Technical University of Crete, GREECE

Identification of Combination Therapy Models using Adaptive Fuzzy Systems operating in conjunction with High Order Neural Networks Functions

Thesis in Systems Biology

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From

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To Giorgos, Anna, Eleni and Giedre…

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1 Introduction

Systems biology is a new field of biology that aims to develop a system-level understanding of biological systems and provide information that will be useful to molecular biology and medicine. System-level understanding of course, requires a set of principles and methodologies that links the behaviors of molecules to system characteristics and functions. Ultimately, cells, organisms, and human beings will be described and understood at the system-level grounded on a consistent framework of knowledge that is underpinned by the basic principles of physics.

In this thesis, we will simulate and identify combination (or multicomponent) therapy models using a neuro fuzzy approach. Systems Biology provides a perspective from which to understand at a molecular level the basis for the efficacy of some multicomponent drugs. Combination therapy is a very important field in modern medicine and Systems Biology can provide the help for finding answers and solutions in many problems that exist - and mostly in how to predict this efficacy we mentioned.

Starting this thesis, in Chapter 2 we make an introduction to molecular biology and its most basic concepts. Cells, DNA, genomes and proteins are presented in order to introduce biological issues that will help the reader to comprehend better our work. We also make an introduction to the field of Systems Biology, relevant to the application of systems theory to biology.

In Chapter 3, we make a presentation of combination therapy and multicomponent drug; drugs that act selectively on a specific combination of target activities found in diseased cells. We examine the Systems Biology approach of how to predict what we call synergy of multicomponent drugs and we simulate four models of combination therapy pathways.

Chapter 4 is referred to fuzzy systems. We examine basic concepts of the fuzzy theory, such as membership functions, fuzzy sets and fuzzy rules through the presentation of the procedure needed for designing a fuzzy controller. Of course, we conclude presenting a neuro fuzzy identification scheme we use in our work based on Addaptive Fuzzy Systems and High Order Neural Network Functions.

Finally, in Chapter 5 we applicate this identification scheme to the combination therapy models mentioned in Chapter 3. Our work is implemented in Matlab code and the results as we will see are really successful. Of course, we end with the conclusions and possible future work in the field of Systems Biology.

2 Biological Background – Systems Biology

Since this is a thesis in the field of systems biology, we will start with an introduction to molecular biology and its most basic concept; the cell and its general structure. The development of multicellular organisms makes it necessary to have mechanisms for communication between cells, which gives to these organisms the organized structure and ability to maintain different tissues. The basis for intercellular communication is intracellular signaling; the cells' ability to receive and compute stimuli reaching the cell membrane. In this chapter we also discuss about DNA, genomes and proteins since they are the basis for a better understanding of biological issues and will help the reader to comprehend better our work.

Continuing in this chapter, we will examine opportunities and challenges for the application of systems theory to biology in the post-genomic era - an area of research also referred to as *Systems Biology*. While the developments in genomics and bioinformatics have brought tremendous advances in our understanding of molecular biology, it is increasingly recognized that it is the temporal interaction amongst large numbers of molecules that determines phenomena observed at higher (metaboli, cellural, or physiological) levels. Systems Biology is the field which takes a closer look to this dynamic or systems perspective and integrative approach (combining data from the genome, transcriptome, proteome, metabolome etc.), offering control theorists and engineers a great variety of opportunities and challenges.

2.1 Molecular Biology

2.1.1 Cell

Cells are the smallest units of living organisms, capable of the basic life processes: growth, sensitivity, movement, respiration (turning 'food' into energy), nutrition (taking in nutrients), excretion (getting rid of waste) and reproduction. All

living things in this world are composed of cells. Some microscopic organisms, such as most bacteria and protozoa, are unicellular (consist of a single cell). Other organisms, such as humans, plants and animals are multicellular (composed of a great many cells working in concert). But whether it makes up an entire organism or is just one of trillions in a human being, the cell is marvel of design and efficiency. Cells implement thousands of biochemical reactions per minute and reproduce new cells that give life a continuity.

Cells have a great variety of size and shape. A typical cell size is 10 μm, while the bacterium mycoplasma, the smallest cell, measures 0.0001 mm in diameter. The largest biological cell is often cited as the ostrich egg, which is about 15 cm long and weighs about 1.4 kg. This is actually a myth, since there exist nerve cells in especially long animals, such as the Giant Squid and Colossal Squid, which may have nerve cells as long as 12 m. This variety of sizes we can find it also in human cells; from small red blood cells that measure 0.00076 mm to liver cells that may be ten times larger. About 10,000 average – sized human cells can fit on the head of a pin. As about cells' shape, there is an amazingly great variation from organism to organism and even from cell to cell in the same organism. In humans, the outermost layers of skin cells are flat, while muscle cells are long and thin. Some nerve cells, with their elongated, tentacle – like extensions, suggest an octopus. Plant cells typically resemble boxes or cubes, and the amoeba, a protozoan, has an irregular form that changes shape as it moves around. In multicellular organisms shape is typically tailored to the cell's job. For example, flat skin cells pack tightly into a layer that protects the underlying tissues from invasion by bacteria. Long, thin muscle cells contract readily to move bones. The numerous extensions from a nerve cell enable it to connect to several other nerve cells in order to send and receive messages rapidly and efficiently.

A remarkable characteristic of cells is their independence. Each cell is at least somewhat self-contained and self-maintaining. The cell constantly is moving lively, shuttling essential molecules from place to place to carry out the business of living. Despite their individuality, however, cells also display a great ability to join, communicate and coordinate with other cells. The human body, for example, consists of an estimated 100 trillion cells. Cells of different kind are organized into specialized groups called tissues. Different tissues, in turn, are assembled into organs (e.g. brain, heart, liver etc.), specialized to perform a specific function or group of functions. A

group of related organs forms an organ system (or biological system) - such as the circulatory, muscular, or nervous system - which in turn, form the human body.

Cells are consisted of molecules, non-living structures formed by the union of two or more atoms held together by covalent bonds. A molecule may consist of atoms of the same chemical element (e.g. oxygen - O_2), or of different elements (e.g. water - H_2O). Small molecules serve as building blocks for larger molecules. There are four major molecules that underlie cell structure and also participate in cell functions; proteins, nucleic acids, carbohydrates, and lipids. For example, a tightly organized arrangement of lipids, proteins and protein – sugar compounds forms the plasma membrane of certain cells. The organelles, membrane – bound compartments in cells, are built largely from proteins. Biochemical reactions in cells are guided by enzymes, specialized proteins that speed up chemical reactions. The deoxyribonucleic acid (DNA) is the nucleic acid that contains the hereditary information for cells. It works with ribonucleic acid (RNA) to build the thousands of proteins the cell needs.

Cells fall into one of two categories: prokaryotic or eukaryotic. Prokaryotic cells are usually independent and they lack a cell nucleus, or any other membrane-bound organelles, while eukaryotic cells, which have a cell nucleus, are found in animals, plants, fungi, and protists. Most of prokaryote organisms are unicellular, but a few prokaryotes (e.g. myxobacteria) have multicellular stages in their life cycles.

The prokaryotes are divided into two domains; the bacteria and the archaea, which share a similar overall structure. Prokaryotic cells are the smallest, ranging in size from 0.0001 mm to 0.003 mm in diameter. As about their shape, it can be rod like, spherical, or spiral. They are surrounded by a protective cell wall and live in a watery environment. Tiny pores in the cell wall enable water and the substances dissolved in it, such as oxygen, to flow into the cell; these pores also allow wastes to flow out. In spite of their simplicity in construction, prokaryotic cells display extremely complex activity, having a much greater range of biochemical reactions than those found in the eukaryotic cells.

A prokaryotic cell has three architectural regions. Flagella and pili, on the outside, project from the cell's surface. Enclosing the cell is the cell envelope - generally consisting of a cell wall covering a plasma membrane. However, some bacteria also have a further covering layer called a capsule. The plasma membrane, composed of two layers of flexible lipid molecules, is both supple and strong. Unlike the cell wall, whose open pores allow the unregulated traffic or materials in and out of

the cell, the plasma membrane is selectively permeable. Thus, the plasma membrane actively separates the cell's contents from its surrounding fluids. Inside the cell is the cytoplasmic region that contains the cell genome (DNA). Cytoplasm is the semi fluid that fills the cell. Composed of about 65 % water, the cytoplasm is packed with up to a billion molecules per cell, a rich storehouse that includes enzymes and dissolved nutrients, such as sugars and amino acids. The water provides a favorable environment for the thousands of biochemical reactions that take place in the cell. The DNA is about 1000 times the length of the cell, and to fit inside, repeatedly twists and folds to form a compact structure called a chromosome. The chromosome in prokaryotes is circular, and is located in a region of the cell called the nucleoid. Also immersed in the cytoplasm are ribosomes, the only organelles in prokaryotic cells like structures, which we can say that play the role of the cell's protein factories. Following the instructions encoded in the DNA, ribosomes produce hundreds of proteins every minute.

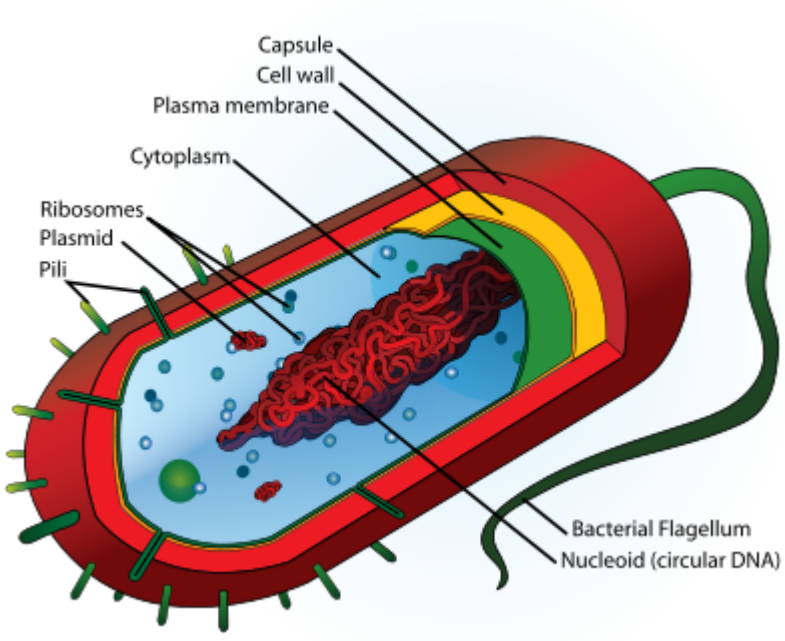


Figure 2.1 Diagram of a typical prokaryotic cell (from Wikipedia, the free encyclopedia)

The eukaryotic cell is about 10 times the size of a typical prokaryote and can be as much as 1000 times greater in volume. It is much more complex than a prokaryote cell, having a nucleus and a lot of other membrane-enclosed organelles that prokaryotes don't have. Like separate rooms of a house, these organelles enable specialized functions to be carried out efficiently. The eukaryotes' plasma membrane

resembles that of prokaryotes in function, with minor differences in the setup. In animal cells, the plasma membrane, rather than a cell wall, forms the cell's outer boundary. With a design similar to the plasma membrane of prokaryotic cells, it separates the cell from its surroundings and regulates the traffic across the membrane. The major difference between prokaryotes and eukaryotes is that eukaryotic cells contain membrane-bound compartments in which specific metabolic activities take place. Most important among these is the presence of a cell nucleus, a membrane-delineated compartment that houses the eukaryotic cell's DNA, which is organized in one or more linear molecules, called chromosomes. The nucleus is the largest organelle in an animal cell. Unlike the circular prokaryotic DNA, long sections of eukaryotic DNA pack into the nucleus by wrapping around proteins. The nucleus is surrounded by a double-layered membrane that protects the DNA from potentially damaging chemical reactions that occur in the cytoplasm. Messages pass between the cytoplasm and the nucleus through nuclear pores, which are holes in the membrane of the nucleus. In each nuclear pore, molecular signals flash back and forth as often as ten times per second. For example, a signal to activate a specific gene comes in to the nucleus and instructions for production of the necessary protein go out to the cytoplasm.

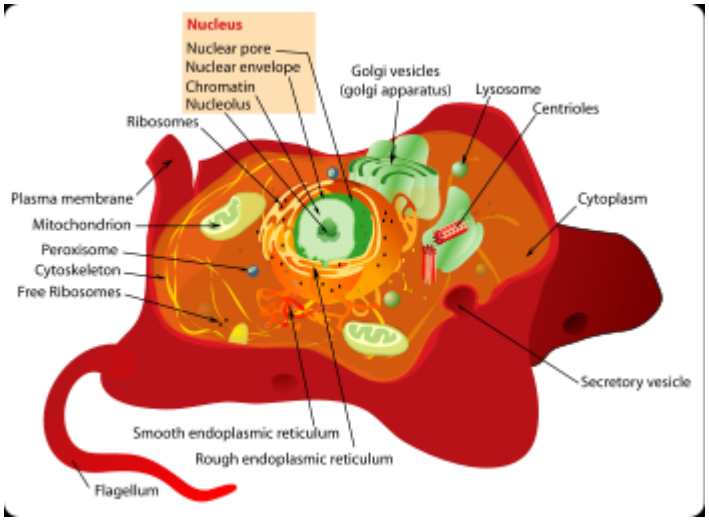


Figure 2.2 Structure of a typical animal cell (from Wikipedia, the free encyclopedia)

The nucleus is surrounded by a double membrane (nuclear envelope), with pores that allow material to move in and out. Various tube- and sheet-like extensions of the nuclear membrane form what is called the endoplasmic reticulum (ER), which

is involved in protein transport and maturation. This organelle tunnels through the cytoplasm, folding back and forth on itself to form a series of membranous stacks. It takes two forms; the rough ER (RER), where ribosomes are attached, and smooth ER (SER), which lacks ribosomes and has an even surface. The ribosomes in eukaryotic cells have the same function as those in prokaryotic cells - protein synthesis - but they differ slightly in structure. Eukaryote ribosomes that are bound to the endoplasmic reticulum help in assembling proteins that typically are exported from the cell. The ribosomes work with other molecules to link amino acids to partially completed proteins. These incomplete proteins then travel to the inner chamber of the endoplasmic reticulum, where chemical modifications, such as the addition of a sugar, are carried out. Chemical modifications of lipids are also carried out in the endoplasmic reticulum. The endoplasmic reticulum and its bound ribosomes are particularly dense in cells that produce many proteins for export, such as the white blood cells of the immune system, which produce and secrete antibodies. There are also some ribosomes that manufacture proteins, which are not attached to the endoplasmic reticulum (free ribosomes). They typically make proteins (many of them enzymes), that remain in the cell. The SER, in turn, has some winding channels where are the enzymes needed for the construction of molecules, such as carbohydrates and lipids. It is extremely important in liver cells, where it also serves to detoxify substances such as alcohol, drugs, and other poisons.

The proteins synthesized from the RER generally enter vesicles, which bud off from the SER. In most eukaryotes, these proteins are transported from free and bound ribosomes to the Golgi apparatus (also called the Golgi body, Golgi complex, or dictyosome), an organelle that resembles a stack of deflated balloons. The primary function of the Golgi apparatus is to process and package the macromolecules such as proteins and lipids that are synthesized by the cell. It has great importance for the processing of proteins for secretion, since it is packed with enzymes that complete this processing. The completed protein then leaves the Golgi apparatus for its final destination inside or outside the cell. During its assembly on the ribosome, each protein has acquired a group of from 4 to 100 amino acids called a signal. The signal works as a molecular shipping label to direct the protein to its proper location.

In general, there are several types of organelles within an animal cell. Lysosomes and mitochondria for example, are of great interest and can be numerous (from hundreds to thousands). Lysosomes are small, often spherical organelles that

function as the cell's recycling centre and garbage disposal. They contain powerful digestive enzymes that break down the contents of food vacuoles and ship their building blocks to the cytoplasm where they are used to construct new organelles. It was believed they can only be found in animal cells, but there is new evidence that supports that they may also exist in plant cells. Lysosomes also decompose and recycle proteins, lipids, and other molecules.

Mitochondria are self-replicating organelles that occur in various numbers, shapes, and sizes in the cytoplasm of nearly all eukaryotic cells. They are the powerhouses of the cell (described sometimes as 'cellular power plants') because they generate most of the cell's supply of adenosine triphosphate (ATP), used as a source of chemical energy. Within these long, slender organelles, enzymes convert the sugar glucose and other nutrients into ATP. This molecule, in turn, serves as an energy battery for countless cellular processes, including the shuttling of substances across the plasma membrane, the building and transport of proteins and lipids, the recycling of molecules and organelles, and the dividing of cells. Muscle and liver cells are particularly active and require dozens and sometimes up to a hundred mitochondria per cell to meet their energy needs. In addition to supplying cellular energy, mitochondria are involved in a range of other processes, such as signaling, cellular differentiation, cell death, as well as the control of the cell cycle and cell growth. Mitochondria are unusual in that they contain their own DNA in the form of a prokaryote-like circular chromosome; they have their own ribosomes, which resemble prokaryotic ribosomes; and they are only formed by the fission of other mitochondria. They are now generally held to have developed probably from proteobacteria. The few protozoa that lack mitochondria have been found to contain mitochondrion-derived organelles, such as hydrogenosomes and mitosomes.[1],[2],[3],[4],[5]

2.1.2 DNA

Inside the nucleus of every eukaryotic cell, or in the cytoplasm for prokaryotes, we can find the genetic instructions that provide almost all the information necessary for a living organism (and for some viruses) to grow and function. These instructions are encrypted in a nucleic acid called Deoxyribonucleic

acid (DNA). The main role of DNA molecules is to preserve, copy and transmit information within cells and from generation to generation. We could say that DNA is something like a set of blueprints or a recipe, or a code, since it contains the instructions needed to construct other components of cells, such as proteins and RNA (Ribonucleic acid) molecules. The DNA segments that carry this genetic information are called genes, but other DNA sequences have structural purposes, or are involved in regulating the use of this genetic information.

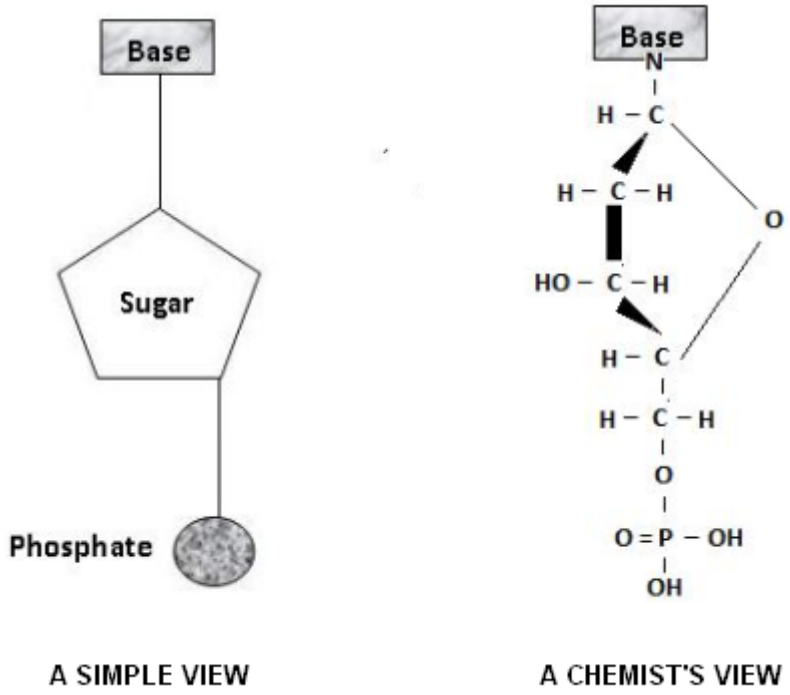


Figure 2.3 Nucleotides of DNA

In most of these cases DNA molecule consists of two ribbon–like strands and wrap around each other, resembling a twister ladder. A single strand of DNA is a biomolecule consisting of many linked, smaller components called nucleotides. Each nucleotide has three components: a phosphate group, a sugar and a nitrogen-containing base (alkaline chemical substance, in particular the cyclic nitrogen compounds found in DNA and RNA). In DNA, the sugar is always deoxyribose. The different types of nucleotide differ only in the nature of the nitrogen-containing base. In DNA there are four alternative bases: adenine, thymine, guanine and cytosine. When writing out genetic information these bases are designed by the letters A, T, G and C respectively. The phosphate groups and the deoxyribose sugars form the

backbone of each strand of DNA. The bases are joined to the deoxyribose and stick out sideways. Each nucleotide has two distinct ends, the 5' End and the 3' End; so that the 5' End of a nucleotide is linked to the 3' End of another nucleotide by a strong chemical bond, thus forming a long, one–dimensional chain (backbone) of a specific directionality. Therefore, each DNA single strand is represented by a character string, which, by convention specifies the 5' to 3' direction when read from left to right.

To understand how nucleotides are joined, we must clarify the situation by numbering the carbon atoms of the sugar molecule. Figure (2.4) shows the convention for numbering nucleotides.

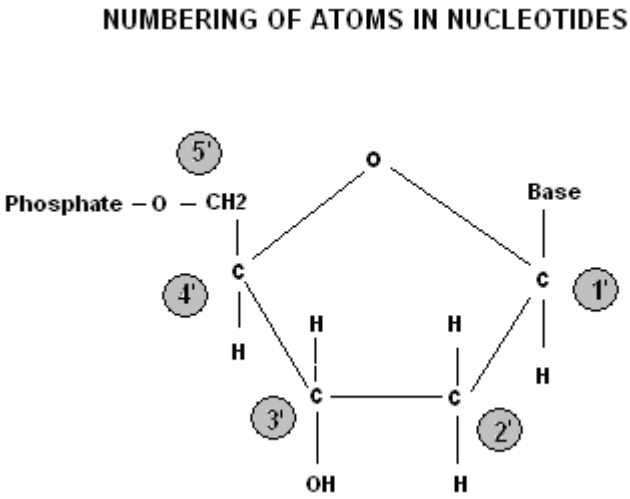


Figure 2.4

Nucleotides are joined by linking the phosphate on the 5' end of the deoxyribose of one to the 3' position of the next, as it's shown in Figure (2.5).

In practice, DNA is normally found as a double stranded molecule. Single DNA strands tend to form double helices with other single DNA strands. Thus, a DNA double strand contains two single strands called complementary to each other because each nucleotide of one strand is linked to a nucleotide of the other strand by a chemical bond, so that A is linked to T and vice versa, and C is linked to G and vice versa. The bases A and G are referred to as the purine bases as they contain a double ring structure known as a purine ring. The other two bases, C and T, are the pyrimidine bases, since they contain a single, pyrimidine ring. Each base pair consists

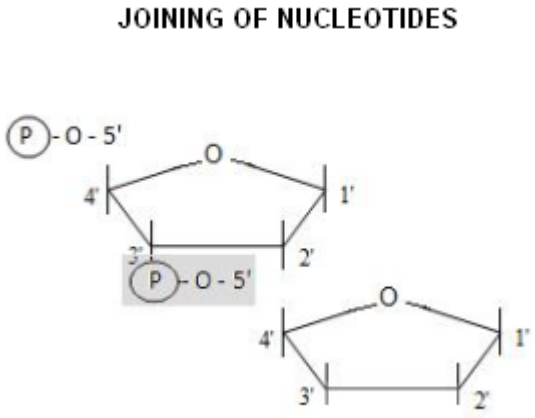


Figure 2.5

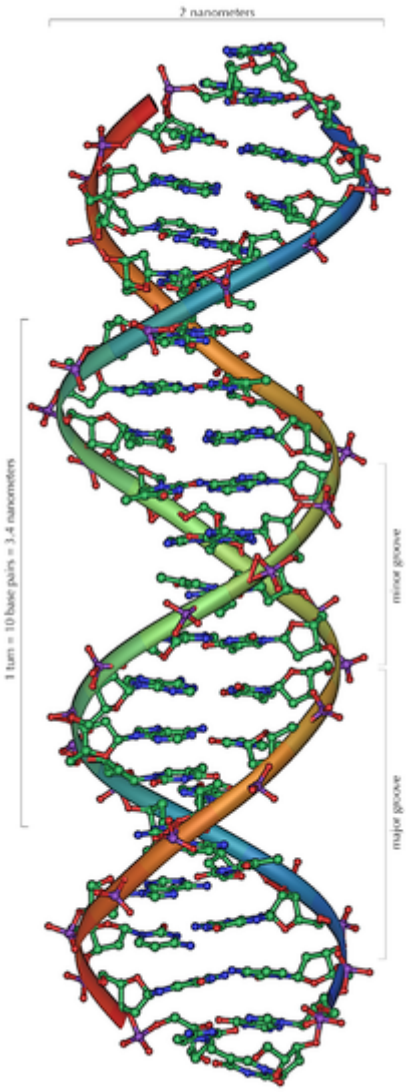


Figure 2.6 Structure of part of a DNA double helix (from Wikipedia, the free encyclopedia)

of one double size purine base paired with a smaller pyrimidine base. In double stranded DNA each base pair is held together by linkages known as hydrogen bonds. The A – T base pair has two hydrogen bonds and the G – C base pair is held together by three. Hydrogen bonds are very weak, but since a molecule of DNA usually contains millions of base pairs, the added effect of millions weak bonds is strong enough to create a stable, double helical structure.

The hydrogen bonding in DNA base pairs uses either oxygen (O) or nitrogen (N), giving three alternative arrangements. In each case the hydrogen (H) is held between the other two atoms and serves to link them together. Before hydrogen bonds form and the bases pair off, the hydrogen atom is found attached to one or the other of the two bases.[6],[7],[8]

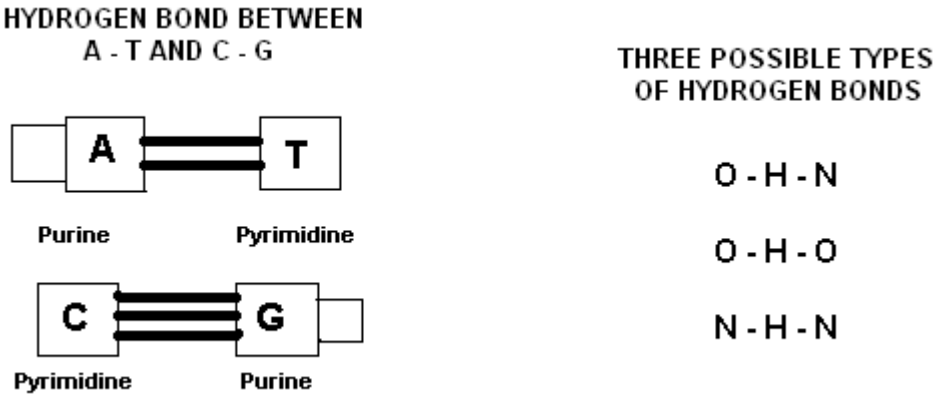


Figure 2.7 Hydrogen bonds

2.1.3 Genes

The fundamentals of modern genetics were laid when Gregor Mendel found that hereditary information is made up of discrete fundamental units which we now call genes. The realization that genes are made up of molecules that obey the laws of chemistry has opened the way both to a deeper understanding of life and to its artificial alteration by genetic engineering. Genes are the basic unit of heredity in living organisms. All living organisms depend on genes. They hold the information to build and maintain their cells and pass genetic traits to offspring.

Genes consist of a length of DNA that contains instructions (codes) for making a specific protein. Through these proteins, our genes influence almost everything about us; for example how tall we will be, how we process food, and how we respond to infections and medicines. Each gene is responsible for a single inherited property or characteristic of the organism. Certain properties of higher organisms, such as height or skin color, are due to the combined action of multiple genes. Consequently, in these cases there is a gradation of the property. Such multi-gene characteristics they are still very difficult to analyze, especially if more than two or three genes are involved. Although most of our cells have the same genes, not all genes are active in every cell. Heart cells for example, synthesize proteins required for the heart's structure and function or liver cells make liver proteins. In other words, not all the genes are switched 'on' and expressed as proteins within every cell. Within an individual cell, the same genes may be switched 'on' at some times and switched 'off' at other times.

The complete set of genes in an organism or cell is called a genome. Each gene is found in linear order and is a major component of structures known as chromosomes. In prokaryotes, the vast majority of genes are located on a single chromosome of circular DNA, while eukaryotes usually possess multiple individual linear DNA helices. Each chromosome has some accessory protein molecules which help maintain its structure and is an exceedingly long single molecule of DNA. Genes that appear together on one chromosome of one species may appear on separate chromosomes in another species. With the exception of identical twins, the sequence of the bases is different for everyone, which makes each organism unique. In sexually reproducing organisms, one copy is normally inherited from each parent.

Although we all look quite different from one another, we are surprisingly similar at the DNA level, considering that the DNA of most humans is 99.9% the same. Only about 3 million base pairs are responsible for the differences among us, which is only 1‰ of our DNA. However, these DNA base sequence variations influence most of our physical differences and many other of our characteristics. Sequence variations that occur in our genes, and the resulting difference forms of the same gene are called alleles. Humans can have two identical or two different alleles for a particular gene.[6],[7],[9]

2.1.4 Proteins

Proteins are among the fundamental molecules of biology, being common to all life present on Earth today. They can be considered as the major structural constituent of living beings. According to the Central Dogma of Molecular Biology, proposed by Francis Crick in 1958, information is transferred from DNA to RNA and from RNA to proteins. Molecules whose primary role is to carry information (nucleic acids like DNA and messenger RNA) are basically linear molecules with a regular repeating structure. Molecules that form cellular structures or have active roles carrying out reactions are normally folded into three-dimensional (3-D) structures. These include both proteins and certain specialized RNA molecules (rRNA and tRNA).

Like other biological macromolecules such as nucleic acids, proteins are essential parts of organisms and participate in every process within cells. Virtually, all the complex biochemical functions of the living cell are performed by protein–based catalysts called enzymes. Moreover, most of scaffolding that holds cells and organelles together is made of proteins. In addition to their catalytic functions, proteins transmit and commute signals from the external environment, duplicate genetic information, transform the energy in light and chemicals with astonishing efficiency, convert chemical energy into mechanical work, carry molecules between cell compartments. Proteins are also necessary in animals' diets, since animals cannot synthesize all the amino acids they need and must obtain essential amino acids from food. Through the process of digestion, animals break down ingested protein into free amino acids that are then used in metabolism.

All proteins are biomolecules consisting of many linked, smaller components called amino acids. Broadly speaking, amino acids are molecules that contain both amino (-NH2) and carboxylic acid (-COOH) functional groups. In biochemistry, amino acids consist of a primary amine bound to an aliphatic carbon (-carbon) atom, which in turn is bound to carboxylic acid group. At least one hydrogen atom is bound to the –carbon; in addition, the –carbon bears a side chain, which is different for different amino acids.

We can subdivide proteins into four main categories:

1) structural proteins,

2) enzymes,

3) regulatory proteins, and

4) transport proteins.

Structural proteins are found making up many subcellular structures. The flagella with which bacteria swim around, the microtubules used to control traffic flow inside cells of higher organisms, the fibers inside a muscle cell, and the outer coats of viruses are some few examples of structures built using proteins.

Enzymes are proteins that carry out chemical reactions. An enzyme first binds another molecule, known as its substrate, and then performs some chemical operations with it. Some enzymes bind only a single substrate molecule; others may bind two or more, which they react together to make the final product. In any case, the enzyme needs an active site, a pocket or cleft in the protein, where the substrate binds and the reaction occurs. The active site is produced by folding up the polypeptide chain correctly so that amino acid residues that were spread out at great distances in the linear chain now come together and will cooperate in the enzyme reaction.

Regulatory proteins vary enormously. Many of them can bind both small signal molecules and DNA. The presence or absence of the signal molecule determines whether or not the gene is switched on. Although regulatory proteins and transport proteins are not enzymes, they also bind other molecules and so they also need ‘active sites’ to accommodate them.

Transport proteins are found mostly in biological membranes where they carry material from one side to the other. Nutrients, such as sugars, must be transported into cells of all organisms, whereas waste products are deported. Multicellular organisms also have transport proteins to carry materials around the body.

While there are theoretically billions of possible amino acids, most proteins are formed of only 20 amino acids, the natural or proteogenic amino acids. These amino acids are connected with strong bonds, one after the other, forming a long chain (backbone) of a specific directionality. Protein molecules tend to fold into complex three–dimensional (3-D) structures forming weak bonds between their own atoms, and they are responsible for carrying out nearly all of the essential functions in the living cell by properly binding to other molecules with a number of chemical bonds connecting neighboring atoms. Although we do not yet know how to reliably

predict protein 3-D structures from their one-dimensional amino acid sequences, we do know that nearly all proteins in the living cell are uniquely determined by these sequences. Therefore, the amino acid character strings determine the functions of proteins. In fact, protein functions are ultimately determined by the DNA character string because it is the digital information in the DNA nucleotide sequences that determine the amino acid sequences; each protein character string is generated based on information in genes, which are regions in the DNA character strings. This process is shown schematically in Figure (2.8) in which, for simplicity, the intermediate role of another biomolecule (RNA) is omitted, as it is the fact that sometimes the same gene may code for multiple proteins through a process called alternative splicing.

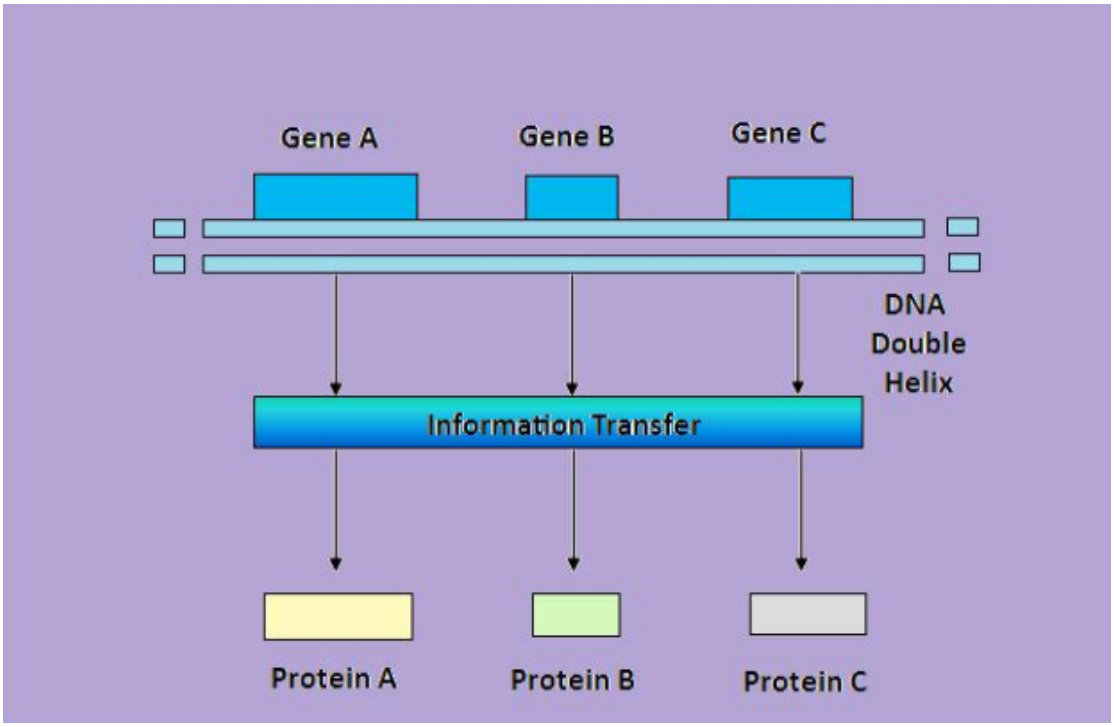


Figure 2.8 Information is transferred from each gene to make a protein

Protein synthesis is governed by the genetic code which maps each of the 64 possible triplets (codons) of DNA characters into one of the 20 possible amino acids (or into a punctuation mark, like a stop codon, signalling termination of protein synthesis). Figure (2.9) shows the genetic code in which the 20 amino acids are designated by both their one-letter and three-letter symbols. A particular triplet, ATG, serves as the START codon and it also codes for the M amino acid (methionine): thus, methionine appears as the first amino acid of proteins, but it may also appear in other locations. We also see that there are three STOP codons indicating termination of

amino acid chain synthesis, and the last amino acid is the one generated by the codon preceding the STOP codon.[6],[10],[11]

AAA: K (Lys)	GAA: E (Glu)	TAA: STOP	CAA: Q (Gln)
AAG: K (Lys)	GAG: E (Glu)	TAG: STOP	CAG: Q (Gln)
AAT: N (Asn)	GAT: D (Asp)	TAT: T (Tyr)	CAT: H (His)
AAC: N (Asn)	GAC: D (Asp)	TAC: Y (Tyr)	CAC: H (His)
AGA: R (Arg)	GGA: G (Gly)	TGA: STOP	CGA: R (Arg)
AGG: R (Arg	GGG: G (Gly)	TGG: W (Trp)	CGG: R (Arg)
AGT: S (Ser)	GGT: G (Gly)	TGT: C (Cys)	CGT: R (Arg)
AGC: S (Ser)	GGC: G (Gly)	TGC: C (Cys)	CGC: R (Arg)
ATA: I (Ile)	GTA: V (Val)	TTA: L (Leu)	CTA: L (Leu)
ATG: M (Met)/START	GTG: V (Val)	TTG: L (Leu)	CTG: L (Leu)
ATT: I (Ile)	GTT: V (Val)	TTT: F (Phe)	CTT: L (Leu)
ATC: I (Ile)	GTC: V (Val)	TTC: F (Phe)	CTC: L (Leu)
ACA: T (Thr)	GCA: A (Ala)	TCA: S (Ser)	CCA: P (Pro)
ACG: T (Thr)	GCG: A (Ala)	TCG: S (Ser)	CCG: P (Pro)
ACT: T (Thr)	GCT: A (Ala)	TCT: S (Ser)	CCT: P (Pro)
ACC: T (Thr)	GCC: A (Ala)	TCC: S (Ser)	CCC: P (Pro)

Figure 2.9 The genetic code

2.2 Systems Biology

2.2.1 General Overview

The ultimate goal of biology is to understand every detail and principle of biology systems. Almost fifty years ago, Watson and Crick indentified the structure of DNA; the beauty of their work was that they grounded biological phenomena on a molecular basis[12]. This made it possible to describe every aspect of biology, such as heredity, development, disease and evolution, on a solid theoretical ground. Biology

became part of a consistent framework of knowledge based on fundamentals laws of physics.

Since then, the field of molecular biology has emerged and enormous progress has been made. Molecular biology enables us to understand biological system as molecular machines. Today, we have in-depth understanding of elementary process behind heredity, evolution, development and disease. Such mechanisms include replication, transcription, translation and so forth. Large numbers of genes and the functions of their transcriptional products have been indentified, with the symbolic accomplishment of the complete sequencing of DNA. Methods to obtain extensive gene expression profiles are now available that provide comprehensive measurement of the mRNA level. Measurement of protein level and their interactions is also making process. In parallel with such efforts, various methods have been invented to disrupt the transcription of genes, such as loss-of-function knockout of specific genes and RNA interference (RNAi).[13],[14]

Systems biology is a new field of biology that aims to develop a system-level understanding of biological systems. System-level understanding requires a set of principles and methodologies that links the behaviors of molecules to system characteristics and functions. Ultimately, cells, organisms, and human beings will be described and understood at the system-level grounded on a consistent framework of knowledge that is underpinned by the basic principles of physics.[15]

It is not the first time that system-level understanding of biological system has been pursued; it is a recurrent theme in the scientific community. Norbert Wiener was one of the early proponents of system- level understanding that led to the birth of cybernetics, or biological cybernetics. Ludwig von Bertalanffy proposed general system theory in 1968 in an attempt to establish a general theory of the system, but the theory was too abstract to be grounded[16]. A precursor to such work can be found in the work of Cannon, who proposed the concept of homeostasis[17]. With the limited availability of knowledge from molecular biology, most such attempts have focused on the description and analysis of biological systems at the physiological level. The unique feature of systems biology that distinguishes it from past attempts is that there are opportunities to ground system-level understanding directly on the molecular level such as genes and proteins, whereas past attempts have not been able to sufficiently connect system level description to molecular-level knowledge. Thus, although it is not the first time that system-level understanding has been pursued, it is

the first time to have an opportunity to understand biological systems within the consistent framework of knowledge built up from the molecular level to the system level.

The scope of systems biology is potentially very broad and different sets of techniques may be deployed for each research target. It requires collective efforts form multiple research areas, such as molecular biology, high-precision measurements, computer science, control theory and other scientific and engineering fields. Research needs to be carried out in four key areas: genomics and other molecular biology research, computational studies such as simulation, bioinformatics and software tools, analysis of dynamics of the system and technologies for high precision, comprehensive measurements. This constitutes a major multi – disciplinary research efforts that will enable us to understand biological system as systems. To understand the system, it is essential that it can be not only to describe in detail, but also it to comprehend what happens when certain stimuli or disruptions occur. The ultimate goal should be the ability to design the system to meet specific functional properties. It takes more than a simple in – depth description; it requires more active synthesis to ensure that it is completely understood.

The functions of a cell do not reside in the molecules themselves but in their interactions, just as life in emergent, rather than immanent or inherent, property of matter. Although life, or the function of the cell, arises from the material world, they cannot be reduced to it. Systems biology therefore signals a shift, away from molecular characterization and cataloguing of the components in the cell, towards an understanding of functional activity.

The term systems in systems biology refers to systems theory, or more specifically, to dynamic systems theory. Thus, systems biology focuses on dynamics and transient changes occurring within cells. These changes, which in most cases will be molecule concentrations, carry information and are at the root of cellular functions that sustain and develop an organism.[18]

Several methods of modeling such intracellular signal transduction pathways have appeared such a reaction systems using ordinary differential equations, stochastic models, Petri-nets, neuronal networks, rule based systems, and Boolean networks. The dominant concept by which scientists use to organize these processes is chemical kinetic models known as transduction pathways, i.e., networks of biochemical reactions. A pathway is an abstraction, a model, of an observed reality. In

most of cases, these chemical reactions are represented mathematically as differential equations where the changes in the concentrations of reactants and post reaction products are recorded based on the reaction rates, as we will see in more detail further down in this document. Normally such a system of differential equations is too complex to be solved explicitly. Moreover, in most of the cases, such a mathematical model of a biological system is too simple to code the entire detail which the real system encapsulates.[19]

2.2.2 Cells as I/O Systems

One may view cell life as a collection of "wireless networks" of interactions among proteins, RNA, DNA and smaller molecules involved in signaling and energy transfer. These networks process environmental signals, induce appropriate cellular responses, and sequence internal events such as gene expression, thus allowing cells and entire organisms to perform their basic functions.

Research in molecular biology, genomics, and proteomics has provided, and will continue to produce, a wealth of data describing the elementary components of such networks, as well the mapping of intra and inter-cellular signaling networks. The genome encodes, through a particular ordering of the four possible (A,T,C,G) bases in its DNA sequence, a parts list for the proteins that are potentially present in every cell of a given organism. Genomics research has as its objective the complete decoding of this information, both the parts common for a species as a whole as well as the cataloging of differences among individual members. The shape of proteins is what largely determines their function, and thus the elucidation of their three-dimensional structure is a goal of proteomics research. Proteins, which interact with each other through lego-like fitting of parts in lock and key fashion, are the primary components of living things. Among other roles, they form receptors that endow the cell with sensing capabilities, actuators that make muscles move (myosin, actin), detectors for the immune response, enzymes that catalyze chemical reactions, and switches that turn genes on or off. They also provide structural support and help in the transport of smaller molecules, as well as in directing the breakdown and reassembly of other cellular elements such as lipids and sugars. (An intermediate link between genetic

information and the proteins that DNA encodes for is RNA. Until recently, RNA was not believed to be a direct player in cell control mechanisms, but research into microRNA conducted within the past two years is forcing a complete rethinking of their role.) Massive amounts of data are being generated by genomics and proteomics projects, facilitated by sophisticated genetic engineering tools (gene knock-outs and insertions, PCR), and measurement technologies (green fluorescent protein, microarrays, FRET), and there is a widely recognized need to organize and interpret these data.

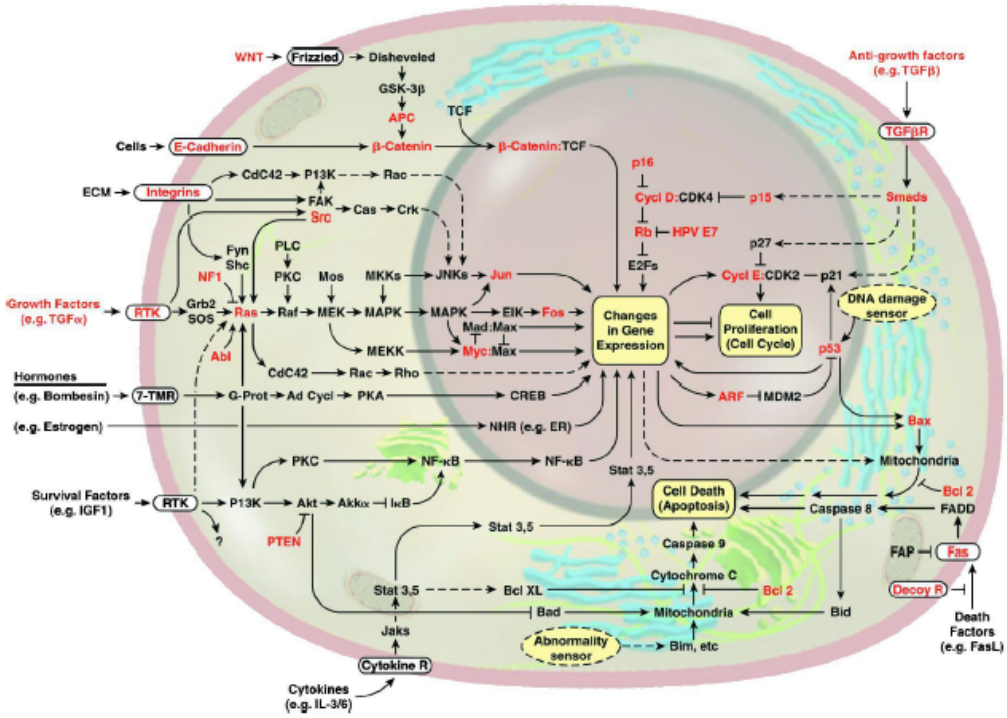


Figure 2.9 Part of the signal transduction network in human cells (Reproduced from [21] with permission from Elsevier)

The control and systems-theory paradigm of input/output systems, built out of simpler components that are interconnected according to certain rules, is a most natural one in this context. Cells receive external information through inputs that may be physical (UV or other radiation, mechanical, or temperature) as well as chemical (drugs, growth factors, hormones, nutrients), and their measurable outputs include chemical signals to other cells, the movement of flagella or pseudopods, the activation of transcription factors, and so forth. Each cell can be thought of, in turn, as composed of a large number of subsystems, involved in processes such as cell growth and

maintenance, division, and death. Indeed, an important theme in the current molecular biology literature is the attempt to understand cell behavior in terms of cascades and feedback interconnections of elementary 'modules'[22],[23]. For example, in Figure (2.9) we can view the wiring diagram of the growth signaling circuitry of the mammalian cell. Of course, such a figure leaves out a lot of information, some known but omitted for simplicity, and some unknown: much of the system has not been identified yet, and the numerical values of most parameters as well as the functional forms of interactions are only very approximately known. However, data is being collected at an amazing rate and better and better models are being constantly obtained.[20]

2.2.3 Cell Chemistry

The cell is the basic building block of which higher organizational levels such as tissues and organs and entire organisms are composed. It is a rather complex environment, consisting of many different components. Because cells are about 70% water, life depends mostly on aqueous chemical reactions. These reactions occur between molecules, where a molecule is a cluster of atoms, held together by so called covalent bonds. The weight of a molecule is its mass relative to that of a hydrogen atom. The mass of a molecule is specified in Daltons; 1 Da being an atomic mass unit approximately equal to the mass of a hydrogen atom.

$$moles = \frac{weight}{molecularweight} \text{ (a quantity)}$$

One mole (1 M) corresponds to NA = 6.001 * 10^{23} molecules of a given substance. NA is referred as the Avogadro's number. The molarity of a solution is defined by a concentration of 1 moles of the substance in 1 liter of solution:

$$1\ molar \equiv 1M \equiv 1\frac{mol}{L} \text{ (a concentration)}$$

If molecules are cluster of atoms, held together by bonds, these bonds can be broken by violent collisions amongst molecules. Average thermal motion does not break these bonds and thus the breaking and making of bonds is the fundamental process that determines the concentrations of chemical species in a reaction. This process requires energy to place and is carefully controlled by highly specific catalysts, called enzymes. How fast a reaction occurs is a matter of kinetics, defined by the rate of a reaction. In general, kinetics energy is the ability of a system to perform work; therefore whether or not a reaction can proceed is determined by its energetic.

There are two principle types of reactions: catabolic pathways, breaking down foodstuff and thereby generating energy and smaller building blocks. Secondly, biosynthetic or anabolic pathways use energy to synthesize molecules. Both sets of reactions together constitute what is called the metabolism of the cell.

Proteins are particularly versatile, having various roles in maintaining the function of a cell and the organism as a whole. Many proteins serve as enzymes that are catalysts that control kinetic (bond-breaking and –making) reactions. Other proteins are used to build the structural components that make up the cell, or they act as motors and produce fore and movement. Enzymes catalyze reactions by binding one or more ligands which are also called substrates, and converting them into one or more chemically modified products, without changing themselves. Enzyme-catalyzed reactions happen faster by a factor of a million or more and are therefore an important mechanism by which the cell can respond to changes and regulate functions. A typical enzyme will catalyze the reaction of thousands substrate molecules every second. The enzyme therefore requires sufficient amounts of substrate around it. The motion caused by collision and thus heat energy ensures that molecules are rapidly moving about a confined area but can also move (diffuse) wider distances. The cell is a crowded environment and yet a small organic molecule can diffuse the entire distance across a cell in a fraction of a second.

Enzymes move much more slowly than substrates, and the rate of encounter of each enzyme molecule with its substrate will depend on the concentration of the substrate molecule. For example, an abundant substrate may have a concentration of 0.5 mM and since water is 55 M, there is only about one such substrate molecule in the cell for every 105 water molecules. Nevertheless, an enzyme that could bind this substrate would collide with it about 500,000 times a second. The biological

properties or function of a protein is determined by its physical interaction with other molecules. The substance that is bound by a protein is referred to as a ligand for that protein.

Antibodies, or immunoglobulins, are proteins produced by the immune system in response for foreign molecules. A specific antibody binds tightly to its particular target (called an antigen), and thereby inactivates it. Antibodies can therefore be used in experiments to select and quantitate proteins. For example, considering a population of antibody molecules which suddenly encounter a population of ligands, diffusing in the fluid surrounding them. The frequent encounters of ligands and antibody will increase the formation (association) of antibody-ligand complexes. The population of such complexes will initially increase but eventually complexes will also break apart (dissociation). Eventually, a chemical equilibrium is reached in which the number of association events per second is equal to the number of dissociation events. From the concentrations of the ligand, antibody and the complex at equilibrium, one can calculate the equilibrium constant Keq of the strength of binding. The same principle described here for antibodies, applies to any binding of molecules.

For example consider two proteins A and B, the corresponding complex they form AB and the reversible reaction

$$A+B \leftrightarrow AB$$

For association the reaction diagram is

$$A+B \xrightarrow{k_a} AB,$$

where the association rate is the product of k_a , A and B.

For disassociation respectively, the reaction diagram is

$$A+B \xleftarrow{k_d} AB,$$

and the disassociation rate equals the product of k_d and the complex concentration AB. At equilibrium

$$k_a\ [A][B] = k_d\ [AB],$$

which leads to the definition of the equilibrium constant

$$k_{eq} = \frac{[AB]}{[A][B]} = \frac{k_a}{k_d}$$

The equilibrium constant has a unit of liters per mole. The larger the equilibrium constant, the stronger the binding between A and B.[18]

2.2.4 Mathematical Modeling using ODEs

As we saw, the area of cellular signaling investigates intracellular communication. Modern scientists are trying to establish a mathematical and computational framework in order to investigate dynamic interactions within cells. In other words, they are concerned with dynamic pathway modeling since they do not simply map or list proteins in a pathway. Spatial-temporal sequences of reaction events in a biochemical network form the basis for signals, a non – physical concept used to describe the information processing, regulation and control in cells. The objective of dynamic pathway modeling is to establish mathematical models that allow scientists and researchers to predict the spatio-temporal response of protein concentrations and gene expression to pathway stimulation.

Thus, mathematical modeling and simulation of molecular or cellular biological systems is challenging. Such systems are indeed “complex” for the following reasons. A collection of cells, but also an individual cell consists of many interacting subsystems. For example, choosing any particular pathway there will be other pathways that cross talk. Due to the complexity of experiments to generate data and the sometimes complicated maths involved, it is usually easier to consider one pathway or particular aspect of one pathway at a time.

Modeling implies a process of abstraction and is often also a form of generalization. In this process numerous assumptions about the natural system under consideration are being made in order to simplify the mathematical approach, without losing the ability to make predictions. It is therefore, possible to build predictive models without them being precise. Modeling and simulation should in this sense complement the in biologists reasoning, help them to generate and test hypotheses in

conjunction with the design of experiments and experimental data. System biology requires an iteration of the modeling loop as shown in Fig (2.10).

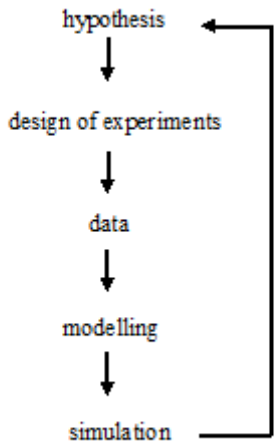


Figure 2.10 Iteration of the modeling loop

The diagram shows the role of mathematical modeling and simulation in testing hypotheses but also in generating hypotheses through prediction. The purpose of modeling is to support experimental design, helping to identify which variables to measure and why. As we have previously stated, the dominant mathematical modeling tool for intracellular signaling networks is their description with the help of ordinary differential equations (ODE). As a simple example of the way by which such a model is built, consider the following enzymatic reaction:

$$E+A \leftrightarrow EA \rightarrow E+P \quad (2.1)$$

In the first step, enzyme E binds reversibly with substrate A, and in a second step, the enzyme releases the modified substrate P. the second step is assumed to be irreversible. This is a reasonable assumption for phosphorylation, because phophorylation consumes energy in the form of ATP and it cannot be reserved, once the ADP is released. And also dephosphorylation can be modeled by this, because the phosphorylated state of a protein is on an energetically higher level, therefore the probability of returning to the phosphorylated state is very small. Such enzymatic

reaction schemes are in the most of the cases the fundamental building blocks of large intracellular signaling pathways.

The reaction scheme of Equation (2.1) is called Michaelis – Menten mechanism. The rates for the three reactions (v1, v2, v3) are:

$$E+A \rightarrow EA \ : \ v1 = \alpha[E][A] \quad (2.2)$$

$$E+A \leftarrow EA \ : \ v2 = d\,[EA] \quad (2.3)$$

$$EA \rightarrow E+P \ : \ v3 = V[EA] \quad (2.4)$$

where [E], [A] and [EA] are the concentrations of enzyme E, substrate A, and enzyme-substrate complex EA, while α, d and V are rate constants. Thus, there are two unimolecular and one bimolecular reaction. The ordinary differential equations for this system are given by:

$$\frac{d[A]}{dt} = -v2 + v1 \quad (2.5)$$

$$\frac{d[EA]}{dt} = v1 - v2 - v3 \quad (2.6)$$

$$\frac{d[E]}{dt} = -v1 + v2 + v3 \quad (2.7)$$

$$\frac{d[P]}{dt} = v3 \quad (2.8)$$

with the initial conditions:

$$[E](0) = [Etot] \quad (2.9)$$

$$[A](0) = [Atot] \quad (2.10)$$

$$[P](0) = 0 \quad (2.11)$$

$$[EA](0) = 0 \quad (2.12)$$

Given the two conservation laws for the molar concentrations,

$$[Etot] = [E] + [EA] \quad (2.13)$$

$$[Atot] = [A] + [EA] + [P] \quad (2.14)$$

the system can be reduced to two equations:

$$\frac{d[EA]}{dt} = a[A]([Etot] - [EA]) - d[EA] - V[EA] \quad (2.15)$$

$$\frac{d[A]}{dt} = - a([Etot] - [EA])[A] + d[EA] \quad (2.16)$$

The Michaelis-Menten equation can be derived in case that the amount of enzyme-substrate complex is in pseudo steady-state (d[EA]/dt = 0). Collecting terms in [EA] and rearranging leads to:

$$[EA] = \frac{a[Etot][A]}{d+V+a[A]} = \frac{[Etot][A]}{\frac{d+V}{a}+[A]} \quad (2.17)$$

Conventionally, a parameter called Michaelis-Menten constant, K_m is introduced:

$$K_m = \frac{d+V}{a} \quad (2.18)$$

with the dimension of a concentration, and the parameter V is called V_{max}- . Introducing these parameters, the steady-state of [EA] can be written as

$$[EA] = \frac{[Etot][A]}{Km+[A]} \quad (2.19)$$

the rate of production of P is given by:

$$v3 = V\,[EA] = \frac{Vmax[Etot][A]}{Km+[A]} \quad (2.20)$$

Equation (2.20) is known as Michaelis-Menten equation. Thus, the pseudo steady – state is reached in the time scale of

$$EA = (a[A] + d + V)^{-1} \quad (2.21)$$

Another detailed analysis of the steady – state is made by Schnell and Mendoza: Assuming that [A] >> [Etot], the timescale is estimated as

$$EA = \frac{1}{a[Atot]+d+V} = \frac{1}{a(Km+[Atot])} \quad (2.22)$$

Furthermore, they also estimate the time for the change of the substrate concentration as

$$A = \frac{[Atot]}{\left|\frac{d[A]}{dt}\right|_{max}} = \frac{Km + [Atot]}{Vmax\ [Etot]} \quad (2.23)$$

Therefore, the ratio of the two timescales is given by

$$\frac{\varepsilon A}{A} = \frac{Vmax\ [Etot]}{\alpha(Km+[Atot])^2} \quad (2.24)$$

A more general condition for the pseudo steady – state assumption is given by Segel:

$$\frac{[Etot]}{Km + [Atot]} \ll 1 \quad (2.25)$$

Therefore, the steady – state assumption is reasonable, if the enzyme concentration is small compared to the concentration of the substrate. A problem might occur, if an enzyme acts in different reactions. In this case, the two conservation equations (2.13) and (2.14) are no longer valid. Nevertheless, if the saturation of the enzyme is small, they might be a good approximation, because the concentration of bound enzyme is small. [24]

3 Combination Therapy and Systems Biology

Therapeutic treatments that combine two or more active ingredients are commonly used in clinical medicine and increasingly important in probing biological systems. It is what we call combination or multicomponent therapy. In this chapter we are presenting this kind of therapy, so that we will completely understand the models we will identify later.

We start with the drugs used in this kind of therapy; their first applications, their main categories and, of course, the way they interact to produce such effective results. How we reach in what is called synergy between two components and how we can predict it.

And after we examine the two main reference models of synergy, we continue with the Systems Biology approach. Systems biology provides a perspective from which to understand the basis of the efficacy of most multicomponent drugs. Using basic principles, we simulate four signaling pathways characterized from amplification, ultrasensitivity and feedback control, which helps us to understand better the behaviour of a full-drug interaction network.

3.1 History of Combination Therapy and Multicomponent Drugs

Drug treatments with more than one active component are not in fact something new in medicine, since they have been used in several forms for many years, starting from a lot of historical and traditional approaches to medicine. For example, Chinese have used mixtures of naturally occurring herbs and herbal extracts, and such mixtures are considered integral to the therapy[28]. Many of the natural product extracts that have been tested, have brought in activities that later disappeared when the extracts were used as individual chemical components[29],[30],[31]. Numerous combinations of active compounds have been found to be produced by

natural sources. In Western medicine, we can find the first attempts to examine interactions between purified single compounds only at the end of the nineteenth century, when Thomas Richard Fraser investigated the interaction between physostigma and atropia[32]. After this first step, we had to reach 1928, for further studying of the interactions between other defined drug combinations from Loewe and others[33],[34]. At the beginning of the twentieth century, most therapeutic regimens were composed of cocktails or complex extracts, such as the application of polyclonal antibody therapy.

The gradual shift from the use of complex extracts to the use of purified single compounds started in the early part of the twentieth century with Paul Ehrlich's pioneering one-gene-one-drug approach. Ehrlich proposed the – known as - 'magic bullets', chemicals that selectively target the constituents of infectious organisms relative to the host's constituents[35]. For his work, he was awarded the Nobel Prize for Physiology or Medicine in 1908. Ehrlich's first 'magic bullet' was Salvarsan or arsphenamine, which provided the only cure for syphilis until it was superseded and replaced by penicillin. What occurred in the following years can be described as a 'bombing' of researches and significant discoveries in the direction given by Ehrlich. Except from syphilis, compounds were subsequently found that were effective against many other diseases, such as sleeping sickness, malaria, trypanosomes, pneumonia, sepsis, schistosomiasis and babesiasis. These findings were followed in subsequent years by the monumental discoveries of the sulphonamides and penicillin[36].

However, most diseases of interest to contemporary drug discovery involve physiological processes controlled in a combinatorial fashion. These diseases are frequently difficult to treat using Ehrlich's approach. In the second half of the twentieth century, the evolution described above reached its logical zenith in the search for single compounds that affect single targets. It was the time to revisit past experiences to identify multicomponent therapeutics for the treatment of complex diseases. There was already a lot of widespread evidence that combinations of compounds can be more effective than the sum of the effectiveness of the individual agents themselves; and in addition to that, more recent findings with the great help of sciences like modern systems and molecular biology came to support these theories.[25],[26]

3.2 Combination Drugs

As we mentioned above, most disease processes present a combinatorial behaviour, while redundancy and multifunctionality seem to be the most obvious features of this behaviour. The solution to this can be the use of a multicomponent drug; a drug that will act selectively on a specific combination of target activities found in diseased cells. For example, it is now known that not only one, but several mutations are required for the development of colorectal cancer[37]; the correction of these defective pathways will probably require several interventions. Except from colorectal cancer, this phenomenon can be observed and to other kinds of cancer. Furthermore, we can already notice that oncological chemotherapeutic regimens most often involve combination therapies, such as doxorubicin, cyclophosphamide, vincristine and prednisone[38]. In noticing the success of such combinations, some companies have converted clinically used drug combinations into single-pill formulations.

The most common way for developing multicomponent drugs is from the combination of single-compound drugs that already exist to treat the target disease. For example, Advair for asthma combines a steroid, which affects an inflammatory component of asthma, with a long-acting β2-adrenoceptor agonist, which acts as a bronchodilator to relax constriction of the airways. It has been proved from trials that this combination provides greater benefit to the patient than either agent alone[39]. There are many other examples of such drugs (e.g. Advicor for hypercholesterolaemia, Combivir for HIV) which have shown how successful multicomponent products can be.

We can find two main categories of combination drugs; congruous and syncretic drugs. A congruous drug is composed of two or more active ingredients, each of which has been individually used to treat the target disease indication. Advair, which described above, is such a congruous combination, built on the basis of clinical observations, a combination that would be logical to test because both of the component drugs are already being used to treat the target disease. Syncretic drugs on the other side, they have components with discrete mechanisms of action into a single

intervention, at least one of which is not used individually to treat the target disease indication. Syncretic and congruous drugs both belong to what is called multicomponent therapeutic, an optimized combination and formulation of multiple active ingredients. In general though, it's not easy to systematize a set of principles that can account for combination drugs that exist today, but this exactly can make easier the discovery of future effective combination drugs and this is the direction researchers are facing. [25]

3.3 Theoretical Evaluation of Combination Therapy

In the field of Biology there have been many studies focusing on interactions between specific drugs. In this thesis we focus on the system properties of a full drug interaction network. There are three main types of interactions among multiple drugs: additive, synergistic and antagonistic[25],[34],[40],[41]. In other words, drugs may not interact at all (additive); their interaction may have a smaller-than–additive effect, suppressing their individual effects (antagonistic – negative interaction); or it may have a larger-than-additive effect, increasing their individual effects (synergistic – positive interaction) (Fig. 3.1). Synergistic combinations are those with the greatest interest, since they provide greater effect than would be predicted by simply adding together the effects of the components. In one word, the 'key' of synergy is effectiveness; benefit of the combination of the drugs that could not be achieved by the components on their own. There are two main reference models of synergy, two methods that calculate the expected dose-response relationship for combination therapy as compared to monotherapy: Loewe Additivity [33],[34],[42],[43],[44] and Bliss Independence[41],[45],[46].

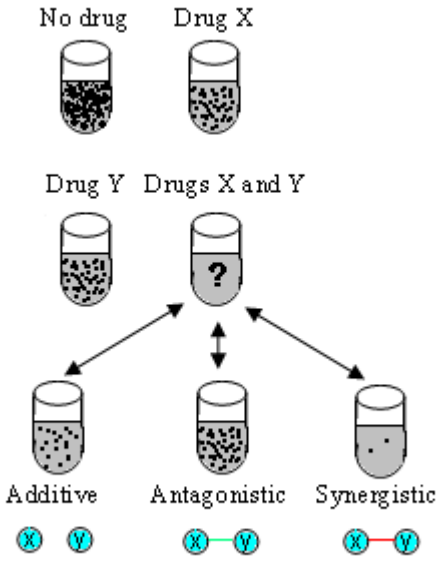


Figure 3.1 Schematic illustration of additive, synergistic and antagonistic interactions between two drugs X and Y

Loewe additivity, which for most is the preferred additive reference model, assumes that two inhibitors act on a target through a similar mechanism (Fig. 3.2). Let's suppose we have the concentrations of two inhibitors ($[I_1]$, $[I_2]$), that individually achieve X% target inhibition. Then the concentration of inhibitors theoretically required to produce the same X% effect when used in combination ($[CI_1],[CI_2]$), can be calculated by taking into account the potency of the inhibitors.

$$1 = \frac{[CI_1]_{X\%}}{[I_1]_{X\%}} + \frac{[CI_2]_{X\%}}{[I_2]_{X\%}} \quad (3.1)$$

For denoting whether or not inhibitors interacted with each other, Loewe additivity uses the Combination Index method of Chou and Talalay[42],[43],[47], a generalized method for analysing combination effects on the basis of the principle of mass action:

$$Combination\ index = \frac{[CI_1]}{[I_1]} + \frac{[CI_2]}{[I_2]} \begin{cases} > 1 \text{ antagonism (negative interaction)} \\ = 1 \text{ additive (no interaction)} \\ < 1 \text{ synergy (positive interaction)} \end{cases} \quad (3.2)$$

The combination index compares the doses of inhibitors that experimentally produce the same level of inhibition, and individually and in combination. By finding the dose required for equal effect, we can determine whether the combination is effective at a lower total dose. An experimentally determined dose–response surface is synergistic when its combination index is less than 1 and antagonistic when it is greater than 1.

Furthermore, the method gives us the opportunity to calculate the expected combination's degree of inhibition. For example, if we consider a mass action kinetic enzyme inhibition with constant substrate, where *E* is the enzyme activity, E_{MAX} is the maximum activity, *m* is the hill coefficient and K_I is the concentration of inhibitor I required to decrease enzyme activity by 50%, we have the following equation that gives the effect of the inhibitor (F_{UA}):

$$F_{UA} = \frac{E}{E_{MAX}} = \frac{1}{1+(\frac{[I]}{K_I})^m} \quad (3.3)$$

From equations (3.1) and (3.3) we take the following equation which relates the expected combined effect of the two inhibitors (F_{UA}) to the concentrations of the inhibitors:

$$1 = \frac{[CI_1]}{K_{I_1}(\frac{1-F_{UA}}{F_{UA}})^{\frac{1}{m_1}}} + \frac{[CI_2]}{K_{I_2}(\frac{1-F_{UA}}{F_{UA}})^{\frac{1}{m_2}}} \quad (3.4)$$

From equation (3.4), we can determine F_{UA} for any combination of inhibitor concentrations, which helps in the evaluation of clinical dose-response curves relative to a simple computed standard of additivity. When the combination is better than additive, it means that we have found a beneficial case of synergism, without caring about the - usually unknown - mechanism.

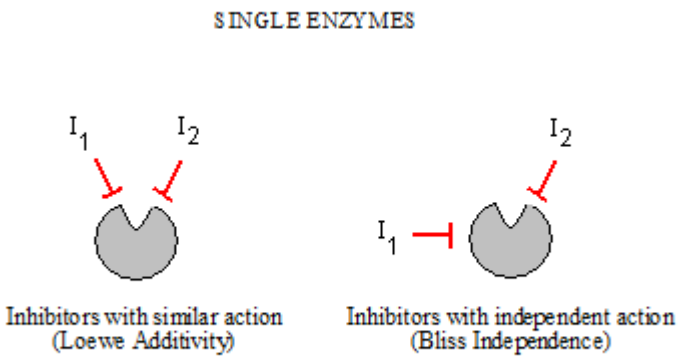


Figure 3.2 Loewe additivity and Bliss independence in single enzymes

The advantage of Loewe additivity compared to the Bliss independence method is that it can correctly predict the trivial case in which the two inhibitors are actually the same compound, since it is based in the assertion that a compound, when combined with itself, must by definition be additive. A theoretical Loewe additive response surface for a combination of two agents can be calculated from the fitted dose–response curves of the individual compounds. Bliss independence on the other hand, does not require the determination of dose–response curves for the individual compounds to generate the theoretical reference case, but its expectation for a combination is simply the product of the activity ratios of the individual inhibitors (F_{UA_1}, F_{UA_2}) at the same compound concentrations (equation (3.5)). That's why it is also called the fractional product approach.

$$F_{UA} = F_{UA_1} \times F_{UA_2} \quad (3.5)$$

What we should take into account is that Bliss independence as a method describes the case for two active agents, which, when combined, do not directly interfere with each other (Fig. 3.2). It assumes that the two inhibitors act through independent mechanisms but, as we saw, they can both contribute to a common result. Another characteristic of the method, in contrast with Loewe additivity as described before, is that a compound that is tested in combination with itself will not generally seem to be independent, so it cannot be considered as additive. It is logical so, that the two methods provide different outcome. This can be more specific if we combine the

fractional product (equation (3.5)) with the enzyme kinetic relations (equation (3.3)). The combined effect result is different from that Loewe additivity produces, except from some specific circumstances of non-exclusivity:

$$F_{UA} = \frac{1}{1+(\frac{[CI_1]}{K_{I_1}})} \times \frac{1}{1+(\frac{[CI_2]}{K_{I_2}})} \quad (3.6)$$

Loewe additivity and Bliss independence are two methods with great differences, with advantages and disadvantages, each of which can be characterized 'better' in various occasions. And their 'conflict' can be extended and in different level; for example which method performs better with noisy clinical data. There will always be 'for' and 'against', ideal and undesirable cases; this is not goal of this thesis though. What is clear from what we examined until now is that the methods are developed to describe simple enzyme reactions. It is easy to justify them theoretically, but we cannot be certain that they can completely represent the biochemistry of complex cell-signaling networks (Fig. (3.3)).

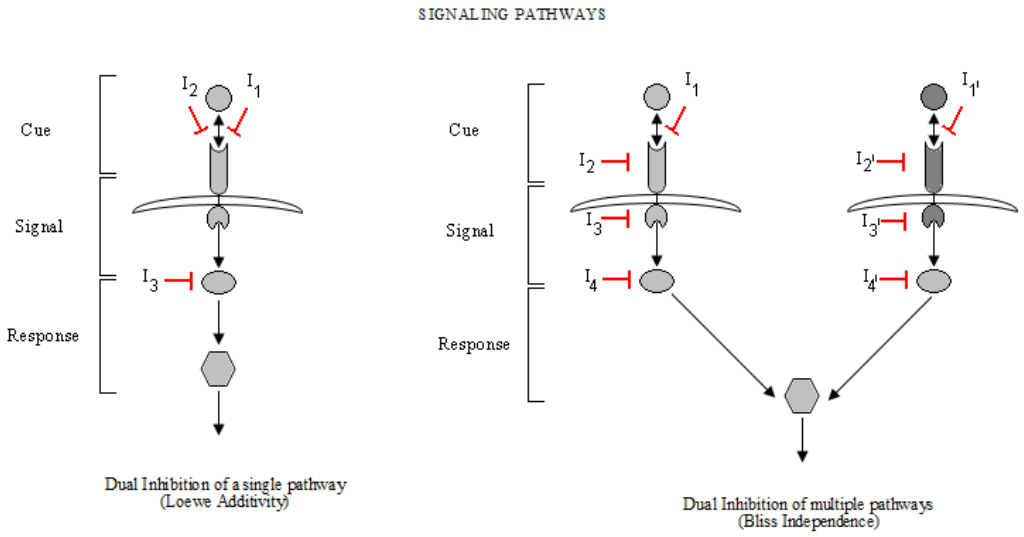


Figure 3.3 Loewe additivity and Bliss independence in signaling pathways

We can say though, that these methods mostly seem to constitute black-box approaches, extremely valuable in clinical trials, considering the importance of synergy in combination therapy (Fig. (3.4)).

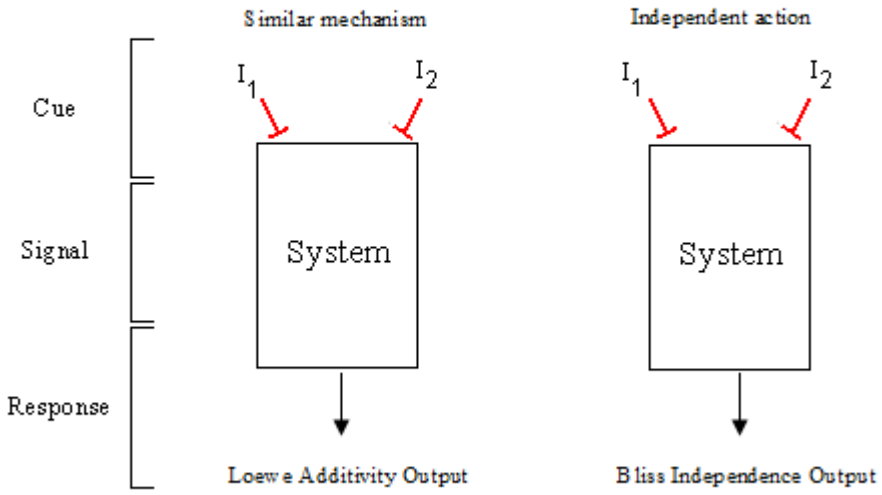


Figure 3.4 Loewe additivity and Bliss independence as a black-box approach

However, synergy determination remains one very difficult and complicated procedure. Sometimes, researchers have to think in a higher level than the conventional way Loewe additivity and Bliss independence provide. A combination for example, can be synergistic over one range of doses and antagonistic over another. In such case the answer they have to find is not if the combination is synergistic, but the dose range which optimizes the synergy of this combination. It is obvious that the methods we analysed are very helpful tools but certainly they don't solve all the problems.[25],[26],[27]

3.4 A Systems Biology Approach

After analyzing theoretical methods for the evaluation of combination therapy, in this section we will examine models of cell signaling networks to simulate the effects of multicomponent drugs. We use ODE-based models, which contain one or more cell surface receptors and a downstream signaling cascade. These models cannot

completely represent real biological networks, but they are quite close to reality, especially considering the fact that they are characterized from amplification, ultrasensitivity and feedback control[48]. The greatest advantage of this analysis is that a big number of drug combinations can be explored computationally at much lower cost than in preclinical or clinical experiments. We simulated four signaling pathways using Matlab code (models and parameter values taken from[26]), in each of which we calculated dose-response curves for a single downstream signaling protein when combined inhibitors are used.

3.4.1 Combination of Inhibitors Targeting Two Converging Pathways

In the first system that we simulate, two ligands (A_1 and A_2) and their receptors (B_1 and B_2) converge on a single downstream signaling kinase C which is activated by phosphorylation (Fig. (3.5)). As we can see in the reaction scheme, activation of B_1 and B_2 by ligands A_1 and A_2 respectively, contributes equally to the activity of C. Phosphatases Ptse1 and Ptse2 ensure recycling of activated C and B.

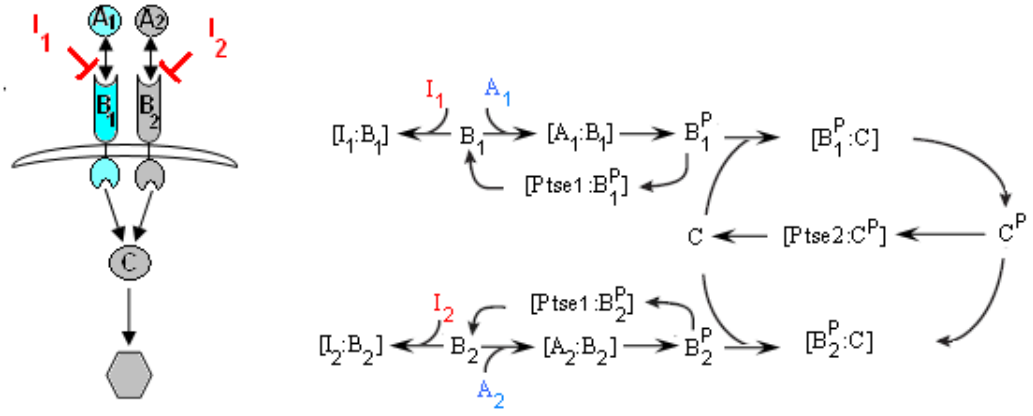


Figure 3.5 Regulatory and reaction scheme for a combination of two inhibitors targeting two converging pathways

By using Systems Biology principles, as described in the previous chapter, we have the following reactions that describe our model:

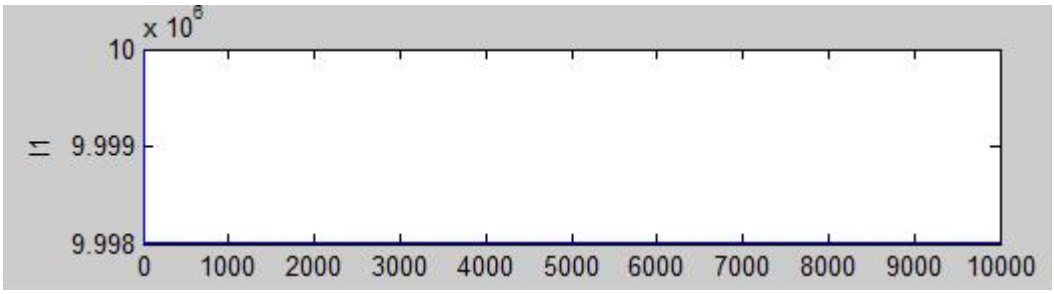
Reaction Description	Reaction	ReactionRate
activation of B1 by A1	A1 + B1 <-> A1:B1	k1*A1*B1 - kd1*[A1:B1]
	B1P + A1 <-> A1:B1	k2*B1P*A1 - kd2*[A1:B1]
	P1 + B1P <-> P1:B1P	k3*P1*B1P - kd3*[P1:B1P]
	P1 + B1 <-> P1:B1P	k4*P1*B1 - kd4*[P1:B1P]
activation of C by B1	C + B1P <-> C:B1P	k5*C*B1P - kd5*[C:B1P]
	CP + B1P <-> C:B1P	k6*CP*B1P - kd6*[C:B1P]
	P2 + CP <-> P2:CP	k7*P2*CP - kd7*[P2:CP]
	P2 + C <-> P2:CP	k8*P2*C - kd8*[P2:CP]
inhibition of B1	I1 + B1 <-> I1:B1	k9*I1*B1 - kd9*[I1:B1]
inhibition of B2	I2 + B2 <-> I2:B2	k10*I2*B2 - kd10*[I2:B2]
activation of B2 by A2	B2 + A2 <-> B2:A2	k11*B2*A2 - kd11*[B2:A2]
	B2P + A2 <-> B2:A2	k12*B2P*A2 - kd12*[B2:A2]
	P1 + B2P <-> P1:B2P	k13*P1*B2P - kd13*[P1:B2P]
	P1 + B2 <-> P1:B2P	k14*P1*B2 - kd14*[P1:B2P]
activation of C by B2	C + B2P <-> C:B2P	k15*C*B2P - kd15*[C:B2P]
	CP + B2P <-> C:B2P	k16*CP*B2P - kd16*[C:B2P]

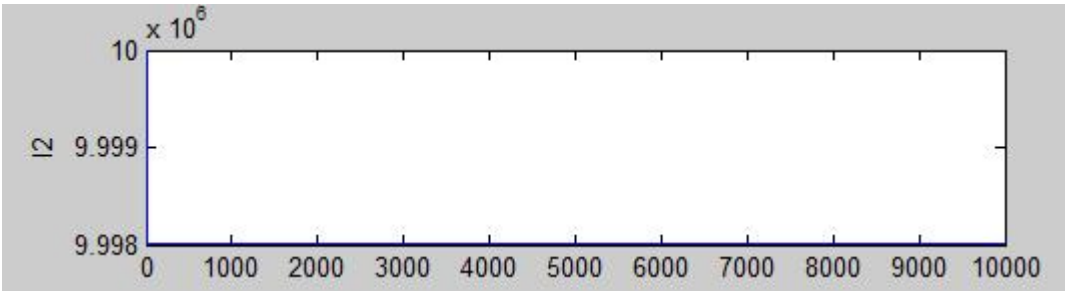
For the simulation, we used the differential equations that we take from the reactions above, as we can see in the following part of our Matlab code:

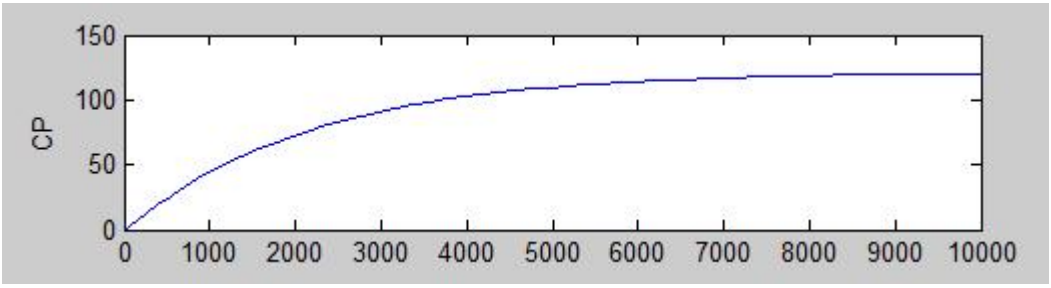
```
A1=x(1);
B1=x(2);
A1B1=x(3);
C=x(4);
CB1P=x(5);
CP=x(6);
P1=x(7);
P1B1P=x(8);
P2=x(9);
P2CP=x(10);
B1P=x(11);
I1=x(12);
I2=x(13);
I1B1=x(14);
B2=x(15);
I2B2=x(16);
A2=x(17);
B2A2=x(18);
B2P=x(19);
P1B2P=x(20);
CB2P=x(21);


xdot(1)=-k1*A1*B1+kd1*A1B1-k2*B1P*A1+kd2*A1B1;
xdot(2)=-k1*A1*B1+kd1*A1B1-k4*P1*B1+kd4*P1B1P-k9*I1*B1+kd9*I1B1;
xdot(3)=k1*A1*B1-kd1*A1B1+k2*B1P*A1-kd2*A1B1;
xdot(4)=-k5*C*B1P+kd5*CB1P-k8*P2*C+kd8*P2CP-k15*C*B2P+kd15*CB2P;
xdot(5)=k5*C*B1P-kd5*CB1P+k6*CP*B1P-kd6*CB1P;
```

```
xdot(6)=-k7*P2*CP+kd7*P2CP-k6*CP*B1P+kd6*CB1P-k16*CP*B2P+kd16*CB2P;
xdot(7)=-k3*P1*B1P+kd3*P1B1P-k4*P1*B1+kd4*P1B1P-
        k13*P1*B2P+kd13*P1B2P-k14*P1*B2+kd14*P1B2P;
xdot(8)=k3*P1*B1P-kd3*P1B1P+k4*P1*B1-kd4*P1B1P;
xdot(9)=-k7*P2*CP+kd7*P2CP-k8*P2*C+kd8*P2CP;
xdot(10)=k7*P2*CP-kd7*P2CP+k8*P2*C-kd8*P2CP;
xdot(11)=-k2*B1P*A1+kd2*A1B1-k3*P1*B1P+kd3*P1B1P-k5*C*B1P+kd5*CB1P-
         k6*CP*B1P+kd6*CB1P;
xdot(12)=-k9*I1*B1+kd9*I1B1;
xdot(13)=-k10*I2*B2+kd10*I2B2;
xdot(14)=k9*I1*B1-kd9*I1B1;
xdot(15)=-k10*I2*B2+kd10*I2B2-k11*B2*A2+kd11*B2A2-
         k14*P1*B2+kd14*P1B2P;
xdot(16)=k10*I2*B2-kd10*I2B2;
xdot(17)=-k11*B2*A2+kd11*B2A2-k12*B2P*A2+kd12*B2A2;
xdot(18)=k11*B2*A2-kd11*B2A2+k12*B2P*A2-kd12*B2A2;
xdot(19)=-k12*B2P*A2+kd12*B2A2-k13*P1*B2P+kd13*P1B2P-
         k15*C*B2P+kd15*CB2P-k16*CP*B2P+kd16*CB2P;
xdot(20)=k13*P1*B2P-kd13*P1B2P+k14*P1*B2-kd14*P1B2P;
xdot(21)=k15*C*B2P-kd15*CB2P+k16*CP*B2P-kd16*CB2P;
```

Here are the results of our simulation for the two inhibitors and the 'output' of the system, the activated C, which is a measure of therapeutic effect:







This model is a simplified implementation of the apparent interaction of insulin-like growth factor 1 (IGF-1) and ErbB signaling in breast cancer. Further analysis of the network has shown the existence of synergy between I_1 and I_2, although neither Loewe additivity nor Bliss independence predicts that correctly[26].

3.4.2 Inhibition of a Single Target by Two Inhibitors

The second system we examine is about dual inhibition of a single target (Fig. (3.6)). As we can see, inhibitors I_1, I_2 and I_3 block binding of ligand A to receptor B and prevent the activation of C. We assume that the kinetic parameters of I_1, I_2 and I_3 are equal.

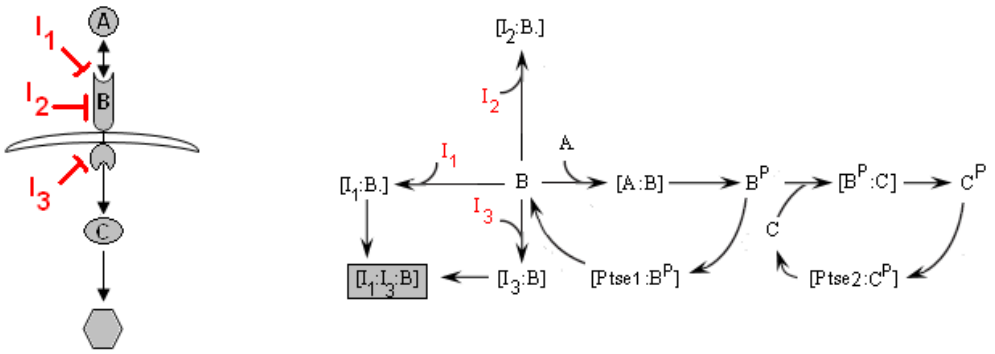


Figure 3.6 Regulatory scheme of a single linear pathway with dual inhibition of a single target and its reaction scheme

We have two occasions, depending on which is the second inhibitor. If it is I_2, it prevents I_1 from binding (I_1 and I_2 mutually exclusive); if it is I_3 though, it does not affect binding of I_1 (I_1 and I_3 mutually non-exclusive). When we have mutually exclusive inhibitors, it is reasonable to assume they act through a similar mechanism and that their combined effect on C is described by Loewe additivity. In the second case, it is reasonable to expect Bliss independence. This is represented in the model

by inclusion of a triplex species [$I_1 : I_3$:B]. There is no change in the effectiveness of the individual inhibitors, but the difference is that in this case we have synergy. This model can be considered as an implementation of dual inhibition of gefitinib (Iressa) and certuximab (Erbitux) to the ErbB1 tyrosine kinase[26].

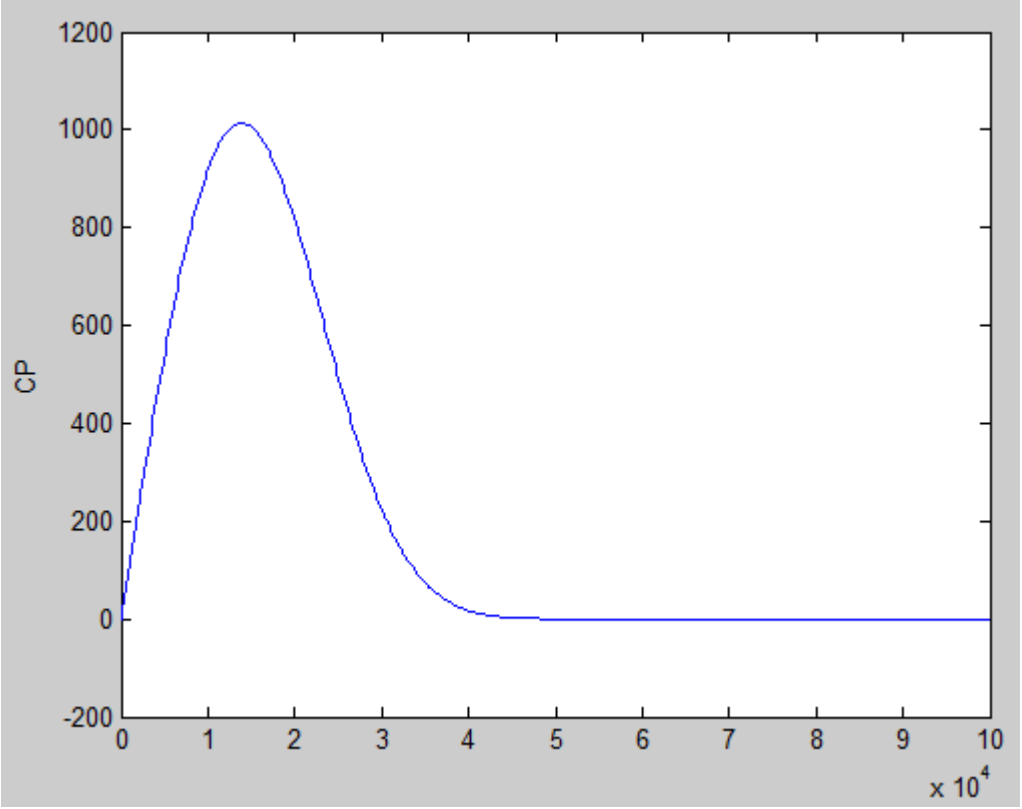
The reactions that describe the model and the differential equations taken from our Matlab code are the following:

Reaction Description	Reaction	ReactionRate
ligand binding to receptor	A + B <-> A:B	k1*A*B - kd1*[A:B]
receptor activation	BP + A <-> A:B	k2*BP*A - kd2*[A:B]
binding of Pase1	P1 + BP <-> P1:BP	k3*P1*BP - kd3*[P1:BP]
deactivation of B	P1 + B <-> P1:BP	k4*P1*B - kd4*[P1:BP]
activation of C by BP	C + BP <-> C:BP	k5*C*BP - kd5*[C:BP]
producing CP	CP + BP <-> C:BP	k6*CP*BP - kd6*[C:BP]
binding of Pase2	P2 + CP <-> P2:CP	k7*P2*CP - kd7*[P2:CP]
dephosphorylation of CP	P2 + C <-> P2:CP	k8*P2*C - kd8*[P2:CP]
inhibition of B by I1	I1 + B <-> I1:B	k9*I1*B - kd9*[I1:B]
inhibition of B by I2	I2 + B <-> I2:B	k10*I2*B - kd10*[I2:B]
non-excusive inhibition of B by I3	I3 + B <-> l3:B	k11*I3*B - kd11*[I3:B]
dual inhibitor non-exclusive binding	I3 + I1:B <-> I3:I1:B	k11*I3*[I1:B] - kd11*[I3:I1:B]
dual inhibitor non-exclusive binding	I1 + I3:B <-> I3:I1:B	k9*I1*[I3:B] - kd9*[I3:I1:B]

```
A=x(1);
B=x(2);
AB=x(3);
BP=x(4);
C=x(5);
CBP=x(6);
CP=x(7);
P1=x(8);
P1BP=x(9);
P2=x(10);
P2CP=x(11);
I1=x(12);
I1B=x(13);
I2=x(14);
I2B=x(15);
I3=x(16);
I3B=x(17);
I3I1B=x(18);

xdot(1)=-k1*A*B+kd1*AB-k2*BP*A+kd2*AB;
xdot(2)=-k1*A*B-k4*P1*B+kd1*AB+kd4*P1BP-k9*I1*B+kd9*I1B-
        k10*I2*B+kd10*I2B-k11*I3*B+kd11*I3B;
xdot(3)=-kd1*AB-kd2*AB+k1*A*B+k2*BP*A;
```

```
xdot(4)=-k2*BP*A-k3*P1*BP-k5*C*BP-
        k6*CP*BP+kd2*AB+kd3*P1BP+kd4*P1BP+kd5*CBP+kd6*CBP;
xdot(5)=-k5*C*BP-k8*P2*C+kd5*CBP+kd8*CBP;
xdot(6)=k5*C*BP-kd5*CBP+k6*CP*BP-kd6*CBP;
xdot(7)=-k6*CP*BP+kd6*CBP-k7*P2*CP+kd7*P2CP;
xdot(8)=-k3*P1*BP+kd3*P1BP-k4*P1*B+kd4*P1BP;
xdot(9)=k3*P1*BP-kd3*P1BP+k4*P1*B-kd4*P1BP;
xdot(10)=-k7*P2*CP+kd7*P2CP-k8*P2*C+kd8*P2CP;
xdot(11)=k7*P2*CP-kd7*P2CP+k8*P2*C-kd8*P2CP;
xdot(12)=-k9*I1*B+kd9*I1B-k9*I1*I3B+kd9*I3I1B;
xdot(13)=k9*I1*B-kd9*I1B;
xdot(14)=-k10*I2*B+kd10*I2B;
xdot(15)=k10*I2*B-kd10*I2B;
xdot(16)=-k11*I3*B+kd11*I3B-k11*I3*I1B+kd11*I3I1B;
xdot(17)=k11*I3*B-kd11*I3B-k9*I1*I3B+kd9*I3I1B;
xdot(18)=k11*I3*I1B-kd11*I3I1B+k9*I1*I3B-kd9*I3I1B;
```

And here is the result of our simulation for the activated C in the mutually non-exclusive case of dual inhibition of a single target:



3.4.3 Targeting Different Levels of a Single Pathway

In the next case we will examine, two inhibitors target different components of a linear amplification pathway (Fig. (3.7)). As we can see, we have the same regulatory scheme as in previous case, but now I_2 binds to a downstream signalling molecule C.

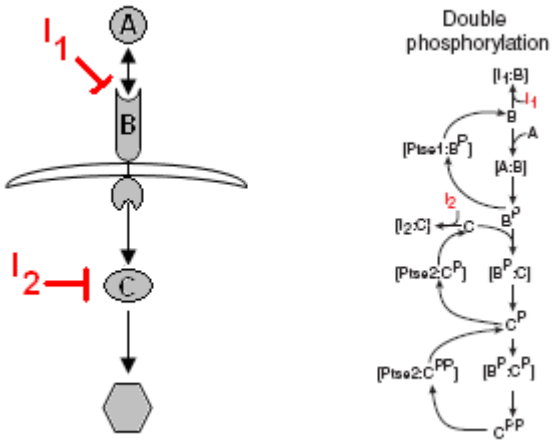


Figure 3.7 Regulatory scheme of a single linear amplification pathway with inhibitors acting at different level of a single target and the reaction scheme of an ultrasensitive signaling cascade (dual phosphorylation)

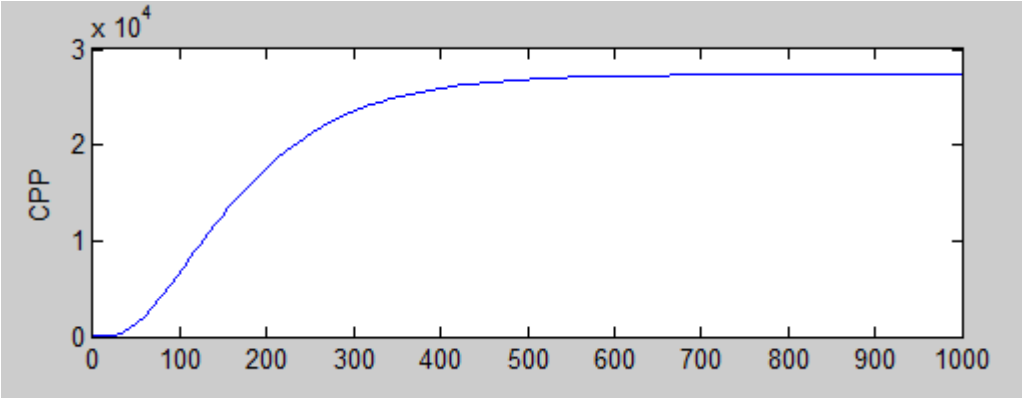
Double phosphorylation makes our model ultrasensitive, similar to the MAPK cascade, where MAPKK is also activated by two independent phosphorylations. The combination of I_1 and I_2 is synergistic, something that wouldn't happen with a single phosphorylation (additive)[26].

Reactions, differential equations and result for the double phosphorylated C are the following:

Reaction Description	Reaction	ReactionRate
ligand binding to receptor	A + B <-> A:B	k1*A*B - kd1*[A:B]
receptor activation	BP + A <-> A:B	k2*BP*A - kd2*[A:B]
binding of Pase1	P1 + BP <-> P1:BP	k3*P1*BP - kd3*[P1:BP]
deactivation of B	P1 + B <-> P1:BP	k4*P1*B - kd4*[P1:BP]
activation of C by BP	C + BP <-> C:BP	k5*C*BP - kd5*[C:BP]
producing CP	CP + BP <-> C:BP	k6*CP*BP - kd6*[C:BP]
binding of Pase2	P2 + CP <-> P2:CP	k7*P2*CP - kd7*[P2:CP]
dephosphorylation of CP	P2 + C <-> P2:CP	k8*P2*C - kd8*[P2:CP]
inhibition of B by I1	I1 + B <-> I1:B	k9*I1*B - kd9*[I1:B]
inhibition of C by I2	I2 + C <-> I2:C	k12*I2*C - kd12*[I2:C]
double phosphorylation of C by BP	CP + BP <-> CP:BP	k5*CP*BP - kd5*[CP:BP]
	CPP + BP <-> CP:BP	k6*CPP*BP - kd6*[CP:BP]
dephosphorylation of CPP	CPP + P2 <-> P2:CPP	k7*CPP*P2 - kd7*[P2:CPP]
	CP + P2 <-> P2:CPP	k8*CP*P2 - kd8*[P2:CPP]

```
A=x(1);
B=x(2);
AB=x(3);
BP=x(4);
C=x(5);
CBP=x(6);
CP=x(7);
P1=x(8);
P1BP=x(9);
P2=x(10);
P2CP=x(11);
I1=x(12);
I1B=x(13);
I2=x(14);
I2C=x(15);
CPBP=x(16);
CPP=x(17);
P2CPP=x(18);


xdot(1)=-k1*A*B+kd1*AB-k2*BP*A+kd2*AB;
xdot(2)=-k1*A*B+kd1*AB-k4*P1*BP+kd4*P1BP-k9*I1*B+kd9*I1B;
xdot(3)=-kd1*AB-kd2*AB+k1*A*B+k2*BP*A;
xdot(4)=-k2*BP*A+kd2*AB-k3*P1*BP+kd3*P1BP-k5*C*BP+kd5*CBP-
        k6*CP*BP+kd6*CBP-k5*CP*BP+kd5*CPBP-k6*CPP*BP+kd6*CPBP;
xdot(5)=-k5*C*BP+kd5*CBP-k8*P2*C+kd8*P2CP-k12*I2*C+kd12*I2C;
xdot(6)=k5*C*BP-kd5*CBP+k6*CP*BP-kd6*CBP;
xdot(7)=-k6*CP*BP+kd6*CBP-k7*P2*CP+kd7*P2CP-k5*CP*BP+kd5*CPBP-
        k8*CP*P2+kd8*P2CPP;
xdot(8)=-k3*P1*BP+kd3*P1BP-k4*P1*B+kd4*P1BP;
xdot(9)=k3*P1*BP-kd3*P1BP+k4*P1*B-kd4*P1BP;
xdot(10)=-k7*P2*CP+kd7*P2CP-k8*P2*C+kd8*P2CP-k7*CPP*P2+kd7*P2CPP-
         k8*CP*P2+kd8*P2CPP;
xdot(11)=k7*P2*CP-kd7*P2CP+k8*P2*C-kd8*P2CP;
xdot(12)=-k9*I1*B+kd9*I1B;
xdot(13)=k9*I1*B-kd9*I1B;
xdot(14)=-k12*I2*C+kd12*I2C;
xdot(15)=k12*I2*C-kd12*I2C;
xdot(16)=k5*CP*BP-kd5*CPBP+k6*CPP*BP-kd6*CPBP;
```

```
xdot(17)=-k6*CPP*BP+kd6*CPBP-k7*CPP*P2+kd7*P2CPP;
xdot(18)=k7*CPP*P2-kd7*P2CPP+k8*CP*P2-kd8*P2CPP;
```



3.4.4 Feedback-Controlled Targets

Finally, we simulated a model, in which the target of inhibitor I_2 is within a negative feedback loop (Fig. (3.8)). In the reaction scheme we can see that C^P deactivates B creating the loop. What is interesting in this case, is that C is inhibited more effectively by I_1 than by I_2, while in the previous model where we didn't have feedback it was happening the opposite[26].

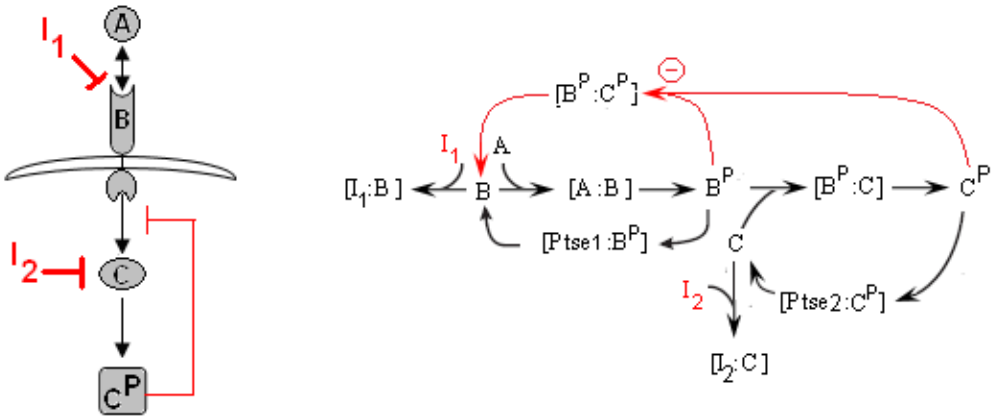


Figure 3.8 Regulatory scheme in which the target of the second inhibitor is within a negative feedback loop and its reaction scheme

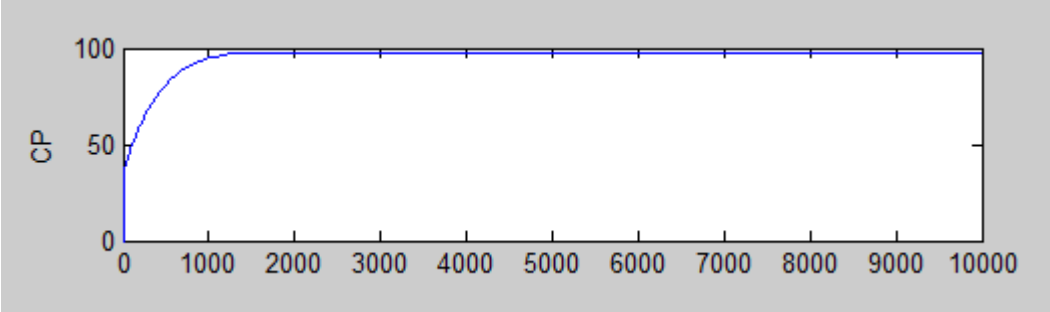
Negative feedback is found very often in biological networks probably because of the robustness it can ensure[49],[50],[51],[52],[53]. However, this makes quite difficult the intuition of combination therapy's effectiveness. Because of that, we must be very careful with the reaction pathways when feedback regulation is involved, if we want to maximize therapeutic efficacy[26].

Reactions, differential equations and result for the activated C are following:

Reaction Description	Reaction	ReactionRate
ligand binding to receptor	A + B <-> A:B	k1*A*B - kd1*[A:B]
receptor activation	BP + A <-> A:B	k2*BP*A - kd2*[A:B]
binding of Pase1	P1 + BP <-> P1:BP	k3*P1*BP - kd3*[P1:BP]
deactivation of B	P1 + B <-> P1:BP	k4*P1*B - kd4*[P1:BP]
activation of C by BP	C + BP <-> C:BP	k5*C*BP - kd5*[C:BP]
producing CP	CP + BP <-> C:BP	k6*CP*BP - kd6*[C:BP]
binding of Pase2	P2 + CP <-> P2:CP	k7*P2*CP - kd7*[P2:CP]
dephosphorylation of CP	P2 + C <-> P2:CP	k8*P2*C - kd8*[P2:CP]
inhibition of B by I1	I1 + B <-> I1:B	k9*I1*B - kd9*[I1:B]
inhibition of C by I2	I2 + C <-> I2:C	k12*I2*C - kd12*[I2:C]
negative feedback from CP to BP	CP + BP <-> CP:BP	k12*CP*BP - kd12*[CP:BP]
feedback dephosphorylation of BP	CP + B <-> CP:BP	k13*CP*B - kd13*[CP:BP]

```
A=x(1);
B=x(2);
AB=x(3);
BP=x(4);
C=x(5);
CBP=x(6);
CP=x(7);
P1=x(8);
P1BP=x(9);
P2=x(10);
P2CP=x(11);
I1=x(12);
I1B=x(13);
I2=x(14);
I2C=x(15);
CPBP=x(16);

xdot(1)=-k1*A*B+kd1*AB-k2*BP*A+kd2*AB;
xdot(2)=-k1*A*B+kd1*AB-k4*P1*B+kd4*P1BP-k9*I1*B+kd9*I1B-
        k13*CP*B+kd13*CPBP;
xdot(3)=-kd1*AB-kd2*AB+k1*A*B+k2*BP*A;
xdot(4)=-k2*BP*A+kd2*AB-k3*P1*BP+kd3*P1BP-k5*C*BP+kd5*CBP-
        k14*CP*BP+kd14*CPBP-k6*CP*BP+kd6*CBP;
xdot(5)=-k5*C*BP+kd5*CBP-k8*P2*C+kd8*P2CP-k12*I2*C+kd12*I2C;
xdot(6)=k5*C*BP-kd5*CBP+k6*CP*BP-kd6*CBP;
xdot(7)=-k6*CP*BP+kd6*CBP-k7*P2*CP+kd7*P2CP-k14*CP*BP+kd14*CPBP-
        k13*CP*B+kd13*CPBP;
xdot(8)=-k3*P1*BP+kd3*P1BP-k4*P1*B+kd4*P1BP;
xdot(9)=k3*P1*BP-kd3*P1BP+k4*P1*B-kd4*P1BP;
```

```
xdot(10)=-k7*P2*CP+kd7*P2CP-k8*P2*C+kd8*P2CP;
xdot(11)=k7*P2*CP-kd7*P2CP+k8*P2*C-kd8*P2CP;
xdot(12)=-k9*I1*B+kd9*I1B;
xdot(13)=k9*I1*B-kd9*I1B;
xdot(14)=-k12*I2*C+kd12*I2C;
xdot(15)=k12*I2*C-kd12*I2C;
xdot(16)=k14*CP*BP-kd14*CPBP+k13*CP*B-kd13*CPBP;
```



3.4.5 Summary

In this section, we simulated four models of combination therapy, which we will use them later for identification. What we have to notice from biological view is that only experimentally validated models that accurately describe actual signaling systems can be used to explore the mechanisms of action of real combination drugs. 'Toy' models, such those we examined, can be very helpful but they can only illustrate possibilities. However, models of biological networks relevant to human diseases that realistically capture, in mathematical form, actual cellular and tissue physiology are not far to be developed. Until then, mathematical models of cellular physiology will be the most reliable guides for drug development despite their limitations.

4 Fuzzy Systems

In the previous chapter we examined the combination therapy models that we will identify in this thesis. Now, it is time to present the neuro fuzzy identification scheme that we will use in this process. It is a Neuro-Fuzzy Dynamical System Definition Scheme, which uses the concept of Adaptive Fuzzy Systems operating in conjunction with High Order Neural Network Functions (F-HONNFs)[54].

In the first section of the chapter we focus on fuzzy systems and mostly on fuzzy control theory. Our goal is to examine basic concepts of the fuzzy theory, such as membership functions, fuzzy sets and fuzzy rules, which we will use in the identification procedure. We show how fuzzy logic provides a methodology for representing and implementing our knowledge about how best to control a process.

The second section of the chapter is about our identification scheme. After a brief introduction, we examine some essential theory in Addaptive Fuzzy Systems and High Order Neural Network Functions that is the basis for the identification scheme we use, which is finally presented in section 4.2.3.

4.1 Fuzzy Control

4.1.1 Introduction

Before starting to examine fuzzy control, we will try to explain the reasons for turning to it from conventional control. It is a fact that the difficult task of modeling and simulating complex real–world systems for control systems development, especially when implementation issues are considered, is well documented. Even if a relatively accurate model of a dynamic system can be developed, it is often too complex to use in controller development, especially for many conventional control design procedures that require restrictive assumptions for the plant (linearity for example). It is for this reason that in practice conventional controllers are often developed via simple models of the plants behavior that satisfy the necessary

assumptions, and via the ad hoc tuning of relatively simple linear or nonlinear controllers. However, it is well understood that heuristics enter the conventional control design process as long as we are concerned with the actual implementation of the control system. What we certainly must admit, is that conventional control engineering approaches that use appropriate heuristics to tune the design have been relatively successful.

The main idea of fuzzy control is to build a model of a human control expert who is capable of controlling a plant without thinking in terms of a mathematical model. It provides a formal methodology for representing, manipulating, and implementing a human's heuristic knowledge about how to control a system. We will try now to approach the design of fuzzy controllers, starting from examining a block diagram of a fuzzy control system (Fig. 4.1).

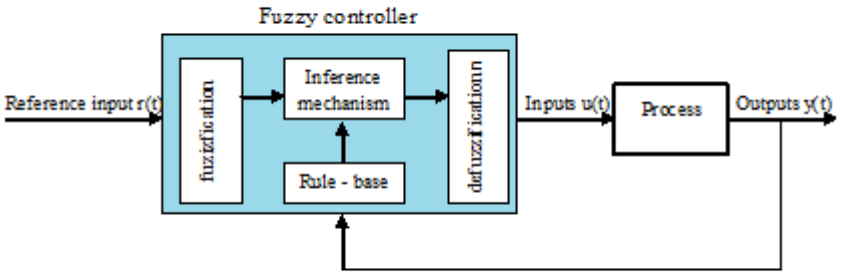


Figure 4.1 Block diagram of a fuzzy controller

In the diagram we can see a fuzzy controller embedded in a closed–loop control system. The plant inputs are denoted by u(t), its outputs by y(t), and the reference input to the fuzzy controller is denoted by r(t). The fuzzy controller is composed of the following four elements:

1) A rule base (a set of If – Then rules), which contains a fuzzy logic quantification of the expert's linguistic description about how to achieve good control.
2) An inference mechanism ('fuzzy inference' module), which emulates the expert's decision making interpreting and applying knowledge about how best to control the plant.

3) A fuzzification interface, which converts controller inputs into information that the inference mechanism can easily use to activate and apply rules.
4) A defuzzification interface, which converts the conclusions of the inference mechanism into actual inputs for the process.

In general, we can describe the fuzzy controller as an artificial decision maker that operates in a closed–loop system in real time. It gathers plant output data y(t), compares it to the reference input r(t), and then decides what the plan input u(t) should be ensure that the performance objectives will be meet.

To design the fuzzy–controller, we need information on how the artificial decision maker should act in the closed–loop system. Sometimes this information can come from a human decision maker who performs the control task, while at other times the control engineer can come to understand the plan dynamics and write down a set of rules about how to control the system without outside help. Trying to describe these 'rules', we could imagine them of the form: 'If the plant output and reference input are behaving in a certain manner, then the plant input should be some value.' After the whole set of such 'If – Then' rules is loaded into the rule–base and an inference strategy is chosen, then the system is ready to be tested to see if the closed–loop specifications are met.

For a better understanding of the above, we will use the problem of balancing an inverted pendulum on a cart (Fig. (4.2)), a very simple and common nonlinear control problem, for which there already are many techniques to provide a solution.

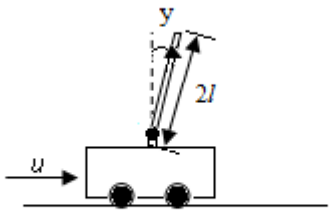


Figure 4.2 Inverted pendulum on a cart

Here, u is the force input that moves the cart, y denotes the angle that the pendulum makes with the vertical, and *l* is the half–pendulum length. We suppose that

r is the desired angular position of the pendulum. The goal is to balance the pendulum in the upright position (r = 0) when it initially starts with some nonzero angle off the vertical (y ≠0).

Continuing in this chapter we will examine general issues in fuzzy control system design, using the inverted pendulum as a convenient problem to illustrate the design and basic mechanics of the operation of a fuzzy control system.[55]

4.1.2 Choosing Fuzzy Controller Inputs and Outputs

The first step in the design of a fuzzy controller is to choose its inputs and outputs. We can describe a fuzzy system as a static nonlinear mapping between its inputs and outputs, which, in fact, are 'crisp' (real numbers) and not fuzzy sets. The fuzzification block converts the crisp inputs to fuzzy sets, the inference mechanisms uses the fuzzy rules in the rule-base to produce fuzzy conclusions, and the defuzzification block converts these fuzzy conclusions into the crisp outputs (Fig. (4.1).

For the inverted pendulum problem we described before, let's consider a human–in–the–loop whose responsibility is to control the pendulum. The fuzzy controller is to be designed to automate how a human expert who is successful at this task would control the system. First, the expert has to decide about the information that will be used as inputs to the decision–making process. Suppose that for the inverted pendulum, we have the following variables on which to base decision:

$$e(t) = r(t) - y(t)$$

$$\frac{d}{dt}e(t)$$

We have to note here, that we can have many other choices and that's something very common in most control problems.

The next step is to identify the controller variable. For the inverted pendulum, we are allowed to control only the force that moves the cart, so the choice here is simple. For more complex applications though, the choice of the inputs to the controller and outputs of the controller (inputs to the plant) can be more difficult. Essentially, we have to make sure that the controller will have the proper information available to be able to make good decisions and have proper control inputs to be able to steer the system in the directions needed for achieving high-performance operation. Practically speaking, access to information and the ability to effectively control the system often costs money. If we believe that proper information is not available for making control decisions, we may have to invest to another sensor that can provide a measurement of another system variable. Alternatively, we can implement some filtering or other processing of the plant outputs. In addition, if it is determined that the current actuators will not allow for the precise control of the process, we may need to invest in designing and implementing an actuator that can properly affect the process. What we want to show is that, while in most academic problems we may be given the plant inputs and outputs, in many practical situations we may have some flexibility in our choice. These choices affect what information is available for making on-line decisions about the control of a process and hence affect how we design a fuzzy controller.

Once the fuzzy controller inputs and outputs are chosen, we must determine what the reference inputs are. For example, in the inverted pendulum the choice of the reference input r=0 is clear. After all the inputs and outputs are defined for the fuzzy controller, we can specify the fuzzy control system, which for the example of the inverted pendulum, with our choice of inputs and outputs, we can see in Figure 4.3.

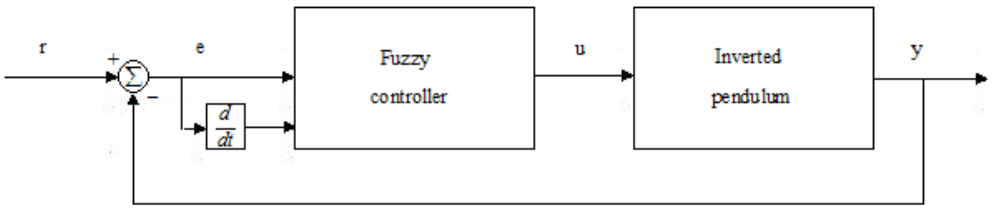


Figure 4.3 Fuzzy controller for an inverted pendulum on a cart

Now, within this framework we seek to obtain a description of how to control the process. In many occasions the choice of the inputs and outputs of the controller

may place certain constraints of the reminder of the fuzzy control design process. If the proper information is not provided to the fuzzy controller, there will be little hope for being able to design a good rule–base or inference mechanism. Moreover, even if the proper information is available to make control decisions, this will be of little use if the controller is not able to properly affect the process variables via the process inputs. From all these, it is clear that the choice of the controller inputs and outputs is a fundamentally important part of the control design process.[55],[56],[57]

4.1.3 Rule-Bases

To specify rules for the rule-base, the expert will use a 'linguistic description'; hence, linguistic expressions are needed for the inputs and outputs and their characteristics. Suppose that the human expert provides a description of how best to control the plant in some natural language (e.g. Greek). Our goal is to take this 'linguistic' description and load in into the fuzzy controller. We will use 'linguistic variables' (constant symbolic descriptions of what are in general time-varying quantities) to describe fuzzy system inputs and outputs. For example, let's suppose we use linguistic variables $\tilde{u}_i$ and $\tilde{y}_i$ to describe the inputs u_i and the outputs y_i respectively. An input to the fuzzy system may be described as $\tilde{u}_i$ = 'position error' and an output from the fuzzy system may be $\tilde{y}_i$ = 'voltage in'.

The linguistic description provided by the expert can generally be broken into several parts. There will be 'linguistic variables' that describe each of the time–varying fuzzy controller inputs and outputs. To make things more clear, for the inverted pendulum as described previously we will have:

'error' describes e(t)

'change–in–error' describes $\frac{d}{dt}e$

'force' describes u(t)

Note that we use quotes to emphasize that certain words or phrases are linguistic descriptions, and that we have added the time index (e.g. e(t)) to emphasize that generally e varies with time. There are many possible choices for the linguistic descriptions for variables. Some designers like to choose them so that they are quite descriptive for documentation purposes. However, this can sometimes lead to long descriptions. Others seek to keep the linguistic description as short as possible (e.g., using 'e(t)' as the linguistic variable for e(t)), yet accurate enough so that they adequately represent the variables. The truth is that the choice of the linguistic variable has no impact on the way that the fuzzy controller operates. It is simply a notation that helps to facilitate the construction of the fuzzy controller via fuzzy logic.

Just as e(t) takes on a value of, for example 1 at t = 2 (e(2) = 1), linguistic variables take on 'linguistic values' that are used to describe characteristics of the variables. That is, the values that linguistic variables take on over time change dynamically. Suppose for the pendulum example that 'error', 'change–in–error' and 'force' take on the following values:

'neglarge'
'negsmall'
'zero'
'possmall'
'poslarge'

We are using 'negsmall' as an abbreviation for 'negative small in size', 'neglarge' for 'negative large in size' and so on for the other variables. Such, abbreviations help keep the linguistic description short yet precise. For an even shorter description we could use integers:

'-2' to represent 'neglarge'
'-1' to represent 'negsmall'
'0' to represent 'zero'
'1' to represent 'possmall'
'2' to represent 'poslarge'

This is a particularly appealing choice for the linguistic values since the descriptions are short and nicely represent that the variable we are concerned with has a numeric quality. We are not, for example, associating '-1' with any particular number of radians of error: the use of the numbers for linguistic description simply quantifies the sign of the error (in the usual way) and indicates the size in relation to

the other linguistic values. We shall find the use of this type of linguistic value quite convenient and hence will give it the special name, 'linguistic – numeric value'.

The linguistic variables and values provide a language for the expert to express her or his ideas about the control decision – making process in the context of the framework established by our choice of fuzzy controller inputs and outputs. Recall that for the inverted pendulum r = 0, and e = r – y, so we have:

$$e = -y \quad \text{, and}$$

$$\frac{d}{dt}e = -\frac{d}{dt}y \quad \text{, since } \frac{d}{dt}r = 0 .$$

. First, we will examine how we can quantify certain dynamic behaviors with linguistics. Let's see for example how to quantify knowledge about how to control the pendulum using linguistic descriptions. Each of the following statements quantifies a different configuration of the pendulum (Fig. 4.2):

- The statement 'error is poslarge' can represent the situation where the pendulum is at significant angle to the left of the vertical.
- The statement 'error is negsmall' can represent the situation where the pendulum is just slightly to the right of the vertical, but not too close to the vertical to justify quantifying it as 'zero' and not too far away to justify quantifying it as 'neglarge'.
- The statement 'error is zero' can be represented the situation where the pendulum is very near the vertical position (a linguistic quantification is not precise, so we have to accept any value of the error around e(t) = 0 as being quantified linguistically by 'zero', since this can be considered a better quantification than 'possmall' or 'negsmall').
- The statement 'error is poslarge **and** change–in–error is possmall' can represent the situation where the pendulum is to the left of the vertical, and since $\frac{d}{dt}y < 0$, the pendulum is moving away from the upright position (the pendulum is moving counterclockwise).
- The statement 'error is negsmall **and** change–in–error is possmall' can represented the situation where the pendulum is slightly to the right of the

vertical and, since $\frac{d}{dt}y < 0$, the pendulum is moving toward the upright position (the pendulum is moving counterclockwise).

Overall, we see that to quantify the dynamics of the process we need to have a good understanding of the physics of the underlying process we are trying to control. While for the pendulum problem, the task of coming to a good understanding of the dynamics is relatively easy, this does not happen usual for most physical processes. Quantifying the process dynamics with linguistic is not always easy, and certainly a better understanding of the process dynamics generally leads to a better linguistic quantification, which in turn leads to a better fuzzy controller, provided that we can adequately measure the system dynamics so that the fuzzy controller can make the right decisions at the proper time.

Next step in the procedure is to map the inputs to the outputs for our fuzzy system. This mapping is in part characterized by a set of *condition* → *action* rules, which are of the form:

If premise **Then** consequent.

For this, we use the previous linguistic quantification to specify a set of rules (a rule–base) that captures the expert's knowledge about how to control the plant. For better understanding, we consider the inverted pendulum in the three positions shown in Figure 4.4, for which we have the following rules:

a) **If** error is neglarge **and** change–in–error is neglarge **Then** force is poslarge

This rule quantifies the situation in Figure (4.4a) where the pendulum has a large positive angle and is moving clockwise; we should apply a strong positive force (to the right) so that we can try to start the pendulum moving in the proper direction.

b) **If** error is zero **and** change–in–error is possmall **Then** force is negsmall

This rule quantifies the situation in Figure (4.4b) where the pendulum has nearly a zero angle with the vertical and is moving counterclockwise; we should apply a small negative force (to the left) to counteract the movement so that it moves toward zero.

c) **If** error is poslarge **and** change–in–error is negsmall **Then** force is negmall

This rule quantifies the situation in Figure (4.4c) where the pendulum is far to the left of the vertical and is moving clockwise; we should apply a small negative force (to the left) to assist the movement, (not large since the pendulum is already moving in the proper direction).

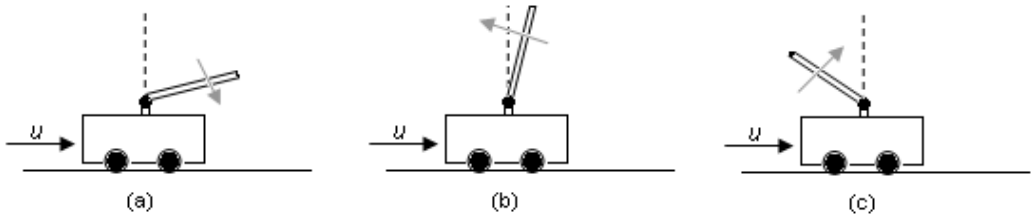


Figure 4.4 Inverted pendulum on a cart in various positions

Each of the three rules listed above is a 'linguistic rule' since it is formed only from linguistic variables and values. Since linguistic values are not precise representations of the underlying quantities that they describe, linguistic rules are not precise either. They are simply abstract ideas about how to achieve good control. Of course, this could mean somewhat different things to different people, but in general humans are often comfortable with such abstraction in terms of specifying how to control a process.

As we can see from the three rules above, the premises are associated with the fuzzy controller inputs and the consequents are associated with the fuzzy controller outputs. The number of fuzzy controller inputs and outputs places an upper limit on the number of elements in the premises and consequents. What we should note is that there does not need to be a premise or consequent term for each input or output in each rule, although often there is.

Using the above approach, we could continue to write down rules for the pendulum problem for all possible cases. Since we only specify a finite number of linguistic variables and linguistic values, there is only a finite number of possible rules. For the pendulum problem for example, with two inputs and five linguistic

values for each of these, there are at most $5^2 = 25$ possible rules (all possible combinations of premise linguistic values for two inputs).

The most common and convenient way to list all possible rules for the case where there are not too many inputs to the fuzzy controller is to use a tabular representation. We present a tabular representation of one possible set of rules for the inverted pendulum in the next table:

"force" u		"change – in – error" e				
		-2	-1	0	1	2
"error" e	-2	2	2	2	1	0
	-1	2	2	1	0	-1
	0	2	1	0	-1	-2
	1	1	0	-1	-2	-2
	2	0	-1	-2	-2	-2

If we view the body of the table as a matrix, we can see that it has a kind of symmetry; also we can see a diagonal of zeros. This symmetry that emerges when the rules are tabulated is no accident and is actually a representation of abstract knowledge about how to control the pendulum; it arises due to a symmetry in the system's dynamics. In general, similar patterns are found when constructing rule–bases for most applications; this symmetry is very useful and it can be exploited in implementing fuzzy controllers.[55],[57]

4.1.4 Membership Functions

Up to this point we have only quantified, in an abstract way, the knowledge that the human expert has about how to control the plant. Next, we will show how to use fuzzy logic to fully quantify the meaning of linguistic descriptions so that we may automate, in the fuzzy controller, the control rules specified by the expert.

First, we quantify the meaning of the linguistic values using 'membership functions'. Let U_i denote a universe of discourse and $\tilde{A}_i^j \in \tilde{A}_i$ denote a specific

linguistic value for the linguistic variable $\tilde{u}_i$. The function $\mu(u_i)$ associated with $\tilde{A}_i^j$ that maps U_i to [0,1] is called a 'membership function'. This function describes the 'certainty' that an element u_i of U_i with a linguistic description $\tilde{u}_i$, may be classified linguistically as $\tilde{A}_i^j$. Membership functions are subjectively specified in an ad hoc (heuristic) manner from experience or intuition.

Trying to make things more clear we will examine the plot of Figure 4.5. This is a plot of a function μ versus *e(t)* that takes on special meaning. The function μ quantifies the *certainty*[2] that *e(t)* can be classified linguistically as 'possmall'. To understand the way what membership function works, it is best to perform a case analysis where we show how to interpret it for various values of *e(t)*:

- If *e(t)* = $-\pi/2$ then $\mu(-\pi/2) = 0$, indicating that we are certain that *e(t)* = $-\pi/2$ is *not* 'possmall'.
- If *e(t)* = $\pi/8$ then $\mu(\pi/8) = 0.5$, indicating that we are halfway certain that *e(t)* = $\pi/8$ is 'possmall' (we are only halfway certain since it could be also 'zero' with some degree of certainly – this value is in a 'gray area' in terms of linguistic interpretation).
- If *e(t)* = $\pi/4$ then $\mu(\pi/4) = 1.0$, indicating that we are absolutely certain that *e(t)* = $\pi/4$ is what we mean by 'possmall'.
- If *e(t)* = π then $\mu(\pi) = 0$, indicating that we are certain that *e(t)* = π is not 'possmall' (actually, it is 'poslarge').

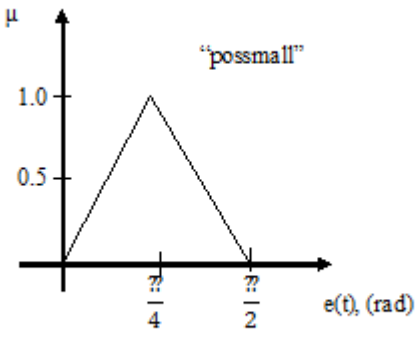


Figure 4.5 Membership function for linguistic value 'possmall'

The membership function quantifies, in a continuous manner, whether values of *e(t)* belong to the set of values that are 'possmall,' and hence it quantifies the meaning of the linguistic statement 'error is possmall'. This is why it is called a membership function. Of course, the membership function of Figure (4.5) is not the only possible definition of the meaning of 'error is possmall'; it could used a bell – shaped function, a trapezoid, a gaussian or many others.

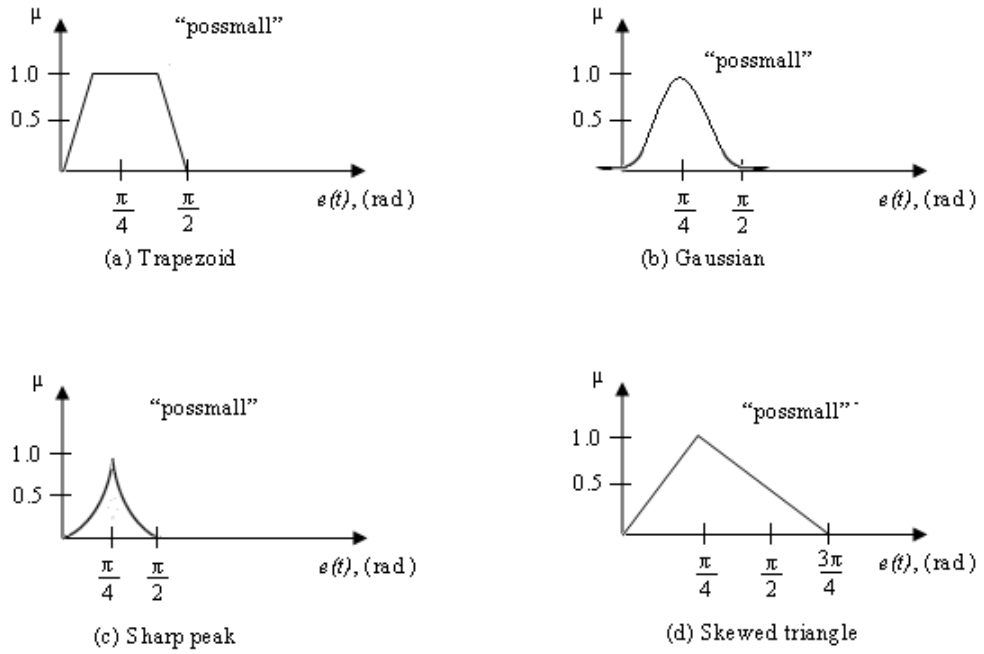


Figure 4.6 Four membership function choices for representing 'error is possmall'

To become more specific, we will examine the membership functions shown in Figure 4.6. For some applications someone may be able to argue that we are absolutely certain that any value of e(t) near $\frac{\pi}{4}$ is still 'possmall' and only when we get sufficiently far from $\frac{\pi}{4}$ do we lose our confidence that it is 'possmall'. One way to characterize this understanding of the meaning of the 'possmall' is via a trapezoid–shaped membership function (Fig. (4.6a)). For other applications we may think of membership in the set of 'possmall' values as being dictated by the Gaussian–shaped membership function (Fig. (4.6b)). And for other applications we may not readily accept values far away from $\frac{\pi}{4}$ as being 'possmall', so we may use the membership function in Figure (4.6c) to represent this. Finally, while we often think about

symmetric characterizations of the meaning of linguistic values, we are not restricted to these symmetric representations. For example, we can represent that we believe that as e(t) moves to the left of $\frac{\pi}{4}$ we are very quick to reduce our confidence that it is 'possmall', but if we move to the right of $\frac{\pi}{4}$ our confidence that e(t) is 'possmall' diminishes at a slower rate (Fig. (4.6d)).

To sum up, we saw that depending on the application and the designer, many different choices of membership functions are possible. What we should notice, however, is that for the most part the definition of a membership function is subjective rather than objective. That is, we simply quantify it in a manner that makes sense to us, but others may quantify it in a different manner.

Now, we will try to examine with simple examples the meaning of the term 'fuzzy set'. The set of values that is described by μ as being 'positive small' is called a 'fuzzy set'. Let A denote this fuzzy set. As we saw in Figure (4.5), we are absolutely certain that e(t) = $\frac{\pi}{4}$ is an element of A, but we are less certain that e(t) = $\frac{\pi}{16}$ is an element of A. Membership in the set, as specified by the membership function, is fuzzy; hence we use the term 'fuzzy set'.

A 'crisp' (as contrasted to 'fuzzy') quantification of 'possmall' can also be specified, but with the help of the membership function shown in Figure (4.7). This membership function is simply an alternative representation for the interval on the real line $\pi/8 \leq e(t) \leq 3\pi/8$, and it indicates that this interval of numbers represents 'possmall'. Clearly, this characterization of crisp is simply another way to represent a normal interval (set) of real numbers.

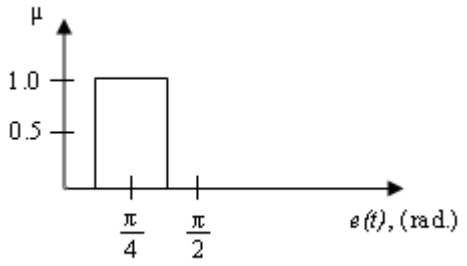


Figure 4.7 Membership function for a crisp set

Returning again to Figure (4.5) we will take a closer look in its horizontal axis. While the vertical axis in represents certainty, the horizontal axis is also given a

special name. It is call the 'universe of discourse' for the input e(t) since it provides the range of values of e(t) that can be quantified with linguistics and fuzzy sets. In conventional terminology, a universe of discourse for an input and output of a fuzzy system is simply the range of values the inputs and outputs can take on.

Now that we saw how to specify the meaning of linguistic value via a membership function (and hence a fuzzy set), we can easily specify the membership functions for all 15 linguistic values (live for each input and five for the output) of our inverted pendulum example (Fig. (4.8)).

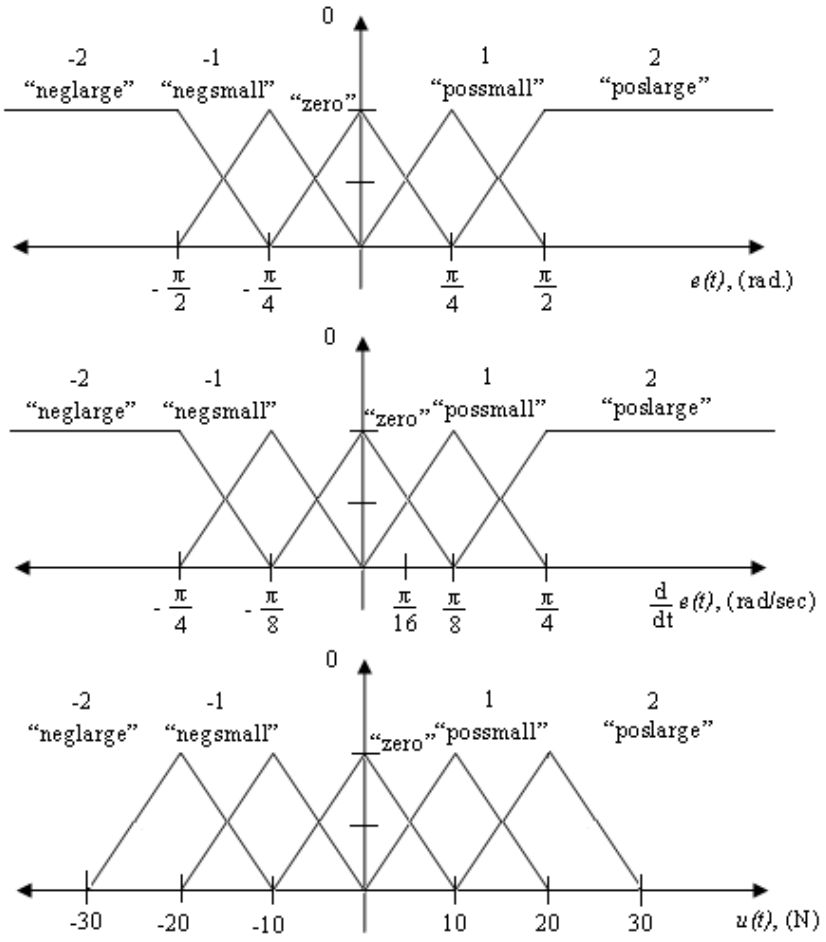


Figure 4.8 Membership functions for an inverted pendulum on a cart

In Figure (4.8) there are listed both the linguistic values and the linguistic–numeric values associated with each membership function. Hence, we see that the membership function in Figure (4.5) for 'possmall', is embedded among several others that describe other sizes of values (so that, for instance, the membership

function to the right of the one for ‘possmall’ is the one that represents ‘error is poslarge’).

The membership functions at the outer edges in Figure (4.8) deserve special attention. For the inputs e(t) and $\frac{d}{dt}e(t)$ we see that the outermost membership functions ‘saturate’ at a value of one. This makes intuitive sense as at some point the human expert would just group all large values together in a linguistic description such as ‘poslarge’. The membership functions at the outermost edges appropriately characterize this phenomenon since they characterize ‘greater than’ (for the right side) and ‘less than’ (for the left side).

For the output u, the membership functions at the outermost edges cannot be saturated for the fuzzy system to be properly defined. The basic reason for this is that in decision–making process of such type, we seek to take actions that specify an exact value for the process input.

In general, in such a procedure it is important to have a clear picture in our mind of how the values of the membership functions change. For instance, as e(t) changes form $-\pi/2$ to $\pi/2$ we seek that various membership functions will take on zero and nonzero values indicating the degree to which the linguistic value appropriately describes the current value of e(t). For example, at $e(t) = -\pi/2$ we are certain that the error is ‘neglarge’, and as the value of e(t) moves toward $-\pi/4$ we become less certain that it is ‘neglarge’ and more certain that it is ‘negsmall’. We see that the membership functions quantify the meaning of linguistic statements that describe time–varying signals.

The rule–base of the fuzzy controller holds the linguistic variables, linguistic values, their associated membership functions, and the set of all linguistic rules (as we can saw them above in the rule table for the inverted pendulum), so it is completed the description of our system (for example of the simple inverted pendulum). Next, follows the fuzzification process, the inference mechanism and finally the defuzzification process. In our work for this thesis we don’t use these procedures, so we will be restricted in a briefly description for each.[55],[57]

4.1.5 Fuzzification

Fuzzy sets are used to quantify the information in the rule-base, and the inference mechanism operates on fuzzy sets to produce fuzzy sets. So, we have to specify how the fuzzy system will convert its numeric inputs into fuzzy sets so that they can be used from the fuzzy system. This process is called fuzzification process and can be described more specifically as the act of obtaining a value of an input variable (for example e(t)) and finding the numeric values of the membership function(s) that are defined for that variable. For example, let's suppose that we have e(t) = π/4 and $\frac{d}{dt}$ e(t) = π/16. With the fuzzification process we find the values of the input membership functions, which as we can see from Figure (4.8) are:

$$\mu_{possmall}(e(t)) = 1 \text{ ,and}$$

$$\mu_{zero}(\frac{d}{dt}e(t)) = \mu_{possmall}(\frac{d}{dt}e(t)) = 0.5\,.$$

As we can see, it is a very simple process; hence for most fuzzy controllers the fuzzification block can be ignored.

The most common fuzzification process which can be found is the, so called, 'singleton fuzzification'. If U_i^* is the set of all possible fuzzy set that can be defined on U_i, given that numeric inputs $u_i \in U_i$, singleton fuzzification produces a fuzzy set $\hat{A}_i^{fuz} \in U_i^*$, with a membership function defined by

$$\mu_{\hat{A}_i^{fuz}}(x) = \begin{cases} 1, \text{ if } x = u_i \\ 0, \text{ otherwise} \end{cases}$$

Any fuzzy set with this membership function form is called a 'singleton' and as we can see, its membership function can be represented by the discrete impulse function. In fact, we can consider the singleton fuzzy set as a different representation for the number u_i. Singleton fuzzification is generally used in implementations when, without the presence of noise, we can be certain that u_i takes on only its measured value. It is preferred from other fuzzification methods because it does not add so

much computational complexity to the inference process and because with its use there can be achieved very good functional capabilities with the fuzzy system.[55]

4.1.6 The Inference Mechanism

We can consider the membership function values as an 'encoding' of the fuzzy controller numeric input values; this encoded information is then used in the fuzzy inference process, which in general involves two steps.

In the first step, the premises of all the rules are compared to the controller inputs to determine which rules apply to the current situation. This 'matching' process involves determining the certainty that each rule applies, and typically we will more strongly take into account the recommendations of rules for which we are more certain that apply to the current situation.

To perform inference we must first quantify each of the rules with fuzzy logic. To do this, we first quantify the meaning of the premises of the rules that are composed of several terms, each of which involves a fuzzy controller input. After this, we have to determine which rules are 'on'. We say that a rule is 'on at time *t*' if for its premise membership function we have $\mu_{premise}(e(t), \frac{d}{dt} e(t)) > 0$. Hence, the inference mechanism seeks to determine which rules are on to find out which rules are relevant to the current situation. In the next step, the inference mechanism will seek to combine the recommendations of all the rules to come up with a single conclusion.

In the second step (inference step), we determine the conclusions (what control actions to take) using the rules that have been determined to apply at the current time. The conclusions are characterized with a fuzzy set (or sets) that represents the certainty that the input to the plant should take on various values. To do this, usually we consider first the recommendations of each rule independently and then we combine all the recommendations together. There are two standard alternatives for performing the inference step; one by determining the implied fuzzy sets and another one by determining the overall implied fuzzy set.[55]

4.1.7 Defuzzification

The final step for the designing of a fuzzy controller is the defuzzification process, in which we convert the conclusions of the inference mechanism into actual inputs for the process. Defuzzification operates on the implied fuzzy sets produced by the inference mechanism and combines their effects to provide the 'most certain' controller output (plant input). It can be considered as 'decoding' the fuzzy set information produced by the inference process (i.e., the implied fuzzy sets) into numeric fuzzy controller outputs.

There exist many defuzzification strategies, and it is believed that it is not hard to invent even more. Each provides a means to choose a single output based on either the implied fuzzy sets or the overall implied fuzzy set, depending on the type of the inference strategy chosen as we saw before. As most common techniques we can mention the 'Center of gravity'(COG), the 'Center-average), the 'Max criterion', the 'Mean of maximum', the 'Center of area' and many others more.[55]

4.2 A Neuro Fuzzy Identification Scheme

4.2.1 Introduction

In this section we will present the identification scheme we use in this thesis for the identification of the combination therapy models we described in the previous chapter. We will use a neuro fuzzy identification scheme, which uses the concept of Adaptive Fuzzy Systems operating in conjunction with High Order Neural Network Functions (F-HONNFs). Since the plant is considered unknown, we first propose its approximation by a special form of an adaptive fuzzy system and in the sequel the fuzzy rules are approximated by appropriate HONNFs. Thus the identification scheme leads up to a Recurrent High Order Neural Network, which however takes into account the fuzzy output partitions of the initial AFS. The proposed scheme does not require a-priori experts' information on the number and type of input variable

membership functions making it less vulnerable to initial design assumptions. There are provided weight updating laws for the involved HONNFs, which guarantee that the identification error reaches zero exponentially fast. Simulations illustrate the potency of the method and comparisons with conventional approaches are given.

We know that a nonlinear dynamical system can be represented by general nonlinear dynamical equations of the form $\dot{x} = f(x, u)$ (4.1). We need this mathematical description of the system, so that we will be able to control it. In most of cases the exact mathematical model of the plant, especially when this is highly nonlinear and complex, is unknown; hence we have to use appropriate identification schemes which can provide an approximate model of the plant.

We saw in the previous section, how a fuzzy system can perform control. Fuzzy systems of course, and neural networks too, can be also universal approximators[58],[59],[60]; they can approximate any nonlinear function to any prescribed accuracy. Essential for this is that sufficient hidden neurons and training data or fuzzy rules are available. The combination of these two different technologies has given rise to neuro fuzzy approaches, which have the advantages of both fuzzy logic and neural networks. Neural and fuzzy approaches are most of the time equivalent, differing between each other mainly in the structure of the approximator chosen. Many recent researchers have brought them even closer introducing adaptive schemes using a class of parameterized functions that include both neural networks and fuzzy systems[61],[62],[63],[64],[65],[66].

The identification procedure is an essential part in any control procedure. In the neuro fuzzy control approaches, it is most common the use of indirect adaptive control and not the direct approach, because it is not always clear how to construct the control law without knowing the system dynamics. Their difference is that indirect adaptive control first identifies the dynamics of the systems and then performs control, while direct adaptive control directly generates the control input to guarantee stability. High order neural network function approximators (HONNFs) have been also proposed for the identification of nonlinear dynamical systems, approximated by a Fuzzy Dynamical System. This approximation depends on the fact that fuzzy rules could be identified with the help of HONNFs.

In the scheme we use in this thesis, HONNFs are also used for the neuro fuzzy identification of unknown nonlinear dynamical systems. In fuzzy or neuro-fuzzy approaches the identification phase usually consists of structure identification and

parameter identification. In structure identification we find the main input variables out of all possible, we specify the membership functions, the partition of the input space and then we determine the number of fuzzy rules which is often based on a substantial amount of heuristic observation to express proper strategy's knowledge. These approaches require that all input-output data are ready before we start to identify the plant (off-line identification). In the approach we use, structure identification is also made off-line, based either on human expertise or on gathered data. However, the required a-priori information obtained by linguistic information or data is very limited, which is a very big advantage, especially for bilogical systems like those we examine in our work. The only required information is an estimate of the centers of the output fuzzy membership functions. Information on the input variable membership functions and on the underlying fuzzy rules is not necessary because this is automatically estimated by the HONNFs. This way the proposed method is less vulnerable to initial design assumptions. Hence, the parameter identification is easily addressed by HONNFs, based on the linguistic information regarding the structural identification of the output part and from the numerical data obtained from the actual system to be modelled. This means that the parameters of identification model are updated on-line in such a way that the error between the actual system output and the model output reaches zero exponentially fast.[54]

4.2.2 Addaptive Fuzzy Systems

The performance, complexity, and adaptive law of an adaptive fuzzy system representation can be quite different depending upon whether the representations is linear or nonlinear in its adjustable parameters. Adaptive fuzzy controllers depend also on the type of the adaptive fuzzy subsystems they use and they can be classified into first-type and second-type adaptive fuzzy controllers, which are both nonlinear.[60]

In the first-type adaptive fuzzy controllers there are used fuzzy logic systems which are linear in their adjustable parameters. Suppose we have to approximate the nonlinear function f(x). Then we can use the following fuzzy logic representation:

$$f(x) = \sum_{l=1}^{M} \theta_l \xi_l(x) = \theta^T \xi(x)$$

where M is the number of fuzzy rules, $\theta = (\theta_1, ..., \theta_M)^T$, $\xi(x) = (\xi_1(x), ..., \xi_M(x))^T$ and $\xi_l(x)$ is the fuzzy basis function defined by

$$\xi_l(x) = \frac{\prod_{i=1}^{n} \mu_{F_i^l}(x_i)}{\sum_{l=1}^{M} \prod_{i=1}^{n} \mu_{F_i^l}(x_i)}$$

θ_l are adjustable parameters and $\mu_{F_i^l}$ are given membership functions of the input variables of any type. If we assume that $\mu_{F_i^l}$ are given and they will not change during the adaptation procedure, we have:

$$f(x) = \frac{\sum_{l=1}^{M} y^l (\prod_{i=1}^{n} \mu_{F_i^l}(x))}{\sum_{l=1}^{M} \prod_{i=1}^{n} \mu_{F_i^l}(x)}$$

In the second-type adaptive fuzzy controllers now, there are used fuzzy logic systems which are nonlinear in their adjustable parameters. In this case, for the approximation of the nonlinear function f(x), we use the following fuzzy logic system:

$$f(x) = \frac{\sum_{l=1}^{M} y^l (\prod_{i=1}^{n} \exp(-(\frac{x_i - x_i^l}{\sigma_i^l})^2))}{\sum_{l=1}^{M} (\prod_{i=1}^{n} \exp(-(\frac{x_i - x_i^l}{\sigma_i^l})^2))}$$

where y^l, x_i^l, σ_i^l are the adjustable parameters.

From the above we can understand that the success of the adaptive fuzzy system representations in approximating the nonlinear function f(x) depends on the careful selection of the fuzzy partitions of input and output variables, the selected type of the membership functions and the proper number of the fuzzy rules, which for complex nonlinear functions may become very large and may lead to parameter explosion.[54]

4.2.3 HONNFs as Fuzzy Rule Approximators

We will see in this section that functions of high order neurons are capable of approximating discontinuous functions; hence, we use high order neural network functions in order to approximate the indicator functions $I_{j_1,\ldots,j_{n+m}}^{l_1,\ldots,l_n}$. However, if we want the approximation problem to make sense, the space $\boldsymbol{y} := \boldsymbol{x} \times \boldsymbol{u}$ must be compact. So, the following assumptions are made:

$\boldsymbol{y} := \boldsymbol{x} \times \boldsymbol{u}$ is a compact set, and

$\boldsymbol{y}^{\boldsymbol{T}}$ is a compact set.

Suppose now we have the following high order neural network functions (HONNFs):

$$N(x,u;w,L) = \sum_{k=1}^{L} w_k \prod_{j \in I_k} \Phi_j^{d_j(k)}$$

where $\{I_1, I_2, ..., I_L\}$ is a collection of L not-ordered subsets of {1, 2, ...,m+n}, $d_j(k)$ are non-negative integers, Φ_j are sigmoid functions of the state or the input and $w := [w_1 ... w_L]^T$ are the HONNF weights. The HONNF can be also written:

$$N(x,u;w,L) = \sum_{k=1}^{L} w_k s_k(x,u)$$

where $s_k(x,u)$ are high order terms of sigmoid functions of the state and input. The next lemma states that a HONNF of this form can approximate the indicator function $I_{j_1,\ldots,j_{n+m}}^{l_1,\ldots,l_n}$.

.

Lemma 1: Consider the indicator function $I^{l_1,...,l_n}_{j_1,...,j_{n+m}}$ and the family of the HONNFs N(x,u;w,L). Then for any ε > 0 there is a vector of weights $w^{j_1,...,j_{n+m};l_1,...,l_n}$ and a number of $L^{j_1,...,j_{n+m};l_1,...,l_n}$ high order connections such that

$$\sup_{(x,u)\in\bar{y}} \{I^{l_1,...,l_n}_{j_1,...,j_{n+m}}(x,u) - N(x,u;w^{j_1,...,j_{n+m};l_1,...,l_n}, L^{j_1,...,j_{n+m};l_1,...,l_n})\} < \varepsilon$$

where $\bar{y} \equiv \boldsymbol{y}$, if $\boldsymbol{y} := \boldsymbol{x} \times \boldsymbol{u}$ is a compact set, and $\bar{y}_T \equiv \boldsymbol{y}$, if $\boldsymbol{y^T}$ is a compact set, as we assumed before.[54]

4.2.4 The Identification Scheme

In this section we will see how from the theory we presented in sections 4.2.1 and 4.2.2 we can be lead to the neuro fuzzy identification scheme we will use. Let's consider affine in the control, nonlinear dynamical systems of the form $\dot{x}$ = f(x) + G(x)・u (4.2), where the state $x \in R^n$ is assumed to be completely measured, the control u is in R^n, f is an unknown smooth vector field (the drift term) and G is a matrix with columns the unknown smooth controlled vector fields g_i, i = 1, 2, ..., n ($G = [g_1, g_2, ..., g_n]$).

The above class of continuous-time nonlinear systems are called affine, because in Equation (4.2) the control input appears linear with respect to G. Most of the systems encountered in engineering, are by nature or design, affine. Furthermore, we note that non affine systems of the form given in Equation (4.1) can be converted into affine, by passing the input through integrators. The existence and uniqueness of solution for any finite initial condition and u∈U is guaranteed.

It is proved that the following affine Recurrent High Order Neural Network (RHONN), which depends on the centers of the fuzzy output partitions $\overline{f}_l$ and $\overline{g}_{i,l}$ approximates the system in Equation (4.2):

$$\dot{\hat{\chi}} = A\hat{\chi} + XWS(\chi) + X_1W_1S_1(\chi)u$$

where A is an $n \times n$ stable matrix which for simplicity can be taken to be diagonal as A = diag[$a_1, a_2, ..., a_n$], X, X_1 are matrices containing the centres of the partitions of every fuzzy output variable of f(x) and g(x) respectively, S(χ), S_1(χ) are matrices containing high order combinations of sigmoid functions of the state χ and W,W_1 are matrices containing neural weights. The dimensions and the contents of all the above matrices are chosen so that XWS(χ) is an $n \times 1$ vector and $X_1W_1S_1$(χ) is a $n \times n$ matrix, while the matrix G is diagonal. All output fuzzy variables are partitioned to the same number, m, of partitions. Under these specifications X is an $n \times n \cdot m$ block diagonal matrix of the form X = diag(X_1,X_2, . . . ,X_n) with each X_i being an m-dimensional raw vector of the form $X^i = [\overline{f}_1^i, \overline{f}_2^i, ..., \overline{f}_m^i]$, where $\overline{f}_p^i$ denotes the centre of the p-th partition of f_i. Also, $S(\chi) = [s_1(\chi)...s_k(\chi)]^T$, where each s_i(χ) is a high order combination of sigmoid functions of the state variables and W is a n · m × k matrix with neural weights. W is of the form $W = [W^1...W^n]^T$, where each W^i is a matrix $[w_{jl}^i]_{m \times k}$. X_1 is an n × n · m block diagonal matrix $X_1 = diag({}^1X^1, {}^1X^2, ..., {}^1X^n)$, with each ${}^1X^i$ being an m-dimensional raw vector of the form ${}^1X^i = [\overline{g}_1^{i,i}, \overline{g}_2^{i,i}, ..., \overline{g}_m^{i,i}]$, where $\overline{g}_k^{i,i}$ denotes the center of the k-th partition of g_{ii}. W1 is a m · n × n block diagonal matrix $W_1 = diag({}^1W^1, {}^1W^2, ..., {}^1W^n)$, where each ${}^1W^i$ is a column vector $[{}^1w_{jl}^i]_{m \times 1}$ of neural weights. Finally, S_1(χ) is a n × n diagonal matrix with each diagonal element s_i(χ) being a high order combination of sigmoid functions of the state variables.

Assuming the existence of only parameter uncertainty, the actual system (4.2) can be modeled by the following neural form:

$$\dot{\chi} = A\chi + XW^*S(\chi) + X_1W_1^*S_1(\chi)u$$

The error between the identifier states and the real states is defined as:

$$e = \hat{\chi} - \chi$$

So, we have the following error equation:

$$\dot{e} = Ae + X\tilde{W}S(\chi) + X_1\tilde{W}_1S_1(\chi)U$$

where $\tilde{W} = W - W^*$ and $\tilde{W}_1 = W_1 - W_1^*$.

Finally, we take the following learning laws:

$$\dot{W} = -X^T PeS^T \text{, and}$$

$$\dot{W}_1 = -X_1^T PEUS_1^T$$

where E and U are diagonal matrices such that $E = diag(e_1,...,e_n)$ and $U = diag(u_1,...,u_n)$, and P > 0 is chosen to satisfy the Lyapunov equation $PA + A^T P = -I$.[54]

5 Identification of Combination Therapy Models

In previous chapter, we saw the importance of combination therapy in medicine nowadays by examining its theory and we also presented four models used for the evaluation of multicomponent drugs, which we also simulated. Furthermore, we presented a new neuro fuzzy dynamical system identifier based on High Order Neural Network function approximators. Now, it is time to use this identifier to see the results it can have in approximating biological systems; and more specific for our thesis, in approximating combination therapy models that show amplification, ultrasensitivy and feedback control. Before though we present the analytical description of the identification procedure it is necessary to prove that this F-HONNF approximator is applicable for these systems.

5.1 Identifying Autonomous Systems

As we saw in previous section (4.2.3), a nonlinear dynamical system of the form $\dot{x}$ = f(x) + G(x) · u, where the state $x \in R^n$ is assumed to be completely measured, the control *u* is in R^n, f is an unknown smooth vector field (the drift term) and G is a matrix with columns the unknown smooth controlled vector fields g_i, i = 1, 2, ..., n can be modeled by the neural form $\dot{\chi} = A\chi + XW^*S(\chi) + X_1W_1^*S_1(\chi)u$. However, it is clear that the systems of combination therapy we want to identify as they are described in Chapter 3 are autonomous, since they don't possess an external control u, having only instant external input stimuli (inhibitors). Hence, we will present now the proof that an autonomous system can behave dynamically as a non-autonomous system and so it can be modeled by the neural form $\dot{\chi} = A\chi + XW^*S(\chi) + X_1W_1^*S_1(\chi)u$.

Lemma 1: An autonomous system, with arbitrary initial conditions described by the differential equation:

$$\dot{\chi}(t) = f(\chi(t)), \text{ with } \chi(0) \text{ arbitrary (5.1)}$$

can behave dynamically exactly as the next system, with given initial conditions:

$$\dot{x}(t) = f(x(t)) + u, \text{ with x(0) given and constant (5.2)}$$

if u is of the form:

$$u(t) = [\chi(0) - x(0)] \cdot \delta(t) \quad (5.3)$$

where δ(t) is a dirac function.

Proof: Consider the two systems described by the Equations (5.2) and (5.3). By integrating these two equations, we get:

$$\chi(t) = \int_{0^-}^{t} f(\chi(t))dt + \chi(0) \quad (5.4)$$

$$x(t) = \int_{0^-}^{t} f(x(t))dt + x(0) + \int_{0^-}^{t} u(t)dt \quad (5.5)$$

If we choose the control u to be equal to:

$$u(t) = [\chi(0) - x(0)] \cdot \delta(t) \quad (5.6)$$

system (5.7) is rewritten as follows:

$$x(t) = \int_{0^-}^{t} f(x(t))dt + x(0) + \int_{0^-}^{t} [\chi(0) - x(0)] \cdot \delta(t)dt = \int_{0^-}^{t} f(x(t))dt + \chi(0) \quad (5.7)$$

since, the integration of the dirac function $\delta(t)$ is $\int_{0^-}^{0^+} \delta(t) = 1$.

Thus the control u brings the systems (5.1) and (5.2) in the same initial state. So for $t > 0$ they present identical behaviour.

We proved how an autonomous system $\dot{\chi}_i(t) = f(\chi_i(t))$, with arbitrary initial conditions can behave similarly to the system $\dot{x}_i(t) = f(x_i(t)) + u$, with constant initial conditions by choosing an external input u as in Equation (5.8).

We will now use the neuro fuzzy identification scheme, to identify the four combination therapy models we described previously (models and parameter values taken from [26]). For external input in the scheme we can now use the function (5.8), where x(0) is constant and χ(0) can vary over a wide range of values, without causing any problems to our autonomous systems.

5.2 Combination of Inhibitors Targeting Two Converging Pathways - Identification

The combination therapy model that we identify is described analytically in Section (3.4.1). For the identification we use the neuro fuzzy identification scheme we presented in Section (4.2.3) and the whole procedure is implemented using Matlab code.

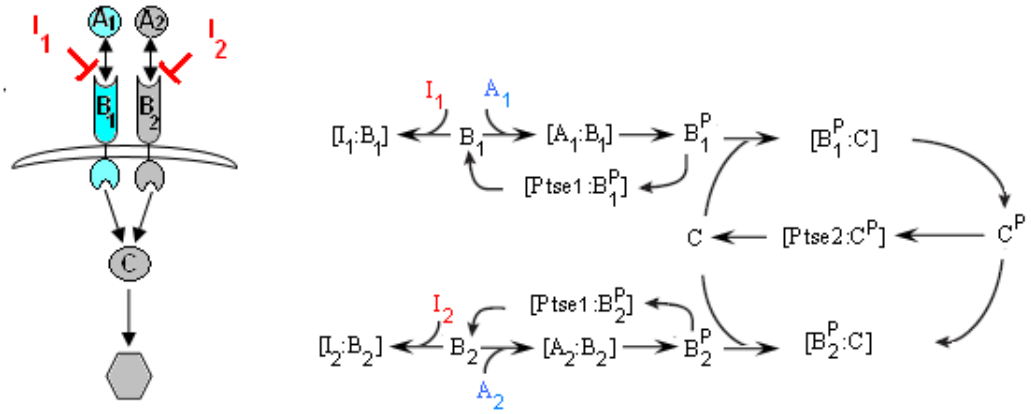


Figure 3.5 Regulatory and reaction scheme for a combination of two inhibitors targeting two converging pathways

Our model has n = 21 states, each of which represents a protein of the pathway shown in Figure (3.5). For the F-HONNF approach we used the following adaptive law:

$$\dot{W} = -X^T PeS^T$$

Hence, the Recurrent High Order Neural Network (RHONN) approximator we used, which depends on the centers of the fuzzy output partition $\overline{f}_l$, is:

$$\dot{\hat{\chi}} = A\hat{\chi} + XWS(\chi)$$

where A is a diagonal $n \times n$ stable matrix, X is a matrix containing the centres of the partitions of every fuzzy output variable of f(x), S(χ) is a matrix containing high order combinations of sigmoid functions of the state χ and W is a matrix containing neural weights. The dimensions and the contents of all the above matrices are chosen so that XWS(χ) is an $n \times 1$ vector.

In order our model to be equivalent with regard to adjustable parameters we have chosen 3 centers for the fuzzy output variables partition in each HONNF. Under these specifications X is an 21×63 block diagonal matrix of the form X = diag (X_1,X_2, . . . ,X_n) with each X_i being a 3-dimensional raw vector of the form $X^i = [\overline{f}_1^i, \overline{f}_2^i, \overline{f}_3^i]$, where $\overline{f}_p^i$ denotes the centre of the p-th partition of f_i. Also, we have $S(\chi) = [s_1(\chi)...s_{42}(\chi)]^T$, where each s_i(χ) is a first or second order sigmoid function of the state variables and W is a 63 × 42 matrix with neural weights of the form $W = [W^1...W^{21}]^T$, where each W^i is a matrix $[w_{jl}^i]_{3\times 42}$. We have selected to use the Log-Sigmoid:

$$s(\chi) = \frac{1}{1+e^{-\chi}}$$

In our training process, our neuro fuzzy model learns to approximate the dynamical behavior of the system in each epoch. The process is consisted from the following steps:

1) Initialization of the W matrix.

2) Initialization of the diagonal matrixes A and P (P > 0, satisfies the Lyapunov equation $PA + A^T P = -I$).

3) Initialization of the block diagonal matrix X.

4) Initialization of the real system and the approximator in the same initial condition.

5) Extraction of the training data from the model we have simulated (Section 3.4.1).

6) The data pass through the Log-Sigmoids to compute S.

7) Evaluation of the approximator's state.

8) Calculation of the error $e = \hat{\chi} - \chi$.

9) Calculation of the weights (W).

10) The final weight values of W are set as initial values for W in the next iteration of the training process.

These steps are performed until a number of maximum epochs to be reached. Our goal is the error to be driven to an acceptable low value (converge to zero), which means that our model 'follows' the real system, which actually happens.

After completing the training process successfully, we proceeded to the validation process, which is similar with before but this time we want results for unknown input stimuli (I_1 and I_2 initial concentrations). So, we changed the initial conditions of I_1 and I_2 using random values.

In the following figures, we can see our results for the errors between our model's and the real system's states:

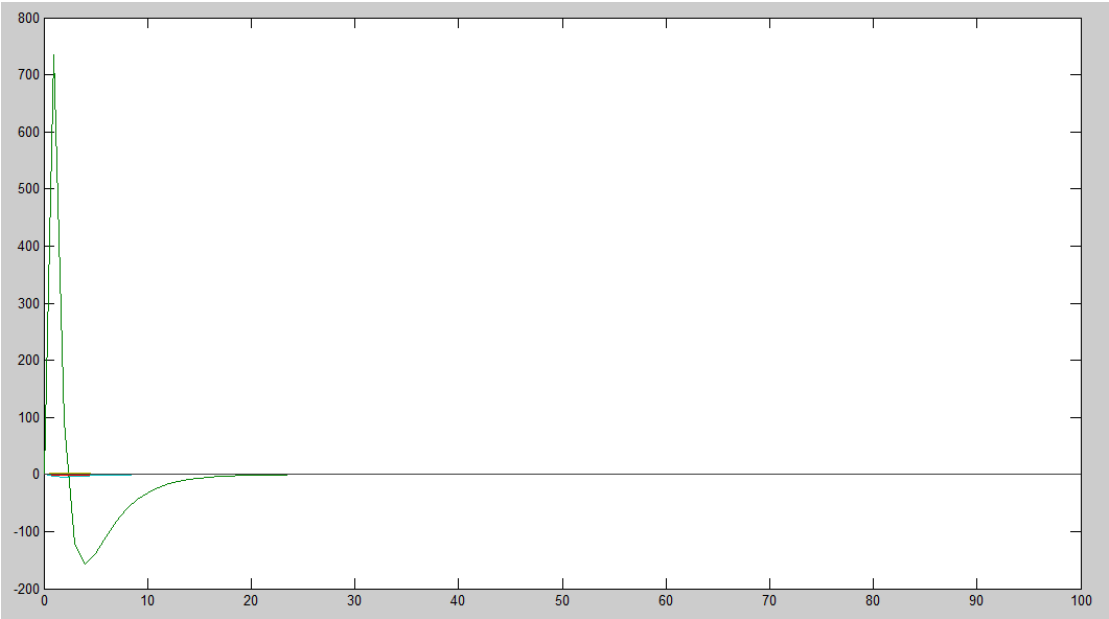


Figure 5.1

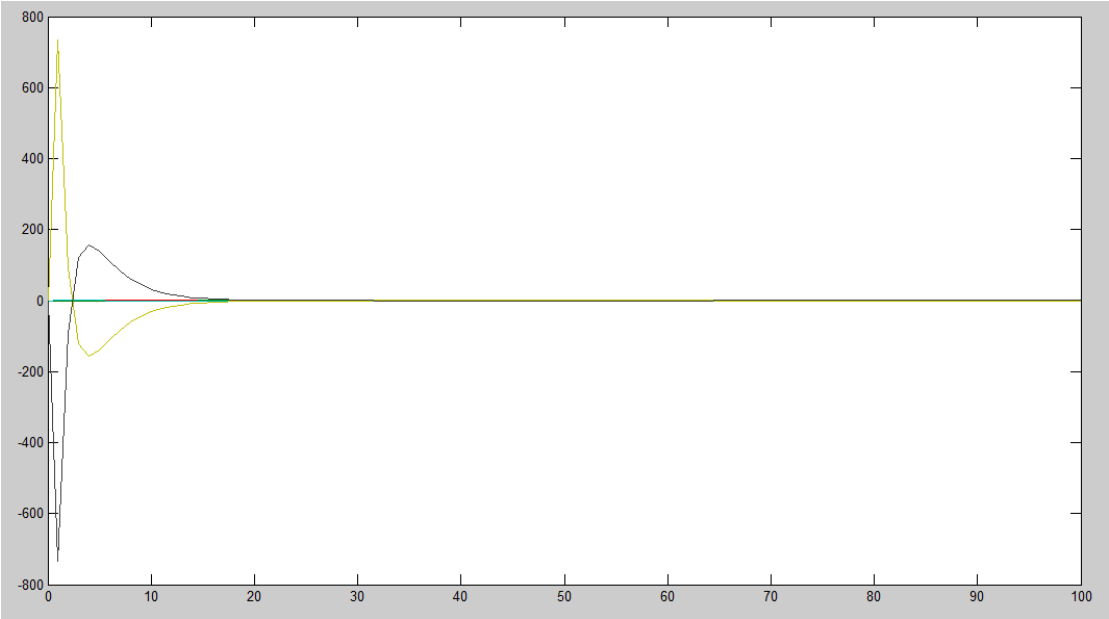


Figure 5.2

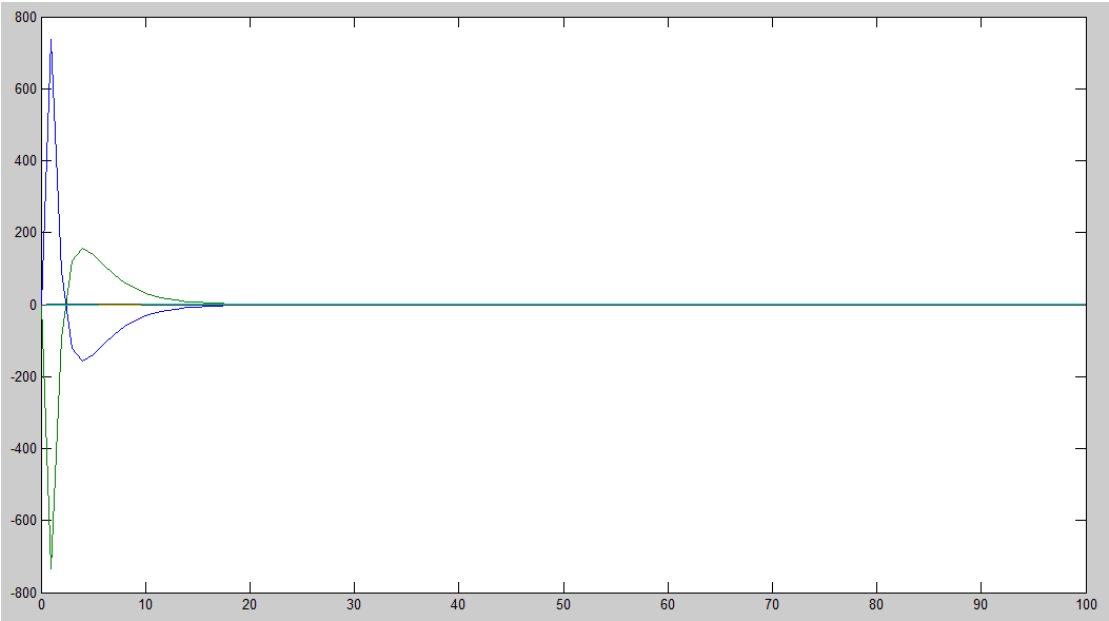


Figure 5.3

In Figures (5.1), (5.2) and (5.3) we can see that the approximating errors for the neuro fuzzy identification scheme indeed converge to zero, and actually this happens very fast. We present our results in three figures, showing from seven errors in each, for the reason to be easier to the reader to understand the success of the identification.

5.3 Inhibition of a Single Target by Two Inhibitors - Identification

The third combination therapy model that we identify is described analytically in Section (3.4.2). For the identification we use the neuro fuzzy identification scheme we presented in Section (4.2.3) and the whole procedure is implemented using Matlab code.

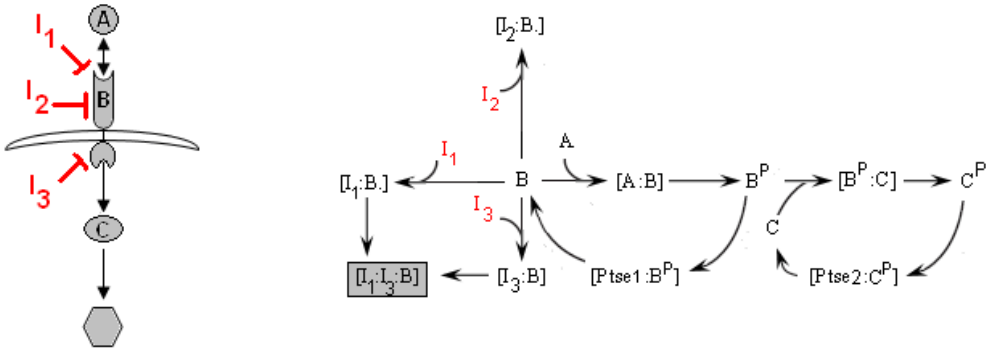


Figure 3.6 Regulatory scheme of a single linear pathway with dual inhibition of a single target and its reaction scheme

This model has n = 18 states, each of which represents a protein of the pathway shown in Figure (3.6). For the F-HONNF approach we used the following adaptive law:

$$\dot{W} = -X^T PeS^T$$

Hence, the Recurrent High Order Neural Network (RHONN) approximator we used, which depends on the centers of the fuzzy output partition $\overline{f}_l$, is:

$$\dot{\hat{\chi}} = A\hat{\chi} + XWS(\chi)$$

as described and in the previous section.

In order our model to be equivalent with regard to adjustable parameters we have chosen 3 centers for the fuzzy output variables partition in each HONNF. Under these specifications X is an 18×54 block diagonal matrix of the form X = diag (X_1,X_2, . . . ,X_n) with each X_i being a 3-dimensional raw vector of the form $X^i = [\overline{f}_1^i, \overline{f}_2^i, \overline{f}_3^i]$, where $\overline{f}_p^i$ denotes the centre of the p-th partition of f_i. Also, we have $S(\chi) = [s_1(\chi)...s_{36}(\chi)]^T$, where each $s_i(\chi)$ is a first or second order sigmoid function of the state variables and W is a 54 × 36 matrix with neural weights of the form $W = [W^1...W^{18}]^T$, where each W^i is a matrix $[w_{jl}^i]_{3\times 36}$. We have selected again to use the Log-Sigmoid:

$$s(\chi) = \frac{1}{1+e^{-\chi}}$$

Following again the training steps we described in Section (5.3) until a number of maximum epochs to be reached, we wanted the error to be driven to an acceptable low value (converge to zero), which means that our model 'follows' the real system. Again the procedure was successful.

After changing the initial conditions of I_1 and I_3 using random values, we have the results for the errors between our model's and the real system's states that we can see in the following figures:

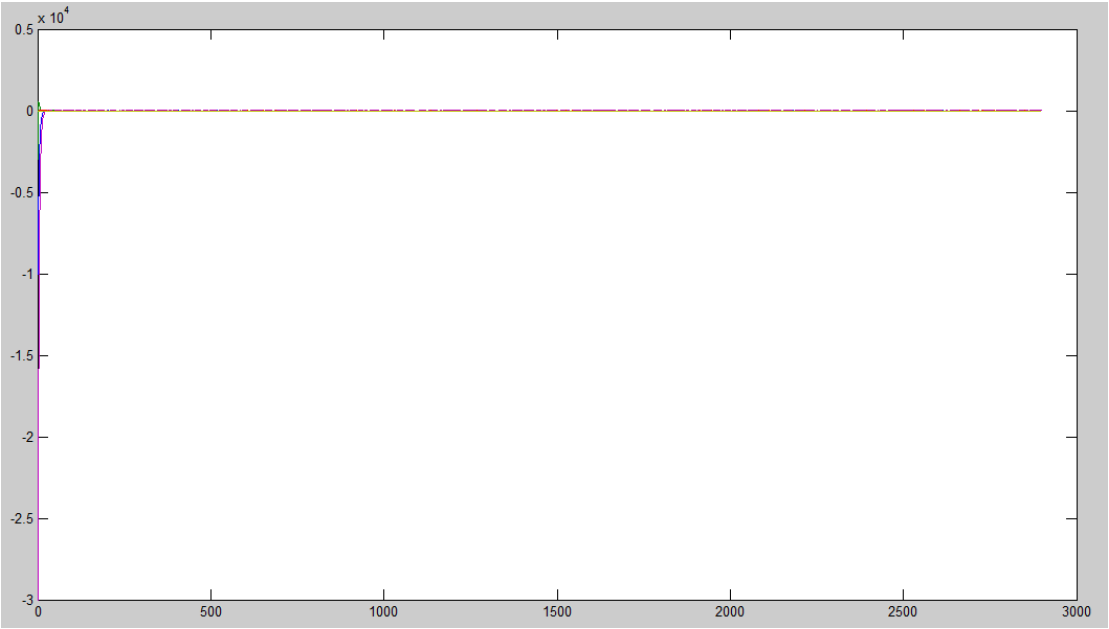


Figure 5.4

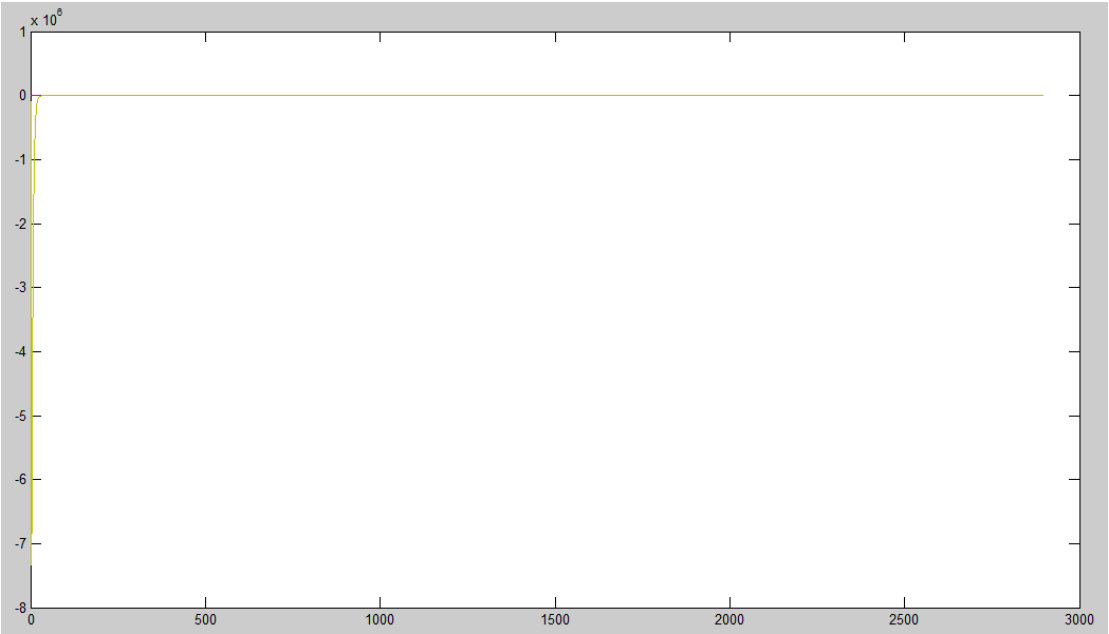


Figure 5.5

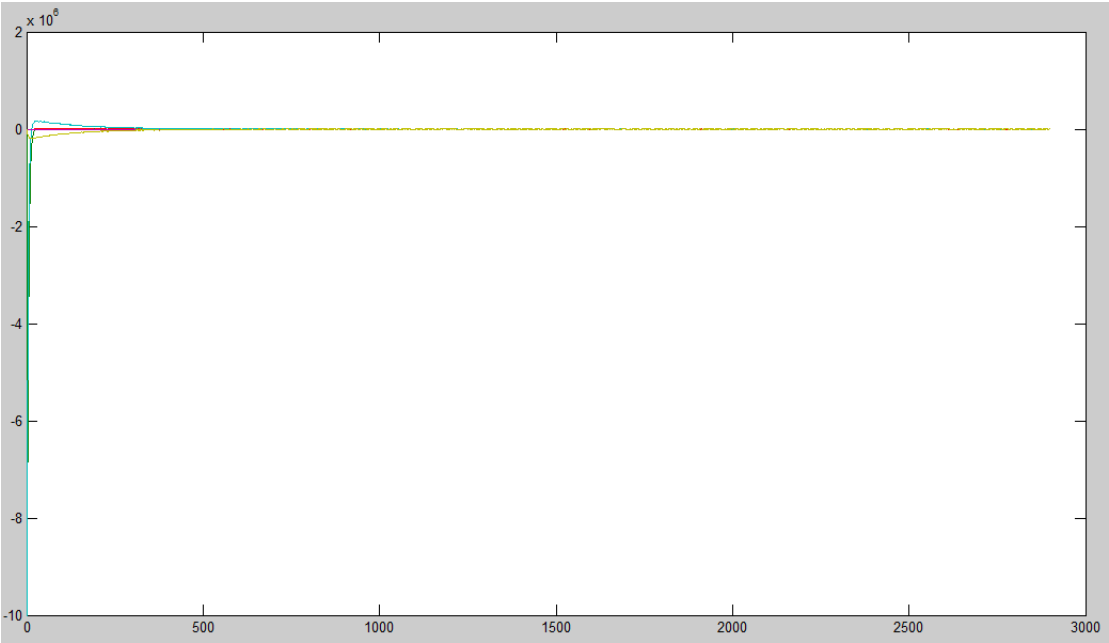


Figure 5.6

In Figures (5.4), (5.5) and (5.6) we can see that the approximating errors for the neuro fuzzy identification scheme indeed converge to zero, and actually again this happens very fast. We present our results in three figures, showing from six errors in each, for the reason to be easier to the reader to understand the success of the identification.

5.4 Targeting Different Levels of a Single Pathway - Identification

The third combination therapy model that we identify is described analytically in Section (3.4.3). For the identification we use the neuro fuzzy identification scheme we presented in Section (4.2.3) and the whole procedure is implemented using Matlab code.

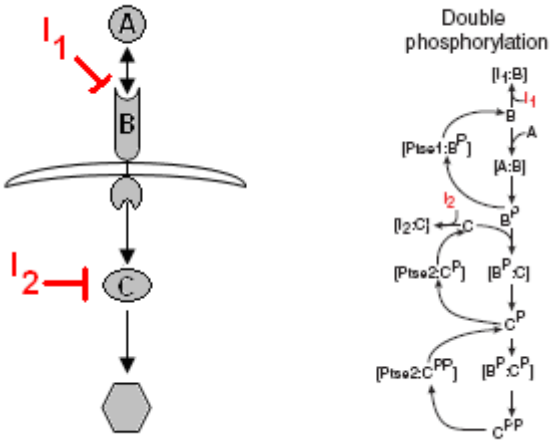


Figure 3.7 Regulatory scheme of a single linear amplification pathway with inhibitors acting at different level of a single target and the reaction scheme of an ultrasensitive signaling cascade (dual phosphorylation)

This model has again n = 18 states, each of which represents a protein of the pathway shown in Figure (3.7). For the F-HONNF approach we used the following adaptive law:

$$\dot{W} = -X^T PeS^T$$

and the following High Order Neural Network (RHONN) approximator:

$$\dot{\hat{\chi}} = A\hat{\chi} + XWS(\chi)$$

as described and in the previous sections.

In order our model to be equivalent with regard to adjustable parameters we have chosen 3 centers for the fuzzy output variables partition in each HONNF. Under

these specifications X is an 18×54 block diagonal matrix of the form X = diag (X_1,X_2, . . . ,X_n) with each X_i being a 3-dimensional raw vector of the form $X^i = [\bar{f}_1^i, \bar{f}_2^i, \bar{f}_3^i]$, where $\bar{f}_p^i$ denotes the centre of the p-th partition of f_i. Also, we have $S(\chi) = [s_1(\chi)...s_{36}(\chi)]^T$, where each $s_i(\chi)$ is a first or second order sigmoid function of the state variables and W is a 54 × 36 matrix with neural weights of the form $W = [W^1...W^{18}]^T$, where each W^i is a matrix $[w_{ji}^i]_{3\times 36}$. We have selected again to use the Log-Sigmoid:

$$s(\chi) = \frac{1}{1+e^{-\chi}}$$

Following again the training steps we described in Section (5.3) until a number of maximum epochs to be reached, we wanted the error to be driven to an acceptable low value (converge to zero), which means that our model 'follows' the real system. Again the procedure was successful.

After changing the initial conditions of the input stimuli, I_1 and I_2, using random values, we have the results for the errors between our model's and the real system's states that we can see in the following figures:

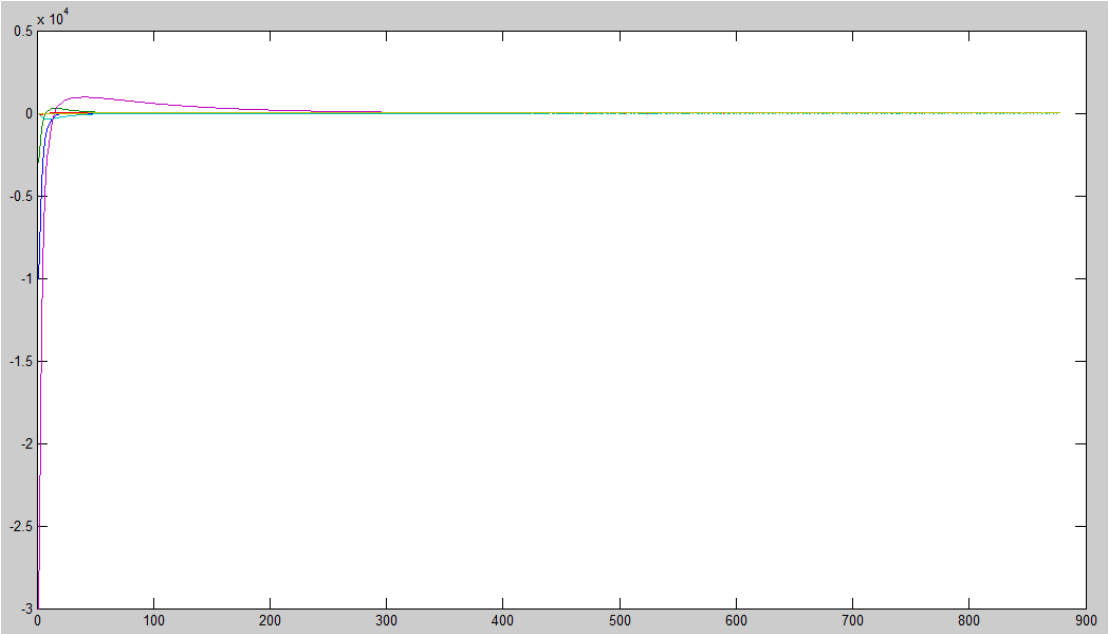


Figure 5.7

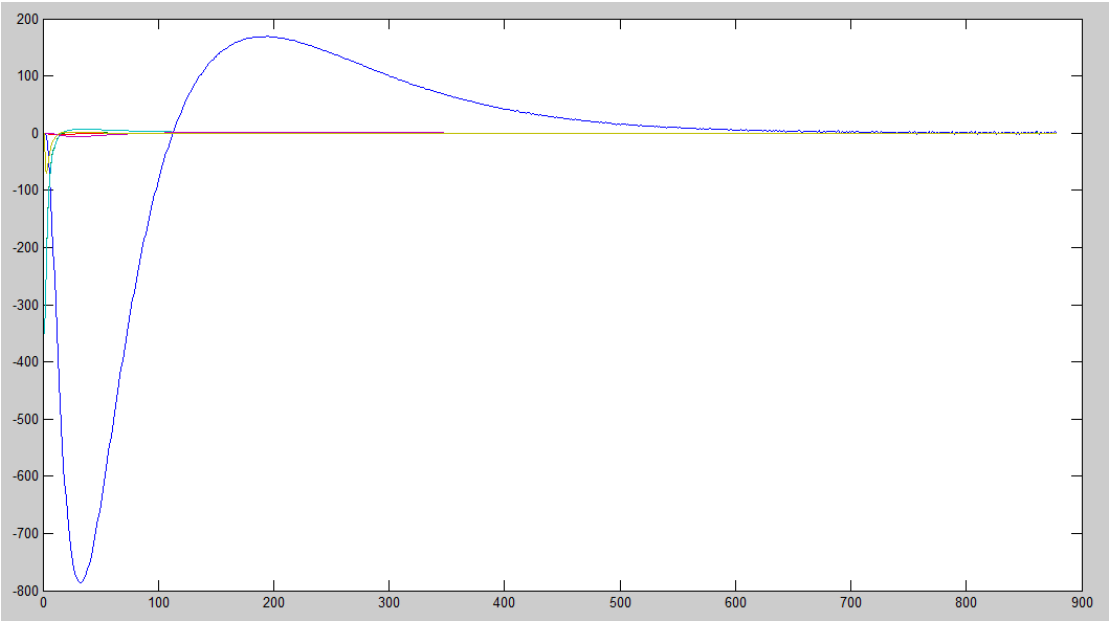


Figure 5.8

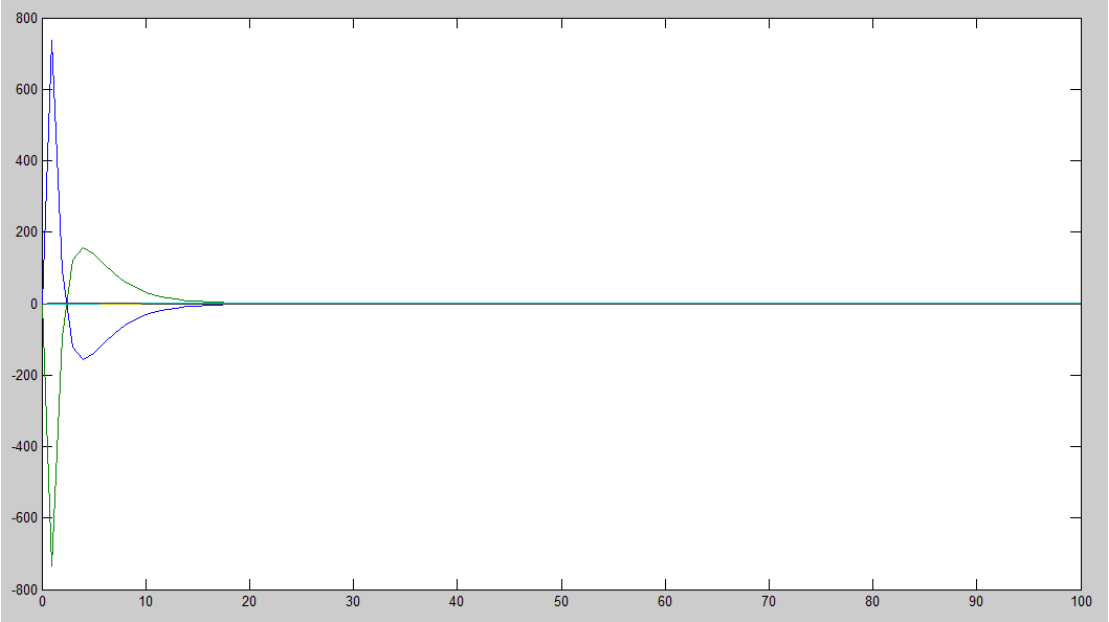


Figure 5.9

In Figures (5.7), (5.8) and (5.9) we can see that the approximating errors for the neuro fuzzy identification scheme indeed converge to zero, and actually again this happens very fast (except one case in Fig. (5.8) where error of our state $\chi(9)$ it needs more time). We present our results in three figures again, showing from six errors in each, for the same reason; to be easier to the reader to understand the success of the identification.

5.5 Feedback-Controlled Targets - Identification

The fourth – and final - combination therapy model that we identify is described analytically in Section (3.4.4). For the identification we use the neuro fuzzy identification scheme we presented in Section (4.2.3) and the whole procedure is implemented using Matlab code.

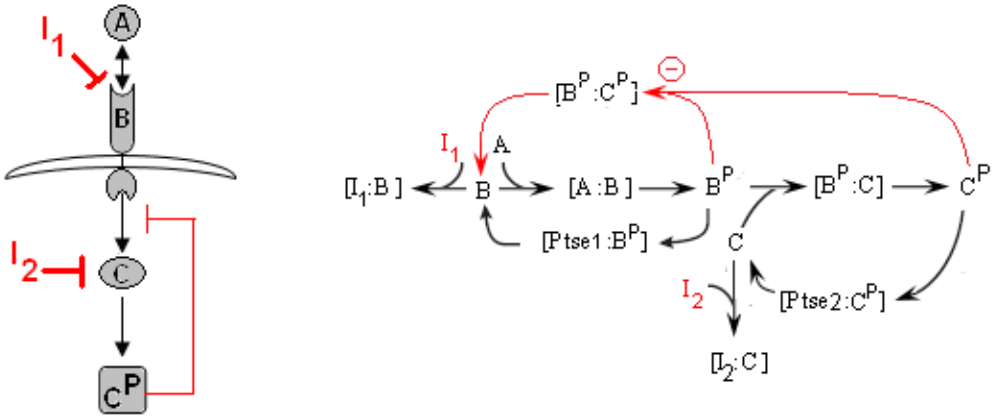


Figure 3.8 Regulatory scheme in which the target of the second inhibitor is within a negative feedback loop and its reaction scheme

Our model this time has again n = 16 states, each of which represents a protein of the pathway shown in Figure (3.8). For the F-HONNF approach we used the following adaptive law:

$$\dot{W} = -X^T PeS^T$$

and the following High Order Neural Network (RHONN) approximator:

$$\dot{\hat{\chi}} = A\hat{\chi} + XWS(\chi)$$

as described and in the previous sections.

In order our model to be equivalent with regard to adjustable parameters we have chosen 3 centers for the fuzzy output variables partition in each HONNF. Under these specifications X is an 16×48 block diagonal matrix of the form X = diag

($X_1, X_2, \ldots, X_n$) with each X_i being a 3-dimensional raw vector of the form $X^i = [\bar{f}_1^i, \bar{f}_2^i, \bar{f}_3^i]$, where $\bar{f}_p^i$ denotes the centre of the p-th partition of f_i. Also, we have $S(\chi) = [s_1(\chi) \ldots s_{32}(\chi)]^T$, where each $s_i(\chi)$ is a first or second order sigmoid function of the state variables and W is a 48 × 32 matrix with neural weights of the form $W = [W^1 \ldots W^{16}]^T$, where each W^i is a matrix $[w_{ji}^i]_{3\times 32}$. We have selected again to use the Log-Sigmoid:

$$s(\chi) = \frac{1}{1+e^{-\chi}}$$

Following again the training steps we described in Section (5.3) until a number of maximum epochs to be reached, we wanted the approximating error to converge to zero, which means that our model 'follows' the real system. Again the procedure was successful.

After changing the initial conditions of the input stimuli, I_1 and I_2, using random values, we have the results for the errors between our model's and the real system's states that we can see in the following figures:

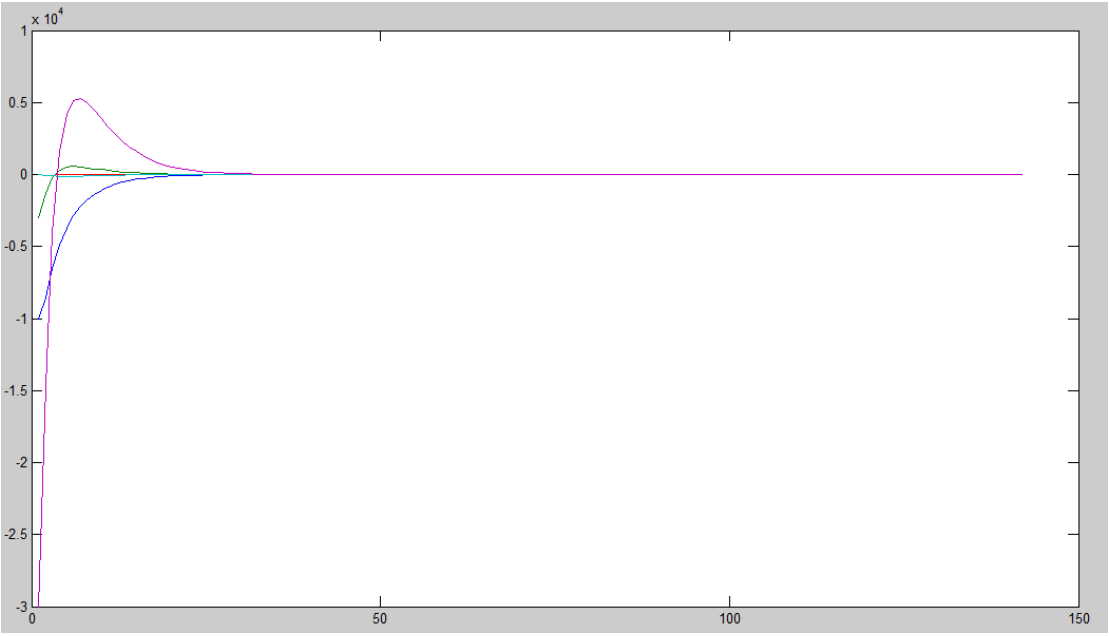


Figure 5.10

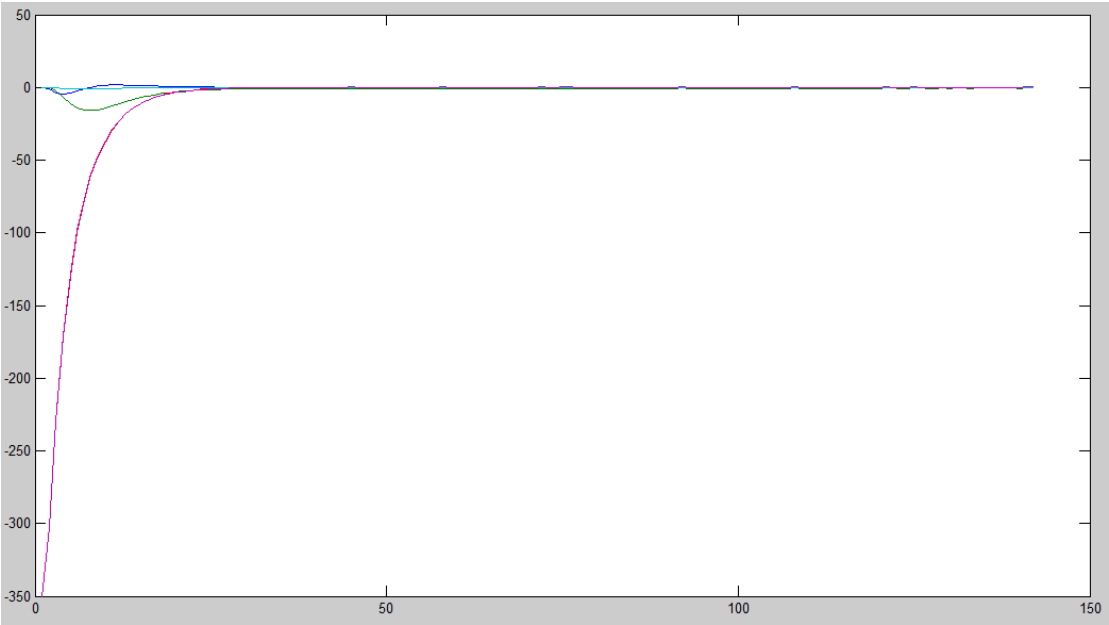


Figure 5.11

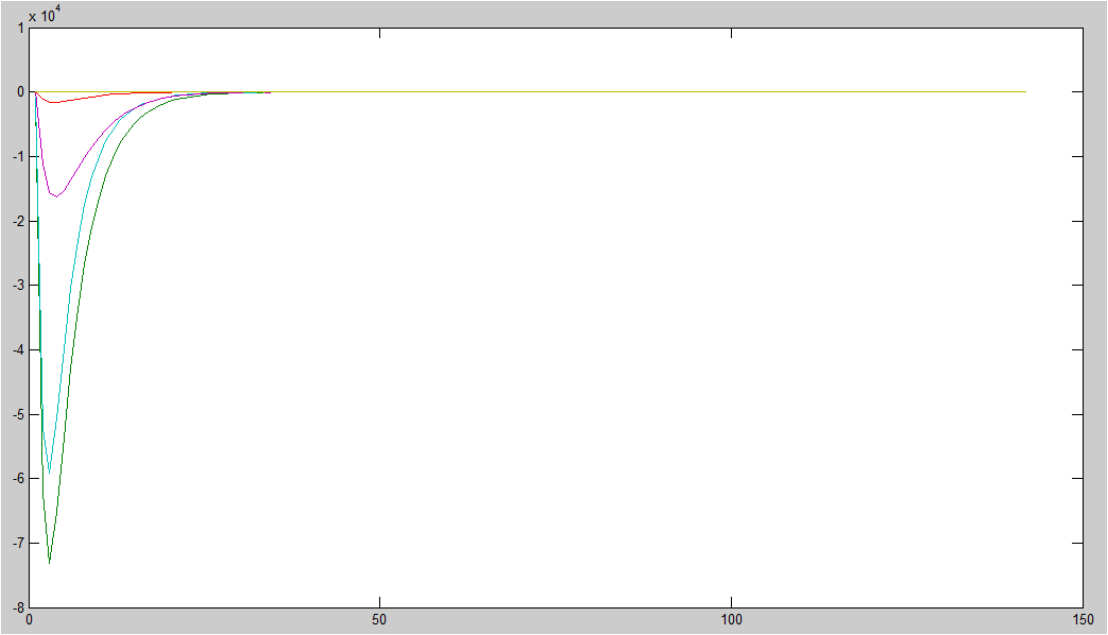


Figure 5.12

In Figures (5.10), (5.11) and (5.12) we can see that the approximating errors for the neuro fuzzy identification scheme indeed converge to zero, and actually again this happens very fast in every case. We present our results in three figures again; for the same reason as before.

As it is clear from the results shown in the previous figures, in all four models, the neuro fuzzy identification scheme we chose to use has effectively learned to approximate the dynamic behavior of the proteins in the four different pathways of combination therapy models we examined. It is important that in every case, the

identifier managed to approximate accurately these dynamic behaviors for input stimulus that had never been seen before. The results of this identification process are very important, showing the great capabilities of the scheme we used, in biological systems of great interest that show amplification, ultrasensitivity and feedback control.

6 Conclusions

In this thesis we presented approaches for modelling and identifying the dynamic behaviour of proteins in combination therapy pathways used for the evaluation of multicomponent drugs. Combination or multicomponent therapy is a very important field in modern medicine and Systems Biology can give it the boost for even greater inventions. A Systems Biology approach to analyzing combination therapy relies on the use of numerical models to simulate the effects of drugs individually and as mixtures. Mathematical analysis is potentially powerful because many pairwise drug combinations can be explored computationally at much lower cost than in preclinical or clinical experiments. Preliminary success has been achieved in formulating mathematical models of signaling pathways and oncogenic processes relevant to human disease. Numerical analysis has also been used to identify critical network nodes and model drug action. The models we use of course, cannot be considered as real representations of biological networks. They can provide though information of great significance, especially in evaluation of multicomponent drugs; they can help in deciding whether a combination of two active ingredients can be synergistic, which is and the desirable in multicomponent therapeutics.

As about the identification scheme we used, it seems to be very powerful. It is an identification scheme for unknown nonlinear dynamical systems using a new definition of Adaptive Fuzzy Systems (AFS); it uses the concept of Adaptive Fuzzy Systems operating in conjunction with High Order Neural Network Functions (FHONNFs). Under this scheme the identification is driven to a Recurrent High Order Neural Network, which however takes into account the fuzzy output partitions of the initial AFS. The big advantage of this identifier - and the greatest reason for choosing it – is that it does not require a-priori experts' information on the number and type of input variable membership functions. This is something that can be very useful in the field of Systems Biology, since the difficulty in finding real data still exists.

This is besides, what we expect in the future from the biological side. The more real data are available the better the identification is. As more and more potent single-agent inhibitors are developed, the question for the biologists will be how to find useful combinations without resorting to large mechanism-blind clinical trials.

The answers, as we saw, can be given from Systems Biology and more available data would make it even more possible; the difficulty though in that must be admitted. The next step relevant to our work, could also be the evaluation of the right control to pathways that do not work properly in order to bring the system to the right dynamical behavior. In medical words, this might mean ‘finding the treatment to a disease’. And we think that it can be very helpful, in such a control procedure, the neuro fuzzy identification scheme we saw this thesis to be used as the first part in a control algorithm.

References

[1] 'Cell (biology)', Microsoft® Encarta® Online Encyclopedia, 2008 http://encarta.msn.com © 1997-2008 Microsoft Corporation.

[2] 'Cell (biology)', Wikipedia®, The Free Encyclopedia, 2009 http://www.wikipedia.org

[3] 'Eukaryote', Wikipedia®, The Free Encyclopedia, 2009 http://www.wikipedia.org

[4] 'Prokaryote', Wikipedia®, The Free Encyclopedia, 2009 http://www.wikipedia.org

[5] 'Cell nucleus', Wikipedia®, The Free Encyclopedia, 2009 http://www.wikipedia.org

[6] D. Anastassiou, 'Genomic Signal Processing', *IEEE Signal Processing Magazine theme article*, vol.18, 2001.

[7] 'Genetics', GlaxoSmithKline, 2005 http://genetics.gsk.com © 2001-2006 GlaxoSmithKline.

[8] 'DNA', Wikipedia®, The Free Encyclopedia, 2009 http://www.wikipedia.org

[9] 'Gene', Wikipedia®, The Free Encyclopedia, 2009 http://www.wikipedia.org

[10] 'Protein', Wikipedia®, The Free Encyclopedia, 2009 http://www.wikipedia.org

[11] 'Enzyme', Wikipedia®, The Free Encyclopedia, 2009 http://www.wikipedia.org

[12] Watson J. D., Crick F.H., 'Molecular structure of nucleid acids: A structure for deoxyribose Nucleic Acid', *Nature*, 171, 1953.

[13] Ito T., Tashiro K., Muta S., Ozawa R., Chiba T., Nishizawa M., Yamamoto K., Kuhara S. and Sakaki Y., 'Toward a protein-protein interaction map of the budding yeast: A comprehensive system to examine two-hybrid interactions in all possible combinations between the yeast proteins', *Proc. Natl. Acad. Sci. USA*, 97, 1143-1147, 2000.

[14] Schwikowski B., Uetz P., Fields S., 'A network of protein-protein interactions in yeast', *Nature Biotechnology*, 18, 1257-1261, 2000.

[15] Kitano H., 'Perspectives on Systems Biology', *New Generation* Computing, 18, 199-216, 2000.

[16] von Bertalanffy L., *General Systems Theory*. New York: Braziler, 1968.

[17] Cannon W. B., *The wisdom of the body*. New York: Norton, 1933

[18] Wolkenhauer Olaf, Systems Biology: Dynamic Pathway Modeling, (www.sbi.uni-rostock.de), 2005.

[19] Systems of life: Systems Biology. 53170 Bonn, Federal Ministry of Education and Research, Public Relations Division, 2002.

[20] Sontag E. D., **'**Some new directions in control theory inspired by Systems Biology', *Systems Biology*, vol.1, 9-18, 2004.

[21] Hanahan D. and Weinberg R. A., The Hallmarks of Cancer, Cell, vol. 100, 57–70, 2000.

[22] Hartwell L.H., Hopfield J.J., Leibler S. and Murray A.W., 'From molecular to modular cell biology', *Nature*, 402, C47-C52, 1999.

[23] Lauffenburger, D.A., 'Cell signaling pathways as control modules: Complexity for simplicity', *Proc. Nat. Acad. Sci. USA*, 97, 5031-5033, 2000.

[24] Bluthgen Nils, Diplomarbeit im Studiengang Physik, 'Dynamical Models of Signal Transduction and the Influence of Feedback Loops', University of Berlin, 2002

[25] Keith C. T., Borisy A. A., Stockwell B. R., 'Multicomponent therapeutics for networked systems', *Nature Reviews. Drug Discovery*, vol.4, January 2005.

[26] Fitzgerald J. B., Schoeberl B., Nielsen U. B. and Sorger P. K., 'Systems biology and combination therapy in the quest for clinical efficacy', *Nature Chemical Biology*, vol.2, September 2006.

[27] Yeh P. , Tschumi A. I. and Kishony R, 'Functional classification of drugs by properties of their pairwise interactions', *Nature Genetics*, vol.38, April 2006.

[28] Yuan R. and Lin Y., 'Traditional Chinese medicine: an approach to scientific proof and clinical validation', *Pharmacol. Ther.*, 86, 191–8, 2000.

[29] Foungbe S., Kouassi G., Kablan J. B. and Marcy R., 'Study of *Costus lucanusianus*: plant juice, fraction combinations and pharmacologic estimation of natural product total activity', *J. Ethnopharmacol*., 33, 221–226 1991.

[30] Turner D. M., 'Natural product source material use in the pharmaceutical industry: the Glaxo experience', *J. Ethnopharmacol*., 51, 39–43, 1996.

[31] Schuster B. G., 'A new integrated program for natural product development and the value of an ethnomedical approach' *J. Altern. Complement. Med.*, 7 (Suppl. 1), S61–S72, 2001.

[32] Fraser T. R., 'An experimental research on the antagonism between the actions of physostigma and atropia', *Proc. R. Soc. Edinburgh* 7, 506, 1871.

[33] Loewe S., 'Die quantitation probleme der pharmakologie' *Ergebn. Physiol.*, 27, 47–187, 1928.

[34] Loewe S., 'The problem of synergism and antagonism of combined drugs' *Arzneimitteforsch*, 3, 285, 1953.

[35] Ehrlich P., 'Chemotherapeutics: scientific principles, methods and results', *Lancet*, 2, 445–451, 1913.

[36] Albert A., 'Selective Toxicity', Methuen & Co., London, 1968.

[37] Kinzler K. W. and Vogelstein B., 'Lessons from hereditary colorectal cancer', *Cell* 87, 159–170, 1996.

[38] DeVita, V. T., in 'Cancer: Principles and Practice of Oncology' (eds DeVita V. T., Hellman S. and Rosenberg S. A.), 333–347, Lippincott–Raven, Philadelphia, 1997.

[39] Nelson H. S., 'Advair: combination treatment with fluticasone propionate/salmeterol in the treatment of asthma', *J. Allergy Clin. Immunol.* 107, 398–416, 2001.

[40] Hartman J.L., Garvik B. and Hartwell L. 'Cell biology - Principles for the buffering of genetic variation', *Science*, 291, 1001–1004, 2001.

[41] Bliss C.I., 'The toxicity of poisons applied jointly', *Ann. Appl. Biol.*, 26, 585–615, 1939.

[42] Chou T.C. and Talalay P., 'A simple generalized equation for the analysis of multiple inhibitions of Michaelis-Menten kinetic systems', *J. Biol. Chem.*, 252, 6438–6442, 1977.

[43] Chou T.C. and Talalay P., 'Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors', *Adv. Enzyme Regul.*, 22, 27–55**,** 1984.

[44] Berenbaum M.C., 'What is synergy?', *Pharmacol. Rev.*, 41, 93–141 1989.

[45] Bliss C.I., 'The calculation of microbial assays', '*Bacteriol. Rev.*', 20, 243–258, 1956.

[46] Greco W.R., Bravo G. and Parsons J.C., 'The search for synergy: a critical review from a response surface perspective', *Pharmacol. Rev.*, 47, 331–385, 1995.

[47] Chou T.-C. and Talalay P., 'Analysis of combined drug effects: a new look at a very old problem', *Trends Pharmacol. Sci.*, 4, 450–454, 1983.

[48] Angeli D., Ferrell J.E. Jr. and Sontag E.D., 'Detection of multistability, bifurcations, and hysteresis in a large class of biological positive-feedback systems', *Proc. Natl. Acad. Sci. USA*, 101, 1822–1827, 2004.

[49] Ferrell J.E. Jr. and Machleder E.M., 'The biochemical basis of an all-or none cell fate switch in *Xenopus* oocytes', *Science*, 280, 895–898, 1998.

[50] Bhalla U. S., Ram P. T. and Lyengar R., 'MAP kinase phosphatase as a locus of flexibility in a mitogen-activated protein kinase signaling network', *Science*, 297, 1018–1023, 2002

[51] Kholodenko B.N., 'Negative feedback and ultrasensitivity can bring about oscillations in the mitogen-activated protein kinase cascades', *Eur. J. Biochem.*, 267, 1583–1588, 2000.

[52] Asthagiri A.R. and Lauffenburger D.A., 'A computational study of feedback effects on signal dynamics in a mitogen-activated protein kinase (MAPK) pathway model', *Biotechnol. Prog.*, 17, 227–239, 2001.

[53] Sauro H.M. and Kholodenko B.N., 'Quantitative analysis of signaling networks', *Prog. Biophys. Mol. Biol.*, 86, 5–43, 2004.

[54] Theodoridis D. C., Boutalis Y. S. and Christodoulou M. A., 'A new Neuro-Fuzzy Dynamical System Definition Based on High Order Neural

Network Function Approximators', preprint submitted to 47th IEEE Conference on Decision and Control, March, 2008.

[55] Passino K. M. and Yurkovich S., 'Fuzzy Control', Menlo Park, CA, Addison Wesley Longman, 1998.

[56] Lewis F. L., Campos J. and Selmic R., 'Neuro-Fuzzy Control of Industrial Systems with Actuator Nonlinearities', Philadelphia, PA, Society for Industrial and Applied Mathematics, 2002.

[57] Nauck D., Klawonn F., Kruse R., 'Foundations of Neuro-Fuzzy Systems', Chichester, Wiley, 1997.

[58] Hornic K., Stinchcombe M. and White H., 'Multilayer feedforward networks are universal approximators', *Neural Networks*, vol.2, 1989.

[59] Wang L., 'Fuzzy systems are universal approximators', *in Proc. Int. Conf. Fuzzy Syst.*, 1992.

[60] Wang L., 'Adaptive Fuzzy Systems and Control', Englewood Cliffs, NJ, Prentice Hall, 1994.

[61] Jang J. S. R., 'ANFIS: Adaptive-network-based fuzzy inference system', *IEEE Trans. Syst. Man. Cyber.*, vol.23, 1993

[62] Lin C. T., 'A neural fuzzy control system with structure and parameter learning', *Fuzzy Sets and Systems*, vol.70, 1995.

[63] Cho K. B. and Wang B. H., 'Radial basis function based adaptive fuzzy systems and their applications to system identification and prediction', *Fuzzy Sets and Systems*, vol.83, 1996.

[64] Juang C. F. and Lin C. T., 'An on-line self-constructing neural fuzzy inference network and its applications', *IEEE Trans. Fuzzy Syst.*, vol.6, 1998.

[65] Li R. P. and Mukaidono M., ‘A new approach to rule learning based on fusion of fuzzy logic and neural networks’, *IEICE Trans. Fuzzy Syst.*, vol.E78-d, 1995.

[66] Lin Y. H. and Cunningham G. A., ‘A new approach to fuzzy-neural system modelling’, *IEEE Trans. Fuzzy Syst.*, vol.3, 1995.