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COMBINING PROTEOMICS and SYSTEMS BIOLOGY to IDENTIFY THEAPEUTIC TARGETS & PATHWAYS OF CANCER DRUGS

A Thesis in

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by

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ABSTRACT

Melanoma is the malignancy of pigment producing melanocyte cells. It is the most lethal form of the skin cancer that takes the life of one person per hour in United States (U.S)[1]. It is estimated that more than 70 thousand new melanoma cases will be diagnosed in the U.S. in 2011 and at least one melanoma diagnosed patient in ten projected to die due to this deathful disease[2]. Moreover, about 40-60% of patients with advanced melanoma will have brain metastasis and after its detection in the brain, median survival time is only 3 months[3]. Unfortunately, melanoma is poorly responsive to chemotherapy. Although there are many drugs in clinical trials, ipilimumab is the second most promising drug which is recently approved by FDA, 35 years after the approval of the dacarbazine (DTIC) [4, 5]. However, the response rate for both DTIC and ipilimumab is lower than ~18% [6, 7]. Thus there is an urgent need for development of therapeutic drugs against malignant melanoma.

We have previously identified leelamine (dehydroabietylamine), a diterpene molecule extracted from pine trees, as a potential melanoma tumor growth inhibitor from natural chemical library screening (unpublished data). We have shown that leelamine inhibits melanoma tumor growth and induces apoptosis both in vitro and in vivo. However, the mechanism of action of leelamine was not revealed. In here, we aimed to illuminate the molecular pathways and targets of leelamine with the help of proteomics and systems biology.

To achieve our objective, we first investigated the activities of the two known targets of leelamine (PDK1 and CB1/CB2 receptors) on melanoma cells. We found that any of these targets directly contribute to the anti-tumor activity of leelamine. Thus to identify the actual target(s), we did antibody array analyses to investigate the expression of more than 800 proteins. We analyzed the data with the Ingenuity pathway analyses tool, which revealed that the PI3K-Akt pathway is the major target of leelamine. After validation of the deregulations in the members of the Akt signaling by western blot analyses we focused on upstream targets that regulate the Akt signaling.

Combinational treatment of phosphotase inhibitor with leelamine eliminated the possibility of the regulation through activation of phosphatases. After that, we ran a second antibody array that was specifically designed to evaluate the activities of the 42 different receptor tyrosine kinases which are one of the major subfamily of proteins that regulate Akt signaling [8]. This array analyses revealed that activity of PDGFR is significantly decreased by leelamine treatment. We validated this inhibition by western blot analyses; however, several groups have reported that inhibition of PDGFR was not enough to decrease melanoma cell growth [9, 10]. Our siRNA mediated knockdown of the PDGFR also supported these reports by not decreasing the growth of UACC903 cells. Thus, PDGFR inhibition seemed to be an indirect effect of leelamine treatment.

We continued our search by using GeneGo's MetaDrug software, which is designed for evaluation of biological effects of small molecule compounds based on their structures. Basically, this tool compared the structure of leelamine with structures in its

own database, which contains more than 600.000 compounds and interactions [11]. This analysis gave rise to two possible targets: NMDA receptors and ALOX5. Combinational treatment of leelamine with agonists or antagonists of these targets revealed that leelamine does not act through NMDA receptors. However, baicalein, an ALOX5/12/15 antagonist protected UACC903 cells from leelamine induced cell death in a concentration dependent manner. Even though, this information draws up us very close to our objective, we finished our thesis by proposing several hypotheses, which we will investigate in future.

In the first chapter of this thesis, we review some of the current proteomic approaches and systems biology tools. In the second chapter we summarize the molecular background of the melanoma development. We briefly give information about the pathways and mutations that regulate melanoma development. The 3rd chapter is the major part of this thesis and include the rationale and results of our studies. It also includes a discussion of the possible future approaches and hypothesis to complete the identification of drugs target. The last chapter is dedicated to future directions in proteomics, systems biology and melanoma therapeutics.

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Chapter 1:

PROTEOMICS and SYSTEMS BIOLOGY

PROTEOMICS

Biological science has witnessed astonishing progress in the last decade. The completion of the human genome project and the discovery of high throughput technologies, especially microarray technology, have advanced the field of molecular biology.

Microarrays are used for detecting expression levels of genes at the genome wide level and consist of thousands of oligonucleotide probes attached on a glass surface[12]. However, proteins perform most of the cellular functions; and genes are consequential only when they are translated into functional proteins. Since the level of transcription does not always correlate with the actual protein expression, it is estimated that microarrays are able to represent less than 40% of the actual protein levels [13, 14]. This fact is related to post-transcriptional regulatory mechanisms and can be understood better by the example of MITF protein in the case of melanoma.

According to the published microarray experiments MITF is one of the most highly upregulated genes in melanoma [15-17]. In average it is upregulated 4.14 fold compared to melanocytes, whereas malignant melanoma cells harboring oncogenic B-RAF contains low levels of MITF protein [18]. Moreover, it is reported that, ectopic expression of MITF gene inhibits proliferation of these cell lines [18]. This contradiction could be attributed to high activity of extracellular signal-regulated kinases (ERK proteins). Active ERK phosphorylates MITF and targets it for degradation in a

proteasome dependent manner [19]. Cells struggle with this abnormality by over expressing or amplifying the MITF gene, so that mRNA levels of MITF are upregulated even though protein levels are not. Moreover, regulation of MITF protein is not limited to the Erk pathway. Boyle et al identified miR-21 as a highly differentially expressed microRNA between normal and melanoma cell lines[20]. In their study, they reported that the expression level of MITF might be regulated through miR-211. In summary, even though there is a high expression of MITF gene, highly complex post-transcriptional regulatory mechanisms disturb its correlation with actual protein levels.

There are several factors that disturb the correlation of mRNA and protein levels of the cell. First of all, translational efficiency and mRNA stability are not the same for all of the transcribed mRNAs, even for different splice variants of a gene[21]. Some mRNA molecules are translated very efficiently while others are not. Some are very stable and have a long half life while some are rapidly degraded after transcription [22, 23]. Secondly, stability of proteins is also regulated by several mechanisms. For instance, ubiquitination targets proteins to proteosomal degradation while establishment of multiple disulphide bonds significantly stabilize the proteins. Hence, development of quantitative methods for the proteome, the protein complement of expressed genes, is much more essential than quantification of gene expression levels [24].

Proteomics is the large scale study of proteins that involves their interactions, structures and expression levels inside the cell[25]. The proteome of an organism differs from cell to cell and even from time to time. The regulation of transcription, splicing,

translation, intracellular transportation, and activity of kinases, phosphotases and cellular receptors in summary; all cellular regulatory mechanisms intend to regulate proteome content of the cell. Furthermore, proteins are subjected to more than 200 chemical modifications that regulate their activity or stability[26]. These posttranslational modifications include but are not limited to phosphorylation, ubiquitination, methylation, acetylation, glycosylation, oxidation and nitrosylation [26]. Some of proteins undergo all of these modifications and can be modified by more than one residue giving rise to an incredibly complex system.

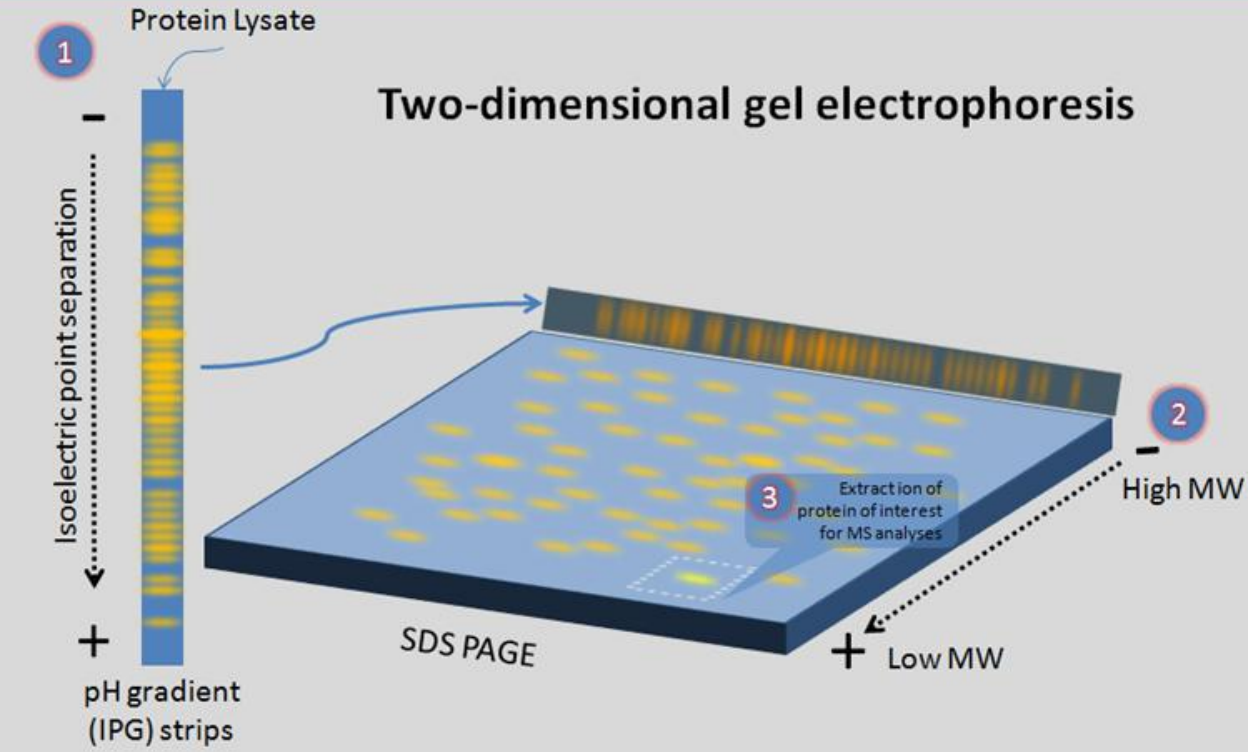
Proteomics deals with the development of experimental methods to analyze the proteome content of the cells. Several proteomics tools and approaches exist for this purpose. In here we will give brief information about several of these tools.

Tools of Proteomics

1) 2D PAGE ANALYZES

Two dimensional polyacrylamide gel electrophoresis (2D-PAGE) is the most widely used proteomics tool for high resolution separation of total cellular proteins[27]. In this method, proteins are separated along a pH gradient after loading into acrylamide gel (Figure 1). This allows migration of each protein to an appropriate isoelectric point. This is followed by a second separation step, which is applied in a direction 90 degrees from the first, to resolve proteins according to their molecular weights. Since it is very unlikely that two proteins will be the same in two distinct properties, proteins will be efficiently separated from each other.

Figure 1: 2D Gel Electrophoresis



1) In 2D-PAGE analyses first proteins are separated along pH gradient.
2) Then, proteins are separated according to their MW by applying electrical potential in the second dimension.
3)Protein of interest is extracted for mass spectrophotometry analyses.

After completing the run, proteins in the gel can be stained by one of the several staining methods. Most commonly, coomassie brilliant blue staining or silver staining can be used [28, 29]. These staining methods are generally adequate for quantification and comparison between experimental and control protein samples. At this point, proteins of interest can be isolated from gel and identified by mass spectrophotometry analyzes. However, there are significant limitations of 2D-PAGE analyzes. First of all, there is a load capacity of gel that limits detection, quantification and comparison of low abundance proteins[30]. Secondly, hydrophobic, acidic, and alkaline proteins and proteins with extreme properties (either mass or isoelectric point), usually cannot be

efficiently resolved [31]. Thus, high molecular weight proteins such as membrane receptors can be underrepresented in this type of analysis[32]. Moreover, 2D gels are difficult to prepare and data reproducibility is very low compared to other proteomic tools[32].

2) Surface Enhanced Laser Desorption/Ionization Time of Flight (SELDI-ToF) Analyzes

Surface Enhanced Laser Desorption Ionization (SELDI) time of flight mass spectrometry method is a one of the recent advancement in mass spectrometry based analyses. This method combines protein chip technology with mass spectrometry. For example, SELDI-ToF allowed analyses of proteome content of body fluids such as saliva and blood serum for the identification of disease biomarkers [33, 34].

In SELDI-ToF analyzes, protein chip arrays are treated with proteome content of complex biological samples to allow the binding and separation of proteins according to the property of protein chips [35]. There are several arrays including cation exchange, anion exchange, immobilized metal affinity and hydrophobic arrays that can be used for separation of proteins. After the incubation of samples with one of these arrays, proteins are bound to the array depending on their biochemical properties which is followed by washing unbound proteins and other extracts (salts, remnants of cell lysates etc.). In the next step, bound proteins are analyzed by time-of-flight mass spectrometry which involves ionization of peptides and further determination of mass to charge the ratio of each ion. The data obtained from SELDI-ToF is analyzed by statistical software that identifies individual proteins by comparing with a hypothetical mass to charge ratio

databases[36].

SELDI-ToF analyses were recently used by several groups for the identification of new disease biomarkers from a variety of body fluids including saliva and blood serum. In a recent study, Seny et al identified four new biomarkers for knee osteoarthritis by analyzing 340 serum samples from patients and controls[37]. In another recent study, Cadieux et al used SELDI-ToF analyses to identify urinary biomarkers of urolithiasis (the process of forming stones in the kidney, bladder or urethra) [38]. Both studies show that this technology has a promising future for novel biomarker discoveries from body fluids.

3) Isotope Coded Affinity Tags (ICAT)

Isotope coded affinity tag (ICAT) technology is a gel free method that is emerging as a powerful tool for quantitative proteomics analysis. This method is based on labeling of cysteine probes of proteins with an isotopically coded linker, and a tag (generally biotin) for the isolation of labeled peptides[39].

As it is illustrated in figure 2, in the first step of ICAT procedure, extracted proteins are treated with one of the two ICAT reagents, which contain either light (d0) or heavy (d8) linkers[39]. These isotopically coded linkers will stably bind to the cysteine residues of the proteins. This is followed by mixing control and experimental protein lysates to minimize error. After that, mixed solutes are digested with protease such as trypsin. Subsequently, proteins containing labels will be purified by affinity chromatography analyses (generally with avidin beads). Purified peptides are analyzed by liquid chromatography and mass spectrometry (LC-MS). A mass difference of 8 Da

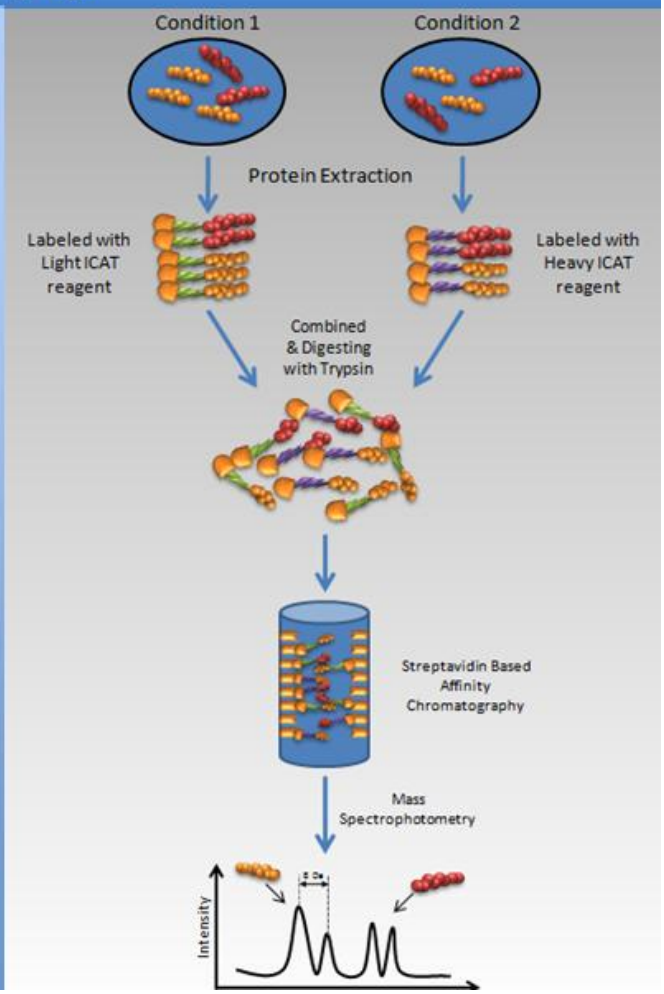
between affinity tags allows the quantification of peptide pairs in the two samples and this provides a ratio of proteins concentration in the original samples[40]. Differentially expressed peptides will be sequenced and determined with the help of bioinformatics.

In a recent study, Chen et al. discovered transcription factor YY1 as a target of microRNA 34a (miR-34a) by using the ICAT technique [41]. In their study, ICAT analyzes identified 335 significantly deregulated proteins by miR-34a. Bioinformatics analysis of biological networks, pointed out a ubiquitously expressed transcription factor YY1. This observation is verified by demonstration of miR-34a-binding site within the 3' UTR of YY1 using a luciferase reporter system.

In another study, Fogle et al investigated the impact of chronic alcohol ingestion on cardiac muscle protein expression by using ICAT technology[42]. Their proteomic analysis identified proteins involved in multiple activities of cardiac function and metabolism. According to their study, they speculated that chronic alcohol consumption modulates fundamental alterations in cardiac muscle protein expression and this would explain alterations in myocardial wall thickness measured under the same circumstances.

Figure 2: Isotope Coded Affinity Tags

ICAT: Isotope coded affinity tag is a gel free method for quantitative proteomics. In this method, first protein lysates are labeled with two different ICAT reagents. After that, to decrease the experimental error, control and experimental lysates are mixed and this is followed by digestion with a protease such as trypsin. Consequentially, labeled peptides are captured by affinity chromatography and analyzed by mass spectrophotometry. Due to a 8 Da weight difference between light and heavy ICAT reagents, MS data will include two peaks for each protein. The intensity of the peak would represent the amount of particular protein in the sample.



4) Reverse phase protein lysate microarray (RPMA)

Reverse phase protein lysates microarray is a combination of laser capture micro dissection and microarray technologies. In conventional protein microarrays, probes (antibodies) are immobilized on the microarray slides which cell lysates are treated (incubated) to investigate the expression of hundreds of genes. However in RPMA, diminutive amounts of cell lysates or body fluids are immobilized on microarrays, which are then incubated with a single specific antibody to detect expression profile of the target protein across the thousands of samples[43].

There are several advantages of RPMA compared to other high throughput approaches. First of all, it is able to provide quantifiable information for post-translationally modified proteins [44]. Secondly, it is highly sensitive, being able to detect protein expression even from an extremely small amount of cell lysates (as low as 10 cells). In their Nature Protocols paper, Brett Spurrier noted that, with highly specific antibodies, RPMA is able to generate 1,000 times more data points using 10,000 times less sample volume compared to western blot analyses or ELISA[44]. RPMA was reported to be able to detect several attograms (10^{-18} grams) amount of proteins compared to ELISA, which requires several micrograms (10^{-6} grams). As a third advantage, RPMA allow analyses of thousands of samples with the same antibody in one experiment.

On the other hand, RPMA has a major limitation regarding antibodies that can be used. It requires antibodies with high specificity, which most lack [45]. If an antibody is not highly specific, it might create false positive signals because of unspecific binding and high sensitivity of the assay. Today, we have a limited number of antibodies that exhibit sufficient sensitivity for use in RPMA analyzes. Thus, extensive screening of many antibodies is required prior to RPMA analyzes.

RPMA analyses are being used for the analysis of protein expression in cancer cells, body fluids or tissues as well as biomarker profiling. In a recent study, Improta et al used RPMA technology to uncover the signaling architecture of brain metastases of

breast and non-small cell lung cancer (NSCLC)[46]. They analyzed expression and activation states of 128 key cancer-signaling proteins, in 42 brain metastases from breast and NSCL cancer patients. From this study, they identified significant differences between breast cancer and NSCL metastases, which assisted them to conclude requirement of prior stratification for a successful targeted therapy.

5) Protein Microarrays

In the last decade, DNA microarrays have proved themselves as powerful tools of genomic research. They have been used to study gene expression profiles, to identify transcription factor binding sites, to map single nucleotide polymorphism and even for genome wide mutation analyses [47, 48]. However, it is a well-known fact that the complexity of the human proteome far exceeds that of the genome, because of variables such as alternative gene splicing events, post-translational modifications and individual coding variants (protein isoforms). Therefore, the number of individual protein species in a cell is likely to be at least several fold greater than the number of genes. Therefore, the complexity of the proteome and the success in DNA microarray technology encouraged development of protein microarrays to achieve global analyses of protein expression.

Protein arrays are composed of antibodies, proteins, aptamers, or affibodies which are deposited on a chip surface in a small array[49]. They are used to study the interaction of proteins with other proteins, small molecules, nucleic acids, and lipids in a single experiment. Protein arrays can be subdivided into two groups as analytical microarrays and functional microarrays.

Analytical protein microarrays are used to measure the expression levels, binding affinities and specificities of the proteins. Most common analytical microarrays are antibody microarrays, which may contain up to several thousands of antibodies for measuring expression levels of proteins. Today, there are commercially available antibody arrays for characterization of special signaling pathways including apoptotic

pathway arrays, receptor tyrosine kinase arrays and phosphatase arrays.

Recently, Vigil and colleagues have applied protein array technology to identify the feline humoral immune response to Bartonella henselae infection[50]. They developed a protein array containing virtually all expressed proteins, (n=1433), from the predicted open reading frames encoded by the genome of the zoonotic pathogen Bartonella henselae, which is the causative agent for potentially fatal cat-scratch disease in immunocompromised patients. They probed these arrays with infected or uninfected cat sera, to profile the antibody repertoire in response to Bartonella henselae infection. By this method, they developed a classifier algorithm that can discriminate infected animals with 93% accuracy. This study demonstrates the potential of protein array technology as a utility in diagnostics, vaccine development and biomarker discovery.

Functional protein arrays contain full-length functional proteins and are used for the identification of protein interactions with other molecules (proteins, DNA, RNA, lipids or small molecules). In this type of array, thousands of proteins that are spotted on the array slide can be treated with a labeled molecule (such as labeled DNA) to detect interacting partners. This provides highly accurate quantifiable information about the biochemical activities of an entire proteome in a single experiment. In 2006, Jones et al revealed the protein interaction network for the ErbB receptors using functional protein microarrays [51]. They were able to create a quantitative map based on dissociation constants where they measured through analysis of the concentration dependent peptide-binding domain interactions. The accuracy of these calculations is validated by surface

plasmon resonance experiments. Their approach allowed identification of novel interactions between SH2 or PTB domain containing proteins and epidermal growth factor receptor proteins.

Protein arrays are advantageous to conventional proteome analyzes (2D gel electrophoresis and mass spectrometry), because of their ability to detect low abundance proteins, which are generally those of the greatest diagnostic interest. However, antibody specificity is a major issue in this technology. Most of the antibodies are not specific to their targets. When they are used in western blot analyses, it is possible to discriminate targets according to the expected molecular weight of the protein. However, in antibody array technology, antibodies are spotted as small droplets and there is no possibility of separating proteins according their molecular weight. Hence, unspecific signals cannot be distinguished from the true signals.

Kinexus Protein Arrays

Currently, several proteomic companies are offering protein array services. In our study we have used KAM 1.3 antibody arrays from Kinexus, a company located in Vancouver, Canada. KAM 1.3 arrays contain 812 antibodies which 550 are pan specific antibodies to detect protein expression and 262 of the antibodies are phospho specific antibodies to detect phosphorylation status of the targets[52]. This array encompasses 193 protein kinases, 24 protein phosphatases and 150 regulatory subunits of these proteins. As illustrated in figure 12, each antibody is printed on array as duplicates with an average spot size of 135uM. A complete list of the antibodies spotted on KAM 1.3 can be obtained as an excel file from the Kinexus web site (www.kinexus.ca).

Kinexus antibody array services use single dye based approach to analyze cell/tissue lysate samples[52]. This approach is advantageous to the two dye based approaches, which could give misleading results due to differential binding of different dyes to proteins. Kinexus Company claims that, antibodies in the Kam1.3 array are chosen after testing over 3500 antibodies from over 20 suppliers and this cost more than 1 million dollars. However, several problems related to protein arrays also apply to the Kinexus array. First of all, false positives due to antibody specificity problems (cross reactivity) are a major problem for the Kinexus array as well. Furthermore, in the procedure protein lysate is not denatured. Thus, proteins that are complexed with other proteins may cause false positive signals. Beside false positives, epitope of the protein might be blocked due to the native form of protein or protein complexes causing false negative data. However, even though these drawbacks, protein arrays are cost-effective solutions for analyzing the expressions of hundreds of proteins in a single experiment.

Receptor Tyrosine Kinase Array (RTK array)

In our study we have used a special type of protein array that is developed by R&D Systems to screen the relative phosphorylation of 42 receptor tyrosine kinase receptors [53]. In this array, capture antibodies are spotted on nitrocellulose membranes as duplicates. Incubation of cell lysates with array permits binding of both the phosphorylated and unphosphorylated RTKs to the antibodies. After washing away unbound proteins, incubation of the array with horseradish peroxidase (HRP) conjugated anti-phospho-tyrosine antibody allows detection of phosphorylated tyrosines on activated

receptors by chemiluminescence. This principle increases sensitivity of the arrays up to 20 times more when compared to western blots or immunoprecipitations.

Systems Biology of Proteomics:

Systems Biology

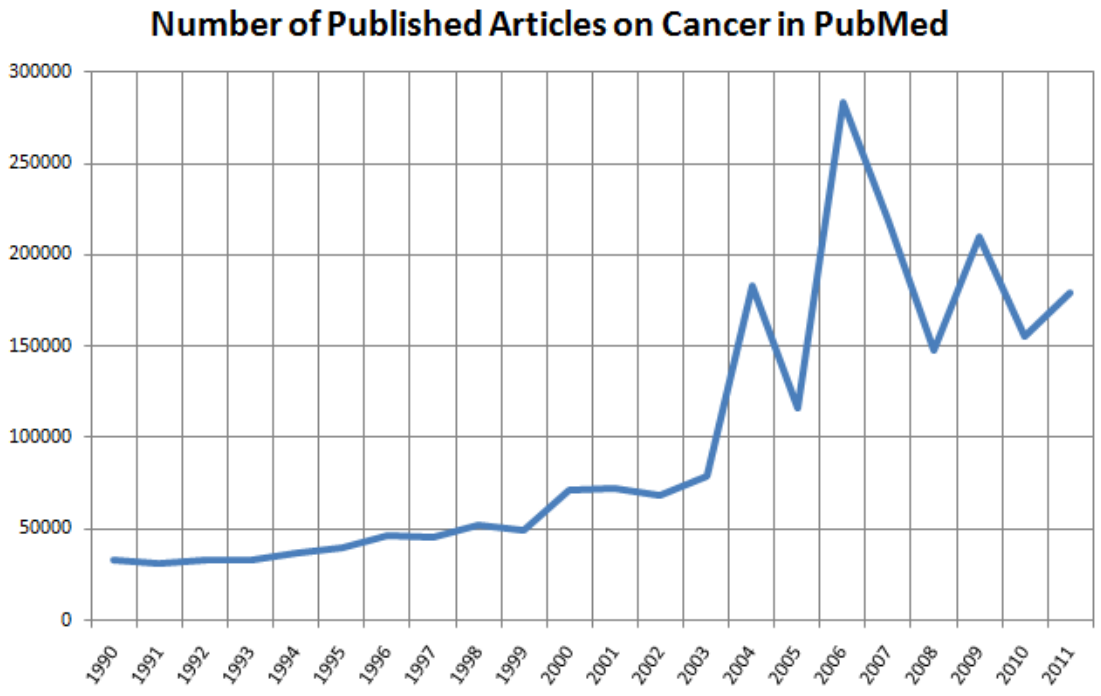
Systems Biology is defined as the analysis of the interactions among the elements of biological systems in response to genetic or environmental changes[43]. It investigates these interactions to understand the function and behavior of the whole system. In general, a biological system may cover everything from molecules to the ecosystem. But in molecular biology, a few protein molecules of a pathway, a network, as well as a single cell can be considered a system.

Life is much more complex than what the word "complex" could begin to describe. The human body consists of more than 210 distinct cell types with each having its own properties [54]. Moreover, every cell has its own proteome content, which is regulated strictly according to intracellular and extracellular stimulus. Thus, every cell is a system with its own properties.

Understanding the molecular basis of biological activity is the central goal of molecular biology. Thousands of laboratories around the world are contributing to this aim by publishing their works every day. Today, we have hundreds of thousands of published papers in the online databases, and these databases are growing with an incredible speed. For instance, the number of published articles per year in pubmed database about cancer, increased more than 3 times in the last decade compared to

previous one (Figure 3). However, a human being is very limited in terms of time and capacity to comprehend all the information. Thus, reading and combining all of the information is impossible even in a particular area of interest. Systems biology steps into science in this phase to integrate the information from the literature, using techniques of information extraction and text mining. Systems biology aims to develop online databases and repositories for sharing, combining and organizing all the accumulated knowledge to increase the understanding of biological systems.

Figure 3: Published Articles on Cancer Related Research



Number of published articles(*) on PubMed is increasing exponentially: In the last ten years number of published studies per year is nearly tripled compared to previous decade.

*Number of published papers on "Cancer" is obtained from search results of PubMed database.

In the last decade development of high throughput technologies, such as microarray technology, high throughput sequencing, and proteomics pushed computer

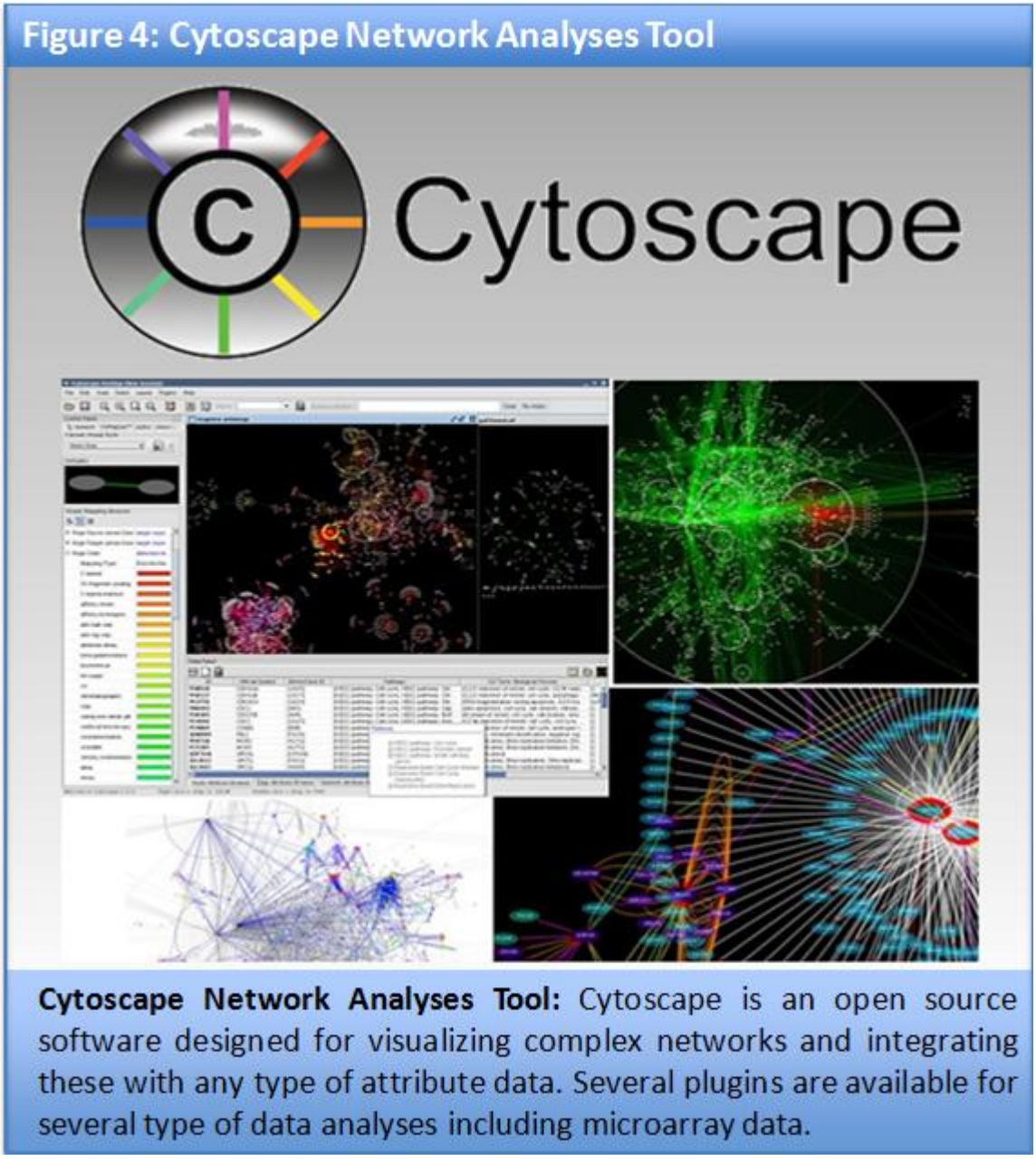
scientists to write softwares to manage the huge amount of data gathered from these techniques. For this purpose, several commercial and non-commercial (with general public license) tools are developed to mine meaningful outcomes from high throughput approaches [11]. We concentrate on three of these tools: Cytoscape, GeneGO and Ingenuity pathway analyses (IPA), which are originally developed for mining and analyzing data from cDNA microarray experiments.

Cytoscape:

Cytoscape is an open source (GPU licensed or freeware) tool for visualizing molecular interaction networks that are generated through analyses of data from high throughput approaches [55]. It integrates biological pathways with gene annotations, expression profiles and mined data from literature to provide best possible information about the alterations in the system. The core distribution of cytoscape provides a basic level data integration and visualization; however, additional features can be added as plugins. Cytoscape plugins, which are generally free, are written by scientific community to provide additional analyses, layouts, and connection with other databases. Cytoscape is able to mine information from several online biological databases including, Pathway Commons, IntAct, BioMart, NCBI Entrez Gene, and PICR.

In the core analyses of cytoscape, gene expression data is screened to create networks according to the gene interactions that are reported in databases. It is possible to filter the generated network according to features of interactions. For instance, one can limit interactions to protein-protein interactions or can set a threshold for expression values.

Figure 4: Cytoscape Network Analyses Tool



Cytoscape Network Analyses Tool: Cytoscape is an open source software designed for visualizing complex networks and integrating these with any type of attribute data. Several plugins are available for several type of data analyses including microarray data.

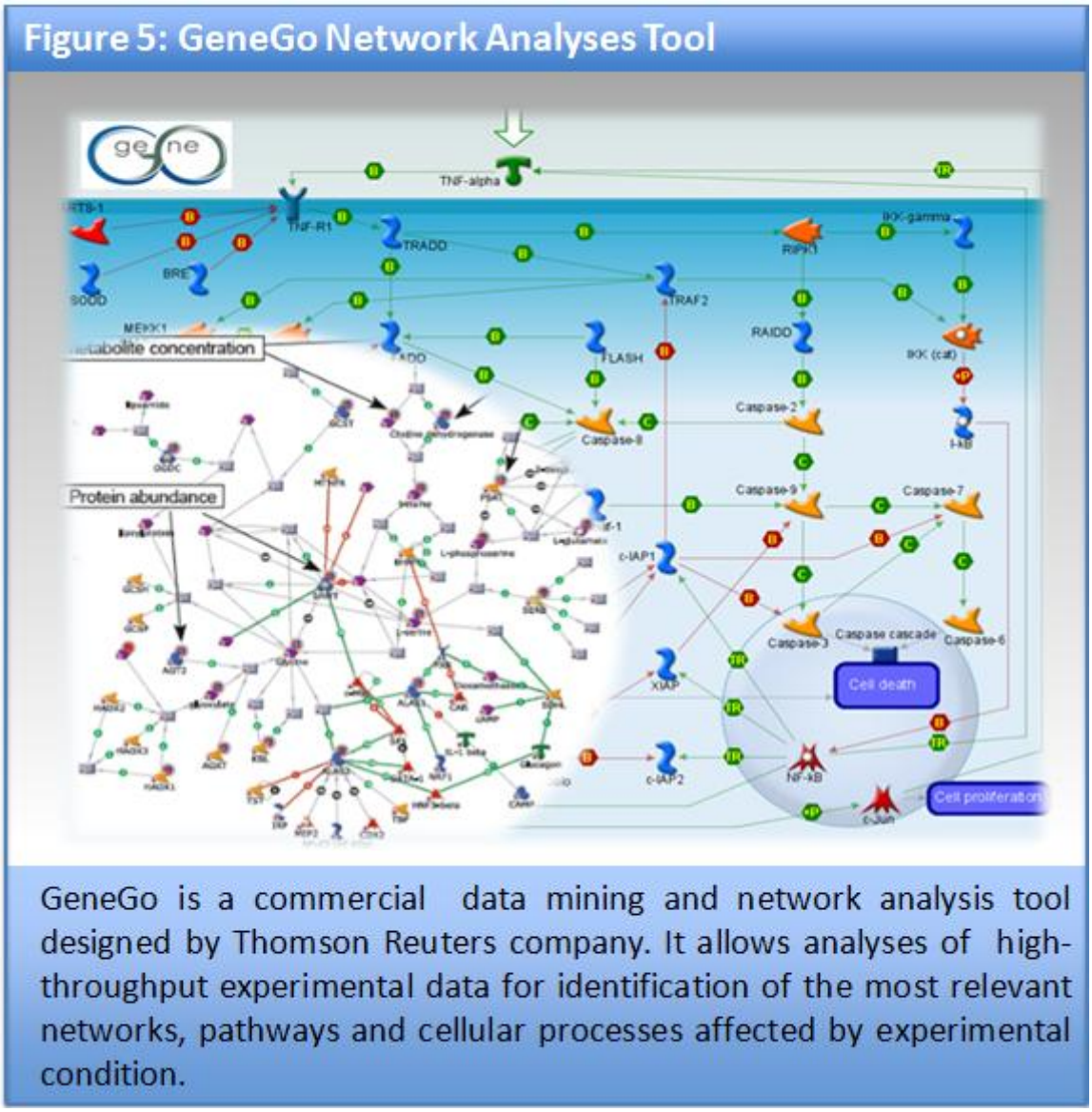
The major advantage of Cytoscape is in the speed of development. It is being developed as open source software to which people all over the world are contributing by writing additional features or plugins. On the other hand, it has a major drawback: it does not have any human curated interaction database. Commercial tools such as GeneGo or IPA have their own human curated databases that store interactions of proteins that are

collected from published papers by a human. But, as we have mentioned above, cytoscape depends on public databases, which are generally generated by computer based data mining algorithms, and this decreases their reliability. Moreover, the guide user interface (GUI) of Cytoscape is not easy to understand and requires extensive computer knowledge to analyze data (Figure 4).

GeneGo

GeneGo is a web-based analyses suit containing more than 10 different types of analyzing software that are developed for the pathway analysis of experimental data and gene lists (Figure 5)[11]. With these tools it is possible to analyze microarray and sequence-based gene expression, SNPs and CGH arrays, proteomics, metabolomics, and Co-IP pull-out data.

The core software in GeneGo suite is called MetaCore, which can handle several types of analyses including canonical pathway analysis, network analysis, enrichment analysis in multiple ontologies, transcription factor analysis, interactome analysis and toxicity analysis. Before briefly describing these analyses, we have to note that all of these analyses are based on biological databases, which is the major advantage of this tool. GeneGo tools are based on the MetaBase knowledge database which is likely to be the most comprehensive human curated molecular biology and medicinal chemistry database available today [56, 57]. It stores over 6 million protein-protein, protein-DNA and protein-compound interactions. It also includes gene-disease interactions for more than 500 human diseases and 4500 genes, ligand-receptor and drug-target association data for thousands of compounds and more than 2,000 canonical pathways on cellular signaling and metabolism.



Canonical Pathway Analyses:

In molecular biology, cellular pathways with a well-known structure are referred as "canonical pathways". For instance, the Raf-MAP kinase pathway is a very well known pathway in which Raf activates the Ras protein, that in turn activates Mek kinase [58]. Afterwards, the Mek kinase activates Erk protein. Therefore, the Raf-Mapk pathway is accepted as a canonical pathway. MetaCore (also IPA and others) seeks to identify which canonical pathways are altered in the experimental case by comparing

uploaded dataset to the gene lists of canonical pathways. During these analyses, each pathway is ranked according to the "fitness" to the uploaded data. A p value is calculated according to the probability of hitting an element of each pathway by chance. For instance, if one uploads a data with thousands of deregulated gene information, you would expect to hit more genes in a canonical pathway with 200 genes compared to a canonical pathway with 30 genes. By considering several criteria, a ranked canonical pathway list will be produced as output.

Network analyses:

Intra-connected networks of protein interactions regulate the metabolism of the cell. Identifying these networks, or in other words, solving the mystery of the cell is the ultimate goal of all pathway analyses tools. Even though our current knowledge is very limited in terms of molecular networks, network analyzes tools aim generate cellular networks according to the uploaded gene lists and known protein-protein interactions with the help of complex algorithms.

MetaCore has 10 different algorithms, each written with a different purpose such as generating a network with the shortest paths, with direct interactions, with self-regulation mechanisms or centered on transcription factors. During these analyses it is possible to set several filters according to the "system" that you are working on. For instance it is possible to limit the interactions to a specific specie, tissue, cell type or even sub-cellular localization.

Gene Set Enrichment analysis:

Enrichment analyses determine whether a Gene Ontology (GO) property is statistically significantly enriched in an uploaded gene list. Gene Ontology database holds information (a controlled vocabulary of terms) about gene product characteristics such as molecular function, biological function or sub-cellular localization [59]. There are several freely available online tools (e.g. David tools) that are capable of doing this type of analysis [60]. However, MetaCore has also its own annotation database that increases the quality of analyses.

Transcription factor analysis:

Transcription factor analysis aims to identify common transcription factors that regulate the genes in the uploaded data. This type of analysis is especially useful for analyzing cDNA microarray experiments since gene expression is regulated through activity of transcription factors. There are several freely available online tools, such as DIRE (Distant Regulatory Elements of co-regulated genes[1]) for this type of analysis also. However most of them lack the power to produce statistically significant results due to complex regulation of gene transcription.

Interactome analysis

Interactome analysis identifies the most relevant proteins (transcription factors, kinases, receptors, signaling proteins, phosphatases and enzymes) that are somehow interacting with the uploaded dataset.

[1] http://dire.dcode.org/

Toxicity analysis:

Toxicity analyses seek to identify alterations in pathways that would result in adverse effects to the liver, kidney, heart and lung cells. Even though these analyzes may not be biologically significant, they might help to have an idea after perceiving toxicity in in-vivo experiments.

MetaDrug and Eureka

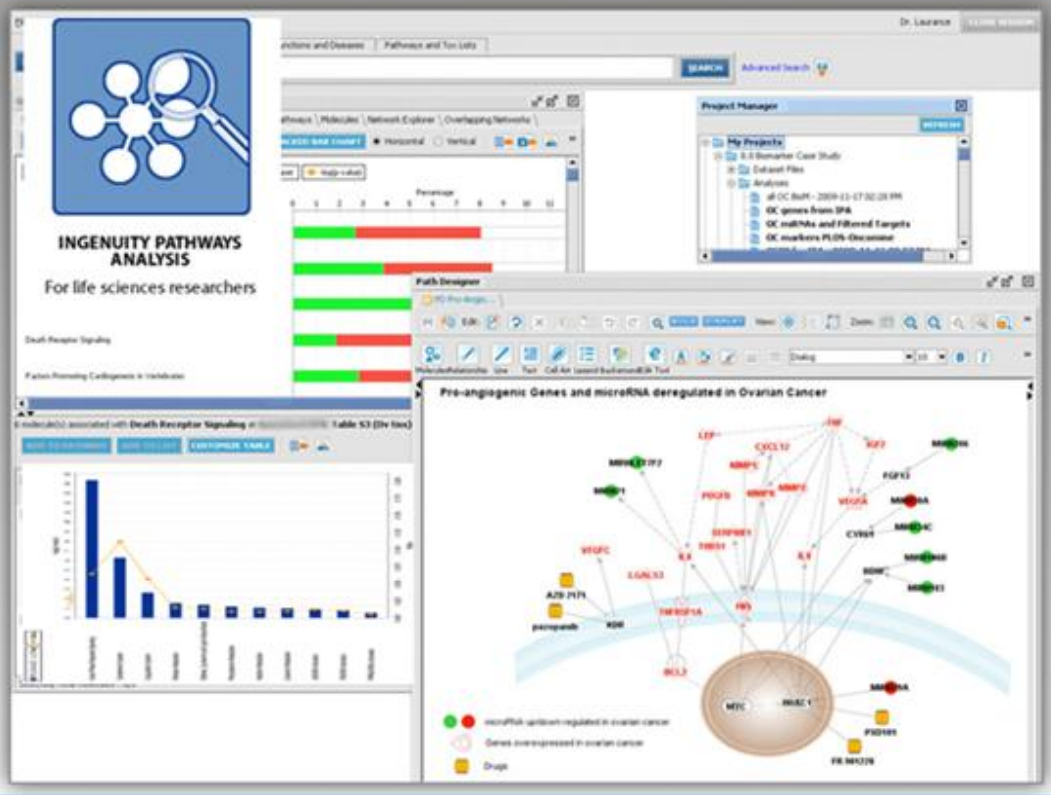
It would be useful to mention two other tools, MetaDrug and Eureka of GeneGo. MetaDrug is a chemical structure based pathway analyses tool that evaluates potential protein and pathway targets of substances according to their chemical structure[11]. This tool compares the structure of the input molecule with over 600,000 biologically active compounds in its database [57].According to the similarity it predicts possible targets and regulated pathways. Thus, it might be helpful for the identification of a drug's mechanism of action, toxicity and off-target effects. It is able to predict human metabolites based on empirical rules.

Eureka is a knowledge-mining tool to find biological information from the manually curated GeneGo databases [61]. In this tool it is possible to build biological goals and questions such as "Find compounds that inhibit IGF1R but not INSR with activity less than 100nM" or "What are the genes that are upregulated in malignant melanoma but not in melanocytic nevi?" or "Which inhibitors have anti-angiogenic activity?". This tool is able to answer these types of questions as a list of compounds or genes, which is exportable as an excel file.

Ingenuity Pathway Analyses tool (IPA)

IPA is a tool with very similar properties to GeneGo software. It is also able to do canonical pathway analysis, network analysis, enrichment analysis and toxicity analysis. However, it has only one algorithm for generating networks. Its knowledgebase seems to be smaller than GeneGo's database. It lacks transcription factor and interactome analyses. However, it has user-friendlier interface (Figure 6), which is an important factor for people who are not get used to these types of tools. In our analyses we have used this tool because of our institutional license that we have.

Figure 6: Ingenuity Pathway Analyses Tool



Ingenuity pathway Analyses Tool: IPA is a commercial data analysis tool designed to understand the complex biological systems at multiple levels by integrating data from a variety of biological databases. IPA is the user-friendliest network analyses tool available today.

Chapter 2:

MELANOMA

"You can't solve a problem without knowing the source"
Parables of Zen

MELANOMA

Melanoma, the sixth most common cancer in United States, is a malignancy of melanocytes[62]. Melanocytes are pigment-producing cells that originate from the neural crest or ectoderm[63]. They are located primarily in the bottom layer of the skin epidermis but also in the middle layer of the eye, GI tract and even in bones and several other tissues[64]. There are several types of skin cancers but melanoma is the deadliest one even though it is accounting for 4% of them[65]. Risk for developing melanoma increasing every year even it already increased more than 2,000% in the last 80 years [66]. The current lifetime risk for melanoma development is 1 per 58 people and roughly 68,000 people developed melanoma in United States in 2010[1].

Melanoma is classified as a solid tumor and resists most of the conventional therapies such as radiotherapy and chemotherapy [67]. Early detection and surgical excision remain to be the best solution for reducing mortality [68]. Several factors are involved in melanoma development. Genetic mutations that alter cellular pathways, which regulate cell proliferation, differentiation and death, stand in the center of melanomagenesis (Figure 8). These mutations are triggered by numerous environmental factors, such as ultraviolet (UV) light exposure, chronic sun damage and using tanning beds. Associated with these environmental factors, a number of risk factors also exist such as skin color [69].

MOLECULAR BIOLOGY OF THE MELANOMA

Melanoma can be separated into two groups as familial melanoma and sporadic melanoma. Familial melanoma is the melanoma that is genetically inherited (or at risk of being inherited) in families, which gives rise to melanoma in two or more first-degree relatives of the patient[70]. Five to 10 percent of the melanoma patients have the same disease in at least one of their first-degree relatives indicating a genetic factor (as a cause or risk factor)[70]. However, most cases of the melanoma are sporadic. Sporadic melanomas may develop from precursor melanocytic nevi such as congenital, atypical or dysplastic lesions[71]. Nevertheless, more than 50% of them are completely de nova and arise from normal melanocytes (not from preexisting lesions)[71]. Here we will summarize important molecular mechanisms of melanoma under these two sub groups.

Molecular biology of familial melanoma:

To date, linkage analyses identified two high penetrance melanoma susceptibility genes: *CDKN2A* and *CDK4*[72]. In addition to these two genes, several other genes encoding proteins that regulate cell growth (EDNRB, EGF), skin pigmentation (*MC1R* and *OCA2*), DNA repair (XPC, XPD and XRCC3) and detoxification of electrophilic compounds (*GSTM1* and *GSTT1*) are identified as low-penetrance susceptibility genes[72].

CDKN2A locus contains leading melanoma susceptibility genes that code for two alternative reading frames for tumor suppressor proteins: p16 ^{INK4A} and p14ARF (alternate open reading frame).

p16 ^{INK4A}, which is also known as Cyclin dependent kinase inhibitor 2A, regulates the cell cycle by inhibiting the activity of CDK4 and CDK6 kinases[73]. In non-transformed cells, CDK4/6 phosphorylates the pRb protein to allow the release of E2F transcription factors, which is coupled to pRb. E2F proteins are active when they are disassociated from pRb protein and induce transcription of S phase promoting genes. This process is strictly controlled by several pathways but primarily through inhibition of CDK4/6 proteins by p16 ^{INK4A}. However, as it is depicted on figure 7, in the case of familial malignant melanoma, the p16 ^{INK4A} gene has mutations that preclude binding to the CDK4 protein[73]. This leads to over activation of the pathway leading to uncontrolled cell proliferation and growth.

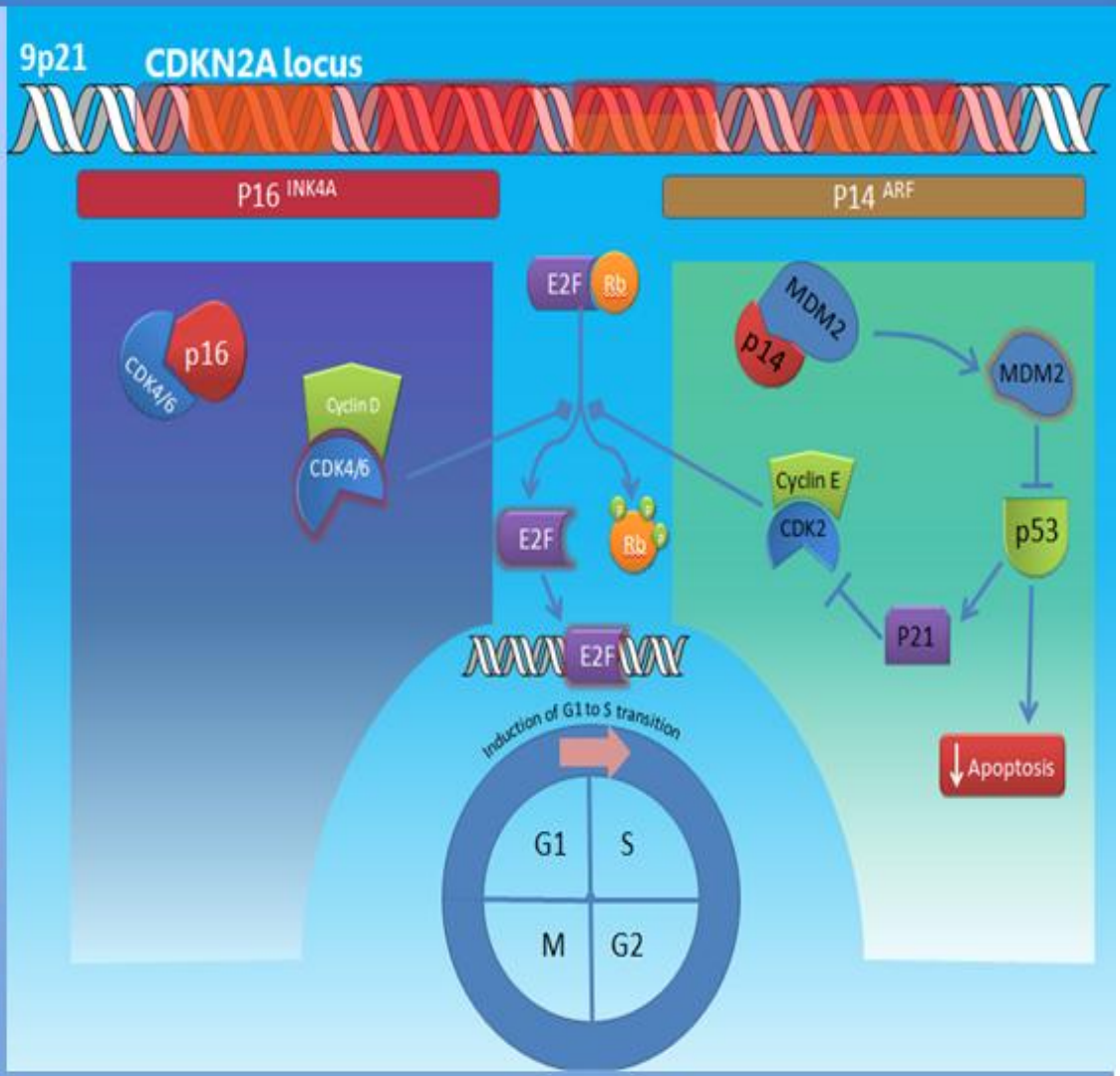
p14ARF, an alternative transcript of CDKN2A locus, also regulates cell cycle progression but through a different pathway. p14ARF protein binds and hinder the activity of murine double minute 2 protein (MDM2) which is a negative regulator of tumor suppressor protein p53 (tumor protein 53)[74]. Consequently, inhibition of MDM2 mediated proteosomal degradation of p53 tumor protein give rise to stabilization and accumulation of the tumor suppressor. p53 is a key regulator of apoptotic pathways. It abolishes cell cycle progression by reducing pRb phosphorylation through activation of p21 protein [75]. In the case of familial melanoma, p14^{ARF} is inactivated by mutations leading to p21 mediated pRb phosphorylation, therefore initiating the S phase-specific gene transcription (Figure 7).

Figure 7: CDKN2A Locus and Melanoma

Molecular mechanism of action of tumor suppressors $p16^{INK4a}$ and $p14^{ARF}$ on melanoma development: The CDKN2a locus on chromosome 9p21 encodes for 2 different tumor suppressors, $p16^{INK4a}$ and $p14^{ARF}$. Normally p16 inhibits interaction of CDK4/6 protein with Cyclin D1 and keeps pRB in un-phosphorylated state.

In the case of melanoma, the absence of p16 allows binding of CDK4/6 to the Cyclin D1. CDK4/6-CyclinD1 complex phosphorylates pRb and allows release of the E2F transcription factors, which promote the G1-to-S phase transition of the cell cycle (Left hand side)

On the other hand, in normal cells, $p14^{ARF}$ inhibits the p53-specific ubiquitin ligase MDM2. However in the case of melanoma, absence of $p14^{ARF}$ allows ubiquitination and proteosomal degradation of p53



protein. This allows hyper-phosphorylation of pRB protein through p53-p21-CDK2 signaling pathway(Right hand site).

The CDK4 gene is accepted as the second high-penetrance melanoma susceptibility gene. Its function was mentioned in detail above. Arginine to cysteine mutations (R24C) in the second exon of the CDK4 prevents interaction of the protein with $p16^{INK4A}$ tumor suppressor[76]. This leads increased phosphorylation of the pRb protein and uncontrolled cell cycle progression. However, CDK4 mutations are rare and seen only in a few familial melanoma cases worldwide [72].

Beside identification of the CDKN2A and CDK4 as high penetrance melanoma susceptibility genes, several other genes are also reported as potential low penetrance genes. Shahbazi et al. reported epidermal growth factor receptor gene as a melanoma susceptibility allele [77]; and two other groups support that [78, 79]. However there are also conflicting reports to these findings [80, 81].

Inherited polymorphism of XPD, a gene coding for a DNA repair enzyme, is also associated with melanoma risk [82]. In addition, two related counterparts of the XPD gene; XPC and XRCC3 are also reported by two distinct groups [83, 84]. Nevertheless, the results of the two other studies were not able to confirm these findings [85, 86]. More work is required to establish if there is an association between variants of these genes and melanoma or not. On the other hand, the vitamin D receptor, endothellin receptor B (EDNRB) and oculocutaneous albinism 2 (Oca2) were also reported as potential melanoma susceptibility genes [87-89].

Molecular biology of the sporadic melanoma:

CDKN2A locus mutations

Sporadic melanoma develops randomly without any family history. Analogous to familial melanomas, homozygous deletions [90, 91], inactivating mutations or epigenetic silencing [92, 93] of the CDKN2A locus are the most significant alterations in sporadic melanomas (hereafter referred to as melanoma). Up to 75-96% of the melanoma cell lines carry abnormality in this locus [94, 95]. However, a defect in CDKN2A is not enough for the development of melanoma and requires supporting genetic alterations in other pathways [96, 97].

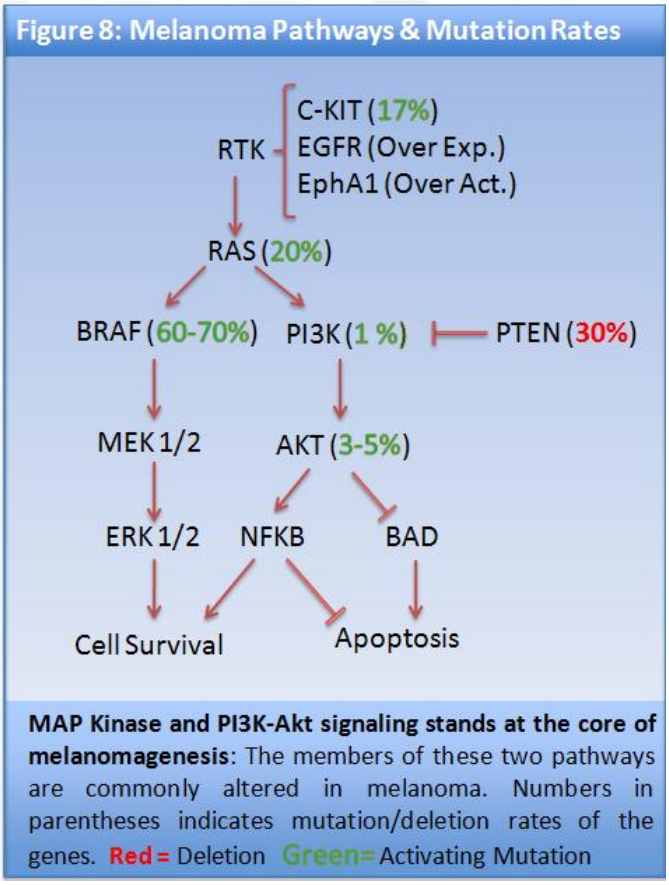
N-Ras Mutations

Activating mutations in the Ras subfamily of genes (H-Ras, K-Ras and N-Ras) are detected in more than 20% of all human tumors[98]. Furthermore, some tumor types, e.g. pancreas tumors carry Ras mutations up to 90%, indicating the important role of Ras in tumorigenesis [99]. On the other hand, approximately 20-30% of the melanoma cell lines carry mutations in the Ras gene, which is exclusively in N-Ras [100, 101]. However, the Ras gene regulates two important signaling pathways: Raf-MAPK and PI3K-Akt, which are almost always abnormal in melanoma cells[102].

MAPK Pathway

Ras regulates cell proliferation through Raf-MAPK pathway. In melanocytes, Ras is activated by either receptor tyrosine kinases or G-protein coupled receptors in response to stimulation by an extracellular ligand, such as growth factors [103-105]. As illustrated

in figure 8, activated Ras induces the Erk1/2 protein (MAPK1/2) through the Ras-Raf-Mek-Erk pathway[104]. Erk1/2 proteins are key kinases which directly regulate more than 70 substrates [106], (indirectly more than 900 proteins (IPA data)) including several transcription factors such as Mitf, Myc, Elk1, Atf1, Creb and Srf [107-109]. These transcription factors regulate the expression of several genes that is required for cell proliferation. As a note, almost all melanoma cells show constitutive activation of Erk proteins [110].



Sixty to seventy percent of melanoma cell lines carry the V600E mutation in the B-Raf gene which renders protein to become constitutively active and induces downstream signaling pathways even in the absence of growth factors[111]. Thus B-Raf activation is one of the most frequent genetic events in melanoma genesis. However, it is also important to note that 70-80% of the melanocytic nevi also carry the V600E mutation in the B-Raf gene; signifying that B-Raf mutation is not able to drive melanoma genesis alone and requires additional genetic or epigenetic alterations [112-114].

PI3K-Akt Pathway

Ras proteins also regulate apoptosis through Ras-PI3K (Phosphatidylinositol 3-kinase)-Akt signaling pathway [111]. Akt/PKB is a serine-threonine kinase, which regulates the activity of several proteins that are involved in cell metabolism, proliferation and apoptosis [115, 116]. Activated Akt inhibits apoptosis through phosphorylation of proteins such as FKHR (a transcription factor that regulates transcription of several cell death genes), Bad, PRAS40 and MDM2 [115, 116].

PI3K phosphorylates lipid second messenger molecule PIP2 (Phosphatidylinositol-4,5-bisphosphate) to yield PIP3 (Phosphatidylinositol 3,4,5-trisphosphate)[116]. PIP3 recruits plekstrin homology domain containing Akt proteins to the cell membrane leading to activation of Akt by phosphorylation at two amino acid residues (Thr308 phosphorylated by PDK1 and Ser473 phosphorylated mTORC2) [117]. PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a negative regulator of this pathway. It dephosphorylates PIP3 molecules which results in the inhibition of the downstream signaling cascade [116]. Akt is activated in more than 60% of the melanoma cell lines [111]. As depicted in figure 8, in most of the cases this activation is through PTEN deletion or mutation (30 to 50% of the melanoma tumors) [111]. However, activating mutations in PI3K catalytic subunit (3% of the melanomas) and amplification of the Akt3 gene is also reported in some melanoma cell lines [118].

STAT3 pathway

The signal transducer and activator of transcription 3 (STAT3) is a transcription factor that mediates the expression of a variety of genes in response to cell stimuli [119]. The STAT family of proteins resides in the convergence point of several cell survival pathways including growth factor receptor pathways (EGFR, Her-2, PDGFR, c-Met, EphB1 etc) cytokine receptor pathways (IL-6R, IL-10R etc), and non receptor tyrosine kinases pathways (FAK , Abl etc) [120]. In normal cells, STAT activation is transient and tightly regulated. However, they are constitutively activated and contribute to tumor cell growth, proliferation, metastasis and angiogenesis in various cancers including melanoma [119, 120]. Niu et al reported constitutive activation of STAT3 in 88% of the melanoma

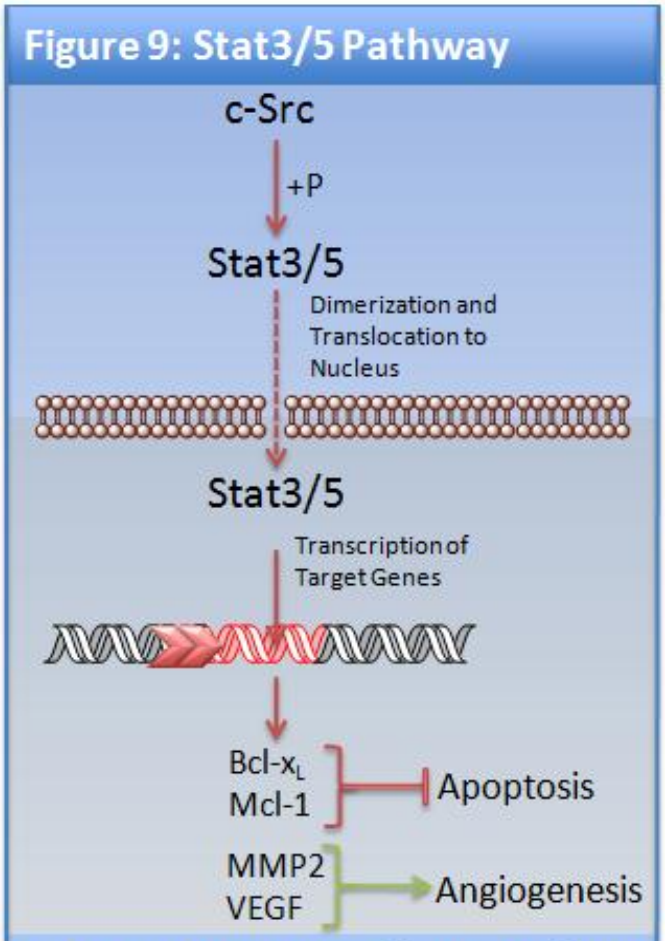


Figure 9: Stat3/5 Pathway

Stat3 transcription factor induces melanomagenesis through inhibition of apoptosis: Upon phosphorylation and activation by c-Src, Stat3/5 homodimerizes and translocate to nucleus to induce transcription of antiapoptotic Bcl-XL , Mcl-1 and pro angiogenic MMP2 , VEGF proteins.

cell lines [120]. They noted that proto oncogenic tyrosine kinase c-Src was responsible for the over-activation of the Stat3 protein.

Beside inhibiting apoptotic pathways through inducing the expression of anti-apoptotic Bcl-x_L and Mcl-1genes, Stat3 promotes angiogenesis by stimulating MMP2 and VEGF genes (Figure 9) [121-123]. It is testified that the activation of Stat3 may promote brain metastasis of melanoma cells [121]. In several studies it was shown that inhibition of Stat3 reduces melanoma invasiveness beside decreasing proliferation [124, 125]. These findings suggest that cSrc-STAT3 signaling plays a critical role in melanoma progression and may be a good therapeutic drug target for melanoma [120].

c-Jun N-terminal Kinase (JNK) Signaling

c-Jun N-Terminal kinases belong to the MAP kinase family of proteins, and are identified as kinases that phosphorylate and activate c-Jun oncogenic transcription factor [126]. The human genome encodes 3 different JNK proteins: JNK1, JNK2, and JNK3. JNK1 and JNK2 are expressed ubiquitously but JNK3 is specific for the brain, heart and testes [127]. JNK proteins regulate cellular response to cellular stress such as UV light exposure, heat shock and cytokines through activating several transcription factors including c-Jun, ATF2, ELK1. p53, and heat shock protein 1 (HSP1) [128].

Since JNK proteins are involved in both apoptosis and cell proliferation their role in tumorigenesis differ from one cell type to another. They behave like a double-edged sword that in some cell lines they enhance apoptotic pathways while repressing in others [128]. However, it is not well understood how JNK suppresses apoptosis; but it is thought to be either through the p53-p21 or Bad-Bcl-xL pathways [128].

In the case of melanoma, the JNK pathway is entwined with Erk signaling [129]. As it was mentioned before, the majority of the melanoma cell lines show increased activity of Erk1/2 proteins due to either upstream B-Raf or N-Ras mutations [101, 110]. Bergami et al reported that active Erk is able to trigger Jnk activity through the Erk-Creb-c-Jun-Rack1-Jnk pathway[129]. In this pathway, Erk increases transcription and stability of c-Jun oncogene through phosphorylation of the CREB and GSK3 proteins, respectively. Consequently, c-Jun induces transcription of RACK1, an adaptor protein that enables phosphorylation of Jnk by PKC protein. In turn, JNK triggers c-Jun activity creating a feed-forward vicious cycle.

Vasileia-Ismini et al reported that inhibition of JNK with the small molecule inhibitor SP600125 decreased the growth of all melanoma cell lines that they tested [130]. In 1205Lu cells, which have wild-type p53 protein, inhibition was through p53-dependent induction of $p21^{Cip1/Waf1}$ expression. However, in the WM983B cell line, which carries P278S mutation in the p53 gene that converts protein to a dominant negative p53 protein [131], the decrease in tumor growth was due to Bad and Bax induction. It is noteworthy that Jnk phosphorylates BAD at Thr201 to reduce its association with Bcl-xL ensuing the inhibition of apoptosis [132].

Other Genetic Alterations

Most recently, while we are writing this manuscript, Wei et al reported a novel common mutation in melanoma [133]. In their study, whole exon sequencing of melanoma cells and normal melanocytes for 14 melanoma patients helped them to

identify that 33% of the melanoma samples were carrying a mutation on the glutamate [NMDA] receptor subunit epsilon-1 (GRIN2A) gene compared to melanocytes. However, this recent study warrants further investigation.

It is worth noting that several other uncommon genetic alterations are reported in distinct melanoma cases: over expression of the apoptosis inhibitor B-cell lymphoma 2 (Bcl2) gene[134-136], loss or silencing of tumor suppressor Adenomatous polyposis coli (APC)[137, 138] or p53[139], rare activating mutations of beta-catenin (CTNNB1) [140], increased expression of nuclear proto-oncoprotein Ski [141, 142], epigenetic silencing of apoptotic protease activating factor 1 (APAF-1) [143] and amplification of CyclinD1[144].

Functional significance of melanoma associated mutations

Functional significance of gene mutations in melanoma genesis was revealed by transgenic mice studies (Table 1) [145-156]. Serrano et al reported that knocking out CDKN2A locus increased predisposition of melanoma development, but still this deletion was not enough and required a secondary abnormality for development of spontaneous melanomas [149]. When these mice were engineered to express constitutively active mutant H-Ras, more than half of the mice developed melanoma in the first six months of their life [153, 157]. On the other hand, single gene mutations of the two CDKNA2 locus genes (either $p16^{INK4A}$ or $p14^{ARF}$) prolonged the required time to one year when co-engineered to express oncogenic H-Ras [146, 158]. Interestingly, 80% of melanomas

developed by p16 knock-out mice showed alterations either in p53 pathway or directly in p14 ^{ARF} protein [146]. On the other hand, UV treatment of p14$^{ARF\ -/-}$ H-Ras^{mut} mice promoted melanoma development by either amplification of Cdk6 or by loss of p16 ^{INK4A} gene [146]. These two observations suggest that both p16 ^{INK4A} and p14^{ARF} are important and cooperate on melanoma genesis [158].

B-Raf is the most highly mutated gene of melanoma [114]. More than 60% of the melanoma cells carry mutation on B-Raf [111]. Virtually all melanoma cells have elevated levels of Raf-Mek-Erk signaling either through B-Raf mutations or upstream N-Ras and receptor tyrosine kinase mutations. Moreover, the PLX4032 compound which targets mutated B-Raf was extensively successful in clinical trials [159]. However, interestingly B-Raf is also mutated in more than 80% of the nevi, which would stay for decades without developing malignancy [112]. This contradictory situation required illumination of the functional significance of B-Raf mutations in melanoma.

Patton et al published the first study that aims to elucidate significance of B-Raf mutations in melanoma with transgenic animals in 2005 [154]. They created a zebrafish model that expresses the most common form of mutant B-Raf (V600E) under the control of the melanocyte specific promoter. They reported that, these animals showed patches of ectopic melanocytes resembling the nevi on human skin. However these animals did not developed melanoma unless a secondary mutation into p53 gene is introduced to their genome. Mutant B-Raf expressing p53 deficient animals rapidly developed malignant melanomas.

Table 1: Knock in/out mouse models of melanoma

Genetic Modification 1	Genetic Modification 2	Affected Genes	Spontaneous Melanoma	Other Cancers	Notes	Refrence	PMID
Arf $^{-/-}$		p14 ARF deleted	NO	low frequency tumor formation		Kamijo et al	9393858
Arf $^{-/-}$	Tyr-RAS	p14 ARF deleted Ras induced	52 % at 81 weeks	YES	50% of the melanomas, show Ink4a lose	Sharpless et al	12902988
Arf $^{-/+}$	Tyr-Ras	p14 ARF decreased	10% at 81 weeks	NO		Sharpless et al	12902988
INK4A $^{-/-}$		p16 INK4A deleted	2.5% of animals	low frequency tumor formation		Sharpless et al	11544531
INK4A $^{-/-}$	Tyr-RAS	p16 INK4A deleted Ras induced	35% of animals at 89 weeks	YES	80% of the melanomas showed inhibition of p53 pathway	Sharpless et al	12902988
INK4A $^{+/-}$	Tyr-RAS	p16 INK4A decreased Ras induced	4% of animals at 89 weeks	low frequency		Sharpless et al	12902988
INK4A $^{-/-}$, Arf $^{+/-}$		p16 deleted p14 decreased	8 % at 17 months	high frequency tumor formation	Tumors showed loss of heterozygosity for Arf gene	Krimpenfort et al	11544530
Cdkn2a $^{-/-}$		p16 INK4A and p14 ARF deleted	NO	high frequency tumor formation		Serrano et al	8620534
Cdkn2a $^{-/-}$	Tyr-RAS	p16 INK4A and p14 ARF deleted Ras Induced	60 % of the animals at 6 months	high frequency tumor formation		Chin et al	9353252
Cdkn2a $^{-/-}$	Pten $^{-/-}$	p16 INK4A, p14 ARF, Pten deleted	7% by ~8 months	high frequency tumor formation		You et al	11818530
p53 $^{-/-}$	Tyr-RAS	p53 deleted Ras induced	29% at 4 months	low viability, hig frequency tumor formation	Almost always retain functional Ink4a,but Rb pathway is inactivated	Bardeesy et al	11238948
Cdk4 $^{R24C/R24C}$		dominant CDK4 resistant to inhibition by p16	NO	high frequency tumor formation		Sotillo et al	11726500
Tyr-B-Raf (V600E)		Mutant B-Raf expression	NO	NA	Zebrafish Model	Patton et al	15694309
Tyr-B-Raf (V600E)	p53 $^{-/-}$	Mutant B-Raf expression in p53 deficient background	YES	NA	Zebrafish Model	Patton et al	15694309
B-Raf (V600E)		Mutant B-Raf expression	54% at 12 months	NA		Dhomen et al	19345328
B-Raf (V600E)	INK4A $^{-/-}$	Mutant B-Raf expression in p16 deficient background	80% at 12 months	NA		Dhomen et al	19345328

The second study on B-Raf with transgenic animals published in Cancer Cell in 2009. Dhomen et al created a mouse model that expresses mutant B-Raf (V600E) in their melanocytes [155]. Interestingly 54% of these animals developed melanomas within 12 months without requiring any secondary mutation. This was contradictory to the most of the previous studies where mice bearing mutant H-Ras was not able to develop melanoma unless tumor suppressor genes such as $p16^{INK4a}$ or $p19^{Arf}$ are also deleted. To investigate this situation they created another mouse model that expresses mutant B-Raf (V600E) in $p16^{INK4a}$ null background mouse. They reported that 80% of these animals developed melanoma within 12 months. Ackermann et al have reported that 28% of the mice expressing activated N-RasQ61K on an INK4a-deficient background develop melanoma within a year [156]. Comparison of these two cases is noteworthy that, it reflects the clinical experience where N-Ras mutations are three times less common than B-Raf mutations [160].

On the other hand, Ras pathway regulate not only Raf-Mek-Erk signaling cascade but also PI3K-Akt signaling. Thus, two distinct groups, Podsypanina et al. studied PTEN inactivation by creating PTEN knock out mouse lines [161, 162]. Homozygous knock out of PTEN ($PTEN^{-/-}$) was intolerable and all mice died at an early embryonic stage. However, heterozygous $PTEN^{+/-}$ mice were viable and developed several types of tumors including thyroid tumors and prostate tumors but not melanoma [162]. When these mice crossed with $CDKNA2^{-/-}$ mice, the ~7 % of the resulting progeny developed melanoma by 8 months [150]. On the other hand, Chin et al reported that 60% of the CDKN2A mice developed spontaneous melanoma when engineered to express

high levels of Ras gene in their melanocytes. These two observations strongly indicate significance of MAPK pathway on melanoma development. Additionally, according to these two studies, neither the loss of PTEN nor the loss of CDKN2A locus was enough for melanoma development, but they cooperate to drive melanoma formation with a low frequency. These data correlates with mutational rates of melanoma cell lines, where approximately ~7% of the cell lines (without N-Ras mutations) carry PTEN mutation without B-Raf mutation and 60% of the cell lines carry B-Raf mutation(s)[111].

Concluding Remarks or Formulating Melanoma

To this point, the roles of several pathways in melanoma development have been described. At this point, we can formulate the most general melanoma driving genetic abnormalities as a following equation.

MELANOMA= (Loss of CDKN2A locus) + ((N-Ras mutation) (B-Raf mutation +/- PTEN deletion) or (N-Ras mutation) or (RTK mutation/over activation))

As aforementioned, up to 96% of the melanomas carry an abnormality in the CDKN2A locus [94, 95]. But as it is supported by transgenic mouse models, this abnormality must be reinforced by additional oncogenic alterations [149, 153]. This reinforcement can be an over activation of a proliferation pathway (such as MAPK pathway) or induction of an anti-apoptotic pathway (such as Akt pathway). In most cases, the mutated N-Ras protein or downstream members of Ras pathway (MAPK and PI3K pathways) reinforce CDKN2A mutation to induce melanoma genesis. However, in only 20 to 25% of the melanoma cases N-Ras is mutated [100]. Thus, usually these supporting

alterations lie in two downstream molecules. In ~60-70 percent of melanoma cases it is B-Raf in the MAPK pathway and in 20% of the cases it is PTEN in the PI3K-Akt pathway [111]. This is supported by two observations. Firstly, activating B-Raf mutations are mutually exclusive with N-Ras mutations; while overlap between N-Ras mutations and uncommon B-Raf mutations, which do not increase, the activity of B-Raf, was detected. Secondly, PTEN mutations are almost always seen together with B-Raf mutations, indicating that concurrent B-Raf and PTEN mutations may function like N-Ras mutations. However, as mentioned above, PTEN is mutated in only 30% of the melanomas compared to 70% in B-Raf mutations. Therefore, at least half of the B-Raf mutations are PTEN deletion notwithstanding and must be being supported by alternative alterations. These alterations might occur in the other members of PI3K-Akt signaling pathways. Until today, mutations or amplifications of Akt gene and mutation of PI3KCa is reported in several melanoma cases [163-165].

Leelamine

As I was discussed above, limited therapeutic agents are available against melanoma development. Natural products are the primary source of pharmacology [166]. More than 60% of the existing chemotherapeutic agents are derived from natural sources such as plants and microorganisms[167]. Thus, to identify novel inhibitors of melanoma cell growth we have screened a natural product library (NPL-480) that consists of 480 compounds which was obtained from Tim Tec Inc., Newark, DE. This screen identified Leelamine as one of the 8 compounds that is able to inhibit the growth of early and advanced melanoma cells at micro molar concentrations. Since compounds other than Leelamine were already reported and studied in melanoma research, we carried our research focusing on Leelamine.

To determine the efficacy of Leelamine on the viability of normal skin cells (melanocytes, keratinocytes and fibroblasts) and melanoma cells, we performed MTS assays which revealed that Leelamine was able to trigger cell death of advanced melanoma cell lines in ~5 times less concentration compared to normal cells (melanocytes). The IC50 values for melanocytes, keratinocytes, fibroblasts and malignant melanoma cell lines were 8.3uM, 9.6uM, 10.1uM and ~2uM respectively. Thus, leelamine was more specific at killing malignant melanoma cells compared to ordinary skin cells. Further research showed that Leelamine inhibits cell proliferation and triggers apoptotic pathways through a G0/G1 block. Moreover, in vivo studies with mice showed that intra peritoneal administration of Leelamine was able to suppress xenograft tumor growth without any detectable toxicity (Figure 10). In the extracted xenograft tumors, beside an

increase in apoptosis; cell proliferation and angiogenesis were decreased. Therefore, Leelamine holds great promise to become a therapeutic agent in melanoma research.

In the literature, there is little information about leelamine. It is reported that it weakly binds to the cannabinoid receptor CB1, exhibits 20% displacement of [3H]-CP55940 (full agonist of CB1 & CB2) at a concentration of 10 μM [168]. Even though this interaction does not stimulate G-protein activity, it shows potent behavioral activity in mice, similar to CB1 & CB2 agonist tetrahydrocannabinol (THC) that is reversed by CB1 antagonist SR141716A [169]. However, CB1 knockout animals show similar behavior upon leelamine treatment suggesting that receptors other than CB1 might be responsible for effects. Additionally, Aicher et al reported that leelamine inhibits pyruvate dehydrogenase kinase (PDK) activity with an IC50 value of 9.5(±1.1) μM [170]. They showed that, oral administration of 300uMol/kg leelamine lowered blood glucose levels 35% after 6hr, but this decrease was independent from the inhibition of PDK [170]. It was also claimed that derivatives of leelamine might also show anti-inflammatory activity through repression of phospholipase-A2 [171, 172]. However, none of these interactions were confirmed by a second study. Thus, identification of the leelamine targets would be important for therapeutic or scientific use. In our study, we showed that treatment of melanoma cells with leelamine inhibits several melanoma driving signaling cascades including PI3 kinase, STAT, Jnk and MAP kinase cascades.

Chapter 3

Combining Systems Biology and Proteomics For Identification of Leelamine Targets

Abstract

Melanoma is the most deadly form of skin cancer with a second place in the increasing cancer trends list[1]. If you are an average reader, before you complete reading this thesis, at least one person in the United States will die from melanoma [173]. If it is detected early, surgery still remains to be the best solution [174]. However if it metastasizes to other organs, only one in five would survive for longer than five years[173]. Despite all the advances in therapeutic cancer research, two drugs, Dacarbazine and Ipilimumab, are currently the only FDA approved chemotherapy agents for metastatic melanoma treatment [4].

In a previous natural compound library screening the Robertson lab identified Leelamine as a potential inhibitor of melanoma growth (unpublished data). Leelamine is able to trigger apoptosis selectively in malignant melanoma cells compared to melanocytes. In this study, we revealed signaling pathways targeted by leelamine treatment in melanoma cells by combining proteomic approach with systems biology tools. According to our findings, Leelamine shows its effect through affecting multiple pathways including, PI3K-Akt, STAT, JNK and MAPK pathways. All of these four pathways support melanoma growth by regulating either cell cycle progression or apoptosis depending on the cellular context [110, 111, 120, 130]. Thus acting as an inhibitor of these pathways, Leelamine has the potential to be used as an anti-cancer agent.

INTRODUCTION

Melanoma, the fastest growing cancer not only in the United States but also in the world, is the most dangerous type of skin cancer that originates from the neural crest [63, 175] . It is the most common form of cancer for women from 25 to 29 years of age[176]. In 2011, it is projected that more than 68,000 people will be diagnosed with melanoma and approximately 8,700 of them will lose their lives due to these skin diseases in the United States [2]. Melanoma resists most of the conventional therapies including radiotherapy and chemotherapy [177]. Thus, early detection and surgical removal of the tumor is the best means of reducing mortality [174]. After the approval of Dacarbazine (DTIC) as a chemotherapy agent in 1975, the only advancement in melanoma chemotherapy was recently achieved with the FDA approval of Ipilimumab (Bristol-Myers Squibb) in March 2011 [5]. However, neither DTIC nor Ipilimumab is absolute solution for metastatic melanoma. Ipilimumab was just able to increase survival length from 6.5 months to 10 months [178]. Thus, there is an urgent need for discovery of new therapeutic agents for melanoma.

A previous natural compound library screening identified Leelamine, a diterpene molecule, as a potential inhibitor of melanoma growth. It was shown that, besides decreasing viability of melanoma cells in vitro; leelamine is able to shrink tumor growth in melanoma cell transplanted xenograft mice. However, the mechanism of action of leelamine is not known. There is very limited information about this small molecule in current literature. It is reported as a weak agonist of cannabinoid receptor 1 (CB1), however this interaction was not able to stimulate downstream molecules [169]. On the other hand, Aicher et al reported that Leelamine inhibits pyruvate dehydrogenase kinase

(PDK) with an IC50 value of 9.5 (+/-1.1) uM [170]. But as it is shown in this study, PDK1 inhibition does not directly show any effect on melanoma cell growth. Other than these two reports, to the best of our knowledge, there is no existing information about the leelamine targets in online databases.

In here, we revealed that Leelamine inhibits the activity of several pathways that are reported to be important for melanoma cell survival. The major effect of Leelamine appears to be through inhibition of the PI3K-Akt pathway, which is commonly activated by loss of PTEN mutations in melanoma[111]. Akt is a key serine/threonine kinase that has been implicated in regulation of apoptotic pathways. It phosphorylates and inhibits action of apoptotic proteins including FKHRL, PRAS40, XIAP and Bad[179]. At least half of the melanoma cell lines show over activity in this pathway indicating therapeutic importance[111]. Other than PI3K-Akt signaling, Leelamine also inhibits three important melanoma-driving pathways: Stat3, Jnk and mitogen-activated protein kinase (MAPK).

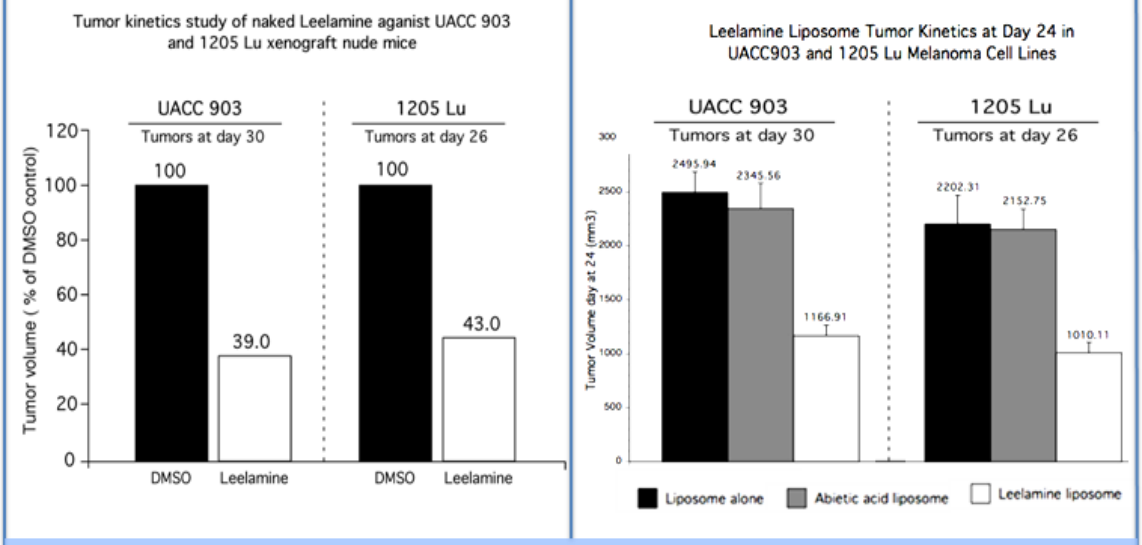
MAP Kinases are also serine/threonine-specific protein kinases that regulate cell behavior in response to the extracellular stimulus such as growth factors, hormones, osmotic stress and cytokines [180, 181]. Almost all of the melanoma cell lines have an over activated MAPK pathway either due to upstream N-Ras mutations or B-Raf mutations [182]. On the other hand, Jnk and Stat3 pathways also have an impact in melanoma tumor growth [120, 130]. However, it is not well understood whether their contribution to melanoma development is a direct effect or if they are being deregulated after the completion of melanomagenesis.

RESULTS

Leelamine Effectively Inhibits Melanoma Tumor Growth

We have previously identified Leelamine as a potential inhibitor of melanoma cell growth. Moreover, in-vivo experiments showed that Leelamine is able to inhibit melanoma tumor growth in xenograft mouse models. Two malignant melanoma cell lines, UACC903 and 1205Lu, were transplanted to the 4-to-6 week old female nude mice. After formation of size matched tumors (~75mm^3), mice were treated with Leelamine (5mg/kg or 7.5mg/kg body weight) or DMSO vehicle intraperitoneally. In both the cell lines, Leelamine treatment reduced tumor size 57 to 61% compared to DMSO treatment (Figure 10A).

Figure 10: Leelamine Melanoma Tumor Inhibition



Leelamine Inhibits Melanoma Tumor Growth In Vivo: A) Intra peritoneal treatment of leelamine significantly inhibits melanoma tumor growth in both UACC903 and 1205Lu xenograft mice . **B)** Intravenous injection of Leelamine loaded liposomes successfully decreased tumor growth. However, abietic acid which is a structural analog of Leelamine, was not able to show any activity on tumor growth

Intravenous (IV) delivery of drugs might yield a better response due to the fact that the drug is delivered to all cells. However, leelamine is not a water soluble molecule,

and requires a carrier for intravenous injection. Thus, we loaded leelamine into neutral liposomes (hereafter will be referred to as Nanolipolee). Intravenous injection of Nanolipolee (30mg/kg body weight) successfully diminished tumor growth (Figure 10B). At day 24, the tumor sizes of Nanolipolee treated mice were less than half of the empty liposome treated animal tumors. Furthermore, as a second control, abietic acid, a structural analog of leelamine, was used. Abietic acid was not able to show any activity on tumor growth. Thus, both in-vitro and in-vivo experiments suggested that Leelamine has a noteworthy potential to inhibit melanoma growth.

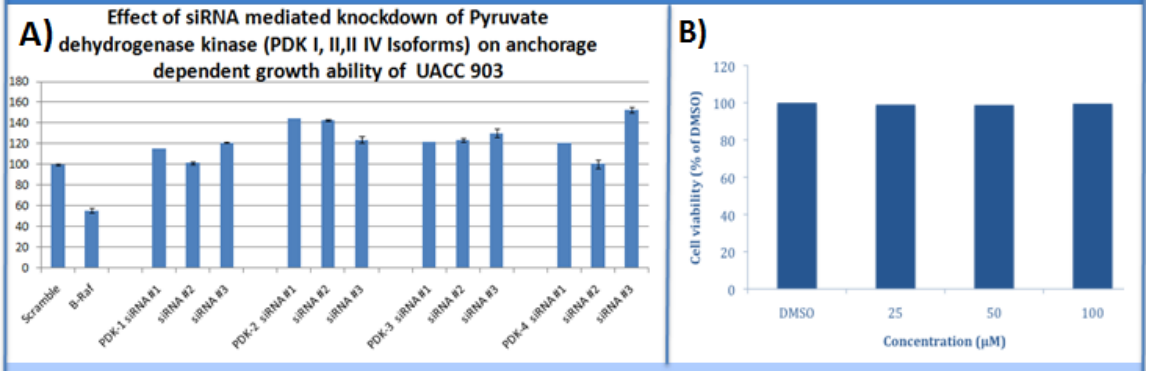
Identification of Leelamine Mechanism of Action

To the best of our knowledge, there are only two known targets of Leelamine in the published literature. It inhibits pyruvate dehydrogenase kinase (PDK) (not 3-Phosphoinositide-dependent kinase 1) with an IC50 value of 9.5 µM [170]. On the other hand, it also weakly binds to cannabinoid receptors CB1 and CB2 as an agonist [169]. However, our studies showed that neither of these targets are directly involved in melanoma tumor growth inhibition.

First of all, neither siRNA mediated knockdown of PDK isoforms nor dichloroacetate (DCA) mediated inhibition of PDK showed significant effect on viability of melanoma cells (Figure 11A- 11B). On the other hand, the affinity of Leelamine to cannabinoid receptors was so weak that 10 µM Leelamine was able to displace only 20% of H^3 labeled cannabinoid agonist CP55940 [168]. Moreover, co-treatment of either CB1 antagonist AM251 or CB2 antagonist AM630 with leelamine did not repressed tumor

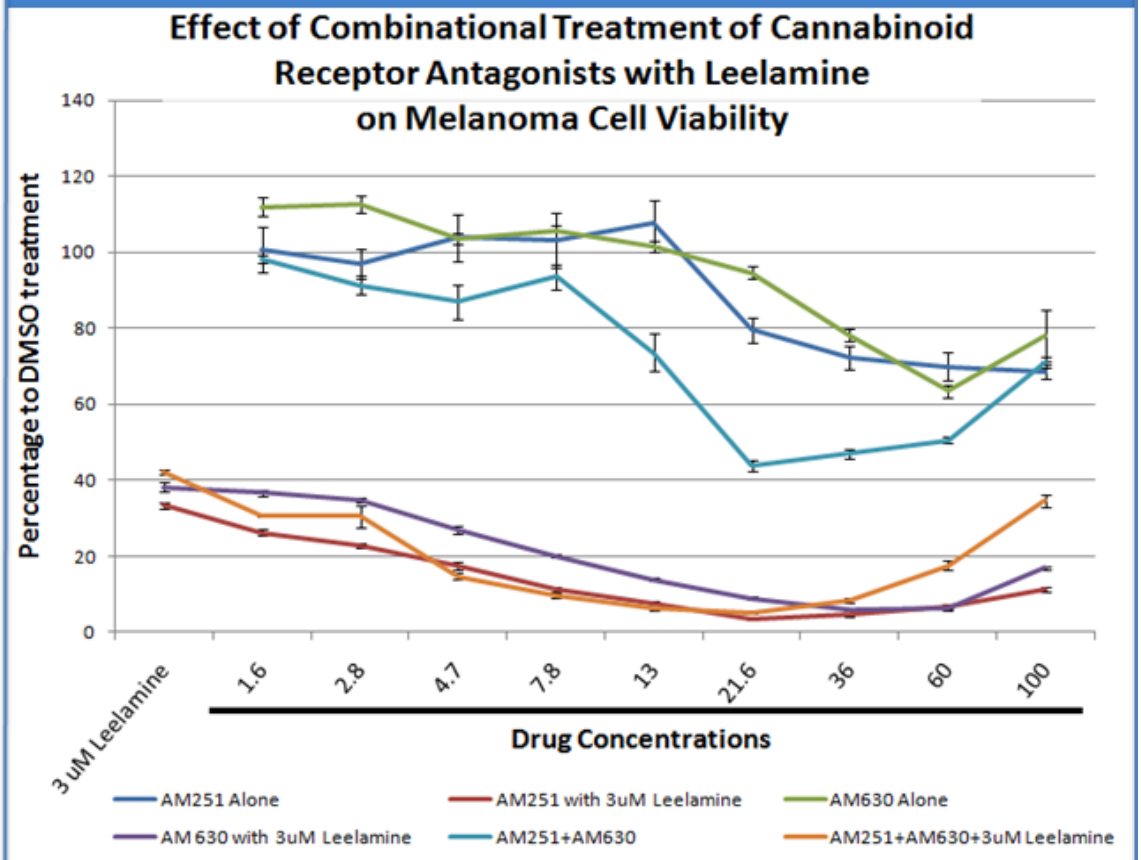
inhibitory action of Leelamine (Figure 12). Thus leelamine should be inhibiting melanoma tumor growth through a target other than CB1, CB2 or PDK.

Figure 11: Pyruvate Dehydrogenase Kinase Inhibition



Inhibition of PDK isoforms had no effect on melanoma cell viability: A) Stealth siRNAs targeting each isoform of PDK were introduced in to UACC 903 cell line via nucleofection using Amaxa nucleofector, K-17 program. 2 days after recovery, 2 X 10^4cells were seeded in a 96-well assay plate, and 3 days later cell viability measured using MTS. The data shows that knocking down PDK isoforms had no effect on cell viability, implying it is not the target for leelamine in melanoma **B)** PDK activity is inhibited by treating 5 X 103 UACC 903 cells with increasing concentration of Dichloroacetate for 24h. DCA treatment had no effect on melanoma cell viability.

Figure 12: Cannabinoid Receptor Antagonists and Leelamine



Inhibition of Cannabinoid receptors did not rescue cells from Leelamine Induced cell death: 5 X 10^4 UACC903 cells were seeded in a 96-well assay plate, 48 hr later cells were treated with compounds either alone or combination with 3uM leelamine. 24 hr after treatment cell viability measured using MTS assay. The data shows that, inhibition of cannabinoid receptors was not able to rescue cells from leelamine induced cell death. Thus leelamine does not show its activity through Cannabinoid receptors.

After elimination of known leelamine targets, I decided to perform a genome wide analysis for identifying the cellular pathways targeted by leelamine. Since chemical substances generally bind and regulate activity of proteins, the primary effect of leelamine would be expected to be on the protein level. Therefore, we performed antibody array analyses with 3uM nanolipolee treated or empty liposome treated UACC903 cells. This antibody array analyses were performed by a proteomics company, Kinexus located at Vancouver, Canada. Kinexus used KAM 1.3 single dye based antibody arrays to analyze the 8 samples as indicated in Table2 (Table 2).

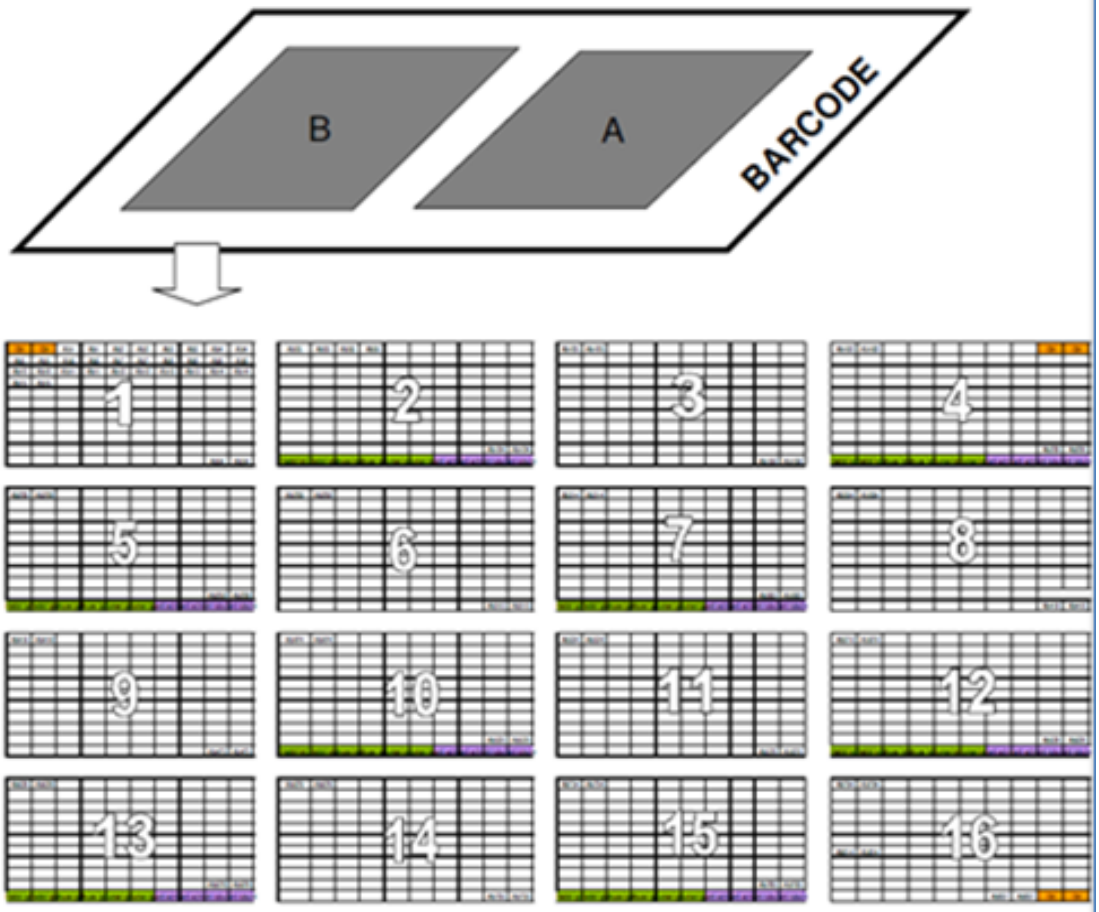
Table 2: Samples analyzed with protein arrays

Sample No.	Time After Treatment	Treatment	Protein Concentration
1	3hr	Empty Liposome	1.8 mg/ml
2	3hr	Nanolipolee-007	1.6 mg/ml
3	6hr	Empty Liposome	1.9 mg/ml
4	6hr	Nanolipolee-007	2.3 mg/ml
5	12hr	Empty Liposome	3.1 mg/ml
6	12hr	Nanolipolee-007	2.1 mg/ml
7	24hr	Empty Liposome	3.9 mg/ml
8	24hr	Nanolipolee-007	3.7 mg/ml

Kinexus used KAM 1.3 single dye based antibody arrays to analyze the 8 samples as indicated in table.

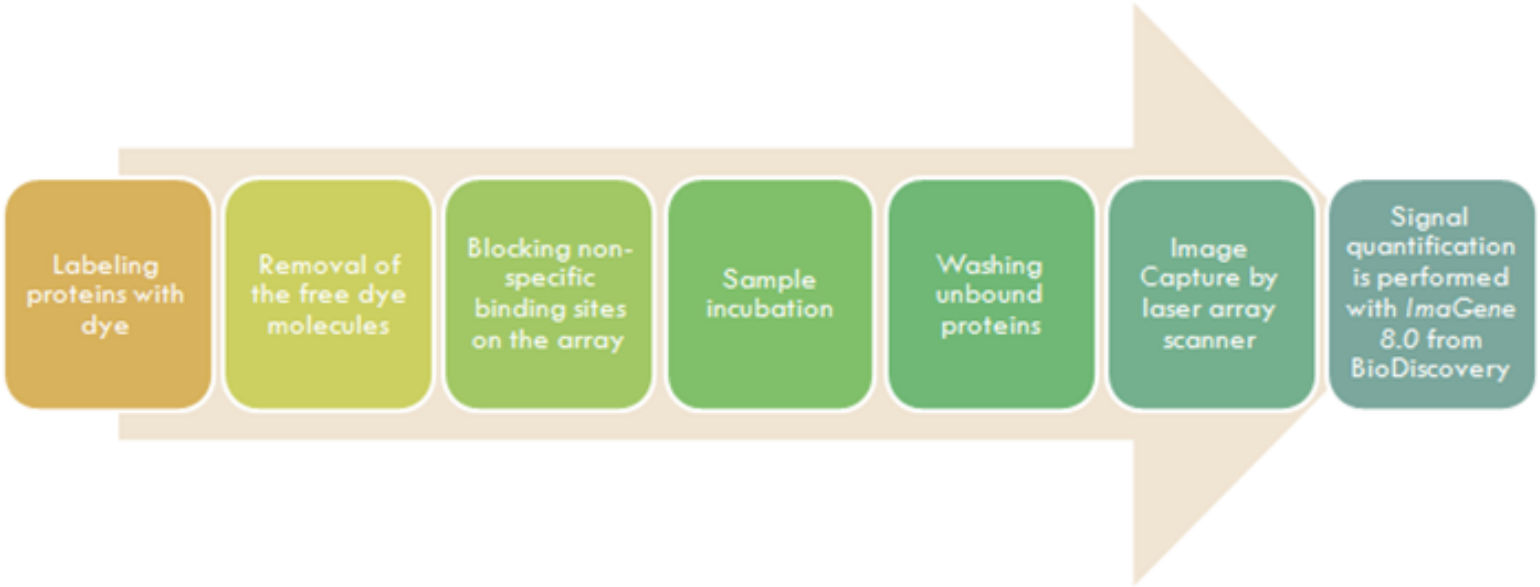
KAM 1.3 arrays contain total of 812 antibodies including 262 phospho-specific antibodies targeting signaling pathways that regulate cell proliferation, apoptosis and stress pathways. Detailed information about the procedure, structure of the array and analyses are depicted in figure 13, 14 and 15. As a result of antibody array analyses, we received a report that contained significantly deregulated protein list in addition to raw data.

Figure 13: Samples analyzed with protein arrays



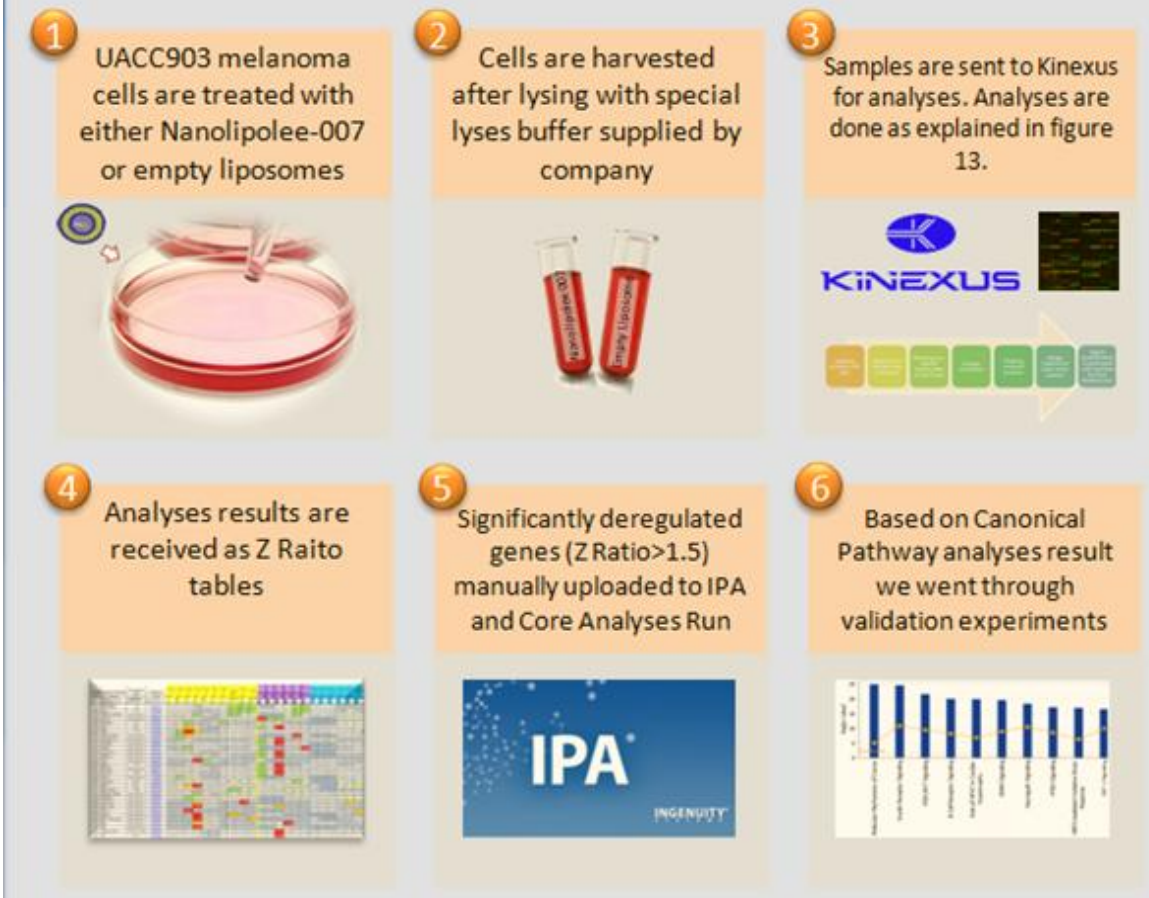
Structure of the KAM 1.3 arrays: 5 Kam 1.3 arrays consists of two identical fields that allows two samples to be analyzed side by side at a time. In each field. There are 16 sub grids of 10 X11 spots . Antibodies are spotted as duplicates. Positive/negative controls (green spots) and anti actin/tubulin antibodies (purple) are spotted through array for local and global normalization.

Figure 14: Procedure of the array analyses



Kinexus antibody arrays require a procedure similar to western blot analyses: In the first step of the procedure of the KAM 1.3 arrays, samples are labeled with single fluorescent dye. Afterwards, unbound dye molecules are washed away and array is blocked for unspecific binding sites. Then, samples are incubated to allow binding of proteins to spotted antibodies. Consequently, unbound proteins are washed away and the image of the array is captured by an array scanner.

Figure 15: Overall Procedure For Protein Array Analyses



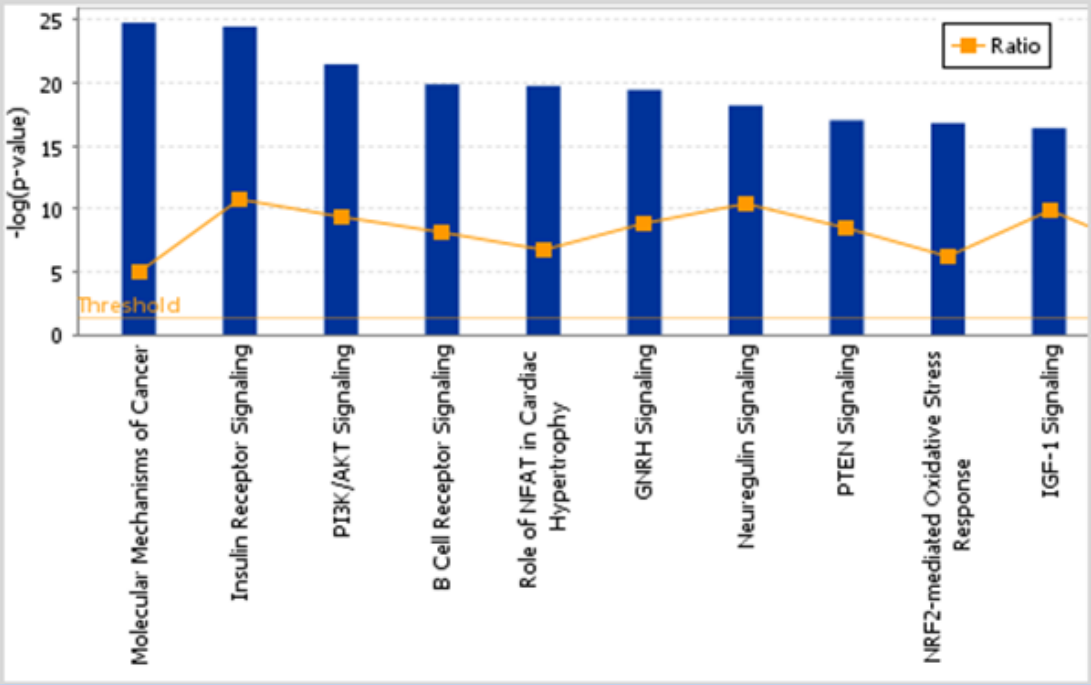
Overall procedure for protein array analyses: 48 hours after plating 1.5 X 10^6 UACC903 cells into p100 plates , cells are treated with either 3uM Nanolipolee-007 or empty liposomes. Cells are harvested and protein lysates are sent to Kinexus for array analyses. Analyses results are received as significantly deregulated protein list. We analyzed this data by uploading to Ingenuity Pathway Analyses tool. Based on analyses we went through validation by western blot analyses.

Systems Biology Approaches for Analyzing Array Data

To analyze the data obtained from antibody array, we went through systems biology approach. As we have mentioned in chapter 2 of this thesis, there is no currently available pathway analysis software that is developed specifically for antibody array data. Tools such as Ingenuity Pathway Analyses (IPA) or GeneGo's MetaCore are developed for analyses of cDNA microarray data and lack power to use protein array specific information such as phosphorylation state of the proteins. However, as it was mentioned before, their databases hold all types of protein interactions (e.g. phosphorylation, expression and degradation) that allow analyses of manually uploaded protein array data, without regarding post-translational modification status of the proteins. I uploaded the significantly deregulated protein list (proteins with Z Ratio>1.5) to the IPA tool.

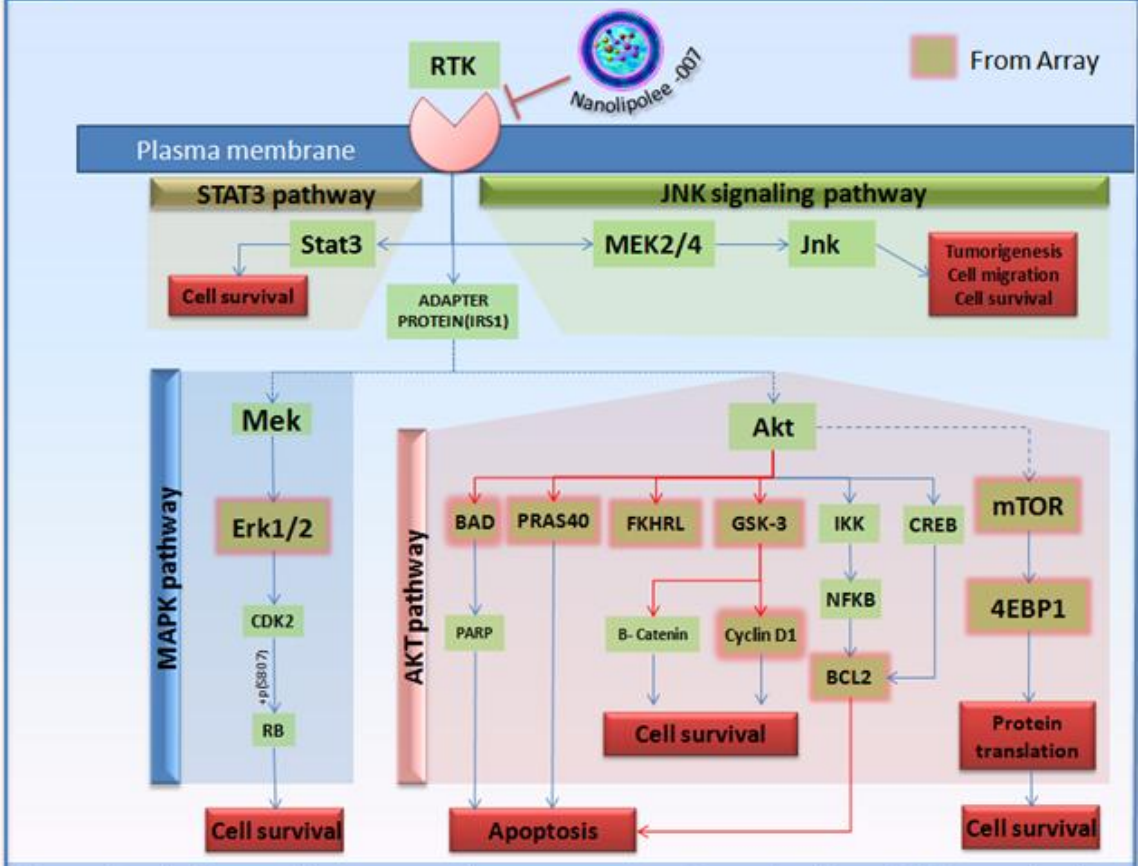
I ran IPA core analyses with default settings or filtering interactions specific to melanoma cell lines. In both cases, insulin receptor signaling and PI3K/AKT signaling pathways were the most prominent pathways deregulated by Nanolipolee-007 (Figure 16). It is well-established knowledge that both insulin receptors and IGF1 receptors regulate activity of PI3K/AKT and MAPK pathways. Thus, by focusing on INSR/IGF1R, PI3K-Akt pathways and array data we created a network model with the help of IPA tool (Figure 17).

Figure 16: Canonical Pathway Analyses Results



Canonical pathway analyses with IPA indicated PI3K-Akt signaling: Significantly deregulated protein list is uploaded to the Ingenuity Pathway Analyses tool. Core analyses run with default parameters. Insulin receptor signaling and PI3K-Akt signaling was promising pathways that might be deregulated by leelamine

Figure 17: Network model according to protein array data



According to the protein array analyses we proposed a network model for mechanism action of leelamine: According to the protein array analyses several members of Akt signaling is deregulated by Nanolipolee. According to the literature and with the help of IPA we proposed above network for mechanism of action of leelamine.

Validation of Network Model

In order to validate the proposed mechanism of action of leelamine, we investigated protein expression levels of the key members of the INSR-AKT pathway by western blot analyses, which confirmed that the Akt signaling pathway is deregulated by leelamine treatment. Western blot analyses for phospho Akt3, phospho PRAS40, phospho Gsk3, CyclinD1, Bcl2, IKKa, phospho p70S6K, phospho Creb revealed that leelamine inhibits Akt signaling pathway (Figure 18). While it was also possible to identify alterations in the members of the Akt signaling pathway from actual protein array spots (Figure 19), no alterations in INSR or IGF1R activities due to low expression of these proteins in UACC903 were observed.

Figure 18: PI3K-Akt signaling western blot analyses

Several PI3K-Akt signaling members were deregulated in protein array: Treatment of leelamine alters several members of the Akt signaling cascade. Leelamine led to significant decrease in phospho Akt and down stream members including phospho PRAS40, phospho GSK3,phospho CREB, pIKKαβ, Cyclin D1, Bcl2, p70S6 kinase with corresponding increase in cleaved PARP.

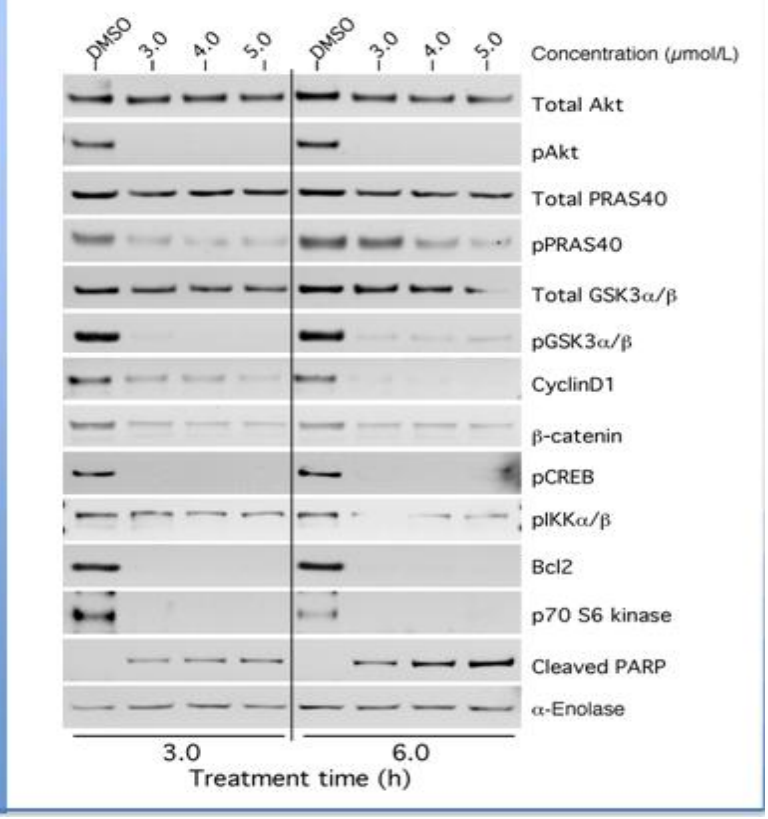
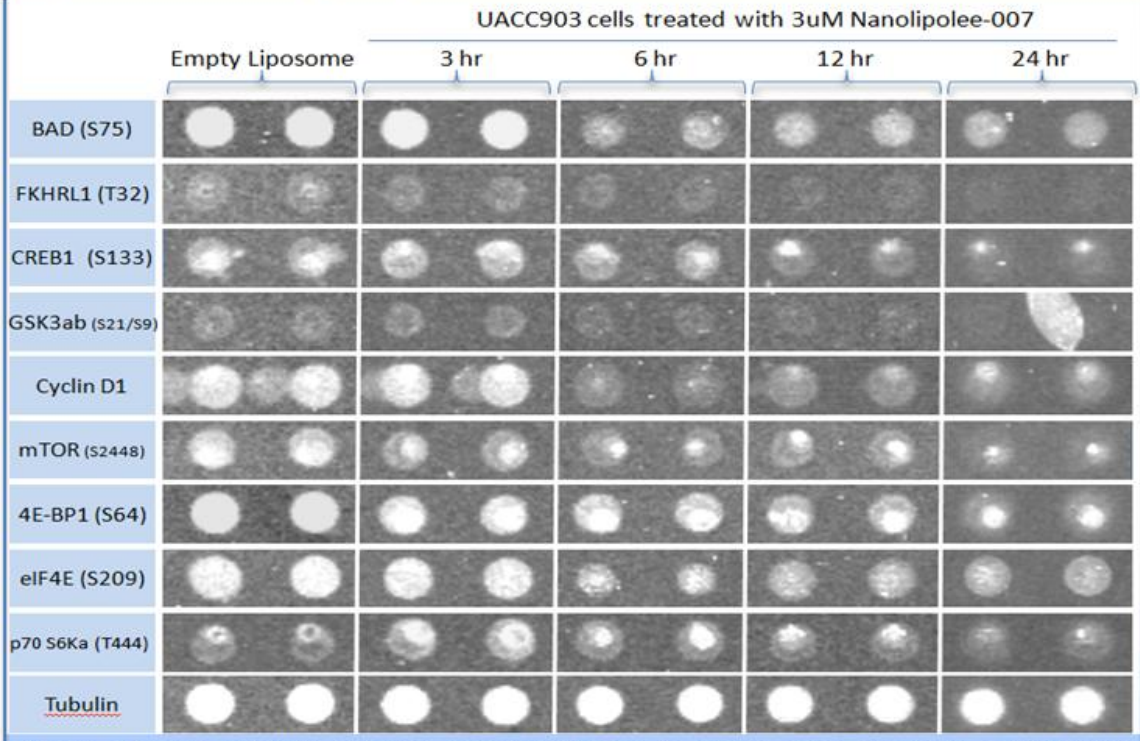


Figure 19: PI3K-Akt signaling members in protein array



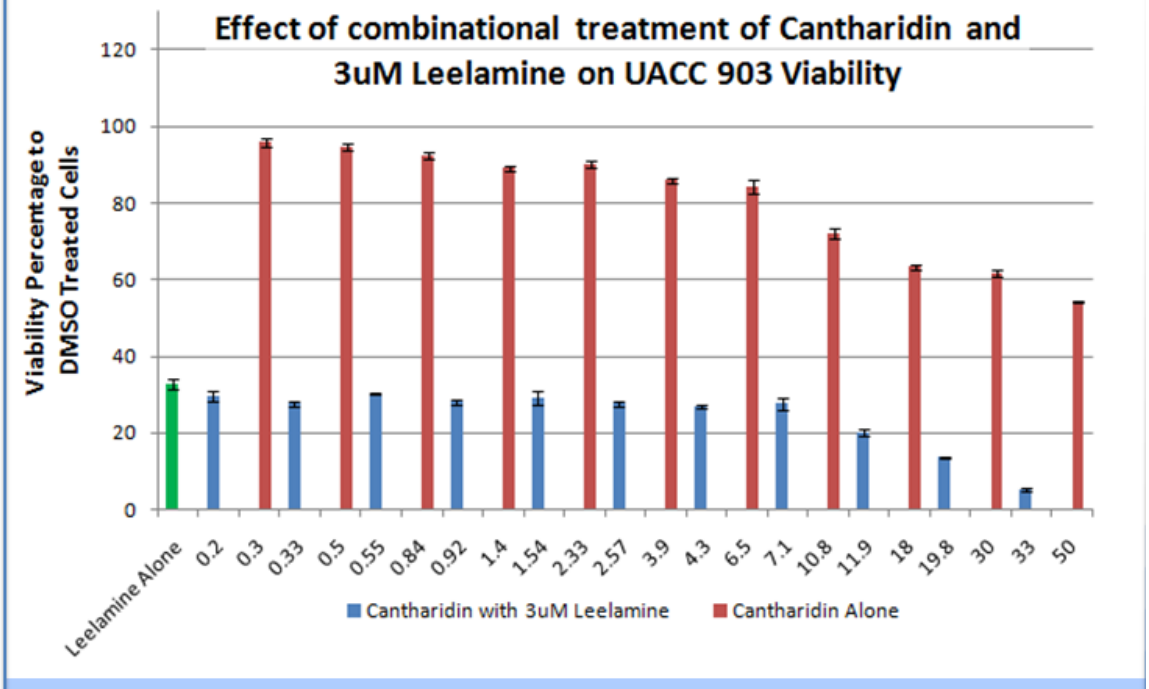
Several PI3K-Akt signaling members were deregulated in protein array: Treatment of Nanolipolee deregulates several members of the Akt signaling cascade. Nanolipolee treatment decreased the phosphorylation of the Akt substrates (Bad, FKHRL1, Creb1, Gsk3, Cyclin D1, mTOR,p70S6K) in a time dependent manner. Images are normalized to local background)

Phosphatases are not involved in Akt inhibition

The PI3K-Akt pathway is a well-studied cascade that is regulated by either upstream receptor tyrosine kinases, G-protein coupled receptors (GPCRs) or protein phosphotases [179]. According to western blot analyses, Akt S473 and T308 phosphorylation decreases upon leelamine treatment. This decrease might be due to increased activity of protein phosphatases. It has been documented that dephosphorylation of Akt is regulated by Ser/Thr phosphatases: PP1 and PP2 [183, 184]. Moreover, according to the protein array analyses, T320 PP1 catalytic alpha subunit (PP1Ca) and total PP1 catalytic beta subunit was upregulated up on 3-hour nanolipolee

treatment. Thus, to illuminate if leelamine regulates Akt signaling through phosphatases UACC903 cells were co-treated with 3uM leelamine and various concentrations of PP1/PP2A inhibitor cantharidin. However, inhibition of PP1/PP2A was not able to rescue cells from leelamine-induced cell death (Figure 20). Cantharidin was toxic for UACC903 with IC50 close to 50uM (Figure 20).

Figure 20: Cantharidin and Leelamine co-treatment



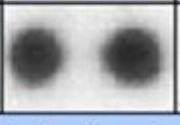
Protein Phosphatase 1 and 2A inhibitor Cantharidin was not able to protect UACC903 cells from leelamine induced cell death : 5 X 10^4 UACC903 cells were seeded in to 96-well assay plates, 48 hr later cells were treated with various concentrations of Cantharidin either alone or combination with 3uM leelamine. 24 hr after treatment cell viability measured using MTS assay. The data shows that, inhibition of PP1/PP2A was not able to rescue cells from leelamine induced cell death. Cantharidin was toxic to cells with an IC50 value of ~50uM.

Revealing the upstream of Akt: Receptor Tyrosine Kinases

After the elimination of phosphatases, to investigate upstream of the Akt cascade we ran a second antibody array that was specially designed by R&D Systems for determining the activity of 42 different RTKs. With this array, we compared the activity

of RTKs in between DMSO treated and 6hr 4uM Leelamine treated UACC903 cells. This study identified decreased activity in several cellular receptors including subtypes of Trk receptors, PDGFRbeta, EphR but not IGF1R or INSR (Table 3). Decreased activity of PDGFR receptors are validated by western blot analyzes. However, low expression of Trk and EphR receptors in this cell line, beside decreasing the importance, was a limitation for validation of the RTK result.

Table 3: Receptor Tyrosine Kinase Array Analyses

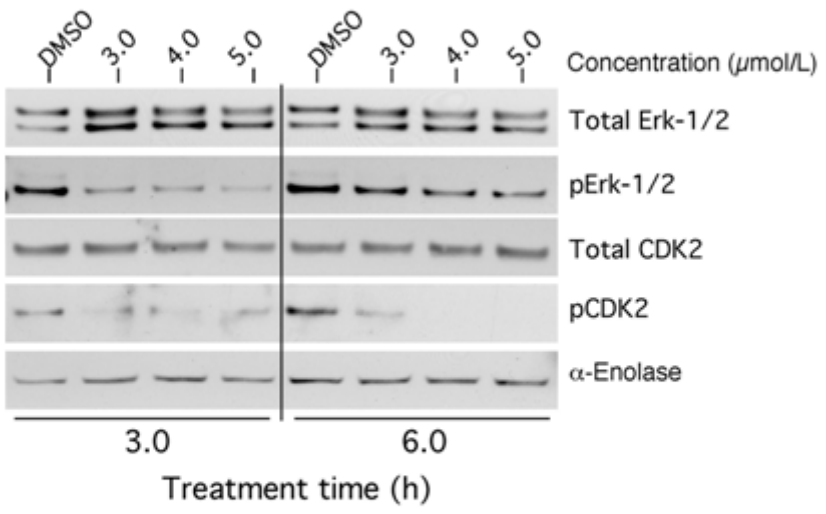
Group	Protein	Ratio	Log 2 Ratio	DMSO	Leelamine
EPHR	EphB1	0.2	-2.3		
NGFR	TrkC	0.4	-1.3		
PDGFR	PDGF Ra	0.4	-1.2		
NGFR	TrkB	0.5	-1.1		
Axl	Axl	0.6	-0.8		
PDGFR	PDGF RB	0.7	-0.6		

Activity of PDGFR beta is decreased by leelamine treatment: To identify the upstream signaling of Akt cascade we run protein array specific for receptor tyrosine kinases. The quantification of the array image was performed by using ImageJ software. As a result we identified alteration in the activities of several RTKs. However, most of these receptors were not significantly expressed in melanoma cells. Thus, most promising change was in PDGFR beta receptors.

Erk; the major melanoma promoting protein: Suppressed.

Upstream signaling pathways of Akt, receptor tyrosine kinases or GPCRs, also regulate MAP kinase signaling through Ras-Raf-Mek-Erk signaling. Activated Erk is one of the major melanoma signatures that virtually all melanoma cells have elevated levels of active phospho Erk protein. Ras-Raf-Mek-Erk signaling also regulates the activity of Cdk2 protein, which induces the cell cycle by allowing release of E2F transcription factors from Rb protein by phosphorylation. Thus, to investigate whether leelamine alters the activity of Erk signaling we did western blot analyses. Western blots revealed that, pCdk2 protein is hypophosphorylated with in concordance with decrease in the activity of Erk protein (Figure 21).

Figure 21: MAPK signaling

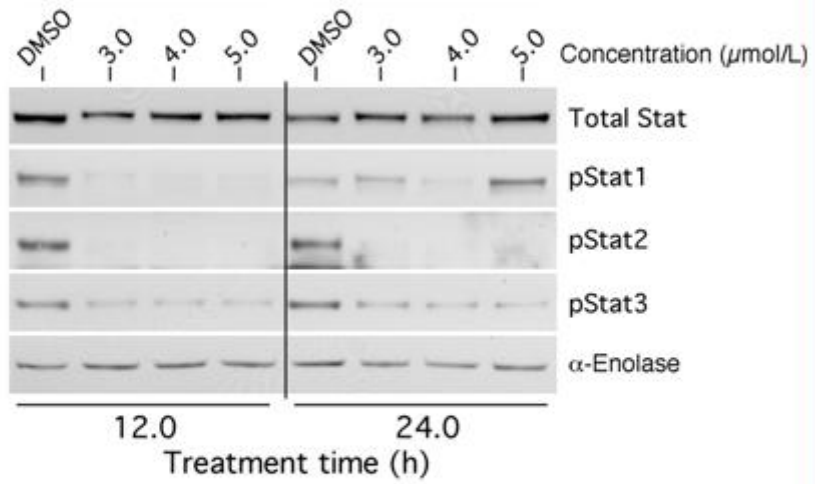


Nanolipolee treatment decreased the activity of Erk pathway: Treatment of UACC903 cells with Nanolipolee-007 decreased the activity of Erk protein in a concentration dependent manner. In concordance with this decrease phosphorylated CDK2 levels also decreased.

Stat3: A melanoma driving transcription factor; deactivated

The canonical PDGFR signaling pathway regulates not only the PI3K/Akt signaling pathway but also Stat3 phosphorylation and activation [185, 186]. Stat3 protein was also significantly deregulated according to Kinexus array. However array results were contradictory to the expected downregulation of Stat3 activity in parallel with inhibition of PDGFR signaling. To reveal the situation, we did western blot analyzes to investigate the activity of several Stat3 proteins upon leelamine treatment. Western blot analyzes showed that, phosphorylation level of the several members of the Stat family, but most critically Stat3 is decreased upon leelamine treatment (Figure 22).

Figure 22: Stat Signaling

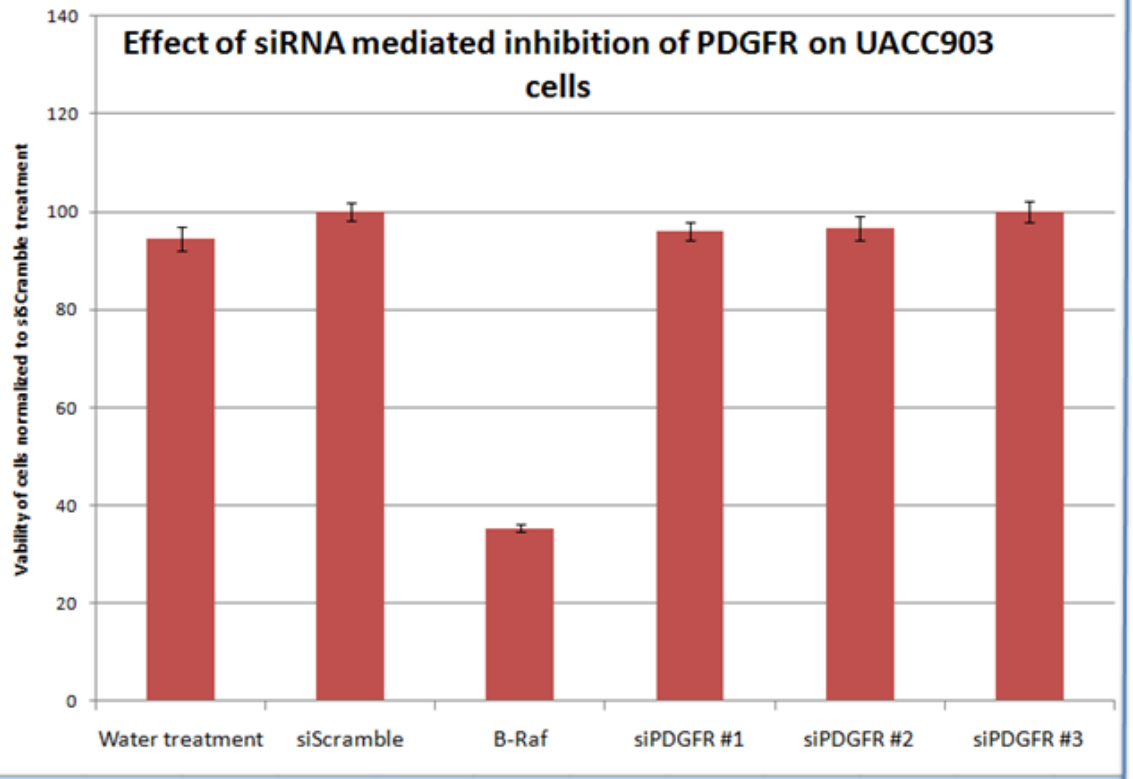


Leelamine decrease the activity of several members of the STAT family: Treatment of UACC903 cells with Nanolipolee 007 decreased the phosphorylation of Stat proteins including Stat3 protein.

PDGFR and Melanoma: Contradictory reports

Several groups have already reported increased expression and activity of PDGFR receptors in melanoma cells [9, 187]. However, McGary et al reported that the inhibition of PDGFR receptors by imitanib mesylate did not show any effect on the proliferation of melanoma cells [9]. Clinical trials with high dose imitanib also showed significant toxicity with no clinical efficacy [10]. We also confirmed these findings by siRNA-mediated knockdown of PDGFR receptors, which did not decrease the viability of UACC903 melanoma cells (Figure 23). On the other hand, resistance to PLX4032, the most promising drug under development for melanoma therapeutics, accounted to the up-

Figure 23: Effect of PDGFR inhibition on UACC903 cells



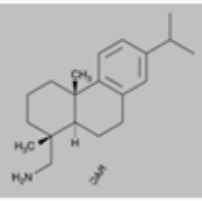
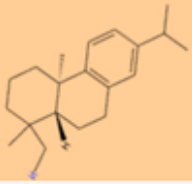
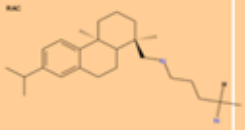
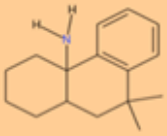
Inhibition of PDGF Receptors did not decreased the viability of UACC903 cells: 3 different stealth siRNAs targeting PDGF receptor were introduced in to UACC 903 cell line via nucleofection using Amaxa nucleofector, K-17 program. 2 days after recovery, 5 X 10^4cells were seeded in a 96-well assay plate, and 2 days later cell viability measured using MTS assay. The data shows that knockdown of PDGF receptors had no effect on viability of UACC903 cells, implying it is not the direct target for leelamine in melanoma

regulation of the PDGFR receptors [188]. Lo and colleagues showed that, PDGFR upregulation is strongly correlated with PLX4032 acquired resistance and siRNA mediated knockdown of PDGFR blocked the cell proliferation in the presence of PLX4032 [188]. PDGFR receptors also regulate PI3K-Akt signaling pathway [189, 190]. It is possible that inhibition of PDGFR signaling in combination with a second pathway might lead cellular apoptosis and growth inhibition. However, it seems that PDGFR is not a direct target for leelamine but might be a downstream-regulated protein.

Alternative approach: Similar compounds with known targets

To identify the molecular targets of leelamine I decided to compare the structure of leelamine to compounds with known targets. GeneGo's MetaDrug tool is designed for deducing drug's mechanism of action, toxicity and off-target effects through comparing the uploaded chemical structure with more than 600,000 biologically active compounds in its database[11]. This tool was used to generate a report that contains similar compounds to leelamine and their known targets (Table 4). In this report, the first two hits, which have 98.21% and 82.09% structural similarity to leelamine, were reported to target the arachidonate 5-lipoxygenase (ALOX5) gene. The report also contained more than 60 related compounds with an average of 74% similarity to the leelamine and they targeted several different subunits of N-methyl d-aspartate receptors (NMDAR).

Table 4 : MetaDrug Analyses

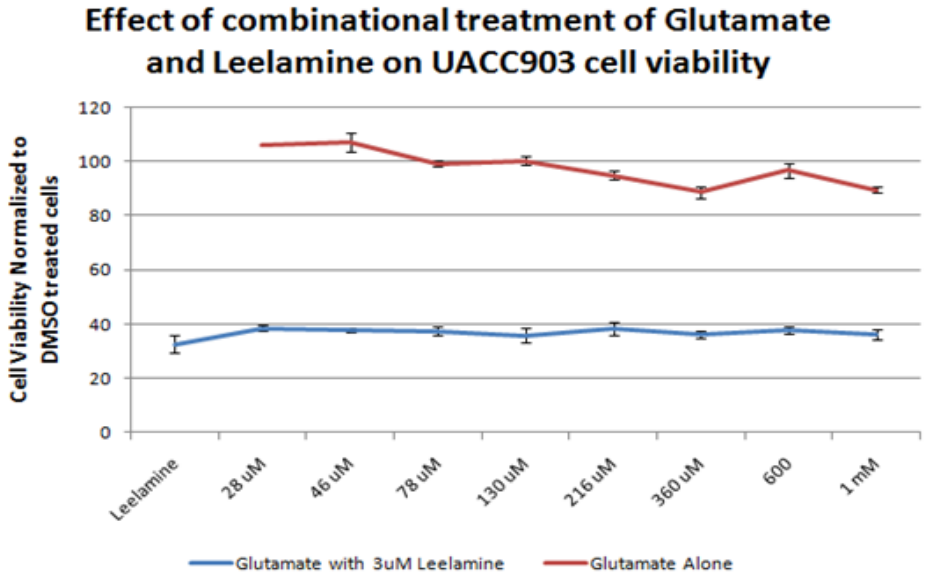
Similarity Rank	Compound Name	Structure	Similarity	Target	Effect
INPUT	Leelamine		100	UNKNOWN	UNKNOWN
1	C-((4aS,10aR)-7-Isopropyl-1,4a-dimethyl-1,2,3,4,4a,9,10,10a-octahydro-phenanthren-1-yl)-methylamine; hydrochloride		98.21	ALOX5	inhibition
2	1N-[12-isopropyl-2,6-dimethyltricyclo[8.4.0.02,7]tetradeca-1(14),10,12-trien-6-ylmethyl]-1,4-pentanediamine		82.09	ALOX5	inhibition
3	9,9-Dimethyl-1,3,4,9,10,10a-hexahydro-2H-phenanthren-4a-ylamine		76.19	NMDA receptor subunits (NR2D / NR3A / NR3B / NR1 / NR2A /NR2B / NR2C)	inhibition

Chemical structure based target analyses: Structure of the Leelamine is uploaded to the GenGo's MetaDrug tool. Analyses run with default options including similar compound search. The first three compounds according to similarity rank is shown in the table.

Glutamate receptors are not involved in leelamine mechanism of action

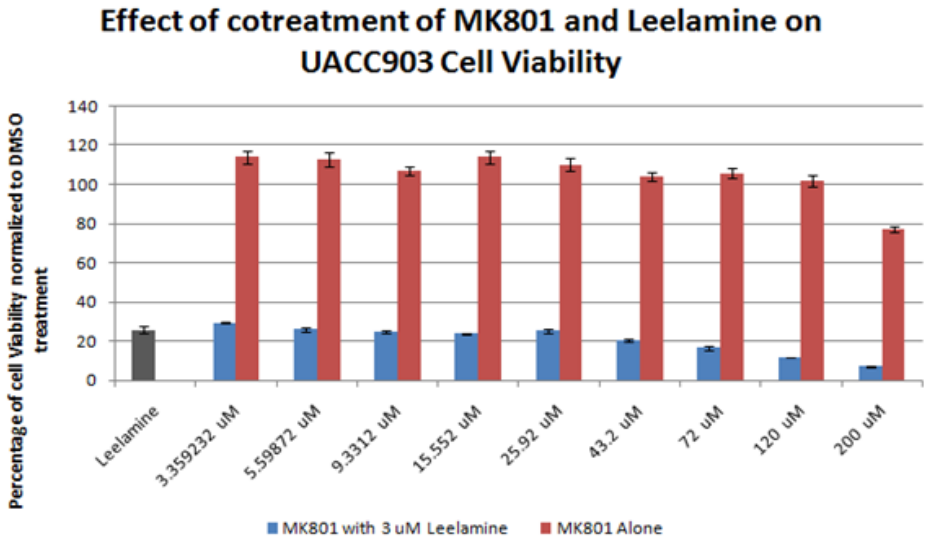
NMDA receptors are glutamate receptors and regulate cell viability through activation of pro-survival kinases including PI3K/Akt and Erk [191]. In a recent study it was revealed that the NMDAR subunit GRIN2A is mutated in more than 33% of the melanoma cases [133]. Moreover, metabotropic glutamate receptor subunit GRM3 is also mutated in 16% of the melanoma cell lines [192]. Thus, it is thought that glutamate receptor signaling might be important in melanoma cell survival. This idea is supported by the finding that ectopic expression of mGlu1 was sufficient to induce spontaneous melanoma development in vivo [193]. Because of these facts we concentrated on the glutamate receptor pathway. However, treatment of cells with up to 1mM glutamate did not inhibit the anti-proliferative activity of leelamine on tumor cells (Figure 24). Moreover, neither of the two NMDA receptor antagonists, dextromethorphan hydrobromide or MK-801, was able to inhibit cell proliferation of UACC 903 cells significantly up to 50uM concentrations (Figure 25, 26).

Figure 24 : Glutamate – Leelamine Cotreatment



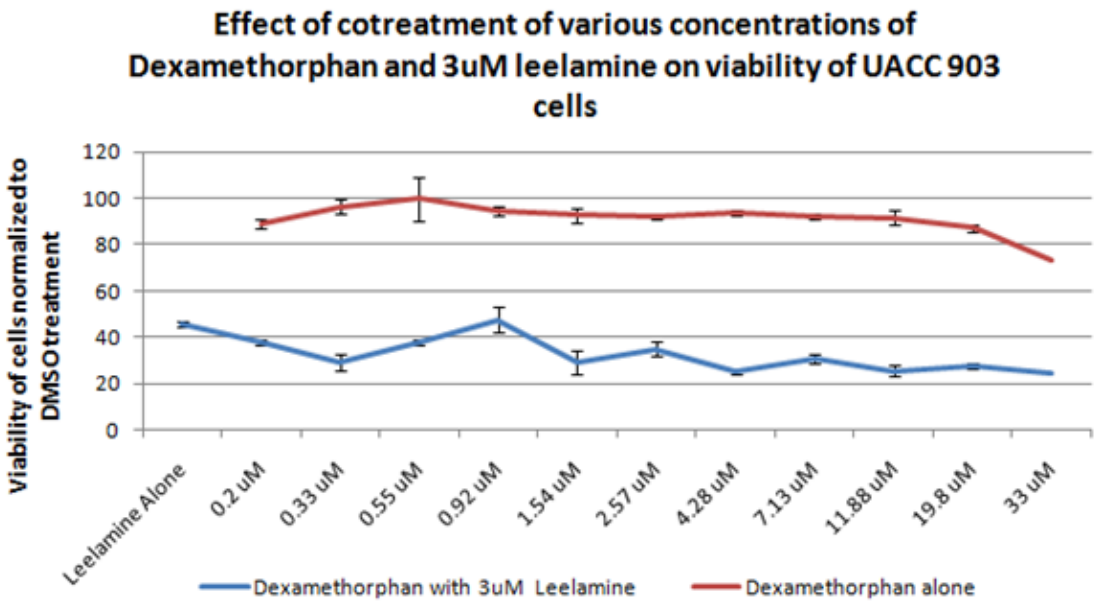
NMDA Receptor ligand, glutamate was not able to protect cells from leelamine induced cell death: 5 X 10^4 UACC903 cells were seeded in a 96-well assay plate, 48 hr later cells were treated with various concentrations of Glutamate either alone or combination with 3uM leelamine. 24 hr after treatment cell viability measured using MTS assay. The data shows that, Leelamine is not an antagonist of NMDA Receptors.

Figure 25: MK801 – Leelamine Cotreatment



NMDA Receptor antagonist, MK801 was not able to protect cells from leelamine induced cell death: 5 X 10^4 UACC903 cells were seeded in a 96-well assay plate, 48 hr later cells were treated with various concentrations of MK801 either alone or combination with 3uM leelamine. 24 hr after treatment cell viability measured using MTS assay. The data shows that, Leelamine is not an agonist of NMDA Receptors.

Figure 26: Dexamethorphan – Leelamine Cotreatment



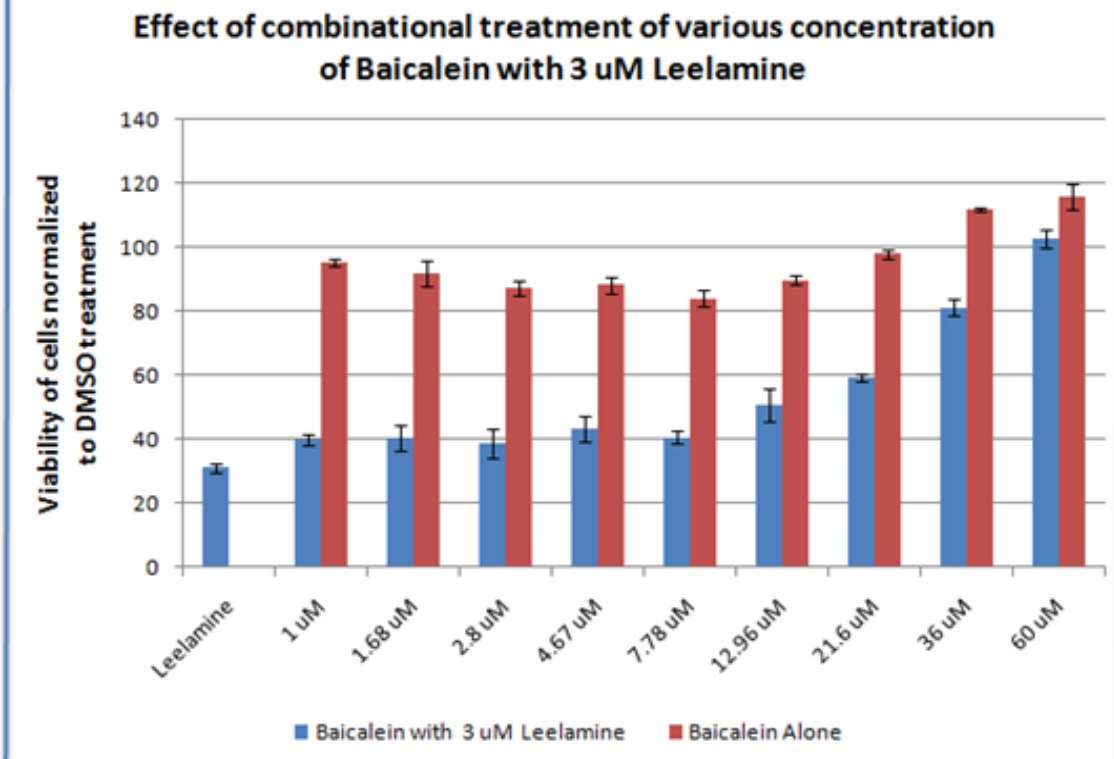
NMDA Receptor antagonist, dexamethorphan was not able to protect cells from leelamine induced cell death: 5 X 10^4 UACC903 cells were seeded in a 96-well assay plate, 48 hr later cells were treated with various concentrations of dexamethorphan either alone or combination with 3uM leelamine. 24 hr after treatment cell viability measured using MTS assay. The data shows that, Leelamine is not an agonist of NMDA Receptors.

Baicalein, an inhibitor of lipoxin pathway suppresses activity of leelamine

The ALOX5 gene is a member of the lipoxygenase family of genes and encodes for an enzyme that synthesize leukotriene A4 (LTA4) from arachidonic acid [194, 195]. Leukotrienes are fatty signaling molecules that are important mediators of inflammatory and allergic conditions. The human genome encodes for six lipoxygenase enzymes that have diverse roles in cell apoptosis, proliferation, angiogenesis and differentiation[195]. These enzymes are implicated in several human diseases including asthma, atherosclerosis and cancer [195]. To identify whether the mechanism of action of leelamine involves the ALOX5 enzyme, I co-treated UACC903 cells with leelamine and ALOX5 inhibitor baicalein. 40 to 60uM baicalein completely inhibited apoptotic activity

of 3uM leelamine (Figure 27). The effect of baicalein on activity of leelamine was concentration dependent. Even though there were several reports about the anti-proliferative activity of baicalein, it was not toxic for UACC903 cells in this concentration range (Figure 27).

Figure 27: Baicalein-Leelamine Cotreatment



Baicalein protects cells from Leelamine induced cell death: 5 X 10^4 UACC903 cells were seeded in a 96-well assay plate, 48 hr later cells were treated with various concentrations of Baicalein either alone or combination with 3uM leelamine. 24 hr after treatment cell viability measured using MTS assay. The data shows that, Baicalein protects UACC903 cells from leelamine induced cell death in a concentration dependent manner.

Even though Baicalein completely inhibits the anti-proliferative effect of leelamine, the mechanism of action was not clear. Baicalein is a well-known inhibitor of lipoxygenases and it inhibits ALOX12, ALOX15 and ALOX5 with IC50 values of 0.64µM, 1.6 µM and 9.5µM, respectively [196]. However, the inhibitory activity of

baicalein is not limited to lipoxygenases. It also inhibits lipid peroxidation, prolyl hydroxylase (PHD2), COX1/2(contradictory reports exist [197]), xanthine oxidase (XAO), IL1-Beta and TNF alpha expression [198-202]. It is also reported that it activates Erk signaling in B16F10 murine melanoma cells [203]. Thus, it is required to illuminate how baicalein inhibits activity of leelamine.

Discussion

Identification of drug targets requires extensive work, which could take even years. Even today, targets of the many well-known drugs are not known[204]. Moreover, most of the compounds are not specific for single target and the response is likely to be due to the combination of the multiple interactions [204]. Even though there is an enormous advance in high throughput technologies, the over-complexity of the cellular networks and the interaction of substances with multiple proteins complicate the identification of drug targets.

This study aimed to identify the molecular target of leelamine compound. Even though we were not able to identify a single target for leelamine, we illuminated the pathways that are regulated up on leelamine treatment. Our study showed that leelamine is able to inhibit several melanoma-driving pathways including PI3K-Akt, MAPK-Erk, Stat3 and Jnk. However, further study is required to elucidate which of these pathways are most critical for the anti-proliferative or pro-apoptotic effect of leelamine. We have to note that; some of the mentioned deregulations may be specific to UACC903 cell line. Therefore, it would be useful to investigate regulation of these pathways in other melanoma cell lines upon leelamine treatment.

The result of the present study suggest that the primary effect of leelamine appears to be through regulation of Akt signaling pathway. Beside alterations in the activity of multiple proteins in this pathway, negative correlation between IC50 values of leelamine and Akt activity levels in various melanoma cell lines supports this idea

(unpublished data). Melanoma cell lines that have more Akt activity were more sensitive to the leelamine treatment.

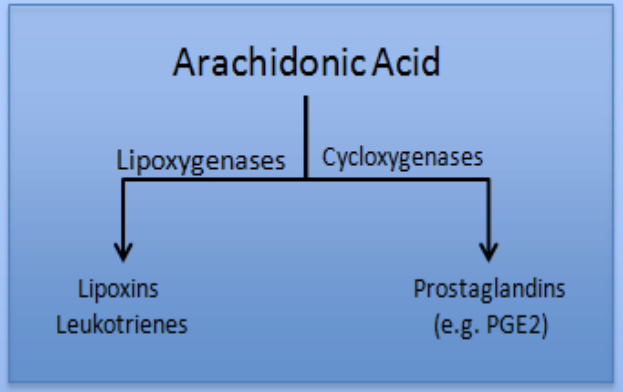
On the other hand, we were able to identify the regulation of Akt signaling pathway partially. Our study revealed that, inhibition of Akt signaling was not due to activation of two well known Akt phosphatases: PP1 and PP2A.However, there may be other phosphatases such as ph domain and leucine rich repeat protein phosphatases (PHLPP) that regulate Akt proteins. The human genome encodes for more than 500 phosphatases [205]. Thus, a genome wide phosphatases RNAi screen could be beneficial for identification of novel phosphatases that regulate Akt leading to leelamine treatment.

On the other hand, our studies identified that, ~40-50 uM lipoxygenase pathway inhibitor baicalein, successfully protected UACC903 cells from 3uM leelamine induced cell death. Even though, baicalein primarily targets lipoxygenase pathway it also regulate activity of NO synthetase, uptake of Ca^{+2} and expression of IL1B and TNF alpha [202, 206, 207]. Moreover, baicalein has been shown to attenuate oxidative stress in neuroblastoma cells [208] and cardiomyocytes [209, 210]. Thus, we propose several hypotheses for the suppression of the leelamine induced cell death. Baicalein may be inhibiting the effect of leelamine through inhibition of lipoxygenases. Wilkerson et al reported that, derivatives of leelamine inhibits the activity of the cytosolic phospholipase A2 (cPLA2) protein that catalyzes the conversion of phospholipids to arachidonic acid and lysophospholipids [172]. Unfortunately, even though they synthesized several

derivatives of leelamine to investigate their activity on lipoxygenases and cPLA2, they did not report the activity of parental compound on these enzymes.

Arachidonic acid is metabolized by either lipoxygenases to lipoxins or leukotrienes; or by cyclooxygenases to prostaglandis [211]. Lipoxins are anti-inflammatory mediators and promote the resolution phase of inflammation. Lipoxin A4 is reported to inhibit PDGFR, ILK, PI3K-Akt and VEGF pathways [212-214]. Leukotrienes act on G-protein coupled receptors (GPCR) to nourish inflammatory reactions [215]. On the other hand prostaglandins, the products of cyclooxygenases, also regulate a subfamily of GPCRs to modulate cell growth, proliferation, apoptosis, angiogenesis and migration [216, 217]. Especially, COX2 derived prostaglandin E2 (PGE2) promotes several type of malignancies including melanoma [217]. Moreover, several groups reported increased expression of Cox-2 protein in 90 to 95% of the melanoma cases compared to controls [218-220]. Thus, in theory it may be that possible inhibition of cPLA2 by leelamine will decrease the available arachidonic acid for the production of PGE2 by COX-2 enzyme, which in turn will lead to inhibition of proliferative pathways. However, baicalein may shut down lipoxygenase pathway, which would lead to an increased amount of arachidonic acid available for cyclooxygenases pathway. Indeed, Cianchi et al showed that MK886 (a 5-lipoxygenase-activating protein (FLAP) inhibitor) mediated inhibition of LOX-5 (5-lipoxygenase) pathway induced PGE2 production 300% from the basal levels [221]. However, inhibition of cycloxygenase pathway by celecoxib (COX-2 inhibitor) doubled the cysteinyl leukotrienes (cysLTs) levels (Figure 28). Moreover, treatment of

Figure 28: Arachidonic Acid Metabolism



Available arachidonic acid determines the activity of downstream signaling: Arachidonic acid is metabolized by both lipoxygenases and cycloxygenases. Inhibition of either pathway induces the activity of other one; possibly due to increased amounts of available arachidonic acid for the second pathway. In this figure, it is showed that treatment of cells with lipoxygenase 5 inhibitor MK866 increases the PGE2 production which indicates increased activity of cycloxygenase pathway. On the other hand, inhibition of cycloxygenase pathway with the treatment of COX-2 inhibitor celecoxib, induces production of cysteinyl leukotrienes which indicates induction of lipoxygeanse pathway.

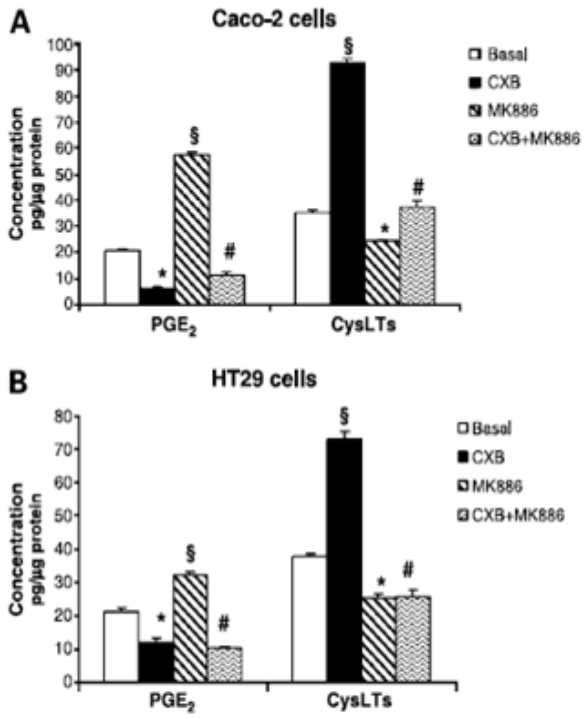
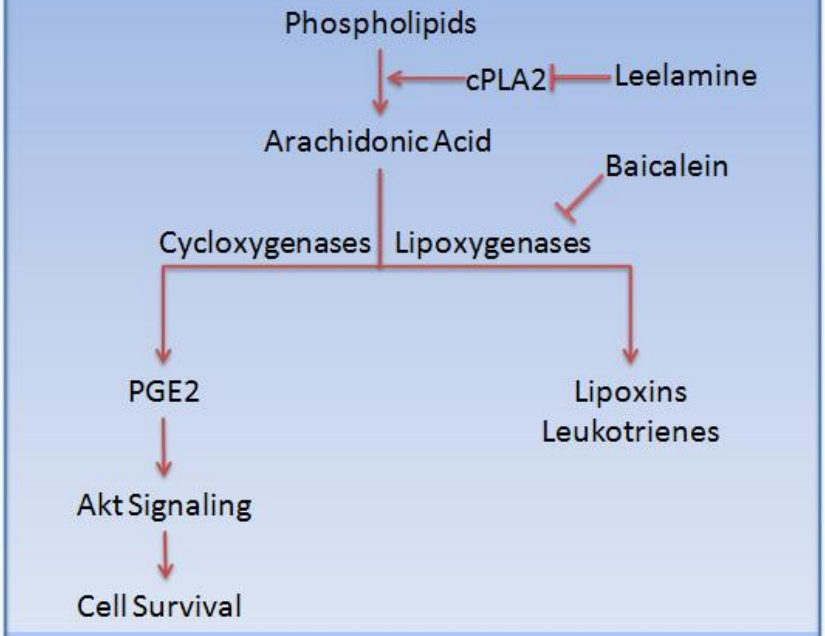


Figure 2. **A** and **B**, variations in PGE_2 and CysLT concentration after treatment with 10 µmol/L celecoxib (*CXB*), MK886, or celecoxib + MK886 in the Caco-2 and HT29 cells, respectively. *Columns*, mean of five determinations; *bars*, SE. *, significant decrease compared with basal condition ($P < 0.05$); §, significant increase compared with basal condition ($P < 0.05$); #, significant decrease compared with celecoxib or MK886 treatment ($P < 0.05$).

Cianchi, F., et al., *Inhibition of 5-lipoxygenase by MK886 augments the antitumor activity of celecoxib in human colon cancer cells.* Mol Cancer Ther, 2006. **5**(11): p. 2716-26.

UACC903 cells with the MK886 showed ~29 % induction (in the range of 1.7 to 20 uM) in cell proliferation, possibly through induction of COX-2 pathway (data not shown); above 20 uM MK886 was toxic for UACC903 cells with an IC50 value of ~80uM. Thus, in summary, leelamine may be targeting cPLA2 protein and decreasing arachidonic acid levels which in turn lead to decrease in the UACC903 proliferation in parallel to decrease in Cox 2-PDGFR- Akt pathway (Figure 29). Alternatively, inhibition of lipoxygenase pathways by baicalein might be leading an increase in available arachidonic acid levels for Cox-2 that sequentially leads to restoration of the leelamine repressed anti-apoptotic and survival pathways. It is possible to test this hypothesis by treating cells with arachidonic acid in combination with leelamine. If the hypothesis is valid, arachidonic acid treatment must reverse the leelamine induced cell death.

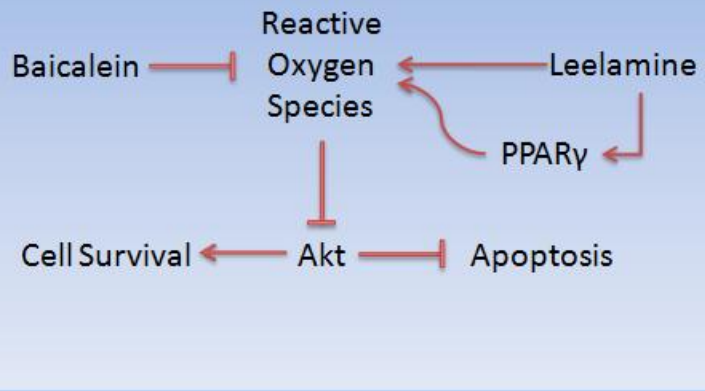
Figure 29: Hypothesis 1



Leelamine might be decreasing arachidonic acid levels: It was reported that derivatives of leelamine inhibits cPLA2 protein. If leelamine is also inhibiting cPLA2 protein this might be leading inhibition of cell survival through decreasing arachidonic acid levels and PGE2 levels. In this case, baicalein might be protecting cells from leelamine induced cell death through inhibiting lipoxygenase pathway and increasing available arachidonic acid levels for cycloxygenase pathway.

An alternative to our first hypothesis is that: leelamine may be inducing cell death through reactive oxygen species (ROS) mediated apoptosis. Athanasiou et al reported that cannabinoid agonists, the phyto-cannabinoid Delta-9-tetrahydrocannabinol (THC) and a synthetic cannabinoid HU 210 increased hydrogen peroxide (H_2O_2) production in H460 cells [222]. As was suggested by S E O'Sullivan, the increase in H_2O_2 upon cannabinoid agonist treatment can be attributed to the direct activation of peroxisome proliferator-activated receptor gamma (PPARγ) by cannabinoid agonists. [223]. Beside cannabinoid agonists, Akt protein itself also increases intracellular ROS levels by stimulating oxidative pathways. It phosphorylates and represses the activity of FoxO type transcription factors, which regulate expression of genes encoding antioxidant enzymes [224]. Thus, cells that have increased activity in Akt pathway are more sensitive to the ROS mediated apoptosis [224].

Figure 30: Hypothesis 2



Leelamine might be increasing reactive oxygen species(ROS): It was reported that cannabinoid agonist may trigger production of reactive oxygen species independently from cannabinoid receptor activation. Leelamine does not stimulate cannabinoid receptor activity, but might be inducing ROS production either through PPRγ or some unknown mechanism. This may lead to induction of apoptosis through inhibition of Akt signaling. This hypotheses also explains the fact that melanoma cells are more sensitive to leelamine compared to melanocytes. On the other hand, baicalein might be protecting cells from leelamine induced cell death through inhibiting ROS production.

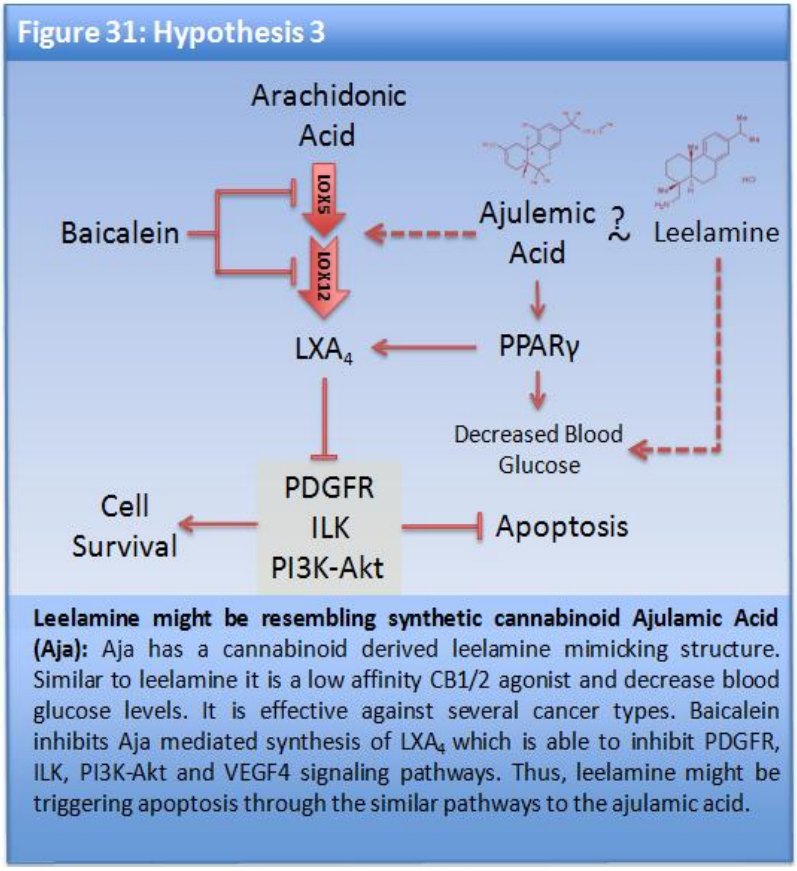
Interestingly, ROS mediated apoptosis involves inhibition of Akt signaling pathway. But this inhibition is not related to upstream molecules of Akt3 and depends on ROS mediated conformational change in Akt protein [225]. Yang and colleagues showed, ROS mediated conformational change in the Akt protein disturbs its binding partners leading to its dephosphorylation [225].

Several groups have reported that baicalein behaves as an antioxidant and suppresses ROS induced apoptosis [209, 226-228]. Moreover, it was also reported that baicalein inhibits PPARγ expression and nuclear translocation [229]. As it is illustrated in Figure 30, leelamine could be inducing cellular apoptosis through production of reactive oxygen species. Increased activity of Akt signaling pathway in melanoma cells might be increasing their sensitivity to the leelamine induced ROS dependent apoptosis. In this case, baicalein mediated suppression of ROS production (either through inhibition of PPARγ or through other intermediates), and might protect cells from leelamine induced cell death. It may be possible to test this hypothesis through combinational treatment of leelamine with antioxidants or PPARγ inhibitors.

A 3rd alternative hypothesis is that, leelamine could be inhibiting cell growth through LXA4 (lipoxin A_4)-PDGFR-Akt signaling pathway. Ajulemic acid (AJA), which has a cannabinoid derived and leelamine mimicking structure, reported to show modest binding to the cannabinoid receptors CB1 and CB2 but does not show psychotropic activity [230]. Several reports suggested that AJA is highly effective in inhibition of several cancer types [231]. Moreover, Robert et al showed that in vivo administration of

AJA resulted in 7-fold increase in LXA_4. However, blockade of 12/15 LOX by baicalein completely inhibited AJA mediated LXA4 synthesis. As it was discussed above LXA4 is reported to inhibit PDGFR, ILK, PI3K-Akt and VEGF4 signaling pathways [213].

Interestingly, it was shown that AJA directly interacts and activates PPARγ receptors [232]. Synthetic ligands of PPARγ, such as thiazolidinediones (TZDs), are used for the treatment of diabetes, hypertension and obesity [233-235]. As discussed above, it was reported that oral administration leelamine decreased the blood glucose levels significantly. Thus leelamine mimics action of thiazolidinediones as an anti-diabetic effect, which may indicate possible regulation of PPAR γ receptors by leelamine.



In summary, as depicted in Figure 31, leelamine resembles ajulamic in several ways. Therefore it might also show similar anti-proliferative mechanism to ajulamic acid. It might be inducing lipoxin A_4 secretion and repressing Akt signaling through LXA_4-

PDGFR-Akt pathway, and this repression might be inhibited by baicalein through blockade of LXA_4 synthesis with the inhibition of ALOX12/15 enzymes.

Chapter 4

Conclusion & Future Directions

Introduction

Although there has been an enormous amount of advancement in pharmacology, chemistry and biological sciences, drug target discovery still remains to be challenging due to the fact that it is unknown if a certain chemical-target interaction is indeed the one that triggers the therapeutic outcome. For many drugs it is suspected that they interact with multiple proteins and show their effect through altering multiple biological pathways[236]. Besides alterations in these pathways, regulatory feedback networks make puzzle incredibly complex. The solution of this puzzle requires a multidisciplinary approach, which at least integrates molecular biology, proteomics, pharmacology, mathematics and computer sciences.

In the present study, although we have identified several molecular alterations in melanoma cells as a response to the leelamine molecule, we were not able to show a direct interaction between a protein and leelamine. Further investigation is required for the identification of actual molecular target(s) of leelamine. We aimed to integrate high throughput proteomic approaches with systems biology tools as an approach to identify drug target(s). We believe that future progress in high-throughput proteomics would rapid up identification of drug target networks with the support of systems biology. In this last chapter, we will elaborate future directions for proteomics, systems biology and melanoma therapeutics.

Future Directions on Proteomics & Systems biology

Proteins are the major functional molecules of cells. Understanding the individual function of each protein and revealing their regulatory circuits is a vital necessity for understanding biological activity. According to current knowledge, the human genome encodes for 20,500 genes [237]. However, the proteome content of a human cell is much richer then genomic content due to alternative splicing mechanism. It is estimated that a human cell contains 50,000 to 500,000 different proteins[238]. Because of this complexity, we are not able to determine individual levels of all proteins with a high throughput approach with current technology. However, several collaborative projects can be established to move proteomics forward.

In determination of the protein expression inside the cell, many proteomic studies rely on antibodies. Several biotechnology companies are developing their own antibody products. However, there is a striking difference in antibody quality, resulting in considerable difference in experiment results. Egelhofer et al tested more than 200 different antibodies raised against 57 different histone modifications and more than a quarter of these failed to pass a validation test [239]. However, high quality antibodies are essential, especially for the reliability of protein arrays and reverse phase protein microarrays (RPMA). Thus, the establishment of government supported projects for production and validation of high quality antibodies is required.

Protein arrays are rapidly advancing as a powerful tool for the global analyses of protein expression levels. Many proteomics companies have established their own system and are producing their own protein arrays. However, there is no common language in terms of reporting and exchanging produced data. Every company has its own data format for expressing protein array data. It is urgently required to establish a standard data type for reporting protein array data. This was also a problem for cDNA microarrays until the establishment of a standard which is called minimum information about a microarray experiment (MIAME) by Brazma et al [240]. Establishment of standard data type for protein arrays would pave the way for writing better tools for analyzing the array data.

At least in theory, when compared to cDNA microarray experiments, protein arrays would yield much more information about the molecular events inside the cell. For instance, it is impossible to know whether a protein is active or inactive according to the mRNA expression level. However, protein arrays are able to measure post-translationally modified levels of a protein which gives quantifiable data for the activity of that particular protein. However, as mentioned earlier in Chapter 2, currently there is no available software specific for analyzing protein array data. We have an enormous amount of information in the literature in terms of protein-protein interactions. For instance, we know of the interaction of kinases, phosphatases, ubiquitin ligases with their substrate proteins. Thus, development of softwares which are capable of analyzing protein array data by considering all of these interactions has significant importance.

Interaction databases are the most critical parts of network analysis tools. However, an enormous amount of published data is needed to be organized into a human curated database. A collaborative project, which would organize published literature according to detailed information such as cell line, experimental method, interaction type (such as protein-protein, protein-DNA, protein-RNA, small molecule-protein, kinase-substrate, phosphatase-substrate e.g.) or western results would lead to the development of much better network analyses tools.

Lastly, our current knowledge for hundreds of genes is very limited. In fact, there is no published study about the function of many genes other than their identification. Therefore, a project which would aim to reveal the function of all genes would move molecular biology one step forward.

Future Directions on Melanoma Therapeutics

Complete identification of molecular mechanisms of melanoma is required. Even though there is an enormous amount of work going on in melanoma biology, not all of the molecular mechanisms are solved. Current melanoma therapeutics is based on the especially mutated B-Raf. In the phase I clinical trials of mutant specific B-Raf inhibitor PLX-4032, 80% of the patients showed partial to complete tumor regression [159]. However, the regression only lasted from 2 to 18 months and the tumor developed drug resistance. Moreover, this gene is also mutated in 80% of the benign nevi [112-114].

Both of these facts development of drug resistance and mutations in benign nevi indicates a requirement of supporting alterations for melanoma development.

In a recent study, a novel common mutation was identified in the GRIN2A gene, which was not previously reported to be involved in melanoma [133]. This study was performed by a whole genome exon sequencing of 14 melanoma patients. Exons are protein-coding sequences and cover only a very small portion of the DNA. This discovery clearly demonstrates that, in the near future, new disease driving mutations and pathways could be discovered in parallel to advances in genome sequencing.

Determination of functional significance of each mutation would be an important step in identification of melanoma cell behavior. As it was mentioned in Chapter 2 of this thesis, genetically engineered mouse models have already produced significant information about the functional contribution of several genes to melanoma development. However, further research is required especially on combinational mutations of these genes. Alternatively, analyses of gene expression and profiling the proteome content of a melanocyte after introducing mutations step-by-step would be helpful for determining the contribution of each mutation to melanoma development. This information would also be supportive for transitioning from disease-based medicine to person-based medicine. In this way, patients would be treated with proper drug combinations according to their genetic background.

Lastly, ***an establishment of melanoma specific database*** is required. Although there are few melanoma specific databases such as the melanoma molecular map project (MMMP) database, they are not able to cover most of the information in the literature[241]. A melanoma database which includes detailed information on melanoma cell lines, their mutational status, gene expression data, and proteomics data would help us understand melanoma biology better.

MATERIALS and METHODS

MATERIALS & METHODS

Cell lines and culture conditions

1205 Lu and UACC 903 were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Hyclone, Logan, UT) in a 5% CO2 atmosphere humidified 37C^{o} incubator.

Identification of Leelamine from natural compound library

Natural product library NPL- 480 (TimTec Inc., Newark, DE) consists of 480 compounds derived from plants, animal, bacteria and fungus was secreed to identify compound with potential to inhibit melanoma cell survival. Compounds that were supplied as powders or oils were dissolved in DMSO to a stock concentration of 10 mM and stored at –20°C. DMSO concentrations in the reaction mixture were controlled to not exceed 0.5% (vol/vol). 5 X 10^3 UACC 903 melanoma cells were plated in 96 well plates for 24 h followed by treatment with each compound at 5 µmol/L. Viability was measured using the MTS assay. Primary screen identified 12 compounds decreasing melanoma cells viability by ~50%.

Cell viability analysis

Viability and IC50 of melanoma cells following treatment with leelamine were measured using the MTS assay (Promega, Madison, WI) (Ref). 5X10^3 cells per well in 100 µL of media were plated and grown in a 96-well plate for 48 hours and treated with

either DMSO vehicle control or drug for 24 hours. IC50 values are calculated in μmol/L for respective cell lines were measured from three independent experiments using GraphPad Prism version 4.01[130] (GraphPad Software, La Jolla, CA).

RTK array analyses

At approximately 70% cell confluence, protein extracts of UACC903 cells, either from 6hr 4uM Leelamine treated or from DMSO treated plates was prepared accordance with the manufacturer's instructions. The extracts were applied to a Human Phospho-RTK Array (R&D Systems, MN, USA), which can detect the phosphorylation level of 42 different RTKs on the same nitrocelullose membrane. Assays were performed in accordance with the manufacturer's instructions. Images of the array is scanned with Epson Perfection 1660 photo scanner and analysed with ImageJ tool.

Kinexus Antibody Microarray Analysis

A Kinexus Antibody Microarray was used to identify the pathways targeted by leelamine. In brief, UACC903 cells were treated with 3 μmol/L Nanolipolee-007 for 3-24 hours. Lysates are collected and sent to Kinexus for analyses. Results were analyzed using the Ingenuity Pathway Analysis (IPA) software.

Ingenuity Pathway Analyses

Significantly up-regulated or down-regulated proteins (Z Score >1.5) with corresponding Swiss-Prot accession numbers and ratio changes were uploaded as an

Excel spreadsheet file to the Ingenuity Pathway Analysis server. Core analyses run either with default settings or with filtering interactions specific to melanoma cell lines.

Western blot analysis

Cell lysates were harvested by addition of RIPA lysis buffers containing 25 mM Tris•HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 10 mM EDTA, 1 mM sodium orthovanadate, 0.1mM sodium molybdate, 1mM phenylmethylsulfonyl fluoride, 20 μg/mL aprotinin, and 5 μg/mL leupeptin. Treatment conditions: 1-1.5 X 10^6 melanoma cells were plated in 100 mm culture dishes, 48 h later, treated with leelamine (3-5μmol/L) for 3 to 24 h. Protein lysates collected for Western blotting. The blots were probed with antibodies according to each supplier's recommendations: antibodies to total Akt, phospho-Akt (Ser473), total PRAS40, phospho-PRAS40 (Thr246), total CREB, phospho-CREB (Ser133), phospho-p70 S6 kinase (Thr389), total Erk1/2, phospho-Erk1/2 (Thr202/Tyr 204), total CDK2, phospho-CDK2 (Thr160), phospho-Rb (Ser807/811), total Stat, phospho-Stat1(Tyr701), Phospho-Stat-2(Tyr690), Phospho-Stat3(Ser727), phospho-Stat3(Tyr705) and cleaved PARP from Cell Signaling Technology (Danvers, MA); total PRAS40 from Invitrogen (Carlsbad, CA); cyclin D1, Bcl-2, α-enolase and secondary antibodies conjugated with horseradish peroxidase from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoblots were developed using the enhanced chemiluminescence (ECL) detection system (Thermo Fisher Scientific, Rockford, IL). Intensity of protein bands was quantified using ImageJ software.

Tumorigenicity assessments.

Animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at Penn State University. Tumor kinetics were measured by subcutaneous injection of 1.0×10^6 UACC 903 or 1205 Lu cells in 0.2 mL of DMEM supplemented with 10% FBS subcutaneously injected above both left and right rib cages of 3- to 4-wk-old female Athymic Nude-Foxn1nu mice (Harlan Sprague Dawley). Six days later, when a fully vascularized tumor (50-75 mm3) had formed, mice were randomly divided into DMSO vehicle control and experimental groups (5 mice/group; 2 tumors/mouse) and treated intra peritoneally with 2.5 mg/kg body weight leelamine daily for 3 - 4 weeks. Body weight, measured in grams, and dimensions of the developing tumors (mm3) were measured at the time of drug treatment.

SiRNA Transfections

100 pmol siRNA was introduced into 1.0×10^6 UACC 903 cells via nucleofection by using an Amaxa Nucleofector (Koeln, Germany) using Solution R/program K-17 . Transfection efficiency was >95% with 80% to 90% cell viability. After siRNA introduction into cells, cells were allowed to recover for 2 days and then plated in to 96-well plates. Five days later, cell viability was measured by using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphe-nyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (CellTiter 96 AQueous Cell Proliferation Assay; Promega, Madison, WI). Stealth siRNA (Invitrogen) were used for these studies:

siPDGFR #1: GAGAGCAUCUUCAACAGCCUCUACA

siPDGFR #2: CGGAAGCAGAGGAUAGCUUCCUGUA

siPDGFR#3: CCCUCAUGUGCAUUGUGAUCGGGAA

siCB1 #1: GGGCUCAAAUGACAUUCAGUACGAA

siCB1 #2: GAAGUCGAUCCUAGAUGGCCUUGCA

siCB1 #3: GCCCUCUGCUUGCAAUCAUGGUGUA

siCB2 #1: CCUGCGCUAUCCACCUUCCUACAAA

siCB2 #2: CCGGAAUCAUCUACACCUAUGGGCA

siCB2 #3: UCCAUGCUGUGCCUCAUCAACUCCA

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