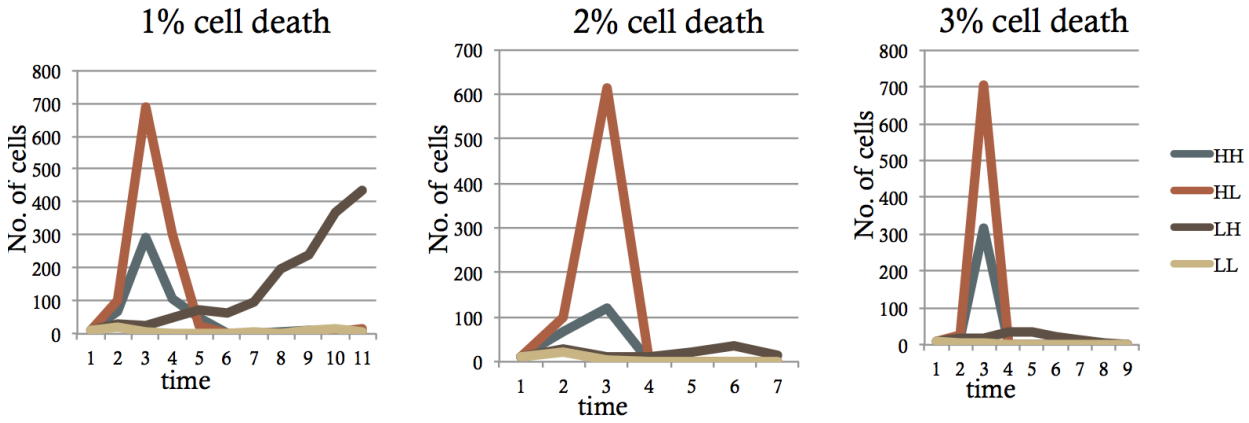


Graph 1: clustered stack chart for the death rate vs phenotype in each phenotypic group

The number of cells in each group is stacked and all the groups are clustered for various death rates under different phenotypic switch rate.

The computational model is used to run the simulation under different cellular stress conditions. In the presence of Lapatinib the death rate and phenotypic switch was regulated and the number of cells in each phenotypic group was tracked. The stacked bars indicate the number of cells in each group at the end of each simulation with various phenotypic switch rates. The bars from each group are then clustered together for different cell death rates. The death rate was varied between 1, 2 and 3% in the presence of Lapatinib and the phenotype switch between different classes of cells was varied between 10, 25, 50,75 and 90% respectively. The graph indicates the significant survival and increase in number of cells with low MAPK and high AMPK levels in comparison to the other phenotypic groups. The cell counts from individual groups have been shown in graph below (figure 16). The simulations show that with high AMPK levels and low MAPK levels in cells they can develop resistance against Lapatinib. These cells can then give rise to a population, which are totally resistant to Lapatinib. These results predict that there is a cross talk between AMPK and MAPK, which leads to the development of resistance in cells.

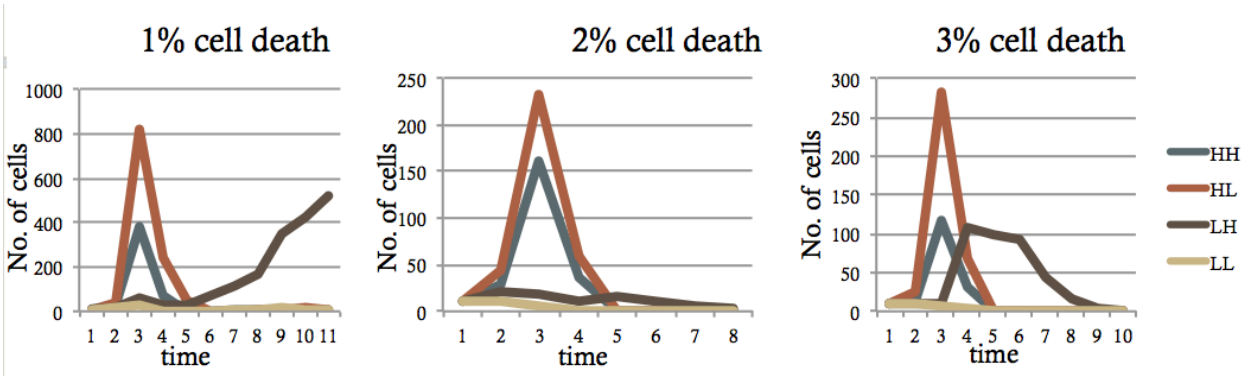
(a) 10% Phenotype switching



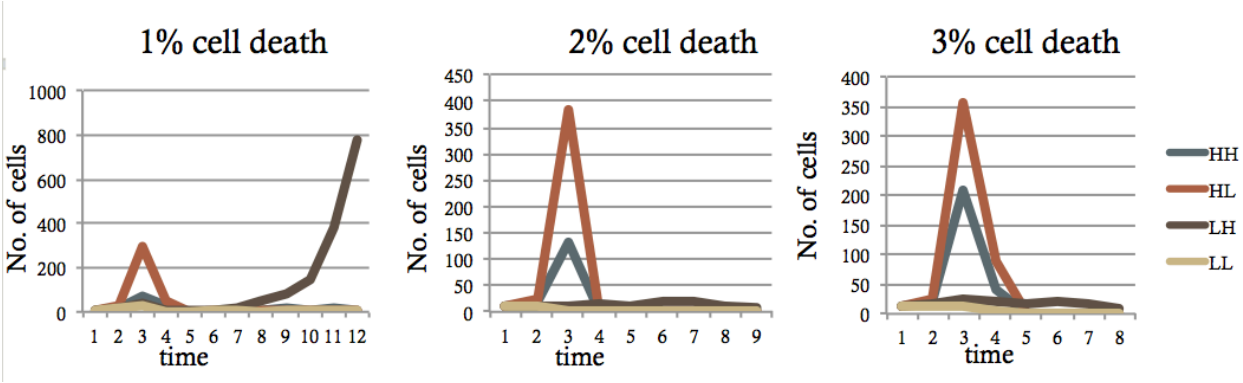
Death Rate

Switch	%	1	2	3
	10	436	0	1

(b) 25% Phenotype switching



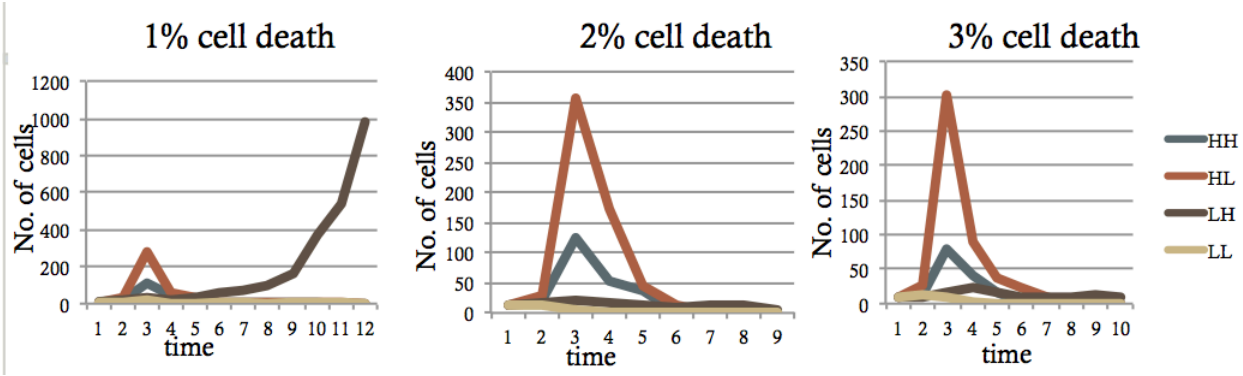
(c) 50% Phenotype switching



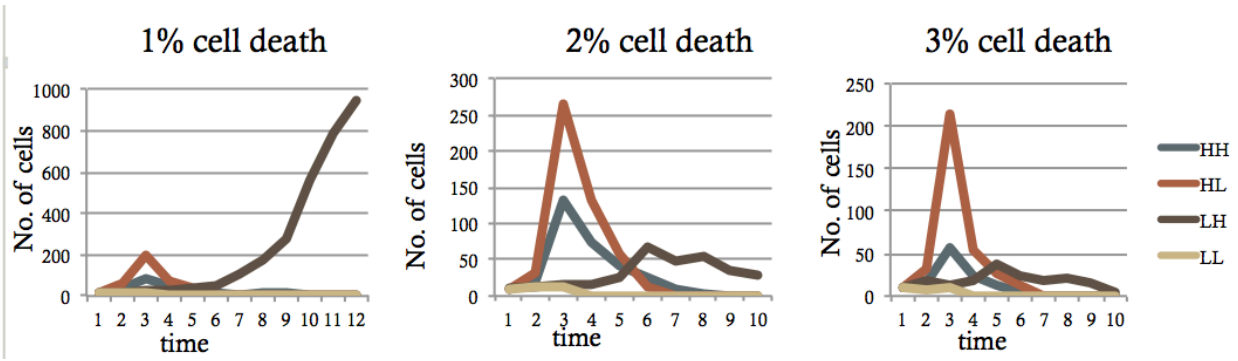
Death Rate

Switch	%	1	2	3
	50	780	8	7

(d) 75% Phenotype switching



(e) 90% Phenotype switching



Death Rate

Switch %	1	2	3
90	945	28	4

Figure 16 (a-e): The simulation at 10,25,50,75 and 90% phenotypic switch with death rates of 1,2 and 3%. The simulation of low MAPK and high AMPK (LH) group of cells is graphed. The table shows the number of cells in LH group in different stress conditions.

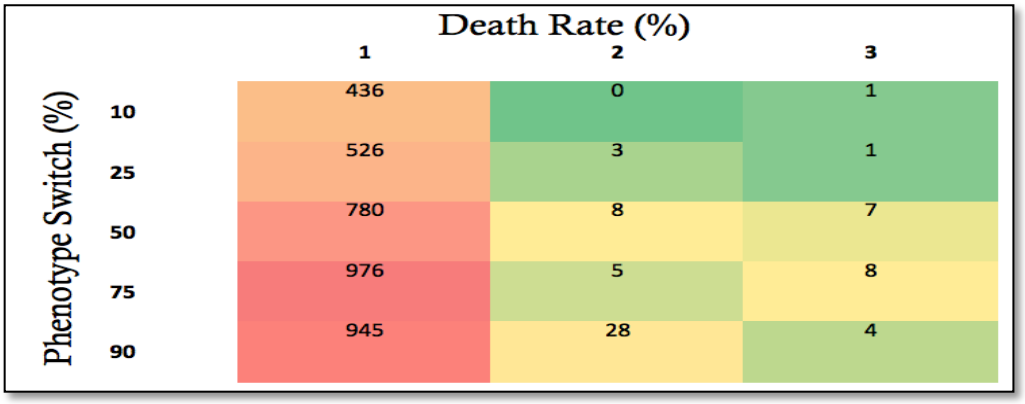


Table 2: Death Rate Vs Phenotype Switch

Cells in high MAPK low AMPK group are the survivors under Lapatinib media and varied cell death and phenotypic switch conditions.

Tracking the cells in high MAPK and low AMPK group, the heatmap shows the number of cells surviving at a given time point with different phenotype and death rate conditions. The model shows that cells with high AMPK and low MAPK have better survival in Lapatinib media condition. These cells can further give rise to the resistant population. The result suggests that high levels of AMPK can contribute to the development of Lapatinib resistance in cells. AMPK is a major player in cellular growth and metabolism regulation. Cells with high AMPK levels signifies high AMP/ATP ratio and activates catabolic pathways inhibiting the anabolic pathways [34]. This allows the cells to survive during the intervals of metabolic stress, which was induced by Lapatinib in this case. From the table 2 it is evident that cells survival rate increases with increase in phenotypic switch rate.

6 Summary and Future Directions

Almost 20-30% of breast cancer cases overexpress Her2 receptor tyrosine kinase. Targeting tyrosine kinase receptors is also front-line therapy along with monoclonal antibodies. Lapatinib is one such clinically approved tyrosine kinase inhibitor for the treatment of Her2-overexpressed breast cancer. However, a major concern with the therapy is that patients develop resistance against Lapatinib within a year. The underlying mechanism of Lapatinib resistance is still under speculation. It has been shown previously that Lapatinib could successfully inhibit Her2 family but it only partially inhibits MAPK signaling. Also, the gene expression data had shown the upregulation of glucose uptake and metabolism pathways. Activity of pAMPK was

upregulated as per the previous work in the lab. This indicated that activities of both MAPK and AMPK could contribute to the development of Lapatinib resistance in cells. To understand the mechanism of resistance, the computational models were developed. The agent-based model is a rule based computational model, which focuses on interactions between agents and with their environments. Agent based modeling system has been extensively used in systems biology to understand various biological behaviours. The modular approach of agent based modeling system gives the flexibility to modify any part of the model based on the biological behavior of the system at any given point without affecting the entire model. SCALA was used as the programming language to code for the model and the simulations were run using the NetLogo software. The various models showed cells in different media conditions. The simulation tracked the number of cells surviving and dividing under different environmental conditions. The model was constrained by different death rates and phenotypic switch between different cellular phenotype. The results showed that variations in death rate and phenotypic switch affect the cellular sensitivity to Lapatinib in the media. At a death rate of 1 resistant cell population is highest in number and they give rise to a population of cells resistant to Lapatinib. This resistant population then grows till the end of the simulation indicating their never-ending growth.

The results support the hypothesis that there is a cross talk between signaling and metabolic pathways, which directs the development of resistance to Lapatinib treatment in Her-2 positive patients. Lapatinib has been previously shown to induce apoptosis and cell death in HER2 overexpressing cells [36].

The agent-based models show the different stress conditions where cells switch phenotypes and die at different percentages. The models show that at higher phenotypic switch

to high AMPK and low MAPK the cells become insensitive to cell death pathways. This can be due to phosphorylation of TSC2 by AMPK, which enhances GTPase activity and decreases growth-signaling pathways [35]. It can also be attributed to the reciprocal relation between AMP and mTORC1, which directly interacts with ERK, activity which allows the cells to coordinate energy requiring anabolic processes with energy availability – under conditions of energy deprivation, activation of AMPK can limit energy-consuming processes such as protein synthesis via repression of mTORC1 [37]. The model shows that cells behave differently in different media conditions. In presence of EGF the cellular division rate increased leading to the increased cellular population in less time making the culture dish confluent. In the presence of Lapatinib, growth inhibitor, there was increase in the cell death. Cells where tracked under different cell death and phenotypic switch conditions in the presence of Lapatinib. The models show a significant increase in the cellular population with the phenotypic switch to high AMP and low MAPK levels in the presence of Lapatinib media condition. This indicates that under stress conditions (Lapatinib in media) cell can switch phenotype to survive. These cells can give rise to a population of cells, which will be resistant to the drug. The model prediction provides an alternative perspective of the development of resistance suggesting that there is heterogeneity in the cellular population and cells switch phenotypes randomly, which can lead to the development of resistance. The experimental tests following the model should aim at tracing levels of MAPK and AMPK in the heterogeneous cellular population. Because the cell death is involved in the study it is important to track the cell cycle and cell doubling time. The following experiment can be studying cellular population under different media condition with varying environmental factors. This model can be implemented in different cell type studies and can incorporate more conditions to replicate the cellular behavior for the study. This model can used to perform studies

on the 3D cell culture where the cell behavior will differ from inside and outside of the cluster. The model can also be utilized to perform a comparative study between multiple drugs and media conditions. The model also involves time point studies.

7 References

1. Komurov, K., et al., *The glucose-deprivation network counteracts lapatinib-induced toxicity in resistant ErbB2-positive breast cancer cells.* Mol Syst Biol, 2012. **8**: p. 596.
2. Yersal, O. and S. Barutca, *Biological subtypes of breast cancer: Prognostic and therapeutic implications.* World J Clin Oncol, 2014. **5**(3): p. 412-24.
3. Lin, N.U. and E.P. Winer, *Brain metastases: the HER2 paradigm.* Clin Cancer Res, 2007. **13**(6): p. 1648-55.
4. Hynes, N.E. and H.A. Lane, *ERBB receptors and cancer: the complexity of targeted inhibitors.* Nat Rev Cancer, 2005. **5**(5): p. 341-54.
5. Riese, D.J., 2nd, R.M. Gallo, and J. Settleman, *Mutational activation of ErbB family receptor tyrosine kinases: insights into mechanisms of signal transduction and tumorigenesis.* Bioessays, 2007. **29**(6): p. 558-65.
6. Baselga, J. and S.M. Swain, *Novel anticancer targets: revisiting ERBB2 and discovering ERBB3.* Nat Rev Cancer, 2009. **9**(7): p. 463-75.
7. Lemmon, M.A. and J. Schlessinger, *Cell signaling by receptor tyrosine kinases.* Cell, 2010. **141**(7): p. 1117-34.
8. Endres, N.F., et al., *Conformational coupling across the plasma membrane in activation of the EGF receptor.* Cell, 2013. **152**(3): p. 543-56.
9. Jura, N., et al., *Catalytic control in the EGF receptor and its connection to general kinase regulatory mechanisms.* Mol Cell, 2011. **42**(1): p. 9-22.

10. Li, B., et al., *Chronic treatment of astrocytes with therapeutically relevant fluoxetine concentrations enhances cPLA2 expression secondary to 5-HT2B-induced, transactivation-mediated ERK1/2 phosphorylation.* Psychopharmacology (Berl), 2009. **207**(1): p. 1-12.

11. Citri, A. and Y. Yarden, *EGF-ERBB signalling: towards the systems level.* Nat Rev Mol Cell Biol, 2006. **7**(7): p. 505-16.

12. Moasser, M.M., *Targeting the function of the HER2 oncogene in human cancer therapeutics.* Oncogene, 2007. **26**(46): p. 6577-92.

13. Nagata, Y., et al., *PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients.* Cancer Cell, 2004. **6**(2): p. 117-27.

14. Li, B., et al., *Reduced expression of insulin-like growth factor 1 receptor leads to accelerated intervertebral disc degeneration in mice.* Int J Immunopathol Pharmacol, 2013. **26**(2): p. 337-47.

15. Al-Lazikani, B., U. Banerji, and P. Workman, *Combinatorial drug therapy for cancer in the post-genomic era.* Nat Biotechnol, 2012. **30**(7): p. 679-92.

16. Cristofanilli, M., et al., *A randomized phase II study of lapatinib + pazopanib versus lapatinib in patients with HER2+ inflammatory breast cancer.* Breast Cancer Res Treat, 2013. **137**(2): p. 471-82.

17. Smith, M.L., et al., *Examining and predicting drug preferences of patients with metastatic breast cancer: using conjoint analysis to examine attributes of paclitaxel and capecitabine.* Breast Cancer Res Treat, 2014. **145**(1): p. 83-9.

18. Li, Z., et al., *Methylation profiling of 48 candidate genes in tumor and matched normal tissues from breast cancer patients.* Breast Cancer Res Treat, 2015.

19. Liu, L., et al., *Novel mechanism of lapatinib resistance in HER2-positive breast tumor cells: activation of AXL.* Cancer Res, 2009. **69**(17): p. 6871-8.

20. Formisano, L., et al., *Epidermal growth factor-receptor activation modulates Src-dependent resistance to lapatinib in breast cancer models.* Breast Cancer Res, 2014. **16**(3): p. R45.

21. Pearson, G., et al., *Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions.* Endocr Rev, 2001. **22**(2): p. 153-83.

22. O'Neill, H.M., *AMPK and Exercise: Glucose Uptake and Insulin Sensitivity.* Diabetes Metab J, 2013. **37**(1): p. 1-21.

23. Kim, J.W. and C.V. Dang, *Cancer's molecular sweet tooth and the Warburg effect.* Cancer Res, 2006. **66**(18): p. 8927-30.

24. Wu, Y., D. Xing, and W.R. Chen, *Single cell FRET imaging for determination of pathway of tumor cell apoptosis induced by photofrin-PDT.* Cell Cycle, 2006. **5**(7): p. 729-34.

25. Wang, K., et al., *Characterizing breast cancer xenograft epidermal growth factor receptor expression by using near-infrared optical imaging.* Acta Radiol, 2009. **50**(10): p. 1095-103.

26. Zhang, J., et al., *Quantitative FRET measurement using emission-spectral unmixing with independent excitation crosstalk correction.* J Microsc, 2015. **257**(2): p. 104-16.

27. Komatsu, N., et al., *Development of an optimized backbone of FRET biosensors for kinases and GTPases.* Mol Biol Cell, 2011. **22**(23): p. 4647-56.

28. Jares-Erijman, E.A. and T.M. Jovin, *FRET imaging.* Nat Biotechnol, 2003. **21**(11): p. 1387-95.

29. San Martin, A., et al., *A genetically encoded FRET lactate sensor and its use to detect the Warburg effect in single cancer cells.* PLoS One, 2013. **8**(2): p. e57712.

30. Harvey, C.D., et al., *A genetically encoded fluorescent sensor of ERK activity.* Proc Natl Acad Sci U S A, 2008. **105**(49): p. 19264-9.

31. Dalle Nogare, D., et al., *Leading and trailing cells cooperate in collective migration of the zebrafish posterior lateral line primordium.* Development, 2014. **141**(16): p. 3188-96.

32. Caplat, P. and S.R. Coutts, *Integrating ecological knowledge, public perception and urgency of action into invasive species management.* Environ Manage, 2011. **48**(5): p. 878-81.

33. Wang, Z., et al., *Cross-scale, cross-pathway evaluation using an agent-based non-small cell lung cancer model.* Bioinformatics, 2009. **25**(18): p. 2389-96.

34. Towler, M.C. and D.G. Hardie, *AMP-activated protein kinase in metabolic control and insulin signaling.* Circ Res, 2007. **100**(3): p. 328-41.

35. Huang, J. and B.D. Manning, *A complex interplay between Akt, TSC2 and the two mTOR complexes.* Biochem Soc Trans, 2009. **37**(Pt 1): p. 217-22.

36. Nahta, R., et al., *Lapatinib induces apoptosis in trastuzumab-resistant breast cancer cells: effects on insulin-like growth factor I signaling.* Mol Cancer Ther, 2007. **6**(2): p. 667-74.

37. Alexander, A. and C.L. Walker, *The role of LKB1 and AMPK in cellular responses to stress and damage.* FEBS Lett, 2011. **585**(7): p. 952-7.

8 VITA