Design and development of drugs targeting molecular structures of genes and proteins

A thesis paper submitted to the Department of Pharmacy, East West University, in the partial fulfillment of the requirements for the award of the degree of Master of Pharmacy

Submitted by:

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THIS THESIS PAPER

IS DEDICATED

TO MY BELOVED FAMILY...

DECLARATION BY THE RESEARCH CANDIDATE

I, Tajrina Ahmed, hereby declare that this dissertation, entitled **"Design and development of drugs targeting molecular structures of genes and proteins"** submitted to the Department of Pharmacy, East West University, in the partial fulfillment of the requirement for the degree of Master of Pharmacy, is a genuine & authentic research work carried out by me under the guidance of Dr. Repon Kumer Saha, Assistant Professor, Department of Pharmacy, East West University, Dhaka. The contents of this dissertation, in full or in parts, have not been submitted to any other Institute or University for the award of any Degree or Diploma of Fellowship.

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CERTIFICATION BY THE SUPERVISOR

This is to certify that the dissertion, entitled **"Design and development of drugs targeting molecular structures of genes and proteins"** is a bonafide research work done, under our guidance and supervision by Tajrina Ahmed (ID: 2014-3-79-013), in the partial fulfillment of the requirement for the degree of Master of Pharmacy.

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ENDORSEMENT BY THE CHAIRPERSON

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AKt	Protein Kinase B
EGFR	Epidermal Growth Factor Receptor
ErK	Extracellular signal Regulated Kinases
HER2	Human Epidermal Receptor 2
HER2 ECD	Human Epidermal Receptor 2 Extracellular Domain
JAK	Janus Kinase
MAPK	Mitogen Activated Protein Kinase
mTOR	Mechanistic Target of Rapamycin
PI3K	Phosphoinositide-3-kinase
STAT	Signal Transducer and Activator Transcription

Abstract

Genomics and proteomics are the two popular and successful approaches in the field of drug discovery and development. Some new genomic technologies have been developed for the identification of drug target and lead compound optimization over the last two decades. Genomics is the study of genome that stores the information in a cell to predict what can be occurred. This approach identifies new gene targets for drug discovery and also finds associations between specific genetic markers and drug response in a patient. And proteomics is the immature, complex, labor intensive and flourishing technology for drug target identification and drug efficacy and toxicity determination. This is the scientific discipline which studies proteins and searches for proteins that are associated with a disease by means of their altered levels of expression and/or post-translational modification between control and disease states. It describes the global analysis of protein expression and function. Proteomics involves some methodologies including the two-dimensional gel electrophoresis, liquid chromatography, mass spectrometry, isotope-coded affinity tag and protein biochips.

Key Words: Genomics, proteomics, mass-spectrometry, biomarkers, isotope-coded affinity tag

CHAPTER 1

INTRODUCTION

1.1 Genomics

Genomics, especially high-throughput sequencing and characterization of human genes expression. This approach has some methodologies named gene expression, gene sequencing, statistical genetics and analysis in clinical development.

Pharmacogenomics:

Pharmacogenomics has come from pharmacogenetics. Pharmacogenetics is the study of the linkage between an individual's genotype and that individual's ability to metabolize a foreign (Neha and Harikumar, 2013). The potential implication of genomics and pharmacogenomics in clinical research and clinical medicine is that disease could be treated according to genetic and specific individual markers, selecting medications and dosages that are optimized for individual patients. The possibility of defining patient populations genetically may improve outcomes by predicting individual responses to drugs, safety and efficacy in therapeutic areas such as neuropsychiatry, cardiovascular medicine, diabetes and obesity and oncology. Ethical evaluations are also established for the use of genomics in drug discovery and drug development (Emilie *et al*., 2000)

The Gene Microarrays:

Gene microarrays allow the rapid parallel analysis of the expression of thousands of genes against hundreds of tissues, cell types, and conditions whether using oligonucleotide arrays or arrays of gene fragments. Gene microarrays are being used extensively to generate broad and in-depth data on gene expression patterns in normal and diseased tissues, both in human and in animal systems. Although many effective drugs have been developed against targets that are widely expressed in the body e.g. the angiotensin converting enzyme), highly selective tissue expression of a drug target, such as that seen for cathepsin K, is attractive, as the potential for unwanted side effects may be more restricted. Perhaps the most promising application of microarrays is the study of differential expression in disease. They have a number of limitations and that can be addressed by complementary technologies. First, the sensitivity of detection is about 1 in 100,000. More sensitive but lower throughput methods such as quantitative polymerase chain reaction, must be utilized for the analysis of genes that are expressed in low abundance. Second, the labor-intensive in situ hybridization and immunocytochemistry methods will continue to make important

contributions because they provide a critical link to histology and cytology that the best microdissection of cells from tissues is unlikely to provide. Third, mRNA levels may not be paralleled at the protein level (Ratain *et al*., 1996).

1.1.1 Background of Genomics

Scientist Garrod worked on alcaptonuria in 1902 and he constituted the first proof of Mendelian genetics in humans. As a result of these studies, he developed the hypothesis that genetically determined differences in biochemical processes, cause of adverse reactions after the ingestion of drugs. Garrod's original hypothesis was introduces when it was noticed during World War II that "primaquine hemolysis" and it was much more common among African-American soldiers in the United States Army taking the antimalarial primaquine. He described that enzymes were implicated in the detoxification of foreign substances, and this mechanism may not respond in some persons for lack of the required detoxifying enzyme. This study was the pioneer for the development of biochemical genetics and biochemical pharmacology. (Mancinelli *et al*., 2000).

The term ‘genomics’ describes the discipline in genetics concerned with the study of the genomes (the coding region and the non-coding region) and determine the entire DNA sequence of organisms, genetic mapping. The definition of genomics does not include the function of single genes is a primary focus of molecular biology.

In 1970, DNA microarrays, have a common origin in the DNA blotting methods pioneered by Southern. In 1976, Molecular genetics merged with human genetics and the first human genes were cloned. In about 1986, Transgenic methods, ‘knock-outs’ and ‘knock-ins’ began. In 1990, genomics research identified new approaches for determining new drug targets (Neha and Harikumar, 2013). And in about 1996, database searching discovered and became a fruitful way to do genomic research (Nanjwade, 2008).

1.1.2 Types of genomics

Structural Genomics:

Involves using NMR spectroscopy and X-ray crystallography to combine 3- dimensional protein structure information with whole genome sequences. NMR is done on aqueous solution of proteins detects chemical shifts of atomic nuclei. These shifts depend the identities and distances

of nearby atoms and estimates distance between specific pairs of atoms called constraints. The Result is combination of models, not single structure of proteins. X-ray Crystallography determines Structure of proteins, crystallized proteins and model of protein built to fit the map.

Functional genomics:

Functional genomics aims to assign function to the genes of a genome. This can be done by comparing the genes, SNPs, proteins and metabolites of organisms in varying conditions and different stages of development.

Comparative genomics:

Comparative genomics compares the genomes of different species. It Helps to determine the function of the genes and noncoding regions of an organism. It includes bioinformatics tools like BLAST (Audenet *et al*., 2011)

Table 1: Functions of different types of genomics

Types	Functions
Structural Genomics	Involves NMR spectroscopy and X-ray crystallography to combine 3-dimensional protein structure information with whole genome sequences.
Functional genomics	comparing the genes, SNPs, proteins and metabolites of organisms.
Comparative genomics	compares the genomes of different species and determine the function of the genes and noncoding regions of an organism.

1.2 Proteomics:

The proteomics utilizes protein sequences, expression and structure and determine how proteins relate, interact and function in an organism including characterization and cataloguing proteins and protein libraries, comparision of variations in protein expression levels under different conditions, studying of protein interactions and functional roles techniques. It involves automated, bioinformatic analysis and storage of data (Michael, 2007)

1.2.1 Proteomics Pathway

Plant material →→protein extraction→→two dimensional (2D) gel electrophoresis→→image analysis→→differentially express protein selection→→spot excision→→tripsin digestion→→peptides derived→→mass spectrometry (peptide sequence) →→database processes (proteome/genome) →→protein identification

Figure 1: Describing proteomics steps (Rose *et al*., 2004)

1.2.2 Background of proteomics

Proteomics is a recent member of the 'omics' family (Michelle L et Al., 2006). Now-a-days this is the most popular technologies in drug target identification specially in the area of therapeutics. The word "proteome" was first used by the scientist Wilkins at the symposium "2D Electrophoresis: from protein maps to genomes" in Siena, Italy in 1994 and subsequently published in his PhD thesis in 1995. He used proteome to describe the entire complement of proteins expressed in a cell, tissue or organism in 1995 (Amelia, 2011). The word "proteomics" was first used in 1997 (Hong *et al*., 2006). Proteomics was defined as "the use of quantitative protein-level measurements of gene expression to characterize biological processes (e.g., disease processes and drug effects) and decipher the mechanisms of gene expression control" (Amelia, 2001). This is an emerging field of research facilitated by numerous advancements over the past 35–40 years in protein separation, mass spectrometry, genome sequencing/annotation, and protein search algorithms. The broad dynamic range of protein expression has also contributed to difficulties in efforts towards identifying every protein expressed in the life cycle of any given organism (Corthals *et al*. 2000). For example, identification of every protein expressed in plant leaves would never reveal proteins that are specifically expressed. In 2003, the successful genome project speed up the discovery of new drug targets and the experts believe that proteins are ultimately the key 'workhorses' in our body developing the drug target more rapidly (Michelle *et al*., 2006). By the help of three dimensional structure, the function of protein can be dictated which may change the interaction with another components inside the cellular tissues. Because of the great excitement surrounding proteomics, there has been a noticeable emphasis for pharmaceutical and its biotechnological and academic partners to expend tremendous resources to refine and review their strategies to make drug development more efficient and successful. In recent years, businesses

have also flocked to the thriving field of proteomics, with more than 100 companies now offering proteomics-related technologies, tools, data and services in the hope of gaining a competitive advantage and finding the best targets for successful drug candidates (Michelle *et al*., 2006).

1.2.3 Types

Profiling approach:

This is the primary approach by which target is identified and the providing information is validated. E.g. protein separation methods such as two-dimensional gel electro phoresis (2DGE) isotope coded affinity tag (ICAT) and protein identification methods such as mass spectrometry (MS) (Hong. *et al*., 2006)

Functional approach:

It is used to characterize protein activities, multiprotein expression and signaling pathways. This approach monitors and analyzes the spatial and temporal properties of the molecular networks and fluxes of living cell (Monti. *et al*., 2005)

Structural approach:

This approach determines the three dimensional (3D) structure of all proteins. The main goal is to identify the best technologies and the most efficient processes to convert gene sequence into 3D structure. X-ray crystallography is the potential workhorse for this approach to determine 3D structure within a single hour (Yee *et al*., 2001)

Table 2: Functions of different types of proteomics approaches (Yee *et al*., 2001) (Monti. *et al*., 2005)

Approaches	Functions
Profiling approach	target is identified and validated
Functional approach	characterize protein activities, multiprotein expression and signaling pathways
Structural approach	determines the three dimensional (3D) structure of all proteins

1.2.4 Methodologies

1.2.4.1 Gel-based proteomic approach for protein separation:

Two-dimensional polyacrylamide gel electrophoresis

Two-dimensional fluorescence difference gel electrophoresis (Amelia, 2011)

1.2.4.2 Gel free proteomic approach for protein identification:

Matrix-Assisted Laser Desorption Ionization-Time of Flight/Time of Flight (MALDI-TOF/TOF) (Kapoor *et al*., 2012)

Mass spectrometry-based proteomics (Amelia, 2011)

1.2.4.3 Protein structure determination:

X-ray crystallography or NMR spectroscopy

1.2.4.4 Protein-protein interaction:

Yeast two hybrid method (Hadjiargyro., 2001)

1.2.5 Challenges of proteomics:

Splice variants create an enormous diversity of proteins such as: 25,000 genes in humans give rise to 200,000 to 2,000,000 different proteins.

Splice variants may have very diverse functions. For example, calcitonin gene – Gene variant 1 encodes for calcitonin (increases Ca++ uptake in bones) – Gene variant 2 encodes for calcitonin gene-related polypeptide (causes blood vessels to dilate).

Proteins expressed in an organism will vary according to age, health, tissue, and environmental stimuli.

Proteomics requires a broader range of technologies than genomics (Hadjiargyro, 2001)

1.3 Relation between Genomics and Proteomics

Genomics and proteomics are related to each other as follows:

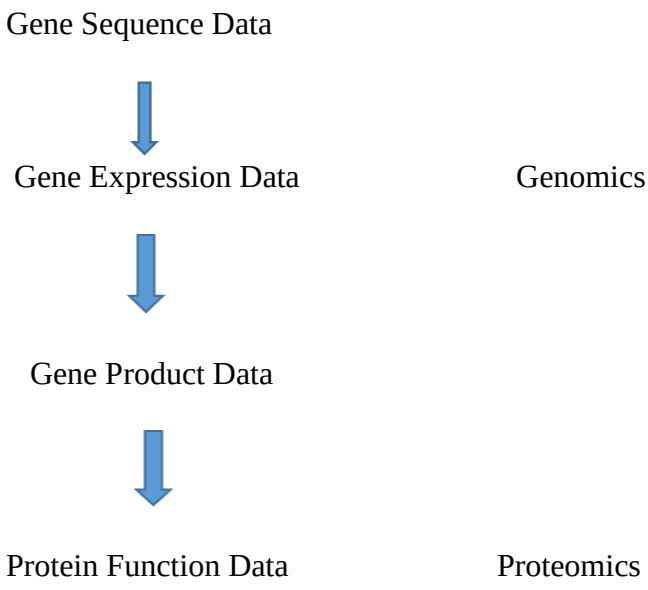

Figure 2: Genomics and Proteomics

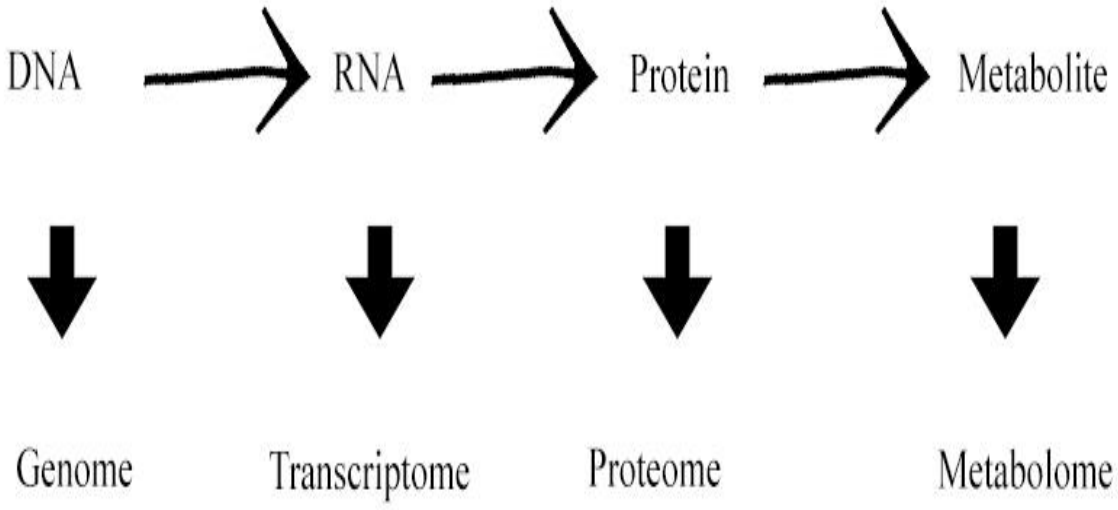

Figure 3: Origin of Genomics and Proteomics

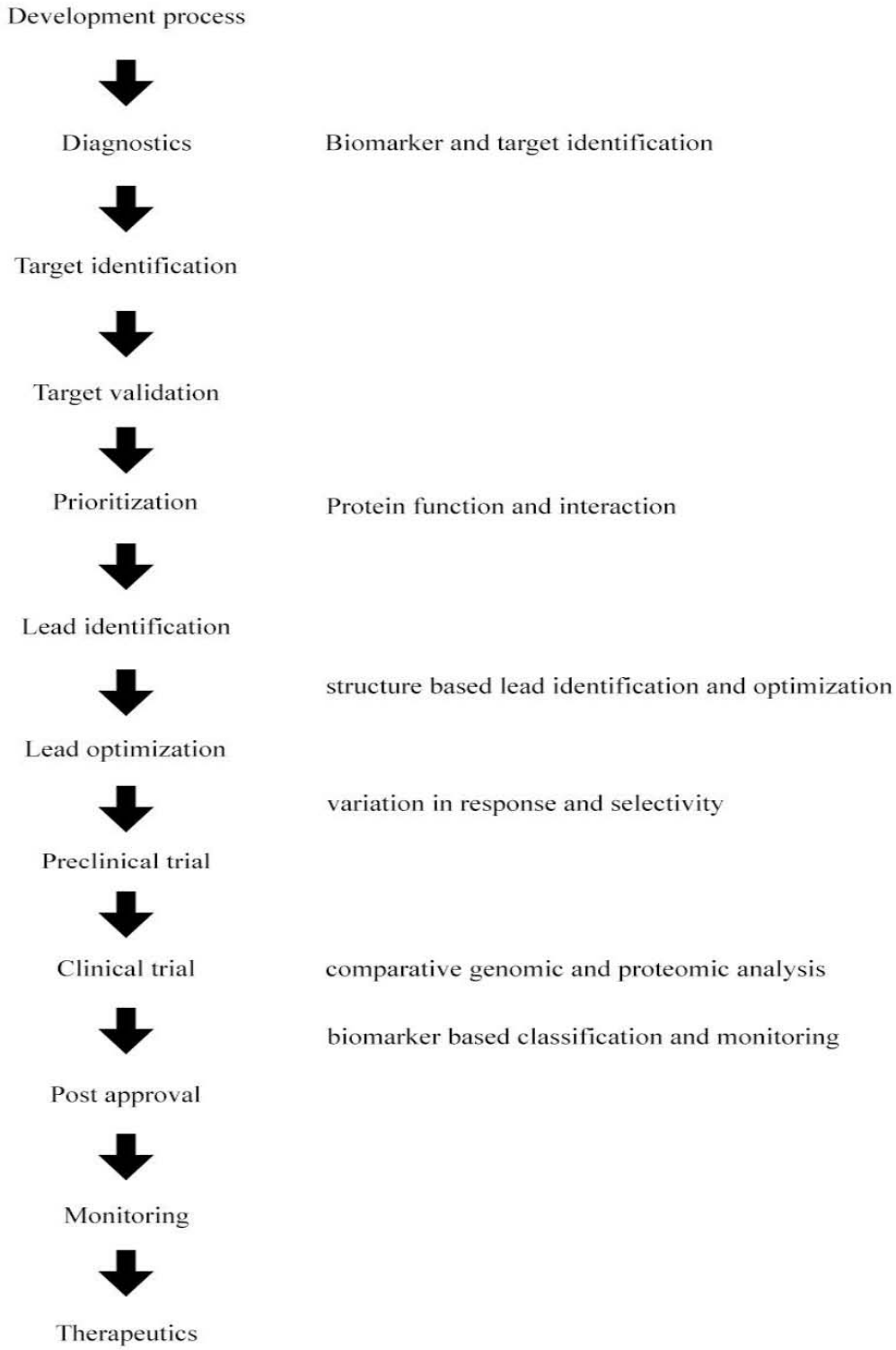

Figure 4: Role of Genomics and Proteomics in drug discovery (Hong. M *et al*., 2006)

1.4 Application of Genomics and Proteomics

In environmental research:

The application of proteomic approaches in environmental research can be divided into two groups including: study of proteomic response to stress, which provides information on the mechanism of the response and helps to understand why some organisms could resist extreme environmental stress (biomarkers of effect) and screening environmental samples by a proteomic method to monitor exposure to certain pollutants (biomarkers of exposure). Traditionally, the pollution status of aquatic or terrestrial ecosystem has been assessed by the chemical analysis of environmental samples (e.g. water, soil). However, given the large number, complexity and in some cases low toxicity thresholds of the chemicals present, chemical analysis alone is not able provide a satisfying assessment of the environmental quality of an ecosystem80. In other words, chemical analysis of environmental samples does not provide complete information regarding the health state of the ecosystem, and therefore it is important to monitor the response of biota to the pollutants as well. In a complementary approach, the number and physiological state of individual species inhabiting a given ecosystem is used as ‘‘indicators’’ of chronic chemical pollution, which lead to the development of biomarkers of toxicity (e.g. DNA fragmentation, level of acetylcholine esterase and cytochrome P450)

In terms of environmental science, biomarkers can be defined as “the measurements of body fluids, cells, or tissues that indicate in biochemical and cellular terms the presence of contaminants or the magnitude of the host response”. In that sense, a proteomic approach capable of detecting changes in the expression of multiple proteins in response to environmental stress has obvious applications in the field of ecotoxicology. While conventional biomarkers of pollution are a good tool in the assessment of environmental conditions, they require a deep knowledge of toxicity mechanisms and characterize only a number of well-known proteins, excluding those that are also altered but whose response to pollutants is currently unknown. Simultaneously, it has become evident that instead of searching for a single “ideal” biomarker, investigation of toxicological effects within ecosystems would benefit from the use of multiple biomarkers. The combined use of sets of marker proteins associated with a given pollution impact is expected to be more reliable, as they are based not only on several unique markers measured independently, but reflect the complexity of a

toxicological response. Environmental proteomics provides a more comprehensive assessment of toxic and defensive mechanisms triggered by pollutants and does not require prior knowledge.

Importantly, they may help to detect subtle pollution, such as a mixture of pollutants at low concentrations, where clear signs of toxicity are absent. Proteomics analysis allows isolating sets of proteins within the proteome that are specific to different stressors: biological, physical or chemical (Amelia, 2011)

In aging research:

Biological aging can be represented as a function of several closely interrelated parameters such as metabolic rate, caloric intake, lifestyle and environmental factors. Several theories trying to explain the mechanisms of aging process have been developed, such as the "oxidative damage/free-radical theory", the "cross-linking/glycosylation theory", the "replicative senescence hypothesis", the "rate-of-living theory" and the "somatic mutation theory". Some of these theories are based on common phenomena and essentially complement each other. E.g. the "rate-of living theory" is based on the fact that higher metabolic rates correlate with shorter life span. High metabolic rates are usually associated with a high level of reactive oxygen species (ROS) produced, which in turn can cause chemical modifications of biological macromolecules. These modifications can lead to post-translational glycation of proteins and DNA, supporting the "cross-linking theory". Actually the "free-radical theory of aging" is built on a similar argument including age-dependent increase of oxidative modifications of biomolecules due to increased level of ROS. Aging represents a complex developmental phenomenon, depending on a global interplay between genes and gene products. The rate of aging depends on the identity of the gene, rate and regulation of translation process, the residence time of gene products in the organism. A thorough understanding of the aging process requires evaluation of all these processes, and that is where aging research will greatly benefit from proteomics and genomics (Amelia, 2011)

CHAPTER2

APPLICATION OF GENOMICS AGAINST DIFFERENT DISEASES AND DISCOVERY OF DRUGS

2. Genomics approach

Genomics, specially high-throughput sequencing and characterization of human genes expression. This approach has some methodologies named gene expression, gene sequencing, statistical genetics and analysis in clinical development.

Table 3: Drugs name discovered by genomics (Griffin et al., 2008)

Diseases	Drugs invented
Hypercholesterolemia	Simvastatin
Osteoporosis and has some antitumor activity	Bisphosphonate
Anticoagulant	Ximelagatran, warfarin
Macrolide antibiotic	Flucloxacillin
Epilepsy	Carbamazepin
Gout	Allopurinol

Table 4: Discovered drugs with molecular targets by Genomics approach (Miller, 2013)

Diseases	Drugs	Target
Breast cancer	Herceptin	HER 2
Kidney and lung cancer	Sorafenib	Protein kinase B
Chronic myeloid leukemia	Femera with spygel Femera with palbocicib	Src/ non receptor tyrosine kinase

2.1 HMG-coA Reductase inhibitors (Statins) discovered against hypercholesterolemia by genomics approach

2.1.1 Hypercholesterolemia

Hypercholesterolemia, defined as excessively high plasma cholesterol levels and high LDL (low density lipoprotein) as emerged as a strong risk factor for cardiovascular disease. Hypercholesterolemia, in which there is a high level of cholesterol (Harikumar *et al*., 2013).

It is the condition of increased cholesterol level than normal level. It can be genetic or it can be appeared over time. Now it is very common disease.

In general, adults older than 20 should try to keep their total cholesterol level below 200 milligrams per deciliter (Krauss *et al*., 2000)

2.1.1.2 Causes of Hypercholesterolemia

- Diet
- Hypothyroidism
- Nephrotic syndrome
- Anorexia
- Obstructive liver disease
- Obesity
- Acute hepatitis
- Systemic lupus
- Obesity
- Diabetes mellitus (Adams *et Al*., 2001)

2.1.1.3 Risk Factors

In addition to elevated LDL levels, major risk factors including:

- Family history of premature death in first-degree relative (male relative <55 years, female relative <65 years)
- Hypertension (blood pressure ≥140/90 mm Hg or on antihypertensive medication)
- Diabetes mellitus (or impaired glucose tolerance)
- Cigarette smoking
- Low HDL cholesterol (<35 mg/dl)
- Overweight or obesity
- Physical inactivity (Adams *et al*., 2001)

2.1.1.4 Pathophysiology of hypercholesterolemia

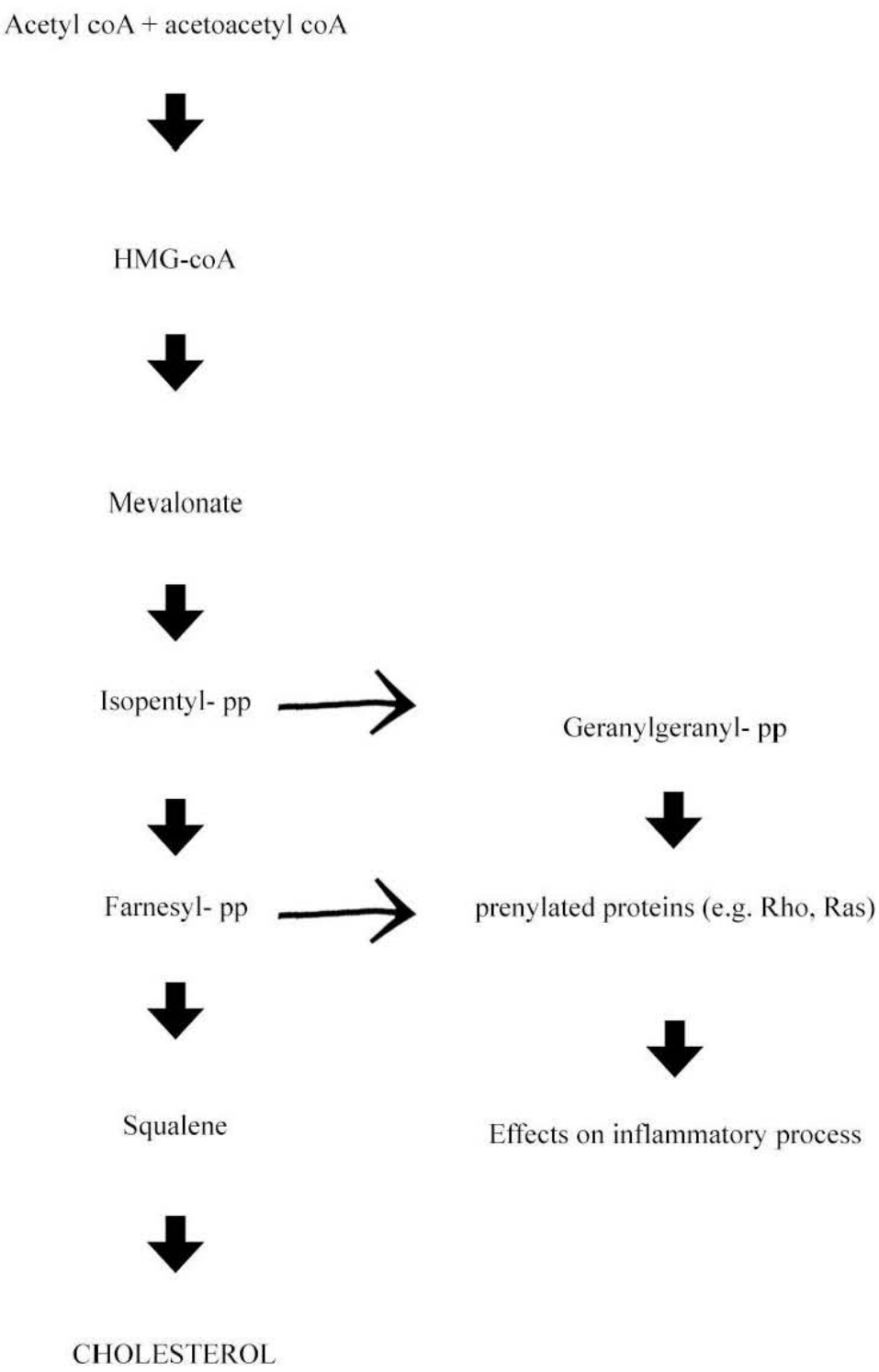

Figure 5: Pathophysiology of hypercholesterolemia (Vasconcelos *et al*., 2004)

2.1.1.5 Treatment of hypercholesterolemia

Diet:

Daily food calories from plant sources, especially fruits and vegetables, grains, beans, nuts, and seeds

Table 5: Diet chart used during hypercholesterolemia (Pullinger *et al*., 2014)

Food type	Quantity
Saturated fat	Less than 7% of calories
Monounsaturated fat	About 20% of calories
Polyunsaturated fat	About 10% of calories
Protein	About 15% of calories
Carbohydrates	About 50% of calories
Fiber	About 25 grams of soluble fiber per day
Cholesterol	Less than 200 milligrams per day

Treatment option:

HMG CoA reductase inhibitors

The enzyme 3-hydroxy-3-methyl glutaryl coenzyme-A (HMG CoA) reductase regulates the rate of cholesterol synthesis in humans. The drugs that inhibit this enzyme lower total and LDL cholesterol by reducing the rate of cholesterol synthesis. Formerly known as mevinolin, lovastatin is the first HMG CoA reductase inhibitor (Baker *et al*., 2008)

Bile Acid Sequestrants:

Cholestyramine interrupts the circulation of bile acids in the body and cause the liver to synthesize new bile acids from cholesterol, are commonly used to reduce the LDL-cholesterol level. These drugs can reduce total serum cholesterol by 20 percent (20%) and LDL-cholesterol by 27 percent (27%) taken in full doses (Sirvent *et al*., 2005)

Fibric Acid Derivatives

Two fibric acid derivatives are currently used in the United States to treat hyperlipidemia. The first, clofibrate, was initially hailed as an important drug. Gemfibrozil, a newer fibric acid derivative, primarily lowers triglyceride levels. It also lowers LDL and raises HDL levels. Another one is fenofibrate (Goldman, 1989)

2.1.2 Statins

2.1.2.1 Mechanism of action:

- Statins inhibit HMG co-A reductase decreases the concentration of cholesterol within the cell
- Low intracellular cholesterol stimulates the synthesis of LDL (low density lipid) receptors
- Increased number of LDL receptors promote uptake of LDL from blood
- Low intracellular cholesterol decreases the secretion of VLDL (Very Low Density Lipid)

Statins mainly inhibit the function of HMG-coA receptor. Steps are given below:

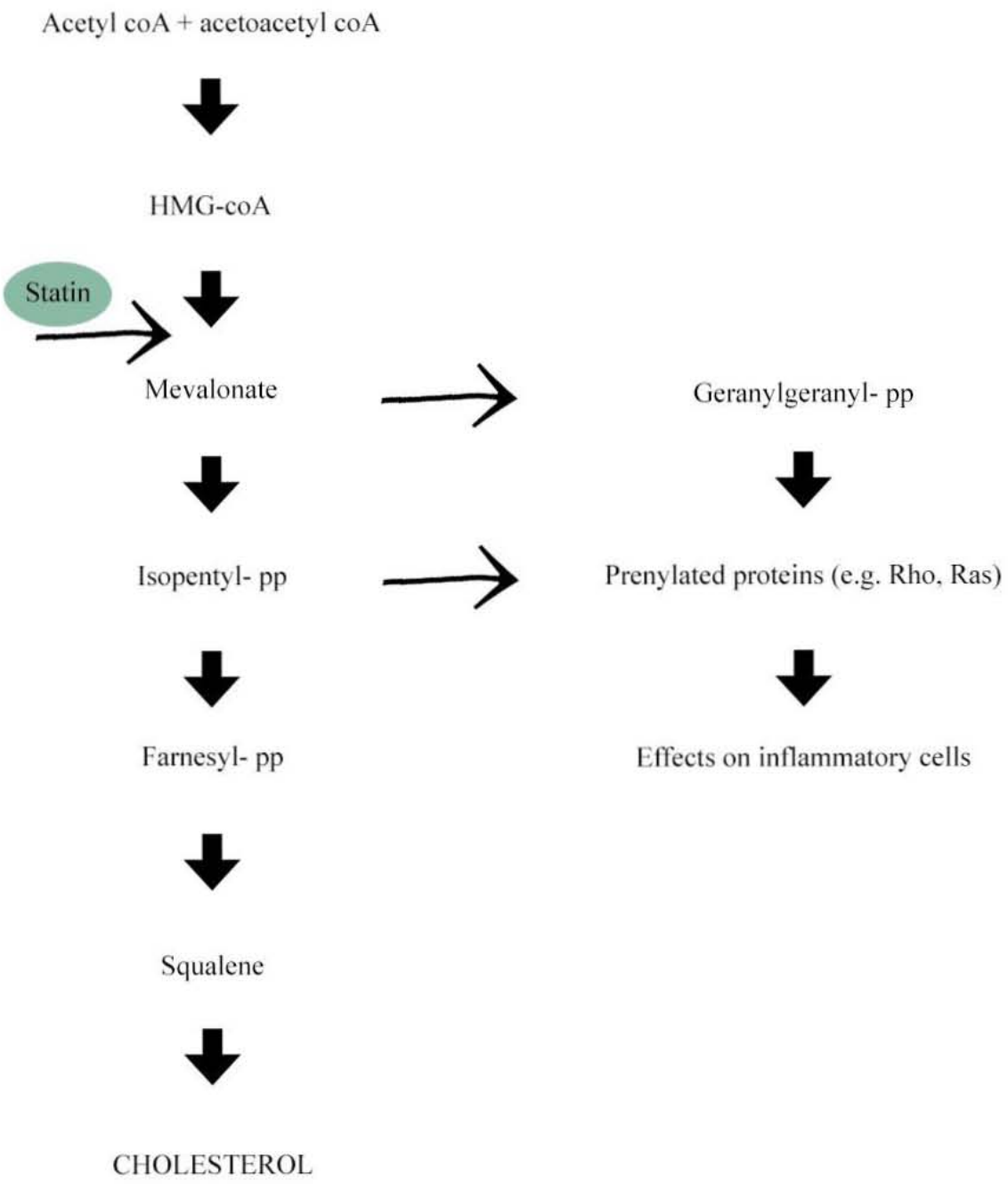

Figure 6: Mechanism of action of statins (Ness *et al*., 1996) (Noel *et al*., 2001)

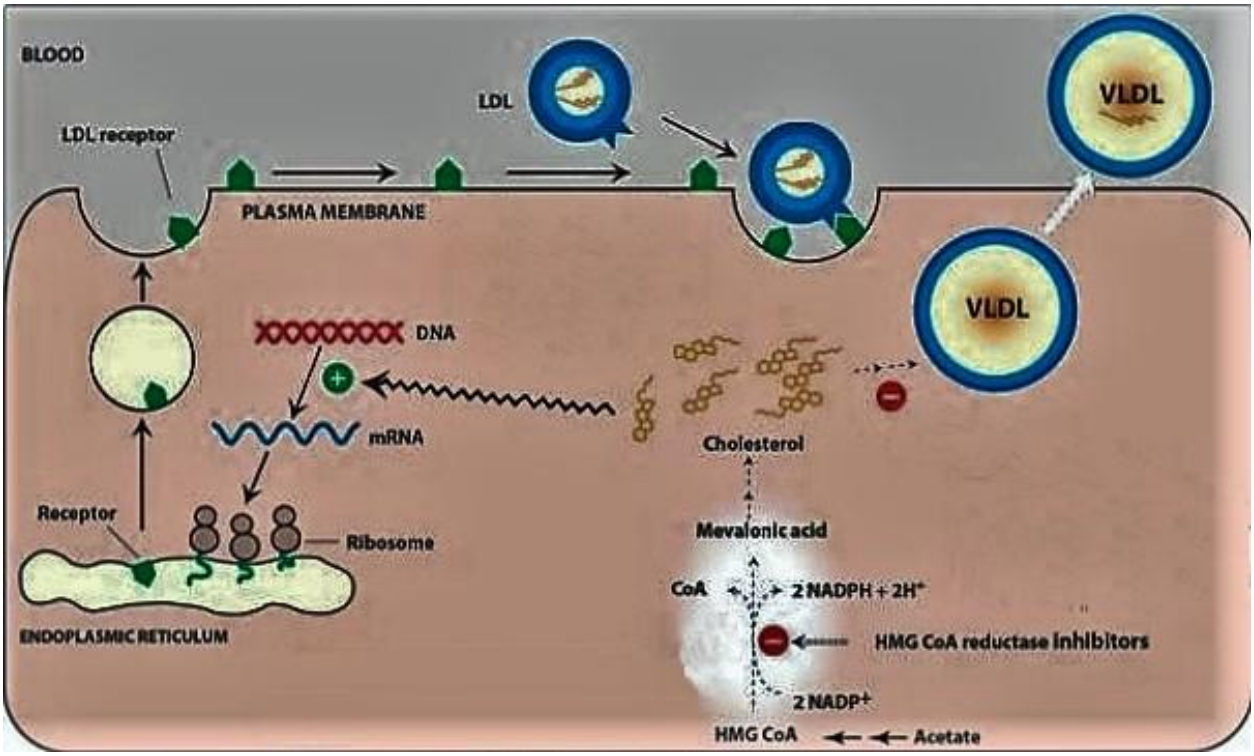

Figure 7: Binding site of statins

2.1.2.2 Effects of statins

Table 6: Effects of statins (Duplaga *et al*., 1999)

Antioxidant activities	Anti-thrombotic activity	Lipid lowering activity	Anti-inflammatory activity
1. Decreased lipid peroxidation 2. Decreased ROS production 3. Increased glutathione production	1. Increased fibrinolytic activity 2. Decreased endothelin production	1. Decreased cholesterol biosynthesis 2. Increased LDL receptors	1. Decreased chemokines, cytokines secretion

2.1.2.3 Type of interaction Examples of drugs

Inhibition of CYP3A4:

- Azole antifungals:

E.g. Itraconazole, ketoconazole, miconazole (Can *et al*., 2008)

- Macrolide antibiotics:

E.g. Erythromycin, telithromycin, clarithromycin

- Protease inhibitors:

E.g. Amprenavir, atazanavir, fosamprenavir, indinavir, lopinavir, nelfinavir, ritonavir, tipranavir

- Fibrates:

E.g. Gemfibrozil, bezafibrate, fenofibrate, ciprofibrate (Elster *et al*., 1994)

- Verapamil, Diltiazem
- Warfarin

Inhibition of CYP2C9:

- Amiodarone
- Omeprazole (Neuvonen *et al*., 2004)

2.1.3 Target gene

HMGCR is the responsible gene for HMG-coA receptor function. So the target site of statin is HMGCR.

Table 7: Sequence of HMGCR gene (Elster *et al*., 1994)

Target gene	Sequence
HMGCR	GCTCTCTTCATCTACTTTCTTATCTAAGCA

There are some genetic variants of statins response. These variants are described below:

Table 8: Genetic variants (Dixon *et al*., 2001)

Gene	Mutated variant	Effects
CYP2D6	CYP2D6 mut	Alterations on statins metabolism and efficacy
ABCB1	3435CT 1236CT 2677GA/T	Impaired efflux function
HMG-coA	SNP 12 SNP 29	Reduced response to therapy

2.2 Bisphosphonate discovered by Genomics approach

2.2.1 Therapeutic effect:

Bisphosphonates are potent inhibitors of osteoclast-mediated bone resorption. They are widely used in the management of osteoporosis and other diseases of high bone turnover

2.2.2 Target gene:

Potential target site of bisphosphonate for treating osteoclast mediated bone desorption is GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) and it has also some direct and indirect antitumor effect (Valenti *et al*., 2004)

2.2.2.1 Properties of GAPDH gene

- It is constitutive housekeeping gene
- This gene is used in transcription activation, initiation of apoptosis
- Used to normalize changes in specific gene expression.
- Used as a control RNA in RT-PCR (Reverse transcriptase-polymerase chain reaction) and northern blotting (Bertoldo *et al*., 2004)

2.2.2.2 Gene sequence of GAPDH

Table 9: Gene sequence (Bertoldo et al., 2004)

Gene	Forward primer	Reverse primer	Annealing (°C)	Extension (°C)
GAPDH	GGCGTGAACCACGAGAAGTATAA	CCCTCCACGATGCCAAAGT	59	72

2.2.3 Bisphosphonate

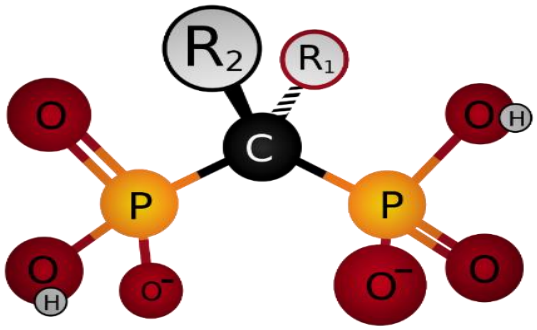

Figure 8: Bisphosphonates

2.2.3.1 Classification and mechanism of action of Bisphosphonate:

Bisphosphonates can be broadly classified into two major classes with distinct mechanisms of action:

- **The non-nitrogen containing class:** This class mainly acts by incorporation into ATP. E.g Clodronate, tiludronate and etidronate
- **The nitrogen-containing class:** This class acts by inhibiting farnesyl pyrophosphate synthase (FDPS) in the mevalonate pathway. E.g. Pamidronate, alendronate, ibandronate, riserdronate and zoledronate (Fraser *et al.*, 2006)

Table 10: Bisphosphonate Formulations

Generic name	Class	Mechanism of action
Etidronate disodium	Non nitrogen containing class	
Clodronate disodium	Non nitrogen containing class	Incorporated into intracellular analogous of ATP (Adenosine Tri Phosphate)
Tiludronate disodium	Non nitrogen containing class	
Aledronate sodium	Nitrogen containing class	
Aledronate sodium and vitamin D3	Nitrogen containing class	
Pamidronate disodium	Nitrogen containing class	Inhibit the prenylation and function of GTP binding proteins require d for osteoclast formation, survival and function
Aledronate disodium	Nitrogen containing class	
Risedronate sodium	Nitrogen containing class	
Risedronate sodium and calcium	Nitrogen containing class	

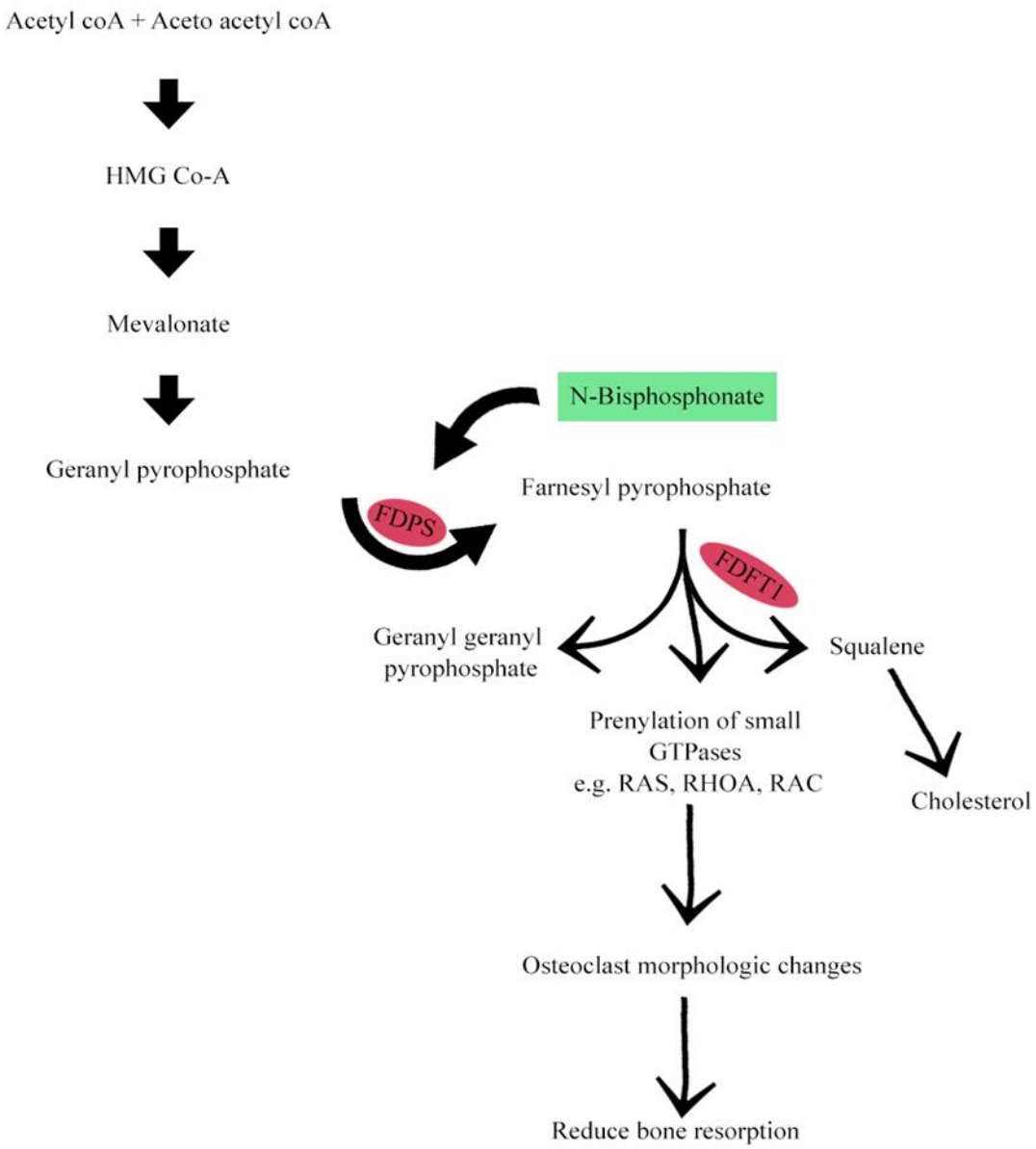

Figure 9: Mechanism of action of Bisphosphonate

CHAPTER 3

APPLICATION OF PROTEOMICS AGAINST DIFFERENT DISEASES AND DISCOVERY OF DRUGS

3. Proteomics approach

Proteomics determines the target of some anticancer drugs, with the determination of biomarkers. This approach can also determine the toxicity profile of some drugs.

Flow chart of proteomics approaches:

Protein extraction by gel electrophoresis → protein separation→ protein staining→ protein identification by mass spectrometry → protein databases analysis

Figure 10: Flow chart of proteomics approaches

Table 11: Ten Monoclonal antibodies approved by FDA (Worrell and Hupp, 2012)

Name	Target	Disease
Trasutuzumab	Erb 2/HER-2	Breast cancer
Rituximab	CD 20	Non-Hodgkin Lymphoma
Gemtuzumab	CD 33	Acute Myeloid Lymphoma
Alemtuzumab	CD 52	Chronic Lymphocytic Lymphoma
Ibritumomab	CD 20	Non-Hodgkin Lymphoma
Tositumomab	CD 20	Non-Hodgkin Lymphoma
Bevacizumab	Vascular endothelial growth factor	Colorectal cancer
Cetuximab	Endothelial Growth Factor Receptor	Colorectal cancer, head and neck cancer
Panitumumab	Endothelial Growth Factor Receptor	Colorectal cancer
Ofatumumab	CD 20	Chronic Lymphocytic Lymphoma, Non-Hodgkin Lymphoma

Table 12: Proteomics approach used against different diseases (Jain *et al*., 2004) (Tchernev, 2005) (Lin *et al*., 2006) (Restori *et al*., 2007) (Griffin, 2008)

Diseases pathology	Technology
Alzheimer's disease, Parkinson's, multiple sclerosis, coronary heart failure	Proteomics (mass spectrometry)
Diabetes, obesity, coronary heart disease, Cohn's disease, ulcerative colitis	Proteomics
Organ transplantation	Proteomics

3.1 HER2 protein inhibitor (Trastuzumab) discovered against breast cancer

3.1.1 Breast cancer:

Breast cancer is a cancer that starts in the tissues of the breast, milk duct and sometimes in the partial parts of breast.

3.1.1.1 Types

There are two main types of breast cancer:

Ductal carcinoma or intraductal carcinoma: It starts in the lining of the milk ducts that spread milk from the breast to the nipple. Ductal carcinoma is more common than Lobular carcinoma. It is of noninvasive type which is not invaded other tissues.

Lobular carcinoma: It starts in the parts of the breast, called milk duct or lobules, which produce milk. This is the marker for an increased risk of invasive cancer in the same or both breasts. Invasive means it can be invaded to other tissues from the milk duct (Lal, 2005)

Another types:

Hormone Receptor-Positive:

This is the most common type of cancer. According to three specific cell surface receptors such as: estrogen receptor (ER), progesterone receptor (PR) and Human Epidermal Growth Factor Receptor (HER)2/ neu receptor. This type grows and spreads with a response to the hormones and the therapies have the action to inhibit the growth effects.

According to the presence or absence of HER2 receptor, breast cancer can also bt divided in two types:

HER2-positive breast cancer: Tumors that express HER2/neu receptors

HER2-negative breast cancer: Tumors that do not overexpress HER2/neu receptors (Sherman et al., 2007)

Table 13: Types of breast cancer (Howe *et al*., 2005)

Cancer types	Description
Ductal carcinoma	It is not invaded in other tissues without breast
Lobular carcinoma	It can be invaded in other tissues from the milk duct

Hormone Receptor-Positive	Presence of different receptor such as estrogen receptors, progesterone receptors and HER2 receptors

3.1.1.2 Main Stages of Breast Cancer

There are three main stages of breast cancer:

Early stage:

This stage refers to cancer that is confined to the fatty tissue of the breast.

Locally advanced:

This stage which has spread to underlying tissue of the chest wall.

Advanced or metastatic:

This stage involves the spreading of symptoms to other parts of the body. Approximately one third of breast cancer cases are diagnosed after the cancer has spread beyond the primary tumor site (Chatterjee, 2004)

Table 14: Goals of different stages of cancer (Carey *et al*., 2006)

Stage	Goal	Treatment option
Stage I	To cure the cancer symptoms	Lumpectomy plus radiation or mastectomy
Stage II	Preventive measures are taken	Lumpectomy plus radiation or mastectomy, with some sort of lymph node removal
Stage III	Preventive measures are taken	Chemotherapy, hormone therapy, and biologic therapy and then surgery if needed
Stage IV	Improvement of symptoms and help the patients to live longer. Curing of cancer is not possible	surgery, radiation, chemotherapy, hormone therapy, or a combination of these treatments.

3.1.1.3 Symptoms

Early breast cancer:

It Usually does not cause symptoms. Regular self-assessment of breast exams is important. But some symptoms are seen including:

Breast lump:

- Hard lump in the breast or armpit with uneven edges
- Does not have any pain

Changes in breast:

- Changes occur in breast size, shape
- Having redness, dimpling, or puckering that looks like orange color skin

Fluid coming from the nipple:

- Fluids may be bloody, clear to yellow, green in color and pus like appearance.

Advanced breast cancer: symptoms become complicated with the advanced stage including:

- Changes in the skin: Symptoms including hardening, dimpling, bumps, redness
- Changes in the nipple: Symptoms including retraction, the secretion of unusual discharge or a rash around the nipple area
- Bone pain
- Breast pain or discomfort
- Skin ulcers
- Swelling of in the armpit
- Weight loss

3.1.1.4 Risk factors

Age and gender: Your risk of developing breast cancer increases as you get older. Most advanced breast cancer cases are found in women over age 50. Men can slowly get breast cancer. But they are 100 times less likely than women to get breast cancer.

Family history of breast cancer: You may also have a higher risk of breast cancer if you have a close relative who has had breast, uterine, ovarian, or colon cancer. About 20 - 30% of women with breast cancer have a family history of the disease.

Genes: Some people have genetic mutations that make them more likely to develop breast cancer. The most common gene defects are found in the BRCA1 and BRCA2 genes. These genes normally produce proteins that protect you from cancer. If a parent passes you a defective gene, you have an increased risk of breast cancer. Women with one of these defects have up to an 80% chance of getting breast cancer sometime during their life (Ford *et al*., 1995)

Menstrual cycle: Women who got their periods early (before age 12) or went through menopause late (after age 55) have an increased risk of breast cancer (Donovan *et al*., 2007)

Other risk factors:

Alcohol use: Drinking more than 1 - 2 glasses of alcohol a day may increase your risk of breast cancer.

Childbirth: Women who have never had children or who had them only after age 30 have an increased risk of breast cancer. Being pregnant more than once or becoming pregnant at an early age reduces your risk of breast cancer.

Having diethylstilbestrol (DES): Women who took diethylstilbestrol (DES) to prevent miscarriage may have an increased risk of breast cancer after age 40. This drug was given to the women in the 1940s - 1960s (Sasco *et al*., 2003)

Hormone replacement therapy (HRT): You have a higher risk of breast cancer if you have received hormone replacement therapy with estrogen for several years or more (Garcia *et al*., 2006)

Obesity: Obesity has been linked to breast cancer, although this link is not completely understood. The theory is that obese women produce more estrogen. This can fuel the development of breast cancer.

Radiation: If you received radiation therapy as a child or young adult to treat cancer of the chest area, you have a very high risk of developing breast cancer. The younger you started such radiation and the higher the dose, the higher your risk. This is especially true if the radiation was given during breast development (Kortencamp, 2006)

Breast implants, using antiperspirants, and wearing underwire bras do not raise the risk of breast cancer. There is also no evidence of a direct link between breast cancer and pesticides (Sasco *et al*., 2003)

3.1.1.5 Diagnosis

Breast self-exam:

- **Breast MRI (Magnetic Resonance Imaging):** It is performed to identify the breast lump or evaluate an abnormal change by mammography. This is more commonly performed on younger women.
- **Breast Ultrasound:** It is performed to show whether the lump is solid or fluid-filled.
- **Breast Biopsy:** It is performed using methods such as needle aspiration, ultrasound-guided, stereotactic, or open CT (Computed tomography) scan to check if the cancer has spread.
- **Mammography:** It is performed to screen for breast cancer or help to identify the breast lump
- **PET scan (Position Emission Tomography):** It is performed to check if the cancer has spread
- **Sentinel lymph node biopsy:** It is performed to check if the cancer has spread to the lymph nodes (perou *et al*., 2000)

Initial assessment:

Biopsy:

Biopsy is done for confirming the diagnosis. After performing MRI, CT scan, mammography and different diagnostic processes, biopsy is performed. It is performed under the microscope. It includes the procedure where a needle is inserted into the lump for some cells to be withdrawn. Sometimes a small operation is performed to get a biopsy sample

Assessing the extent and spread:

If breast cancer is confirmed, then further tests is performed such as chest X-ray, an ultrasound scan of liver, bone scan and blood tests. This assessment is called staging of the cancer (Singletary et Al., 2002). It determines the followings:

How large the tumor size has grown.

Spreading of cancer to local lymph nodes in the armpit

Whether the cancer has spread to other areas of the body (metastasised) (Saunders *et al*., 2006)

3.1.1.6 Breast cancer treatment

Current treatments for breast cancer include surgery, radiotherapy, chemotherapy, hormonal and targeted therapies.

Treatment is based on many factors, including:

- Type and stage of the cancer including size and position of breast
- Sensitivity of cancer to certain hormones
- Overexpression of a gene named HER2/ neu

- Based on the severity of stages of breast

Table 15: Cancer treatment pattern

Treatment	Function	Route of administration
Chemotherapy	Kill cancer cells	Systemic treatment
Radiation therapy	Destroy cancerous tissue	Local treatment
Lumpectomy	Removes the breast lump	Local treatment
Mastectomy	Removes all or part of the breast and possible nearby structures	Local treatment
Hormone therapy	Block certain hormones including estrogen prescribed to the estrogen positive women	Systemic treatment or local treatment

Treatment options are described below:

Surgery:

This is the main treatment option for patients whose breast cancer has not spread to other parts of the body and is also an option for more advanced stages of the disease. The types of breast cancer surgery differ in the amount of tissue that is removed with the tumour; this depends on the tumour's characteristics, whether it has spread, and the patient's personal feelings (Vicini et al., 1997)

etSome of the most common types of surgery including:

- **Breast conserving therapy or Lumpectomy:** It involves the removal of the cancerous area, the surrounding tissue and in some cases the lymph node, aiming to maintain a normal breast appearance after surgery.
- **Partial Mastectomy or Quadrantectomy:** This is compared with lumpectomy where a larger
- portion of tissue is removed.
- **Total Mastectomy**: This is performed to prevent further cancer including removal of the entire breast without removing the lymph nodes.

Radiotherapy:

This is also known as adjuvant treatment or neoadjuvant tharapy. It can be given after surgery or in conjunction with chemotherapy prior to surgery to shrink the tumour. Radiotherapy can also be used without surgery in patients with advanced metastatic breast cancer to help alleviate symptoms (Matthews *et al*., 2006)

Chemotherapy:

Chemotherapy may be given prior to surgery, also called neo-adjuvant therapy with the aim of reducing tumour size and the need for extensive surgery, or after surgery, also called adjuvant therapy to reduce the chances of the recurrence of cancer. When the cancer has spread to other parts of the body (metastatic), chemotherapy may be used to reduce symptoms, improve quality of life and extend survival. Chemotherapy drugs can be given through systemic pathway or in oral form. Adverse side effects such as fatigue, nausea and diarrhea may be seen (Saji *et al*., 2005)

Hormonal therapy:

Medicines that block or inhibit the actions of the hormones estrogen and progesterone are often used in the treatment of patients with Hormone Receptor-Positive breast cancer (Fentiman *et al*., 2006)

Targeted therapy:

Targeted cancer therapies are treatments that target specific characteristics of cancer cells. It is also called biological therapy. It is relatively a new approach. Targeted therapy can include use of monoclonal antibodies, vaccines and gene therapies. These are either given just after chemotherapy as maintenance or in conjunction with other therapies including chemotherapies or hormonal therapies at various stages of advanced disease in accordance with their approved label. Drugs that target the HER2/neu protein

In about 1 in 5 patients with breast cancer, the cancer cells have too much of a growth-promoting protein known as HER2/neu (or just HER2) on their surface. Breast cancers with too much of this protein tend to grow and spread more aggressively without special treatment. A number of drugs have been developed that target this protein: Trastuzumab, Pertuzumab, Ado-trastuzumab emtansine, Lapatinib (Jason *et al*., 2013)

Table 16: Drugs of targeted therapy (Justin *et al*., 2013)

Drugs name	Description
Trastuzumab	treatment of HER2 overexpressing breast cancer. It is used to treat both early- and late-stage breast cancer. It is given intravenously
Pertuzumab	It is given intravenously combined with trastuzumab.
Ado-trastuzumab emtansine	HER2-targeting monoclonal antibody and microtubule inhibitor. It is attached to a chemotherapy drug
Lapatinib	Small-molecule EGFR/HER2 inhibitor. This is not an antibody. It is given as a pill.

3.1.2 Targeted receptor

3.1.2.1 HER2 Overview:

The HER2 gene (ErbB2) is a member of a group of epithelial tyrosine kinase receptors. These receptors also include HER1 (which is an EGFR receptor), HER3 (ErbB3), and HER4 (ErbB4). The HER2 protein tends to dimerize with either HER1, HER3, or HER4.

HER2 has no identified ligand, which allows it to always be in open confirmation to dimerize with HER1, HER3, or HER4.

when the HER2 gene is amplified and overexpressed, it allows for cell growth, survival, and cell differentiation through a signal transduction cascade mediated by the activation of PI3K/Akt (Phosphatidylinositol-3 kinases/protein kinase B) and the Ras/Raf/MEK/MAPK pathways. Before HER2 directed therapies were created, having HER2 positive breast cancer meant hyperactivation of this downstream pathway, which caused high recurrence rates and increased mortality. The development of targeted HER2 therapies, has significantly improved the outcome for patients with HER2 positive breast cancer (Prenzel *et al*., 2001)

The HER2 gene makes HER2 proteins. HER2 proteins are receptors on breast cells. Normally, HER2 receptors help control how a healthy breast cell grows, divides, and repairs itself. But in about 25% of breast cancers, the HER2 gene doesn't work correctly

HER2 gene amplification: Normally, HER2 receptors help control how a healthy breast cell grows, divides, and repairs itself. But in about 25% of breast cancers, the HER2 gene doesn't work correctly and makes too many copies.

HER2 protein overexpression: All these extra HER2 genes tell breast cells to make too many HER2 receptors. This makes breast cells grow and divide in an uncontrolled way (Moghal and Sternberg 2003)

Breast cancers with HER2 gene amplification or HER2 protein overexpression are called HER2-positive in the pathology report. HER2-positive breast cancers tend to grow faster and are more

likely to spread and come back compared to HER2-negative breast cancers. But there are medicines specifically for HER2-positive breast cancers.

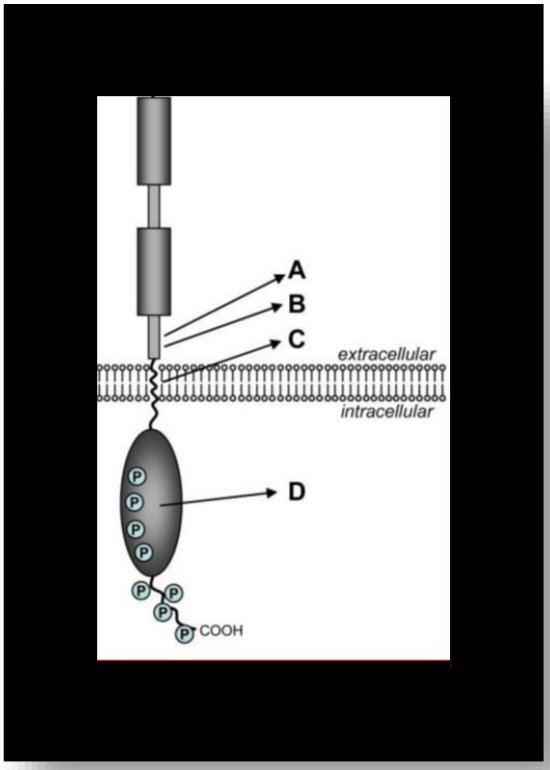

Figure 10: Structure of HER2 proteins, here A= site of somatic mutations found in tumors, B= site of the 48bp deletion in the naturally occuring human ΔHER2 isoform, C= site of the mutation the neuT oncogene initially, D= site of mutations found in rare cases of human lung cancers (Yarden and Sliwkowski 2001)

3.1.2.2 The Signaling Function

The HER family proteins are type I transmembrane growth factor receptors that function to activate intracellular signaling pathways in response to extracellular signals. Their structure consists of an extracellular ligand binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain. The function of this family is simple. Signaling is mediated by a single ligand and a single receptor and slightly more complex in Drosophila where four ligands signal through a single receptor (Lacenere and Sternberg 2000, Moghal and Sternberg 2003). The system is far more

complicated in mammalians where the functions of this family are performed by at least twelve ligands and four receptors. Readers are referred to several recent excellent reviews of HER family signaling and functions (Barnes and Kumar 2004; Mendelsohn and Baselga 2000; Prenzel *et al*., 2001; Olayioye *et al*., 2000). While the reasons behind such multiplicity in this system are not well understood, much is now known regarding the molecular basis underlying their signaling activities. Upon ligand binding to their extracellular domains, HER proteins undergo dimerization and transphosphorylation of their intracellular domains. These phosphorylated tyrosine residues dock numerous intracellular signaling molecules leading to activation of a plethora of downstream second messenger pathways and crosstalk with other transmembrane signaling pathways leading to diverse biological effects (reviewed in (Barnes and Kumar 2004; Prenzel *et al*., 2001; Bazley and Gullick 2005; Yarden and Sliwkowski 2001)). The structural bases for receptor dimerization have been coming to light in the past few years by crystollagraphic data. The extracellular domain of HER proteins can exist in a closed inhibited or an open active conformation. Ligand binding induces a conformational change in their extracellular domain that induces the active conformation and promotes their dimerization and consequent transphosphorylation (Burgess *et al*., 2003). Partner selection appears to be a key determinant of signaling activity among HER proteins and their signaling functions follow a distinct hierarchical order favoring heterodimers over homodimers. HER2 has the strongest catalytic kinase activity and HER2-containing heterodimers have the strongest signaling functions (Tzahar *et al*., 1996). The expansion of the HER family in mammalian systems has been associated with functional differentiation necessitating interdependence rather than promoting independent or redundant functions. This is exemplified by HER2 and HER3 which are functionally incomplete receptor molecules. Unlike the other members of the family, the extracellular domain of HER2 does not pivot between active and inactive conformations and constitutively exists in an activated conformation (Garrett *et al*., 2003; Cho *et al*., 2003). Consistent with its constitutively active conformation, HER2 lacks ligand binding activity and its signaling function is engaged by its ligand-bound heterodimeric partners (Sliwkowski 2003). On the other hand, HER3, unlike the other members, lacks ATP binding within its catalytic domain and is catalytically inactive (Sierke *et al*., 1997). Consistent with this, the signaling functions of HER3 are mediated entirely through the kinase activity of its heterodimeric partners (Kim *et al*., 1998). Even chimeric kinase-active HER3 constructs fail to signal without hetero-partners suggesting that HER3 even lacks the ability to homodimerize and is an obligate

heterodimerization partner (Berger *et al*., 2004). Although individually they are incomplete signaling molecules, a large body of evidence not only establishes HER2 and HER3 as obligate partners but their complex forms the most active signaling heterodimer of the family and essential for many biologic and developmental processes (Sliwkowski *et al*., 1994; Tzahar *et al*., 1996; Britsch *et al*., 1998; Vijapurkar *et al*., 2003; Goodearl *et al*., 2001; Horan *et al*., 1995)

3.1.2.3 Different tests of HER2 protein

IHC test (Immune Histo Chemistry): The Immune Histo Chemistry test finds out if there is too much HER2 protein in the cancer cells. The results of the IHC test can be: 0 (negative), 1+ (also negative), 2+ (borderline), or 3+ (positive — HER2 protein overexpression)

FISH test (Fluorescence In Situ Hybridization): The Fluorescence In Situ Hybridization test finds out if there are too many copies of the HER2 gene in the cancer cells. The results of the FISH test can be positive (HER2 gene amplification) or negative (no HER2 gene amplification)

SPoT-Light HER2 CISH test (Subtraction Probe Technology Chromogenic In-Situ Hybridization): The SPoT-Light test finds out if there are too many copies of the HER2 gene in the cancer cells. The results of the SPoT-Light test can be positive (HER2 gene amplification) or negative (no HER2 gene amplification)

Inform HER2 Dual ISH test (Inform Dual In-Situ Hybridization): The Inform HER2 Dual ISH test finds out if there are too many copies of the HER2 gene in the cancer cells. The results of the Inform HER2 Dual ISH test can be positive (HER2 gene amplification) or negative (no HER2 gene amplification) (Keely and Barrett 1999)

3.1.2.4 Tumor Dependence on HER2 protein

While the tumorigenic potential of HER2 overexpression has been clearly demonstrated in numerous model systems, its suitability as a drug target depends on whether clinically advanced tumors continue to dependent on HER2 for survival and progression. This dependency, recently described as oncogene-addiction, implies that such cancers can be effectively treated and possibly cured with drugs that inactivate the oncogene product (Weinstein 2002). Alternatively, genomic instability can lead to the mutational activation of additional pathways that could compensate for

the pharmacologic inactivation of the tumor-initiating oncogene making such an oncogene less effective as a drug target.

HER2 knockdown models:

Since the identification of HER2 as an oncogene in human tumors, numerous approaches have been undertaken to demonstrate the dependence of HER2 overexpressing tumors on HER2. A number of studies using antisense, ribozyme, or siRNA methodologies to suppress HER2 expression in human cancer cell lines consistently show that HER2 overexpressing tumor cells are dependent on HER2 and undergo growth inhibition and apoptosis in cell culture, or tumor regression in vivo, in the absence of HER2 expression, while tumor types that do not overexpress HER2 are not sensitive to HER2 knockdown (Colomer et al. 1994; Juhl et al. 1997; Roh et al. 2000; Faltus et al. 2004; Choudhury et al. 2004).A kinase-dead mutant of activated HER2 competes with and reverses the transformed phenotype induced by activated HER2 (Messerle et al. 1994). Intracellularly expressed single chain antibodies that target and inactivate HER2 revert HER2 induced transformation or induce apoptotic cell death in HER2 overexpressing tumor cells (Beerli et al. 1994; Deshane et al. 1996).

HER2 withdrawal models

Tetracycline inducible systems offer even more elegant models for analysis of oncogene addiction. NIH3T3 cells transformed by tetracycline regulated overexpression of HER2 revert from the transformed phenotype and their mouse implanted tumors regress when HER2 expression is withdrawn (Baasner et al. 1996; Schiffer et al. 2003). Tetracycline induced expression of activated HER2 in squamous epithelia of mice results in severe hyperplastic abnormalities of squamous epithelial tissues, which reverse upon withdrawal of the HER2 transgene expression (Xie et al. 1999). Tumors in MMTV-neuT mice are also dependent on continued oncogene expression. In the MMTV-rtTA/TetO-NeuNT bitransgenic variant of this model regulated by doxycycline, when expression of the neuT oncogene is induced in the mammary tissue of adult mice, this leads to the formation of multiple mammary tumors and lung metastases, and the entire primary tumor and metastatic disease fully regresses when neuT expression is withdrawn (Moody et al. 2002). Each of these models is subject to specific criticisms. For example, the antisense or siRNA approaches have non-specific effects, the NIH3T3 fibroblast models are not representative of what is principally an epithelial oncogene in humans, and the transgenic models may be too simplistic

and understate the genetic complexity of the human disease. But taken in aggregate, the existing data from all the different models and approaches is highly consistent and collectively makes a highly compelling case that HER2 induced tumors are addicted to HER2. This has made HER2 one of the most sought after targets in cancer drug development.

Table 17: HER2 (Human Epidermal Growth factor Receptor) overview (Keely and Barrett 1999) (Vartanian *et al*., 2000)

Name	Mutated genes	Tests
HER2 protein	TP53, PIK3CA, MUC16, LRP1, ERBB3, DNAH11, LRP2, TTN, ATP1A4	Immune Histo Chemistry, Fluorescence In Situ Hybridization, Inform Dual In-Situ Hybridization, Subtraction Probe Technology Chromogenic In-Situ Hybridization

3.1.3 Drugs

Table 18: Drugs details (Jason *et al*., 2013)

Drugs name	Mechanism of action	Adverse effects
Trastuzumab	treatment of HER2 overexpressing breast cancer. It is used to treat both early- and late-stage breast cancer. It is given intravenously	Fatigue, nausea, thrombocytopenia, cellulitis, elevated liver enzymes, left ventricular dysfunction, neurotoxicity
Pertuzumab	Humanized monoclonal antibody against the extracellular dimerization domain of HER2. It blocks the	Diarrhea, alopecia, neutropenia, nausea, fatigue, rash, peripheral

	heterodimerization of HER2 with other HER family. It is given intravenously combined with trastuzumab.	neuropathy, infusion and hypersensitivity reactions
Ado-trastuzumab emtansine	HER2-targeting monoclonal antibody and microtubule inhibitor. It is attached to a chemotherapy drug	Like trastuzumab
Lapatinib	Dual inhibitor of the intracellular tyrosine kinase domains of both HER1 (EGFR) and HER2.Small-molecule EGFR/HER2 inhibitor. This is not an antibody. It is given as a pill.	Fatigue, nausea, peripheral neuropathy

3.1.5 Binding site of trastuzumab on HER2 tyrosine kinase:

Trastuzumab is a monoclonal antibody targeted against the HER2 tyrosine kinase receptor. The mechanisms by which trastuzumab induces regression of HER2-overexpressing tumors are still being elucidated, but several molecular and cellular effects have been reported in the literature. Trastuzumab reduces signaling mediated by HER2 through the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) cascades. Reduced downstream signaling through these pathways induces the cyclin-dependent kinase inhibitor p27kip1, which promotes cell-cycle arrest and apoptosis (Nahta and esteva, 2006)

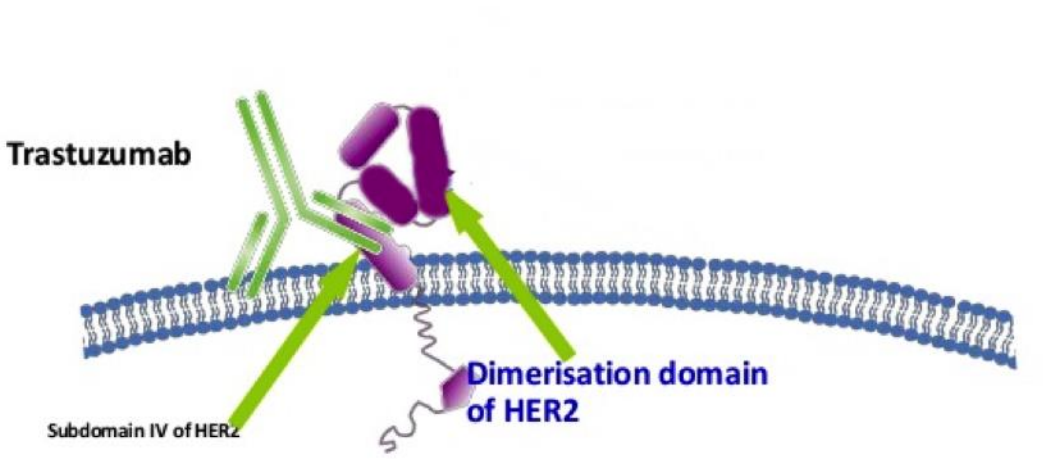

Figure 11: Binding of trastuzumab in HER2 tyrosine kinase receptor

Trastuzumab rapidly dissociates the non-receptor tyrosine kinase Src from HER2, reducing Src activity such that the phosphatase and tensin homolog deleted on chromosome ten (PTEN) is dephosphorylated and translocated to the plasma membrane where it is active. The PI3K downstream effectors (AKt) and mammalian target of rapamycin (mTOR) are then inhibited (Baselga *et al*., 2001)

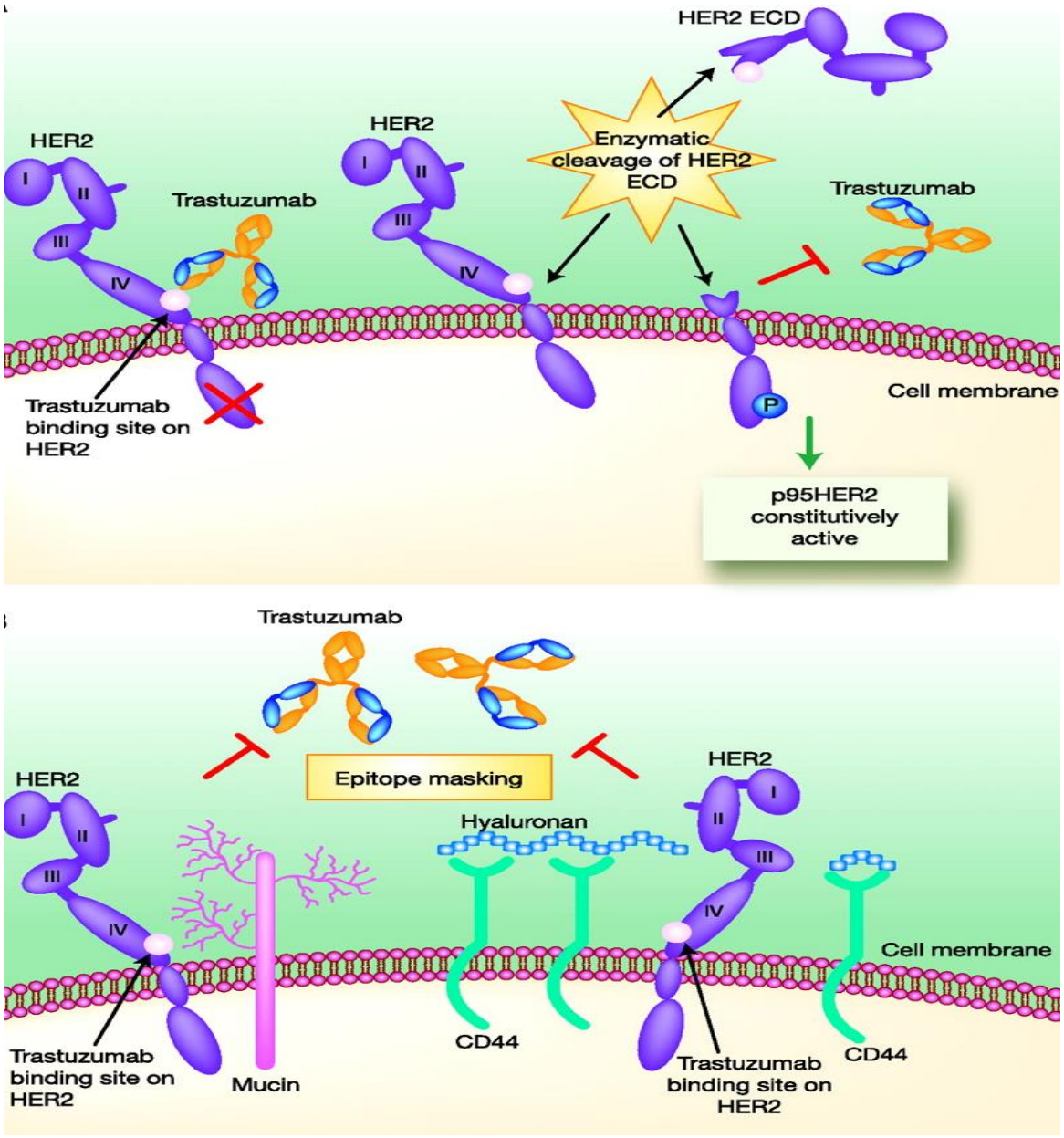

Figure 12: Molecular binding site of Trastuzumab

3.1.3.1 Toxicity profile

Repeated-Dose Toxicity:

The repeated-dose toxicity evaluation of trastuzumab is based on a four-week study in rhesus monkeys and 12- and 26-week studies in cynomolgus monkeys.

In some studies there was a minimal toxic response, with the only noteworthy observations concerning injection-site trauma in the rhesus monkey. Neutralising antibodies were detected from weeks 5-26 in one low-dose female cynomolgus monkey. This represents an incidence of 1/84 animals in repeated-dose studies in which antibodies to trastuzumab were detected.

Cardiotoxicity:

A main safety concern was cardiotoxicity including cardiomyopathy leading to congestive heart failure (CHF).

The original dossier contained a retrospective analysis of cardiac adverse events, which was made by a Cardiac review and evaluation committee (CREC). For this analysis, the clinical data were searched for patients with cardiac-related AEs using specific criteria for symptoms of heart failure. A full reassessment of cardiac-related events was performed on using more broader search criteria. The findings were largely in accordance with those of the original CREC evaluation. The data provided by the applicant as part of the response confirmed that during the clinical studies cardiotoxicity was not prospectively measured as an adverse event and that any data only allow retrospective analysis.

Therefore, the cardiotoxic potential of Herceptin alone or in combination with chemotherapy, in particular with paclitaxel, demands explicit clarification with regard to symptoms and nature of cardiotoxicity, frequency, mechanism, threshold of toxicity, time and dose response relationship, risk factors other than age, major confounding factors, mechanism of interaction between Herceptin

toxicity and chemotherapy toxicity. These data will be submitted through a follow-up measure.

Hepatic and renal toxicity:

WHO Grade III or IV hepatic toxicity was observed in 12% of patients following administration of Herceptin as single agent. This toxicity was associated with progression of disease in the liver in 60% of these patients. WHO Grade III or IV hepatic toxicity was less frequently observed among patients receiving Herceptin and paclitaxel than among patients receiving paclitaxel (7%

compared with 15%). No WHO Grade III or IV renal toxicity was observed in patients treated with Herceptin.

Diarrhea:

The patients treated with Herceptin as a single agent, 27% experienced diarrhoea. An increase in the incidence of diarrhoea, primarily mild to moderate in severity, has also been observed in patients receiving Herceptin in combination with paclitaxel compared with patients receiving paclitaxel alone.

Infection:

An increased incidence of infections, primarily mild upper respiratory infections of minor clinical significance or catheter infections, has been observed primarily in patients treated with Herceptin plus paclitaxel compared with patients receiving paclitaxel alone.

3.2 BCR-ABL Tyrosine Kinase Inhibitors (Imatinib) against Chronic Myeloid Leukemia:

3.2.1 Chronic Myeloid Leukemia (CML)

CML is an acquired abnormality that involves the hematopoietic stem cell. CML is a clonal myeloproliferative neoplasm. CML results from a somatic mutation in a pluripotential lympho hematopoietic cell. It is characterized cytogenetically by the presence of the Philadelphia (Ph) chromosome, which originates from the reciprocal translocations t(9;22) (q34;qll). In the formation of the Ph chromosome, the bulk of the ABL protooncogene is translocated from chromosome 9 onto the BCR gene in chromosome 22 (Junta *et al*., 2015)

3.2.1.1 Pathophysiology

Genetic abnormality:

CML is the result of an acquired genetic abnormality. A translocation between chromosome 9 and 22 (the Philadelphia chromosome). The oncogene BCR-ABL encodes an enzyme named tyrosine phosphokinase (usually p210)

Hematopoietic abnormality:

Expansion of granulocytic progenitors and a decreased sensitivity of the progenitors to regulation, increased white cell count. Megakaryocytopoiesis is often expanded. Erythropoiesis is usually deficient. Function of the neutrophils and platelet is nearly dysregulated and uncontrolled proliferation of mature and maturing granulocyte with fairly normal differentiation.

3.2.1.2 Stages of CML

Chronic phase:

Defined by:

- Increased WBC (White Blood Cell)
- Relative lack of blasts
- Increased Basophils

Accelerated phase:

- Second and intermediate phase of CML
- Blasts quantity increase than chronic phase
- Some symptoms are found including fever, bone pain etch

Blast crisis:

- The final phase of CML
- Maximum blasts are seen (≥30%)

Table 19: Three stages of Chronic Myeloid Leukemia (Amer, 2007)

Chronic phase	Accelerated phase	Blast crisis
Median duration: 5-6 years	Median duration: 6-9 months	Median survival: 3-6 months
WBC count: ≥ 20×10^9/L	WBC count: No	WBC count: No

Platelets: increased or normal	Platelets: increased	Platelets: decreased
Basophils: increased	Basophils: ≥ 20%	Basophils: No
Blasts: 0%	Blasts: ≥10%	Blasts: ≥30%
Bone marrow: Myeloid hyperplasia	Bone marrow: Myeloid hyperplasia	Bone marrow: Myeloid hyperplasia
BCR-ABL: + (present)	BCR-ABL: + (present)	BCR-ABL: + (present)

3.2.2 Imatinib

Some tyrosine kinase inhibitors such as Imatinib is identified by proteomics. This is active against ABL, BCR-ABL, c-KIT, and Platelet Derived Growth Factor Receptor alpha (PDGF-α). Several clinical trials have practiced to evaluate the efficacy and safety of ovarian cancer patients. This is used against chronic myeloid leukemia (CML), ovarian cancer and breast cancer (Patel *et al*., 2008).

3.2.2.1 Proteomics methodologies applied

Human ovarian cancer cells were treated with imatinib for either 6 or 24 h. Two dimensional gel electrophoresis and mass spectrometry-based proteomics approaches were employed to identify protein expression patterns and signaling pathways that altered with the treatment of imatinib. Cells were analyzed for PDGFR alpha expression which were then correlated with imatinib sensitivity (Patel *et al*., 2008)

Using 2D gel electrophoresis of overlapping pH ranges from pH 4 to 11, about 4,000 protein spots could be analyzed reproducibly. Proteins whose levels changed between two fold to 30 fold were grouped according to whether changes were in the same direction at both time points of treatment with respect to the control, or changed their levels only at one of the time points (Patel *et al*., 2008)

Different experiments were applied on the ovarian cancer patients with imatinib, applying mass spectrometry with SILAC (stable isotope labeling by amino acids in cell culture) the comparative study of protein expression in K562 cells were untreated or treated with a clinically relevant

concentration of imatinib. 2-dimensional gel electrophoresis (2-DE) coupled with tandem mass spectrometry (MS/MS) was employed for protein identification and quantification.

SDS-PAGE Separation and In-gel Digestion:

The light and heavy cell lysates were combined at 1:1 ratio (w/w), denatured by boiling in Laemmli loading buffer for 5 min and separated by a 12% SDS-PAGE with 4% stacking gel. The gel was stained with Comassie blue; after destaining, the gel was cut into 20 slices, reduced in-gel with dithiothreitol (DTT) and alkylated with iodoacetamide (IAM). The proteins were digested in-gel with trypsin (Promega, Madison, WI) for overnight, after which peptides were extracted from the gels with 5% acetic acid in H2O and subsequently with 5% acetic acid in CH3CN/H2O (1:1, v/v). The resultant peptide mixtures were dried and stored at −20°C for further analysis.

Benzidine Staining:

The untreated and imatinib-treated K562 cells were collected without removing the media and mixed, at 1:1 (v/v) ratio for 4 min, with an aqueous solution containing 0.2% benzidine dihydrochloride, 0.6% H2O2 and 0.5 M acetic acid. The cells were spotted to a hemocytometer and pictures were taken using a Nikon Eclipse TI microscope.

Mass Spectrometry Identification:

MALDI mass spectra were acquired on a Voyager DE STR MALDI-TOF mass spectrometer in positive reflection mode. The mass spectrometer was equipped with a pulsed nitrogen laser operated at 337 nm with 3 ns duration pulses. The acceleration voltage, grid voltage, and delayed extraction time were set as 20 kV, 65%, and 190 ns, respectively. Each mass spectrum was acquired from an average of 100 laser shots.

Online LC-MS/MS analysis was carried out on an Agilent 6510 Q-TOF system with an Agilent HPLC-Chip Cube MS interface (Agilent Technologies, Santa Clara, CA). The sample injection, enrichment, desalting, and HPLC separation were carried out automatically on the Agilent HPLC Chip with an integrated trapping column (160 nL) and a separation column (Zorbax 300SB-C18, 75 μm×150 mm, 5 μm in particle size)

Data Processing:

The LC-MS/MS raw data were searched against human IPI protein database (version 3.21) and its reverse complement using Turbo SEQUEST with Bioworks 3.2 (Thermo Fisher Scientific, San Jose, CA) for protein identification. Cysteine carbamido methylation was set as a fixed modification. Methionine oxidation (+16 Da) as well as lysine (+8 Da) and arginine (+10 Da) mass shifts introduced by heavy isotope labeling were considered as variable modifications. Peptide filters with appropriate cross-correlation (Xcorr $\geq 1.9, \geq 2.4, \geq 3.5$ for peptide ions that are singly, doubly, and triply charged) and delta correlation ($\Delta Cn \geq 0.1$) scores were used to sort the search results. The protein false discovery rate was less than 1%.

Census was developed (Yates *et al*., 2011) for protein quantification. The Turbo SEQUEST search results were first filtered using DTASelect 15 and ion chromatograms were generated for peptide ions based on their m/z values. Peptide ion intensity ratios were subsequently calculated in Census from peak areas found in each pair of extracted-ion chromatograms. The ratio measurement results were filtered by setting thresholds of Determinant Factor as 0.5 and Outlier p-Value as 0.01.

Result:

Our results revealed that, among the 1344 quantified proteins, 73 had significantly altered levels of expression induced by imatinib and could be quantified in both forward and reverse SILAC labeling experiments, with 26 and 47 being up- and down-regulated respectively (Xiong *et al*., 2011)

3.2.2.2 Mechanism of action of Imatinib

Imatinib is a 2-phenyl amino pyramiding derivative. This is specific for tyrosine kinase domain in ABL (Abelson proto oncogene), c-KIT and platelet derived growth factor receptor (PDGFR). In CML, the Philadelphia chromosome leads to a fusion protein of ABL with BCR. The active sites of tyrosine kinases each have a binding sites for ATP. The enzymatic activity catalyzed by a tyrosine is the transfer of the terminal phosphate from ATP to tyrosine residues on its substrate, known as protein tyrosine phosphorylation. It works by binding close to the ATP binding site of BCR-ABL and inhibit the enzyme activity of the protein

ABL on chromosome 9 is translocated to chromosome 22 and fuses with BCR

↓

Transcription and translation occurred

↓

BCR-ABL fusion protein is formed

↓

This BCR-ABL fusion protein have binding sites for ATP

↓← **Imatinib**

Phosphorylation occurs by transferring the terminal phosphate from ATP (ATP →ADP)

↓

Mitogenic signaling and genomic instability increased

↓

Apoptosis and stromal regulation

↓

Chronic Myeloid Leukemia (CML)

Figure 13: Mechanism of action of imatinib

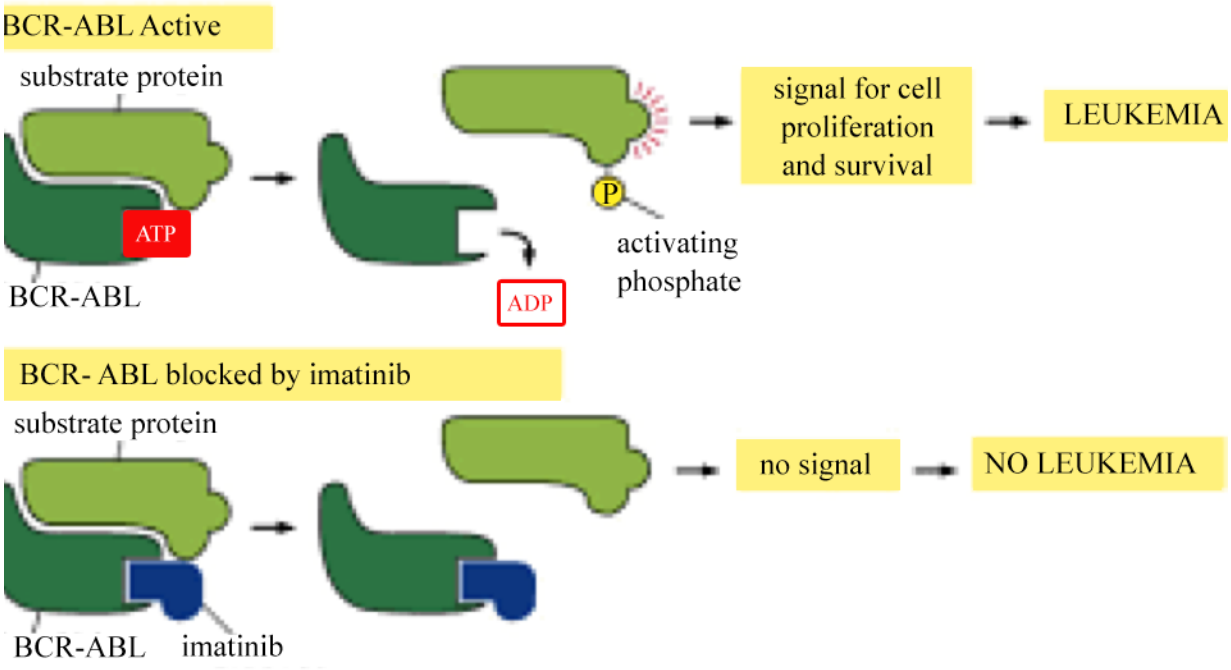

Figure 14: Binding site of imatinib

3.2.2.3 Some other drugs

Dasatanib:

- 300 times more potent than imatanib
- Binds to multiple conformational states (open and closed), unlike imatanib
- Very new drug which was approved in 2006 for further clinical trials
- Side Effects:
- Myelosuppression which can lead to bleeding
- Infection and fatigue,
- Fluid retention,
- Headache,
- Skin rash, nausea
- Can be used in patients who are resistant to imatanib

Nilotonib:

- Structurally similar to imatanib
- 20 to 50 times more potent than imatanib
- Binds in the closed conformation
- Not FDA approved, still under scrutiny

3.2.3 BCR-ABL fused receptor: The target site of imatinib

3.2.3.1 ABL receptor:

- Non receptor tyrosine kinase
- Two isoforms of ABL (Human type 1 and human type 2)
- IN amino terminal of ABL, there are different domains Such as: SH 3, SH2 and tyrosine kinase domain (ɣ-kinase)
- Distributed in both nucleus and cytoplasm
- It induces signals from cell-surface growth factor and adhesion receptors to regulate cytoskeleton structure.

3.2.3.2 BCR receptor:

- A signaling protein containing multiple domains

3.2.3.2 Fusion of BCR-ABL receptors: Philadelphia chromosome

A piece of chromosome 9 and a piece of chromosome 22 break off and trade places. The fusion between BCR-ABL, is called Philadelphia chromosome occurs the changed 22 chromosome is called Philadelphia chromosome

Normal chromosome 9+ normal chromosome 22

Chromosome breakdown

Changed chromosome 22

Fusion between BCR-ABL

Figure 15: Fused BCR-ABL receptors

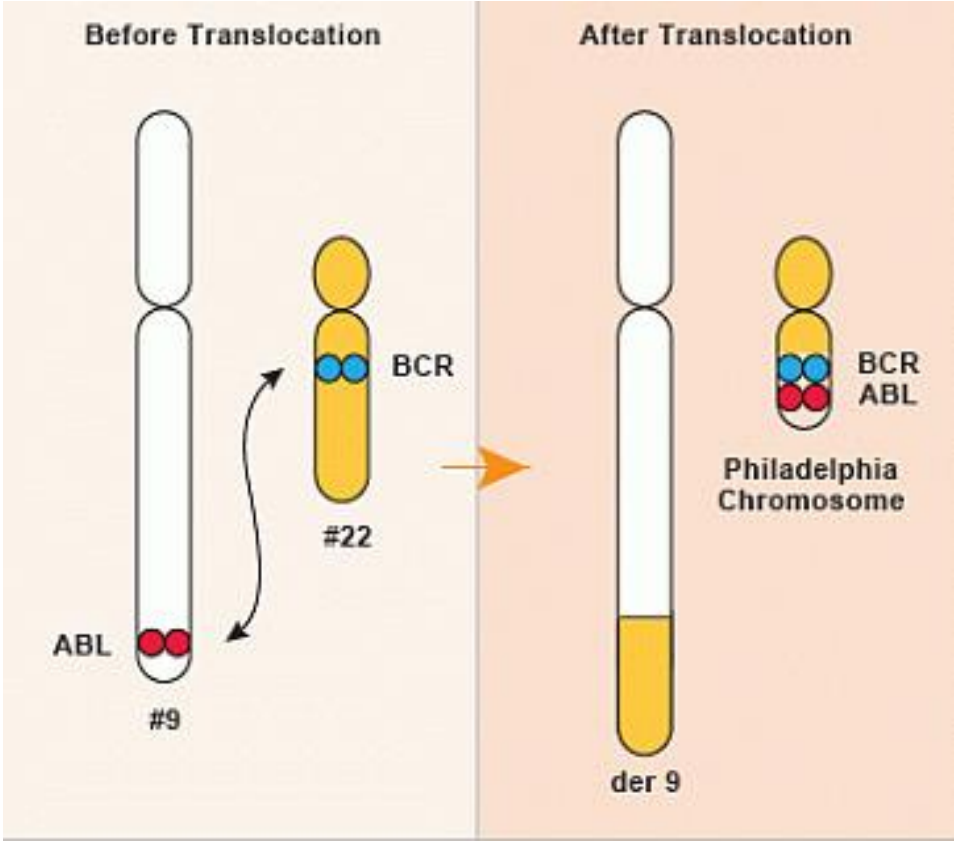

Figure 16: Fusion of BCR-ABL receptors

CHAPTER 4

CONCLUSION

Conclusion

Drug design is the design and optimization of a chemical structure with the goal of identifying a compound suitable for clinical testing named a drug candidate. It is based on knowledge of the drug's three-dimensional structure and how its shape and charge cause it to interact with its biological target, ultimately eliciting a medical effect. Biomarker identification is the new approach of drug design and drug development. This is accomplished by targeting a gene or protein. This gene or protein identification can be approached through genomics and proteomics methodologies. Different drugs such as monoclonal antibodies and tyrosine kinase inhibitors are discovered or developed by genomics and proteomics. But some drugs also gain resistance against diseases. As drug design and discovery is a time consuming process, so it is not so easy to have a drug successfully

CHAPTER 5

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