P53 REVIVAL USING SYSTEM ORIENTED DOSAGE DESIGN TARGETING MDM2

By

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Dedicated to my Parents and Siblings who pray for me and always pave the way to success for me

And

Dedicated to my teachers who have been a persistent source of inspiration and encouragement for me.

CERTIFICATE OF APPROVAL

It is certified that the research work titled **"p53 Revival using System Oriented Dosage Design Targeting MDM2"** carried out by **Shumaila Azam**, Reg. No. **MBI143005**, under the supervision of **Dr. Sahar Fazal** and co-supervision of **Dr. Aamer Iqbal Bhatti**, at Capital University of Science & Technology Islamabad. It is fully adequate, in scope and in quality, as a thesis for the degree of MS in Bioinformatics.

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DECLARATION

It is declared that this is an original piece of my own work, except where otherwise acknowledged in the text and references. This work has not been submitted in any form for another degree or diploma at any university or other institution for tertiary education and shall not be submitted by me in future for obtaining any degree from this or any other University or Institution.

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PUBLICATION AND CONFERENCES

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ABSTRACT

In this study a new paradigm in the drug design is investigated for the revival of the p53 pathway in cancer cells. It is shown that the current strategy of using small molecule based MDM2 inhibitors is not enough to adequately revive p53 in cancerous cells, especially when it comes to the extracting pulsating behavior of p53. This fact has come to notice when a novel method for the drug dosage design is introduced using system oriented concepts. As a test case, small molecule drug MDM2 repressor Nutlin 3a is considered. The proposed method determines the dose of Nutlin to revive p53 pathway functionality. For this purpose, PBK dynamics of Nutlin have also been integrated with the p53 pathway model.

The p53 pathway is the focus of researchers in the last thirty years for its pivotal role as a Frontline cancer suppressant protein due to its effect on cell cycle checkpoints and cell apoptosis in response to a DNA strand break. That is the reason for finding p53 being absent in more than 50% of tumor cancers. Various drugs have been proposed to revive p53 in cancer cells. Small molecule based drugs are at the foremost and are the subject of advanced clinical trials. The dosage design of these drugs is an important issue.

A control systems concepts to develop the drug dosage so that the cancer cells can be treated in appropriate time. We investigate by using a computational model how p53 protein responds to drug Nutlin 3a, an agent that interferes with the MDM2-mediated p53 regulation. The proposed integrated model describes in some detail the regulatory network of p53, including the negative feedback loop mediated by MDM2 and the positive feedback loop mediated by MDM2 mRNA as well as the reversible represses of MDM2 caused by Nutlin. The reported PBK dynamics of Nutlin 3a are also incorporated to see the full effect. It has been reported that p53 response to stresses in two ways. Either it has a sustained (constant) p53 response, or there are oscillations in p53 concentration. The claimed dosage strategy achieves the p53 response in the first case. However, for the induction of oscillations, it is shown through bifurcation analysis that to achieve the oscillating behavior of p53 inhibition of MDM2 is not enough, rather antirepression of the p53-MDM2 complex is also needed which leads to the need of a new drug design paradigm.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ADME	Adsorption, distribution, metabolism, and excretion
ADP	Adenosine diphosphate
ALL	Acute lymphoblastic leukemia
AML	Acute myelogenous leukemia
ATP	Adenosine Triphosphate
BCEC	Brain capillary endothelial cells
BCRP	Breast cancer resistance protein
CNS	Central nervous system
CSF	Cerebrospinal fluid
DDI	Drug–drug interaction
DSBs	Double Stranded Breaks
FDA	Food and drug administration
MDM	Murine double minute-2
MDR	Multiple drug resistance
MRP1	Multidrug resistance protein 1
MXR	Mitoxantrone resistance protein
NB	Neuroblastoma
NBD	Nucleotide binding domain
NDA	New drug applications
PBPK	Physiologically based pharmacokinetic
PhRMA	Pharmaceutical research and manufacturers of America
SSBs	Single Stranded Breaks
17-AAG	17-(allylamino)-17-demethoxygeldanamycin

1. Chapter 1: Introduction

1.1 Overview

In the modern day world, science has made great advancements for the welfare of mankind but still there are things which are continually challenging the researchers. The advancements such as Stem cell and Gene therapy, etc. have provided immense benefits in improving our health care systems and revolutionized the methods of disease treatment (Weatherall*et. al..,* 2006). With the advent of Bioinformatics, humans are capable of storing the exponentially growing DNA, RNA, Protein sequential information about the Human Genome Project and other sources in curated databases. It also provided us with a framework and tools in order to analyze and interpret the massive biological information for their functional assignment which may be later used in biomedical and clinical research (Mwololo*et. al..,* 2010). Despite the availability of such high throughput technologies, researchers remained unsuccessful in finding the permanent cure of certain fatal diseases.

The claims made by Genome Project at the time of its completion that they would transform the performance of disease treatment remained subject to certain doubts. The reason behind these doubts might be the evolution of complex diseases whose remedy is challenging for biomedical researchers. Complex diseases are not caused due to a single gene mutation (as in the case of simple diseases) rather they are controlled by polygenic (Multiple genes) factors along with some environmental factors, lifestyle and are heritable in nature (Plomin*et. al..,* 2009; Mitchell, 2012). In such diseases, genetic factors contribute partially to disease risk and they do not exhibit apparent inheritance patterns. This environment-gene association facilitates in imparting better insights of disease causal and later helps in the development of targeted therapy (Craig, 2008; McCarthy*et. al..,* 2008). Most important examples of complex disease are cancer which is defined as the uncontrolled/abnormal proliferation of cells due to a mutation in certain gene under the control of environmental or inherited factors. Cancer is complex in the sense that it involves a series of the interaction of genetic and environmental factors that directly deregulate various mechanisms of the human body such as Immune system, DNA Repair mechanism, and Apoptosis etc. We know that these mechanisms consist of various signaling pathways so they in

cooperation with epigenetic processes determine the phenotype of cancer (Sarah Knox, 2010). System Biology is a critical field of cancer studies as it provides an aggregated outlook of the modified homeostasis of signaling pathways as a result of aberrations of genome and epigenome among cancerous cells and their local environment at the level of an organ / organism (Werner*et. al..*, 2014). The network analysis provided us with the interacting model to study the individual components that linked with each other to make up complex pathophysiological pathways (Stevens*et. al..*, 2014). Genome-wide association studies are being carried out in order to reveal the architecture of the genome for cancerous phenotypes and development of respective therapeutics (Korpela*et. al..*, 2011).

1.1.1 Significance of p53

p53 gene was discovered in 1979 by Arnold Levine but in the early 1990, its function was eventually identified. Experimental and molecular pathology approaches demonstrated that it is basically a tumor suppressor gene, that is nonfunctional in several cancers detected in human. Hence the p53 gene and protein have earned a central importance in the molecular biology of cancer and have raised the potential for the identification of better cancer management or therapy. p53 gene has special importance in biological network and pathways in at least three aspects. 1) Missense mutations are observed in the majority of the cancers. This is rareoccurrence for the tumor suppressor genes which makes other genes on or off by the deletion or a nonsense mutation(Perez,*et. al..*, 1998; Vogelstein,*et. al..*, 2000; Oren, 2003; Kohn,*et. al..*, 2005;). 2) This alteration is irrespective of an organ or histological type of a significant frequency (between 20 and 80%) in almost every type of cancer (Li,Story,*et. al..*, 2001; Tjebbes,*et. al..*,2002; Wang,*et. al..*, 2003; Magne,*et. al..*, 2006). This observation explains the major role of p53 as one of the leading agent in cellular growth control machinery. 3) p53 itself has central importance in many aspects of normal life e.g in longvety and ageing also normally repairs DNA damage (Weller, 1998; Ritter, *et. al.*, 2002; Tjebbes, *et. al.*,2002; Lindstrom, *et. al.*, 2003; Stark , *et. al.*, 2003; Cuddihy , *et. al.*, 2004). This protein also works in contrast with many other tumor suppressor proteins which in turn regulate other essential proteins. Conspicuously, by the homologous recombination makes mice p53 deficient which show essentially normal development and behavior but after 20 or 30 weeks of age, most of them die from multiple early cancers (Vogelstein *et. al.*,2000; Melino *et. al.*,2002; Vousden , *et. al.*, 2002). Hence M. Oren tags p53 gene "the ultimate tumor suppressor gene", the function is to protect the cell from the events leading normal cells towards cancerous cells. This essential

position of p53 in the control of cell differentiation and proliferation is due to the fact that it is an inducible protein at the post-transcriptional level (Gudkov, *et. al.*, 2003; Oren, 2003). p53 is almost suppressed or missing in vast majorityof the normal cells and tissues however, becomes activated and stabilized because of various kinds of cellular stresses. The damage to the DNA is one of the stresses, inducing p53 to become active in cells. Besides this p53 is able to regulate many overlapping pathways. Tumor suppressor gene at its protein level is a transcription factor for more than 30 known genes in DNA repair, cell cycle control, differentiation and senescence pathways, and apoptosis (Appella, *et. al.,* 2001). Complex formation with other cellular components actively influence the protein to function in p53 signaling pathway. As a result of various stress signal only the p53 protein takes a central importance (in particular to DNA damage) with cell growth. For all of its importance in signaling network p53 is known as "guardian of the genome" (Malkin *et. al.,*1990).Dysfuntional p53 effects the immune system in which cell is capable to proliferate in stressful conditions, while in cancer progression cells may acquire genetic changes (Vousden , *et. al.*, 2002).

1.1.2 Two Modes of p53

As the essential part of p53 was revealed, the excitement achieved a crescendo, and p53 turned into the primary focus of concentrated tumor investigation around the world. There was a ton of theories with respect to the impressive potential for p53-focused cancer treatments, thirty years after, the fact that potential should have still to be figured out.

Part of the troubles emerges from the finding that p53 has the huge potential to play a couple of contradicting parts in the progression of cancer: guardian and the killer. the motivation of ordinary medications, similar to radiotherapy and chemotherapy, depends on the proof that they incite injuries to the cell, that stimulates the p53-subordinate cell demise, destroying the cancerous cells. But, p53 may likewise unconsciously encourage tumor cells to survive by initiating the cell senescence pathways as opposed to apoptotic pathways.

For example, It has been portrayed that a few assortments of breast carcinoma, with the wild-type of p53, indicate imperviousness to chemotherapy medicines, and an expanding number of p53-prompted genes survivals are known. it is vague that how p53 figures out if or not to instigate cell demise or senescence, nonetheless, this range incorporates more prominent exploration interests.

In the late 70s, TP53 gene was discovered and it took more than twenty years to understand its clear functionality. Many experimental and molecular approaches have demonstrated that in

most of the cancers the p53 gene is mutated or its function is lost. Hence the gene and its product have attained a central importance in cancer studies. TP53 gene has taken an important place in human cancers due to three aspects: First, in cancer cells, p53 function is lost due to missense mutation. Secondly, the frequency of this mutation is irrespective of organ site or histological type. Third, p53 itself is important for normal cellular responses.

In many types of human cancers, TP53 gene is altered or it has lost its function. It has been known as the guardian protein for its functionality. It has also given the status of radiation predictor as it can detect multiple stresses to generate its functions (Tjebbes, *et. al.,*2002; Lindstrom, *et. al.,* 2003; Cuddihy and Bristow, 2004; Ritter and Gilchrist, 2002; Weller, 1998; Stark *et. al.,* 2003). The cell is facing the hazards of multiple mutagens at every moment passing. These mutagens are of different types e.g chemicals, radiations etc. These stresses or mutagens are signals for activating p53. Depending upon the type of stress p53 performs its function that either it has to halt cell cycle, cause the cell apoptosis or to take the cell into differentiation. Generally, we categorize the role of p53 in two directions. First in its physiological context and the other in the pathological context. As far as the former is concerned, we study the status of p53 in such a way that how far it responds to the environmental mutagen and for the literal context, we take its functionality for cancerous cells that take us in the direction for proposing a specific therapy. Hence, for cancer management, controlling the function of p53 is in the interest of the present scientific society.

For p53 central importance in a cancer study, many attempts have been made in restoring the p53 function or modulating the p53 protein by pharmacological therapies introducing system medicine in the field of this approach.

1.1.3 p53 Signaling Pathway

P53 signaling pathway comprises of various genes and their translational items that respond to various intrinsic and extrinsic stresses that have a great impact upon normal homeostasis mechanisms of a cell which monitors replication of DNA, cell division and chromosomal segregation (Vogelstein *et. al.,* 2000) in Figure 1.1. The p53 protein reacts to stress signals in a particular way in its post-translational adjustments which causes cell cycle arrest, senescence or towards the death of the cell (Jin, *et. al.,* 2001). An assortment of extrinsic or intrinsic stresses would bring about genome strength loss, fidelity in DNA replication, cell cycle or chromosome isolation can be achieved or, different copies of absconded cells can be expelled from the body.

Moreover, the cellular environment can also be altered by a number of proteins secreted by a cell as a result of the p53 signaling pathway in a single cell to its neighboring cells. Angiogenic motions in a confined area of a tissue and the extracellular grid might be impacted or adjusted by P53-managed and p53-secreted proteins. DNA repair processes can be aided by the set of proteins produced by p53 signaling pathway because of the damage to DNA in a cell. An expounded procedure of auto-regulatory positive and negative feedback loops interfaces the p53 pathway to the other signal transduction pathways in the cell. Overall p53 programmed to response against stresses.

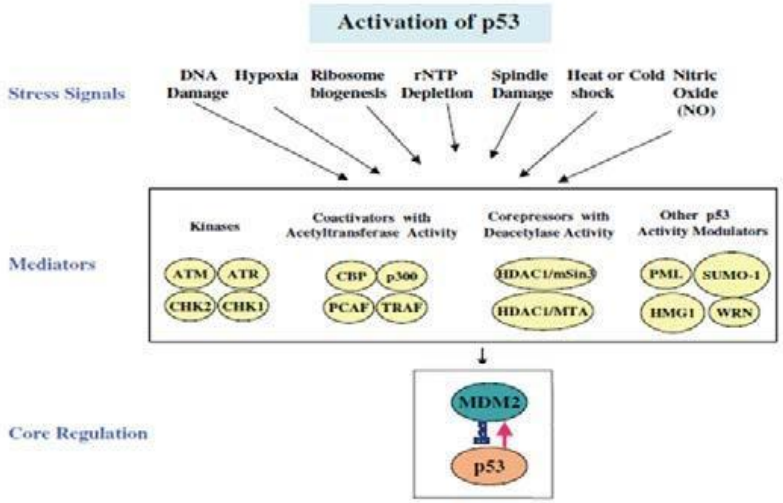

Figure 1. 1 Activation of p53 by different types of stresses

1.2 Problem Statement

Determination of drug dosage rate by means of computational deterministic model for the revival of p53 in cancerous cells with the introduction of small molecule drug (Nutlin etc).

1.3 Purpose

The objective of this research is:

- Introducing system medicine approach in a proliferating cancerous cell for the revival of p53 protein transforming the nonfunctional p53 to a functional p53.
- Integration of network and pathways with the cellular data arising from numerous cancerous cells, hence utilizing a new field of system medicine.

1.4 Scope

The current study has the multi dimensional scope as it covers the bioinformatics, system

biology as well as system medicine.

1.4.1 Bioinformatics

This includes pathway analysis of p53 signaling pathway retrieved from KEGG database.

1.4.2 System Biology

System biology includes ODE derived from the above-mentioned p53 signaling pathway.

1.4.3 System Medicine

System medicine is the emerging field of Bioinformatics which deals with the pharmacological studies of the drug. The present study will have the PBK modeling of Nutlin.

These three fields will be integrated to get the output that defends the application of drug for the p53 revival in the cell.

1.5 Theoretical basis and Organisation

1.5.1 p53 and other Pathways:

p53 has taken an important as well as the central place not only in cancer but it is also involved in many other pathways directly or indirectly. These pathways are associated with the downstream function of p53 e.g. apoptosis, DNA repair, cell cycle arrest etc(Vogelstein *et. al.*,2000; Melino *et. al.*,2002; Vousden & Lu, 2002) (Figure 1.2).

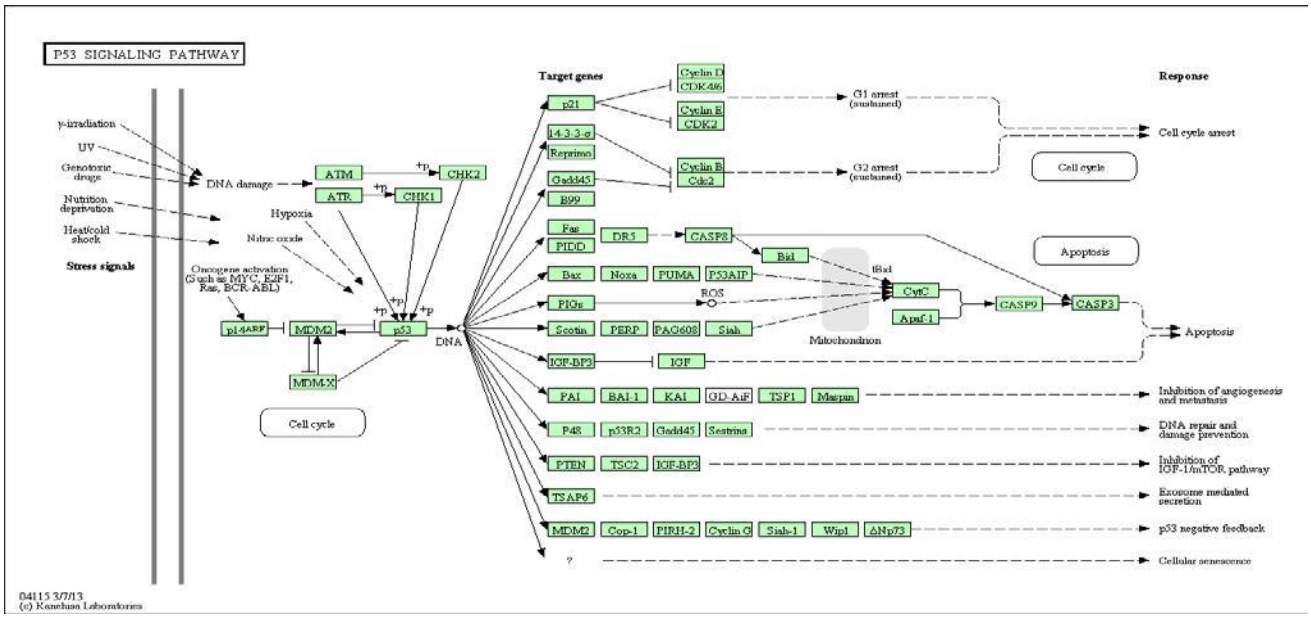

Figure 1. 2 P53 Signaling Pathway

(www.kegg.com)

1.5.2 p53 and Apoptosis

The complexity of p53 regulatory network is needed to be elucidated due to its involvement in most of the non-infectious diseases (cancers). Deregulated levels of apoptosis are noticed due to the dysfunction of p53 protein is strongly evidenced by its pathological conditions (cancer, neurodegeneration, ischemia, cholestasis, and atherosclerosis) (Perez , *et. al.,* 1998; Vogelstein, *et. al.*, 2000;Oren, 2003; Kohn , *et. al.,* 2005).

1.5.3 p53 and DNA Damage Repair

As p53 is a radiation detector. When cancerous cells are treated by using radiotherapy or chemotherapy it posses stress on the unaffected cells though it treats the cancerous cells. The stress is detected as intrinsic stress in the form of double-stranded breaks (DSBs) or single stranded breaks (SSBs). In order to repair this error, p53 is activated by the downstream regulation of mediator complexes depending upon the types of stresses (UV radiation, Gamma radiation or DNA damage itself a stress). p53 is activated till the damage is repaired (Weller, 1998; Ritter , *et. al.,* 2002; Tjebbes , *et. al.,*2002; Lindstrom , *et. al.,* 2003; Stark , *et. al.,* 2003; Cuddihy , *et. al.,* 2004).

1.5.4 p53 and Cell Cycle Arrest

Depending upon the type of stress different mediator complexes are activated by phosphorylation which in turn activates the p53 protein for cell cycle arrest. The downstream pathway is triggered and the cell is not entered into the cell cycle phase. When the damage is repaired and the p53 level is attained its normal state and auto-ubiquitination by its negative regulators the cell is transmitted to cell cycle phase (Tjebbes , *et. al.,*2002).

1.6 Positive and Negative Pathways

The p53 protein is a hub protein for multiple integrated pathways. The regulation of p53 protein is up regulated or down regulated by different types of stresses. There are multiple feedback loops specific to stress signals that trigger p53 signaling pathway. These feedback loops are either positive or negative pathway. Almost ten feedback loops are known in p53 signaling pathway (Li,Story, *et. al.,* 2001; Magne, *et. al.,* 2006). Among them, seven are positive feedback loops incharge of the up-regulation of p53 protein and three are negative feedback loops which are incharge of the down-regulation of p53 protein. Understandings of these feedback loops are important for model developers because these states are modeled in vivo and then required the experimental verifications hence integrate bioinformatics and system biology (Oren, 2003; Kohn,

et. al., 2005; Vogelstein, *et. al.*, 2000).

The vast majority of other transduction pathways are activated by the protein products of p53 responsive genes. There are ten feedback loops, among them three positive and seven negative feedback loops (appeared in Table 1) managing the p53 level and its transcriptional activity (Harris , *et. al.,* 2005). These both negative and positive feedback loops make a network of proteins whose synthesis and or action rates are impacted by phosphorylated p53 and modify the p53 action in the cell also. This versatile quality shows an inconvenience for modelers and makes the regulation of p53 far from being resolved. The other trouble is a direct result of the way that, most of the trial information is accounted for on cancerous cells, which show diverse breakdowns of the p53 framework.

Table 1-1 Feedback loops in p53 signaling pathway

Negative Feedback Loops	MDM-2, Cop-1, Pirh-2, p73 delta N, cyclin G, Wip-1 and Siah-1
Positive Feedback Loops	PTEN-AKT, p14/19 ARF and Rb

1.6.1 p53-MDM2

In most of the cells, the level of p53 is almost undetectable as it is degraded by a proteasome. When it is activated, the level of p53 is increased in the cell which results in the regulation of downstream pathways. MDM2 is the main factor controlling p53accumulation in the cells which it is regulated by p53 a transcriptional factor of MDM2 gene. MDM2 is an ubiquitin ligase, which allows p53 to translocate from nucleus to cytoplasm proteasome and degraded there. In almost all the cancer types this ubiquitin pathway of p53-MDM2 observed. This happens due to the overexpression of MDM2 in the cell. This inhibitory pathway is important because it is involved in most of the cancer types. Thus, the interactive loop amongst p53 and MDM2 and MDM2 ubiquitin ligase activity has turned into the significant target of the drug for a few cancers. Evidences of experiments has demonstrated that approximately 33% of human sarcomas and in leukemias and in glioblastomas there is an over expression of HDM2. This is because although the p53 protein is a functional protein, but due to the overexpression of HDM2 this does not show any function. The drugs are designed so that p53-MDM2 autoregulated loop is broken to increase the level of active p53. This therapy should ultimately induce apoptosis selectively in cancerous cells. Another application would result in the

enhancement of chemotherapy of some drugs that activate p53.

1.7 Restoring p53 as a therapeutic strategy

Important roles in organizing cell defense against cancerous transformation are played by tumor suppressor protein p53 found in studies from the previous thirty years. P53 functions as a strong transcription factor trigger the downstream genes because of the different stresses, for example DNA damages, irradiations and hypoxia brings about cell cycle arrest, angiogenesis and apoptosis (Brown, *et. al.*, 2009). Has been observed a pro-apoptotic function of p53, which is independent of its transcriptional function (Moll,*et. al..*, 2005; Speidel, 2010;).

In half of the human tumors the p53 is mutated (Cheok, C.F., *et. al.*,2011). In those reported tumors p53 is strictly regulated and maintained at its lower concentration. Tumor suppressor p53 protein has a exactly aminor half-life of 20 minutes as its regulation is negatively controlled by E3 ubiquitin ligase MDM2 (murine double minute-2). It not only supports p53 for exportation from the nucleus but also facilitates proteasomal degradation of p53 (Reich, *et. al.*,1983; Tao,*et. al..*,1999; Haupt, *et. al.*,1997). The function of p53 is inhibited directly by the binding of MDM2 at the transcriptional binding site p53 and stops its binding to the transcriptional equipment in the cell (Momand, *et. al.*,1992). There they form an autoregulatory loop where the increased level of p53 regulates the production of MDM2 in the cell while in turn the p53 level is decreased (Bond, *et. al.*,2005) (Figure 1.1).

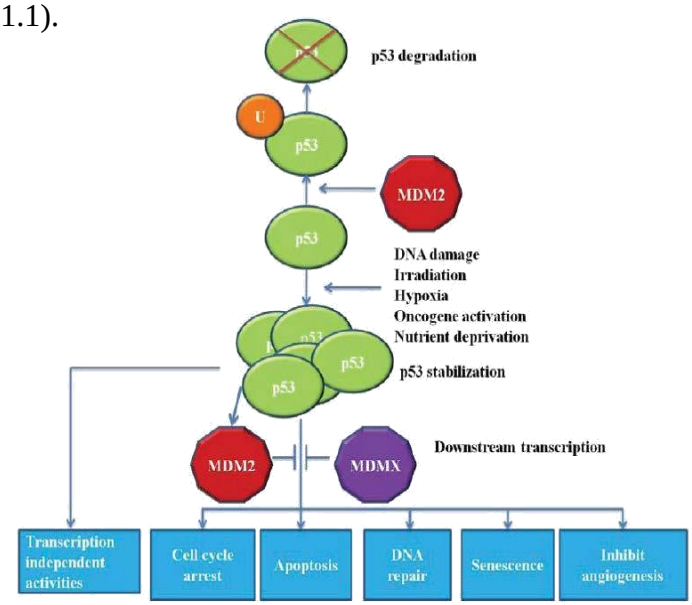

Figure 1. 3 p53 pathway and MDM2/MDMX p53 interaction

MDM2 has the structural homology with MDMX also known as MDM4 or HDMX. This MDM2

has an important regulatory mechanism for p53 (Marine, *et. al.*,2007; Finch, *et. al.*,2002; Wade, *et. al.*,2010). MDMX not only hinders the transcriptional function of p53 but also forms heterocomplex with MDM2 that initiates the degradation and ubiquitylation and of p53 (Linares, *et. al.*,2003; Gu, *et. al.*,2002). P53 is not transcriptionally affected by MDMX as MDM2 does to inhibit p53 transcriptional functions. As heterocomplex of MDMX with MDM2 forms, it causes the degradation and ubiquitination of MDMX.

Considering the important roles of MDM2/MDMX in p53 stability and function, restoration of the impaired function of p53 by inhibiting MDM2/MDMX was considered an attractive strategy to treat tumors with wild-type p53 (Chene, 2003). Several inhibitors of MDM2/MDMX have been discovered and are currently under investigations as mentioned in the table below (Hao,*et al.*,2014).

ID	Company	Status	Mechanism	*In vivo* Test
RG7112 (RO5045337)	Hoffmann-La Roche	Phase I	Inhibition of MDM2-p53 interaction	Advanced malignancies, except leukemia
MI-773 (SAR405838)	Sanofi-Aventis	Phase I	Inhibition of MDM2-p53 interaction	Advanced cancer
CGM097	Novartis	Phase I	Inhibition of MDM2-p53 interaction	Selected advanced and refractory solid tumors
MK-8242	Merck	Phase I	Inhibition of MDM2-p53 interaction	-
RO5503781	Hoffmann-La Roche	Phase I	Inhibition of MDM2-p53 interaction	Soft tissue sarcoma; leukemia
Tenovin	-	Preclinical	Inhibition of Sirt1 and Sirt2 activity	-
Inauhzin	-	Preclinical	Inhibition of Sirt1 activity	-
PRIMA-1^{MET} /APR-246	Aprea AB	Phase I/II	Reactivate mutant p53	Refractory hematologic malignancies and prostate cancer
PK083	-	Preclinical	Reactivate mutant p53 Y220C	-
PK7088	-	Preclinical	Reactivate mutant p53 Y220C	-
NSC319726	-	Preclinical	Reactivate mutant p53 R175H	-

Figure 1. 4 Drugs in Different Phases for The Revival of p53 by Different Mechanisms

1.8 Physiologically-based Pharmacokinetic (PBPK) Modeling and Simulation in Drug Development

Disposition of a drug by the body may be a complicated method. Compounds are often administered by numerous routes like oral, connective tissues, through blood vessels, sub-conjunctival, or intramuscular injection. Through numerous mechanisms of absorption, the drug is absorbed then distributed to the tissues by the body fluids (e.g., blood, vascular system, CSF). The drugs are often metabolized in the liver and/or different tissues to different compounds and/or eliminated directly through the bile or the urine. Protein binding within the plasma and completely different tissues are often diverse because of numerous proteinaceous contents. As mentioned earlier, outflow and uptake transporters expressed throughout the body can influence the ADME properties. Within the same tissue, drug concentrations within the vascular, intracellular, and extracellular compartments are often completely different because of the complexity of this method; drugs concentrations within the blood and in different components of the body are often quite variable. Therefore drug concentrations within the plasma don't continually reflect target tissue concentrations. An acceptable pharmacokinetic model that effectively describes and fairly predicts the time-dependent drug concentrations within the body is vital in each of the preclinical and clinical drug development. Currently, 2 broad approaches to pharmacokinetic analysis are conventional and PBPK models, though; the 2 methodologies do share similar characteristics. The classical pharmacokinetic model focuses principally on the drug concentrations in blood (whole blood, serum or plasma) or on the body fluids (urine, breast milk, CSF, or feces). It generalizes the complicated drug distribution method into multiple imaginary compartments. An example of conventional PK model structure is provided in Figure 1.2. Compartments in classical PK models don't represent real organ compartments and frequently lack mechanistic insight. This limits their application in several areas of drug discovery and development like predicting human systemic and targeted tissues drug concentrations supported in vitro information or animal PK information, and predicting drug-drug interactions.

On the other hand, PBPK models are quite complicated models that map the drug PK method onto physiologically representative compartment structures (Kawai, *et. al.*,1998). In typical PK model, the body is typically modeled as a closed vascular system consisting of tissues that are vital for drug absorption, distribution, metabolism, and elimination, or the other tissues of interest.

Compartments that represent real tissues are connected by blood flow. In general, tissue-specific blood flow is model input into the tissues and comes out of the tissues as venous blood, therefore serving as model output, with some exceptions. For visceral organs like stomach, spleen, pancreas and intestine, venous blood enters into the liver through the vascular system. For the lung, the venous blood flows within the lung and out of the lung. In distinction to the classic PK model, the physiologically-based nature of the PBPK modeling permits us to deal with mechanistic queries with respect to the PK in addition to inferring information obtained from one species to another, as well as human.

However, the difference between the classic and PBPK model isn't perpetually clear. As Nestorov mentioned in his reviews, it's not possible and unwanted to outline formally what a PBPK model is or to specify a quite distinction between the classic model and PBPK model (Nestorov, 2003; Nestorov, 2007). The classic model contains a lot of physiological data (e.g., such as the weight of the body within the subject scaling, using the bile empty time for modeling enterohepatic circulation etc.). Therefore the PBPK model typically incorporates classic PK model elements. For instance, PBPK modeling software package PK-simR incorporates the animal clearance rate from classic PK model into the PBPK model to predict concentration time profiles, each in animal and in human. Also, one necessary step for establishing the whole body PBPK model is to come up with PK model within the plasma by using classic PK modeling, to differentiate between a predominantly classical and predominantly PBPK model, the convention is to seem at the model structure.

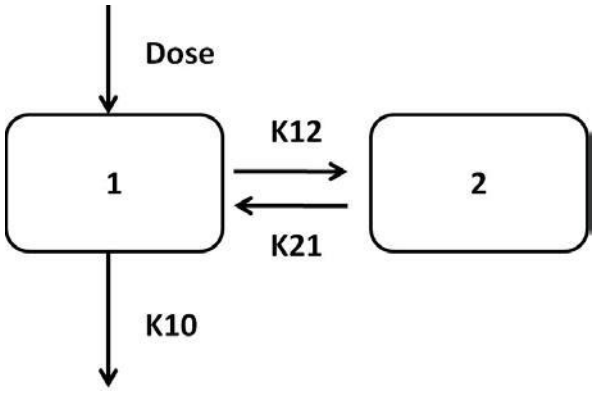

Figure 1. 5 Example model structure of the classical PK model

As mentioned during a review by Nesterov, if the model structure precedes the analysis of the compound-specific information, and predominantly represents actual tissue or organ areas, then

it is classified as a PBPK model (Nestorov, 2003). It must be mentioned that each the classical PK model and the PBPK model belong to the compartment PK model hierarchy as a result, both models classify the body into various subunits referred to as "compartments" (Nestorov, 2003; Aarons, 2005).

1.8.1 PBPK application area

Because of the advances in the computing power, PBPK models have been progressively utilized from several areas (Rowland, *et. al.*, 2011). The dominant part of all PBPK related publications about 60% deals with issues relating to hazard evaluation of natural chemicals (Edginton, *et. al.*, 2008). Since the PBPK model is more anatomically, mechanistically, and physiologically applicable than the conventional compartment modeling techniques, the developed PBPK model from the animal study can be utilized to extrapolate to people. In the drug disclosure and advancement area, there is a specific enthusiasm to utilize the PBPK model for assessing the human PK of drug candidates in silico, in vivo, or in vitro animal PK information and to choose the most encouraging drug candidate for further development. Techniques for anticipating human PK are variable, and numerous strategies are under assessment. For instance, detail scaling strategies can be found in latest published papers from the Pharmaceutical Research and Manufacturers of America (PhRMA), including 24, 29, and 66 different techniques for predicting distribution, drug clearance separately (Poulin, *et. al.*,2011; Jones, *et. al.*, 2011; Ring, *et. al.*, 2011; Vuppugalla, *et. al.*, 2011; Poulin, *et. al.*,2011), in light of in vitro and animal PK information. PBPK model has additionally been utilized for specific population, for example, pediatrics (Bjorkman, 2005; Johnson, *et. al.*,2006; Kersting, *et. al.*, 2011; Edginton, 2011; Johnson, *et. al.*, 2011; Khalil, *et. al.*,2011; Yang, *et. al.*,2006; Ginsberg, *et. al.*,2004; Parrott, *et. al.*,2011), elderly patients (Bjorkman, *et. al.*, 2001; Li, *et. al.*,2003), pregnant women (Hays, *et. al.*, 2000; Gray, 1995; Fisher, *et. al.*,1990), lactating people (Fisher, *et. al.*,1990; Yoon, *et. al.*,2009), and for patients with impaired organ (Edginton, *et. al.*,2008). It has been connected to predict the human drug-drug associations (Johnson, *et. al.*, 2011; Haddad, *et. al.*, 2010; Perdaems, *et. al.*,2010; Bois, 2010; Rekic, *et. al.*,2010; Peck, C.C., 2010; Ito, *et. al.*, 2010; Zhao, *et. al.*, 2011; Rowl, *et. al.*, 2011; Grime, *et. al.*,2009), between individual variability (Edginton, *et. al.*,2011), and the impact of heredity (Gentry, *et. al.*, 2002; Kusama, *et. al.*,2009; Teeguarden, *et. al.*,2008). In addition to small molecules and compounds, the uses of the PBPK model have additionally been reached out to be extensively large molecules (Baxter, *et. al.*,1995; Baxter, *et.*

al.,1994; Puchalski, *et. al.*, 2010; Davda, *et. al.*, 2008; Kletting, *et. al.*, 2009; Urva, *et. al.*, 2010; Boswell, *et. al.*, 2011; Friedrich, *et. al.*, 2002; Kletting, *et. al.*, 2010) and nanoparticles (Fallon, *et. al.*, 2004; Pery, *et. al.*, 2009; Yang, *et. al.*, 2010; Li, *et. al.*, 2010).

1.8.2 Major components of PBPK model.

Inputs given to PBPK models involve drug-independent and drug dependent data. Tissue piece, organ mass or volume, blood stream and the anatomical arrangement of the tissues and organs of the body are the drug independent segments. Drug-dependent data incorporates protein binding, partition coefficients, enzymatic stability, layer penetrability, PK properties and transporter-drug connections. Since the drug independent components and a hefty portion of the drug dependent components are not required in the conventional PK model, it is well known that the PBPK model contains lots of informational contexts .

Other than data collection, the general strategy for creating PBPK models comprises of three major steps (Nestorov, 2003): 1) determination of the whole body structure and the tissue structure, 2) composing differential conditions, and 3) assessing parameters.

1.8.2.1 Specification of whole body structure and tissue structure

To set a PBPK model for a particular compound, the whole body structure should be developed to meet particular study purposes. It is critical to choose which tissues, organs to incorporate into the model. On one hand, the PBPK model ought to contain a substantial number of tissues/organ that is vital for drug ADME and tissues of particular interest. while on the other hand, for practical reasons, an excessive number of tissues, organ compartments not just increase the requirement for a great deal of experimental information and literature data additionally, increases the trouble of the mathematical estimations. At present, there is no clear rule for the determination of the tissues to be incorporated, Generally "core tissue", for example, blood, fat, kidney, liver , and tissues of interest are incorporated into the model (Nestorov,2003). All whatever is left of the tissues can be lumped into "fast equilibrating" or "moderate equilibrating" compartments. At the point, when both a parental drug and its metabolites are studied on, separate PBPK models ought to be produced for both the drugs and the metabolites. The models are then connected through the metabolism compartment (typically liver) with one part of the parent drug elimination output serving as input for the metabolites (Xu, *et. al.*,2003; Clewell, *et. al.*,2001).

After the whole body structure is characterized, the next step is to indicate the structure of a

particular tissue. In several cases, a perfusion restricted model is utilized for the tissues (Figure 1.3A) (Xu, *et. al.*, 2003). The basic assumption is that the drug distributes in the tissues and no concentration gradient exists in the tissues. Despite the fact that making this supposition distorts the real situation, it has the really favorable advantage of lessening the complexity of the model. At the point, when permeability confines the distribution of a drug candidate inside a tissue, a diffusion-limited model can be used (Figure 1.3B) (Xu, *et. al.*, 2003).

One tissue compartment can be isolated further into a few compartments. For a two-compartment model in certain tissues, if the rate limiting step happens in the capillary membrane, then the two compartments refers to as the vascular and additional vascular compartments; if the rate limiting step is expected to happen at the cell layer, then the two compartments refers to as intra-and extracellular compartments. For the three compartment model in a specific tissue, it is expected that both the cell membrane and capillary are rate-limiting steps for the drug. Furthermore, the three compartments refer to as vascular, cellular, and interstitial compartments (Nestorov,2003). For eliminating organs, a component containing eliminating function is added to the model structures (Figure 1.3C, D) (Xu, *et. al.*,2003).

Actuality, a whole body PBPK model can be made out of both diffusion and perfusion limited tissues. For instance, the whole body PBPK model created for 17-(allylamino)- 17-demethoxygeldanamycin (17-AAG) contains both perfusion-limited organs (lung, heart,kidney, brain, spleen, and muscle) and diffusion-limited tumor tissue (Xu, *et. al.*,2003). The whole body PBPK model designed for topotecan comprised of both perfusion-limited organs (lung,skin, heart, muscle, liver, fat, spleen, cerebrum and gut) and diffusion limited organs (kidney and testicles) (Shah, *et. al.*,2011).

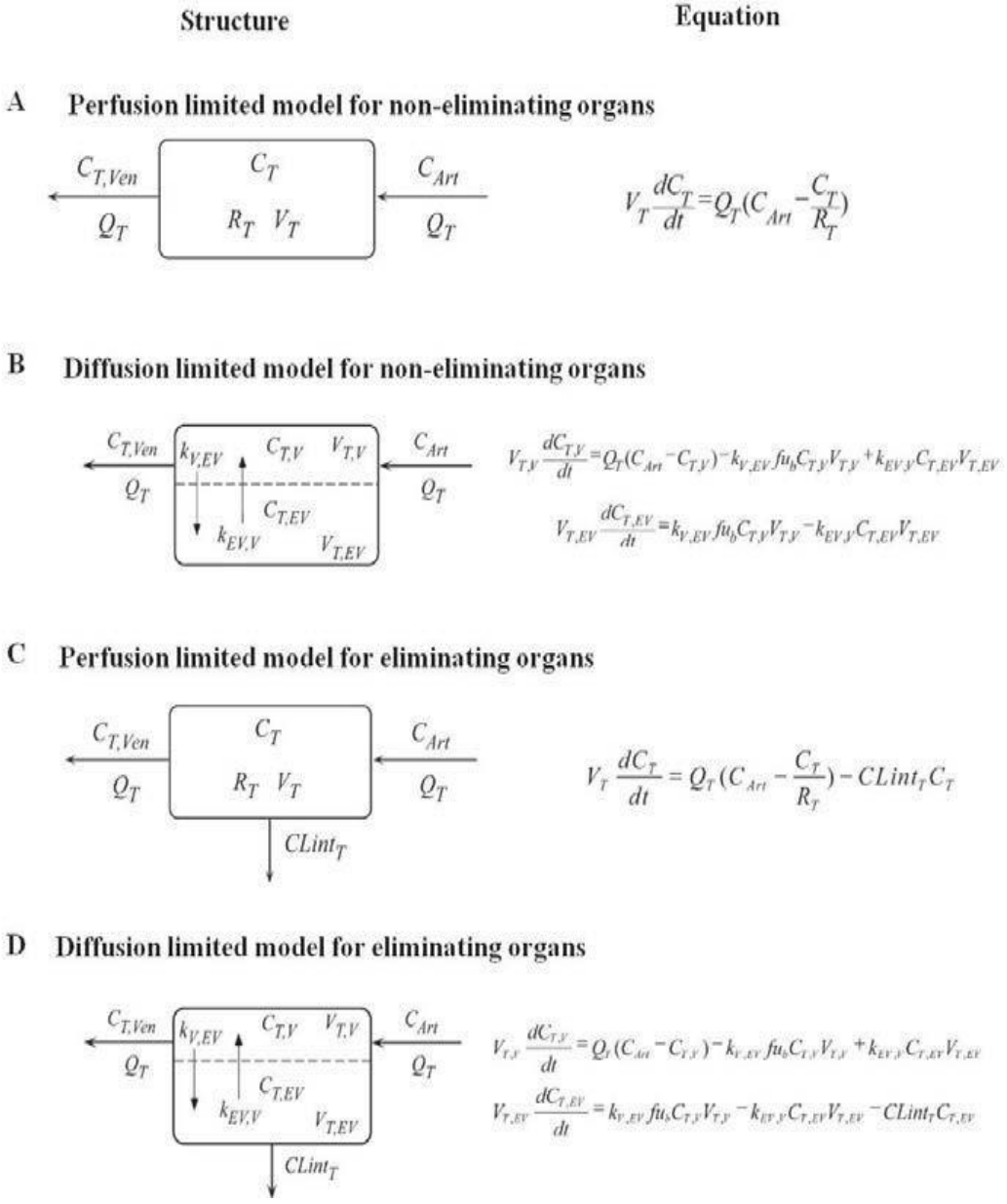

Figure 1. 6 Structures and equations for individual organ models

A. Perfusion-Limited model for the non-eliminating organs. VT is the aggregate volume of organ T; QT is the flow of blood stream to the organ; CT is the concentration of the drug in the organ over time; RT is the partition coefficient; CArt is the drug concentration in the arterial blood; CT, Ven is the venous concentration of effluent drug (CT Ven = CT/RT). In this model, the measured concentration of tissue is CT.

B. Diffusion-limited model for non-eliminating organs. CT,V, and VT,V represent the

concentration and volume of drug for the organ vascular space; CT,EV, and VT,EV demonstrates the consistent terms for the extra-vascular space; kV,EV, and kEV,Vdemonstrates the transport rates of drug between the vascular and extravascular spaces; fub is the fraction of the unbound drug in the vascular space. In this model, the measured concentration of tissue is CT,EV.

C. Perfusion-limited model for eliminating organ. CLintT symbolizes the intrinsic clearance of the drug.

D. Diffusion-limited model for eliminating organs. CLintT denotes the intrinsic clearance of the drug. (Xu, *et. al.*,2003)

1.8.2.2 Writing differential equations

As in conventional PK modeling, PBPK modeling equations are composed on the basis of mass balance equation. Here, the distinction is that the components and structure of the model denote genuine physiology. Examples of equations are shown in Figure 1.3 (Xu, *et. al.*,2003). During the development procedure of model, equations might be changed.

1.8.2.3 Estimating parameters

With a goal to set parameters, in addition to experimental information, anatomical and physiological information must be obtained for modeling. Though, acquiring reliable physiological parameters is not a simple task. Inaccurate physiological parameters utilized as a part of the model will adversely affect the values generated from the final model. Among every one of the parameters, sensitivity analysis demonstrated that flow of blood is the most compelling parameter (Nestorov, 2003). Since different conditions, for example, anesthesia and anxiety can change the flow of blood stream, utilizing of physiological information acquired under these conditions may change the results of modeling. Due to the complexity of the PBPK model, PBPK parameter estimation ordinarily led from the open loop to closed loop. Initially, the Blood concentration is modeled utilizing the empirical approach. The developed blood PK model from the empirical approach is then utilized as a driving capacity to every individual tissue. In this step, data, for example, blood flow, partition coefficient, and organ weight are utilized or evaluated. Since individual tissues are still separate, at this stage, the model is open loop. A the PBPK model is adjusted to a closed circulating framework. Because of the computational difficulties in modeling all the PBPK parameters in the meantime, an open-loop approach followed by closed loop strategy is suggested (Nestorov, 2007).

1.8.3 Predicting human ADME and whole plasma PK profile using PBPK model

In early stages of drug development, the estimations or simulations of drug-particular information, for example, molecular weight, pKa, plasma protein binding, Log P and in vitro hepatic intrinsic clearance can be consolidated into the PBPK model to predict the ADME of animal and human and encourage the selection of drug candidate. Full plasma concentration-time profiles following various schedules of dosing can be anticipated by the PBPK models. For instance, utilizing the PBPK model, Jones, *et. al.,* from F. Hoffmann-La Roche reported better prediction of human plasma concentration contrasted with generally utilized allometric scaling (Dedrick approach) for compounds with particular physicochemical and pharmacokinetic properties (Rowland, *et. al.*,2011, Jones, *et. al.*,2006).

1.8.4 Drug distribution in target tissues and tumors

PBPK models have been utilized to comprehend and anticipate target tissue and the tumor concentrations. In the area of oncology, a PubMed search (09/09/2016) utilizing "physiologically based pharmacokinetic cancer model" or "physiologically based pharmacokinetic tumor model" returned 153 papers. PBPK models had been utilized for radio-immuno-detection and radio-immuno-therapy (Zhu, *et. al.*,1998; Zhu, *et. al.*,1997), immune response (Baxter, *et. al.*,1995; Baxter, *et. al.*,1994; Puchalski, *et. al.*,2010; Davda, *et. al.*, 2008; Urva, *et. al.*, 2010; Boswell, *et. al.*,2011; Friedrich, *et. al.*, 2002; Kletting, *et. al.*,2010; Fang, *et. al.*, 2008; Ferl, *et. al.*,2006; Ferl, *et. al.*,2005), imaging operators (Barboriak, *et. al.*, 2008; Mescam, *et. al.*,2007; Armitage, *et. al.*,2005), liposomal (Qin, *et. al.*,2007), and small molecules, for example; topotecan (Shah, *et. al.*,2011), docetaxel (Hudachek, *et. al.*,2011; Bradshaw-Pierce, *et. al.*, 2008), doxorubicin (Li, *et. al.,2003),* capecitabine *(* Tsukamoto, *et. al.*, 2001*),* temozolomide *(*Gallo,*et. al., 2004;* Zhou,*et. al.*,2007*),* genistein *(*Zager, *et. al.*,2007*;* Schlosser, *et. al.*,2006), gefitinib (Wang, *et. al.*,2008; Wang, *et. al.*, 2011; Zhou, *et. al.*, 2011), 17-AAG (Xu, *et. al.*,2003), moxifloxacin (Edginton, *et. al.*,2009), and methotrexate (Li, *et. al.*, 2002; Devineni, *et. al.*,1996).

Estimations by using non-compartmental modeling. Shah *et. al.*, described a whole body PBPK model for topotecan (Shah, *et. al.*, 2011). *In vitro* measurements. Bradshaw-Pierce, *et. al.*, described a PBPK model for docetaxel(Bradshaw-Pierce, *et. al.*,2007). The PBPK model for docetaxel was established an integrating particular binding of docetaxel to intracellular components, biliary elimination, liver metabolism, and urinary and fecal excretion. Tissue/blood partition coefficients were determined *in vitro* [Bradshaw-Pierce, E.L., *et. al.*, 2007; Jepson, G.W., *et. al.*,1994). The partition coefficient was identified by the proportion of docetaxel

concentrations in the tissue layer to the saline layer. These values were adjusted and applied to the whole body PBPK model.

Sung,, *et. al.,* (2009) described a PBPKPD model for UTF (5-FU, tegafur, and uracil). Partition coefficients for tissues were designed on the basis of n-octanol-water partition coefficient established by Poulin and Theil (Poulin, *et. al.,* 2002; Poulin, *et. al.,* 2002). Bois *et. al,* developed a Generic PBPK models, germane to incalculable, coupled to parameter databases and QSAR modules, for the predictive modeling of drug changeability in the ADME of usually natural and also manufactured synthetic compounds. Their model used Markov chain Monte Carlo methodologies and multilevel populace models that can be commonly used for a Bayesian conformity of a PBPK model (CENTER FOR DRUG EVALUATION AND RESEARCH, 2014). Once a PBPK model is developed, scaling of the concentration from one species to other can be done by altering the physiological data by keeping the structure of the model and supposing equal partition coefficients between species (Xu, *et. al.,* 2003; Wang, *et. al.,* 2008; Gallo, *et. al.,* 2004).

1.8.5 Limitation of PBPK modeling

So far, in contrast with the traditional PK model, the use of the PBPK methodology is still constrained regardless of noteworthy potential. Building up a PBPK model is requesting because of the investment of expansive measure of time and efforts to acquire data required to set up the model. Also, information required to build up the model are not generally accessible. Varieties in physiological parameters acquired from the literature, our present knowledge is restricted about the fundamental instrument about drug ADME process, and wrong suspicions would all be able to affect the nature of the model. The PBPK model is typically complex and more methodologically and computationally difficult contrasted with the conventional PK model. Moreover, PBPK model is not as fully developed as traditional PK model. One restriction of PBPK model is the potential for countless parameters, some of which might be related. This can prompt the issues of parameter identifiability and excess (Krauss, *et. al.,* 2013). After assigning the numerical values to each PBPK model parameter, special computer software is commonly used to numerically incorporate an arrangement of typical differential mathematical equations so as to ascertain the numerical estimation of every compartment at determined estimations of time (Chen, 2010).

In spite of the majority of the constraints of the PBPK model specified over, the PBPK

modeling is growing quickly. The information and strategies for building the model are constantly advancing and improving. The PBPK model breaks the limits of traditional PK by building a model and to comprehend the PK procedure in light of the genuine physiological and anatomical framework, and by consolidating rich information obtained over the years about the impact of dependent and independent factors of the drug on PK. In the drug revelation and development area, pharmaceutical organizations are gaining PBPK modeling software for predicting human PK in light of in vitro or in vivo PK data of animals. Additionally, the administrative power has started welcoming the advantage of the PBPK model and applying the PBPK model to address drug administrative review questions. A key advantage to PBPK models is that variable impacting the ADME of a compound can be joined into a PBPK model in a meaningful manner if its mechanism is properly understood and adequate information is available. This mechanistic perspective is supported by physiological parameters affecting absorption, distribution, metabolism and elimination. PBPK modeling approaches have some importance over other PK modeling approaches (Bois, *et. al.,* 2010). By utilizing PBPK modeling, the results of the different preclinical tests can be coordinated to give quantitative predictions of the human PK profiles (Clewell, *et. al.*, 2013; Jones, *et. al.*, 2006; Luttringer, *et. al.,* 2003).Finally, the PBPK model will be joined with expanded frequency in the areas of toxicology and oncology where understanding target tissue or the tumor concentration is vital yet information is generally difficult to access by the human.

1.9 Summary

Reactivating the p53 pathway is viewed as an engaging non-genotoxic strategy for treating tumors with wild-type of p53. Unlike, grown-up tumors, pediatric malignancies normally hold a high rate of wild-type of p53 at diagnosis. Utilizing neuro-blastoma, for instance, 98% of neuro-blastoma tumors hold wild-type p53 at the time of diagnosis (Tweddle, *et. al.,* 2001; Vogan, *et. al.,*1993). Indeed, even in backslide neuro-blastoma, a greater part of the tumors are still wild-type p53 (Tweddle, *et. al.,* 2003). Nutlin-3a is a small inhibitor compound that targets the MDM2/MDMX-p53 interaction. Currently, it is in the pre-clinical examination in the clinical examination of strong tumor and leukemia models and has demonstrated promising in-vivo and in-vitro activities. Like other anti-cancerous drugs, it is the probability that nutlin-3 will be utilized in a combination with other drugs. Studies have recommended synergistic impacts when nutlin-3 and other chemotherapeutic drugs were co-administered, autonomous of p53 status, by upgrading the capacity of anti-cancerous drugs to enact apoptosis or by turning around P-gp-

intervened drug resistance. ABC transporters assume vital parts in drug resistance and ADME processes. The impact of nutlin-3a on BCRP had not been accounted earlier. Understanding the pharmacokinetics of a compound is basic in the preclinical drug development process. A comprehension of the systemic disposition of nutlin-3a, and in addition the distribution to targeted tissues or tumor destinations, will give a normal premise to the choice of a drug candidates for the preclinical models. To date, the pharmacokinetics of nutlin-3a has not been accounted for. The whole body PBPK model, which depends on anatomical compartments and the flow of blood stream, is an incredible tool to portray and predict the concentrations of drug in blood as well as in target tissues.

P53 an important tumor suppressor can be activated in response to various extrinsic and intrinsic stretch signs prompting to the initiation of various genes consequently suppressing the tumorigenesis by various means like cell cycle arrest, apoptosis, senescence, and metabolic variations. Hence, for the chemotherapeutic development p53 pathway has usually become an ideal target. While researchers have gone through every possible aspect of p53 signaling pathway a very promising field of research have been introduced that is system biology, which along with various challenges gave the solution to the problem related to cancer studies. Acute DNA damage-triggered p53 activation, whereas the tumor suppressive function of p53 remains intact (Brady, 2011; Jiang, 2011; Mello, 2011; Johnson, 2011; Jarvis, 2011; Kozak, 2011; Kenzelmann, 2011; Basak, 2011; Park, 2011; McLaughlin, *et. al.,* 2011; Campisi, 2011). There is still a challenging aspect to find the association of p53 dependent tumor suppression with metabolic target genes. Various strategies are developed for partially diminished p53 activity against DNA damage (Damage producing p53-dependent injurious side effects on normal tissues).

2. Chapter 2: Literature Review

2.1 Inhibiting P53-MDM2 interaction as a target for pharmacological intervention:

About 50% of human cancers are due to the mutations in p53, the revival of p53 to make it a transcriptionally an active factor becomes a hot, most investigating topic and various strategies for the treatment of cancers are adopted for this purpose. For the restoration of tumor suppressor activity of p53 in vitro, a number of molecules have been found by a group of scientists. All of the work is done from the mutant form to an activated form of p53 in a cell. Not a single molecule has shown to induce any significant response biologically but there are few lead compounds identified which are biologically active in restoring the function of p53 to some extent. There are some conventionally designed drugs like such as Adriamycin and Platinum-based drugs that activate p53 pathway but besides that they can also trigger the multidrug resistance by systematic toxicity. That is why strategies are adopted to develop drugs targeting p53 specifically. The core regulation p53-MDM2 has taken the central importance so that MDM2 can be detached from p53 releasing p53.

While p53 is an alluring medication focus for tumor, in light of routine medication advancement forms it is not a perfect target. Notwithstanding, proceeded with enthusiasm for p53 has driven an assortment of unique ways to deal with medication disclosure that could have broad applications to medication advancement.

With an end goal to reactivate mutant or inactivated p53, new techniques for screening, science, and structure-based outline are being utilized to focus on the structure and collapsing of the p53 protein, and its connections with different proteins. Two gatherings of p53-focused on operators have risen up out of this: (1) little particles that quandary to mutant p53 and reactivate its wild-sort capacity; and (2) inhibitors of the communication amongst p53 and its negative controller MDM2, viably keeping its corruption. Among those in preclinical or early clinical improvement are RG7112 (Roche Group), MI-219 (Ascenta Therapeutics), JNJ-26854165 (Johnson and Johnson), reactivation of p53 and instigation of tumor cell apoptosis (RITA), the carbazole derivative PhiKan083, p53 reactivation and impelling of gigantic apoptosis (PRIMA-1) and its more powerful simple mutant p53 reactivation and prompting of fast apoptosis (MIRA-1) (Aprea

AB), and nutlin-3. The most progressive are PRIMA-1, JNJ-26854165, and RG7112, all right now in stage I clinical trials.

2.1.1 Nutlin

The momentous work in this field is the advancement of Nutlin, a little atom upsetting MDM2-p53 connection without creating genotoxicity (Vuppugalla, *et. al.*, 2011). It has been demonstrated that Nutlin copies an MDM2-restricting p53 peptide that intensely takes up with MDM2 and avoids MDM2-p53 collaboration, bringing about powerful non-genotoxic actuation of p53 (Vuppugalla, *et. al.*, 2011). The Nutlin subsidiary RG7112 (RO5045337) has been produced by Hoffmann-La Roche and is the principal particular p53 activator that best in class to clinical trials. It is a more strong inhibitor of MDM2-p53 cooperation, yet through the comparative component, contrasted with Nutlin (Poulin, *et. al.*, 2011). There are likewise another promising intensifies that alienate MDM2 capacity entering Phase I trials (Bjorkman, 2005), including RO5503781, SAR405838, CGM097, and MK- 8242 .

2.1.2 P53-MDM2 in various cancers and treatment by Nutlin

Among the p53-focusing on drugs, a conspicuous part is played by Nutlins (Mihara, *et. al.*,2003), a group of little particles ready to tie MDM2 precisely in the "coupling takes" where p53 ties, so hindering the arrangement of p53-MDM2 edifices and instigating a fast p53 level increment. Since the actuation of p53 may bring about the activating of both the apoptotic pathways and the separation of tumor foundational microorganisms, Nutlins are viewed as conceivably critical anti-tumoral operators. In their study, Vassilev, *et. al.*, (Mihara, *et. al.*, 2003) demonstrated a strong antitumor action of Nutlins on wild-sort p53 tumor cell lines, for example, HCT116, RKO, and SJSA-1 cells, while just a minor impact on mutant p53 cell lines, (for example, SW 480, MDA-MB-435, PC3) was watched. The same exploration bunch (Bond, 2005) later found that diverse Nutlins subtypes may have a differential activity on various tumor cell lines. Various other preclinical studies reported that Nutlin is a viable antitumor drug for essential sorts of tumors conveying broken wild-sort p53. The anti-neoplastic activity of Nutlin on perpetual B-cell lymphocytic leukemia with wild-sort p53 has been indicated (Barak, *et. al.*,1993), recording a progression of collaborations with doxorubicin. Nutlin is dynamic against prostate malignancy cells holding wild-sort p53 and androgen receptor flagging (Fang, *et. al.*, 2000), and works by hindering their multiplication through cell cycle capture and apoptosis. Nutlin-3a is likewise dynamic in Hodgkin lymphoma, where p53 is once in a while transformed (Fang, *et. al.*,2000).

In Ewing's sarcoma cells, Nutlin-3 reestablishes wild-sort p53 capacities, with malignancy development restraint and apoptosis impelling, though no impact was watched for cells with changed p53 (the transformation, be that as it may, influences just 10% of those tumors). Also, Nutlin is dynamic against human glioblastoma multiforme (Vassilev, *et. al.,* 2004), where 14% of patients convey intensifications of MDM2. For this situation, Nutlin was dynamic in the wild-type of p53 glioblastomas, where it likewise brought on cell senescence. In (Vassilev, *et. al.,* 2004), it has been unequivocally seen that phone lines can fundamentally contrast in their apoptotic reaction to comparative levels of p53 actuation. We may watch that the Nutlin-intervened rebuilding of p53 levels does not consequently ensure useful impacts if different modules of the p53-related pathway are broken. For instance, Ma, *et. al.,* (Secchiero, *et. al.,* 2011) demonstrated that Nutlin-3 can't prompt p53-related apoptosis in cells where p53-Ser46 phosphorylation is faulty. In retinoblastoma, p53 is in place, yet it is hushed by MDMX overexpression (Ha, *et. al.,* 2011; Barakat, *et. al.,*2010). A preclinical study (Barakat, *et. al.,* 2010) has reported the solid action of privately managed Nutlin-3a against retinoblastoma, and collaboration with topotecan. As of late, it has been demonstrated that Nutlin beats imperviousness to Vemurafenib in melanoma lines (Geva, *et. al.,* 2006), and to Cisplatin in ovarian growth cells (Ma, *et. al.,* 2005). At long last, in both the aforementioned considers concerning the part of p53 in the separation of foundational microorganisms (Speidel, 2010; Moll, U.M., *et. al.,* 2005), Nutlin was the drug utilized for p53 actuation. The above trial discoveries on the impact of Nutlin on wild sort p53 tumors can be generally outlined as takes after the official of Nutlin to MDM2, by inactivating the primary enemy of p53, prompts expanding the p53 level, which adversely impacts the tumor development, to some degree on account of the onset of cell capture and apoptosis, to a limited extent – for undifferentiated cell based tumors – by setting up in disease immature microorganisms a more physiological pathway of deviated cell division. In any case, the configuration and usage of strong treatments require going past a negligible distinct methodology, which ignores the energy and the quantitative components of the marvels. Legitimate devices can be given by Systems Biology, which can incorporate data from numerous sources in a rational quantitative model by utilizing science and bioinformatics.

2.1.3 Nutlin-3a mechanism of action

In 2004, Vassilev, *et. al.,* reported a group of imidazoline compounds referred to as nutlins, can

hinder the binding of MDM2-p53 with high binding intensity. Nutlin-3 (Figure 2.1) is the most powerful compound among the three nutlins (nutlin-1, nutlin-2, and nutlin-3). As such, nutlin-3 is the most generally published small inhibitor molecule of MDM2/MDMX-p53 interactions. Nutlin-3 is a racemic blend of nutlin-3a: an active enantiomer, and nutlin-3b: an inactive enantiomer. The binding affinity for the nutlin-3a to MDM2 is 150-fold greater than nutlin-3b (Vassilev, *et. al.*, 2004).

Effective development of nutlin depends on comprehension of the basic structural biology of the p53-MDM2 interactions. Kussie and co-workers reported a relative profound binding pocket of p53 on the surface of the MDM2 protein (Kussie, *et. al.*, 1996). In particular, they observed that only the three amino acid residues (Leu26, Phe19, and Trp23) of p53 are vital to the binding and fit firmly in the binding pocket of MDM2. This finding made de novo development of small inhibitor molecules of the MDM2-p53 interaction conceivable. Nutlins were developed by the combing structure-based screening of the three-dimensional database, high-throughput screening of extremely large libraries of chemical compounds, and excessive alterations of the lead compounds. Crystal structure information of MDM2-nutlin complex demonstrated the binding of nutlin to the p53 pocket (Vassilev, *et. al.*,2004; Shangary, *et. al.*,2009). The ethoxy group on the nutlin involves the position of Phe19, the bromophenyl bunch possesses the position of Trp23, and the others involve the position of Leu26 (Vassilev, 2005). Since MDM2 and MDMX share the structure similarities and MDMX binds to the similar region of p53 protein (Bottger, *et. al.*, 1999), nutlin-3 also binds to the MDMX with lower affinity (Figure 2.2) (Vassilev, *et. al.*,2004; Laurie, *et. al.*,2006).

2.1.4 Reactivation of the p53 pathway by nutlin-3a in vitro

Since 2004, numerous in vitro studies have been directed to investigate the impact of nutlin-3 on apoptosis and cell cycle arrest. For instance, the impact of nutlin-3a and nutlin-3b (1.25-10 μM) on cell cycle arrest was analyzed in a board of cancer cell lines from various types of tumor, including lung (H460 and A549), prostate (LnCaP and 22Rv1), colorectal (HCT116 and RKO), breast (MCF7), osteosarcoma (SJSA-1),melanoma (LOX), and renal malignancy (A498) (Tovar, *et. al.*,2006). 24-hour treatment of nutlin-3a incited a depletion of the S-phase division, and also G1 and G2 arrest in all the p53 wild-type cell lines that were tested. Expression of p21, a key component of p53-instigated cell cycle arrest, increased in number after the treatment with nutlin-3a. Interestingly, these impacts were not observed in groups treated with inactive

enantiomer nutlin-3b. Colon malignancy cells with mutant p53 (HT29) did not show any response to nutlin-3a treatment in vitro and in vivo (Tovar, *et. al.*, 2006). Rather than cell cycle arrest, the pro-apoptotic impact is more variable. Apoptosis after nutlin-3a or 3b treatment (24~72 hours) was assessed by Annexin V assay (Tovar, *et. al.*,2006). Annexin V positive portions were varied among wild-type of p53 cells to about 80% (SJSA-1) to 10% (A549 and HCT116). Since the incubation of cells with doxorubicin (250 nM) for 48 hours prompted an extensive increment of the Annexin V-positive cell portions in the majority of the tested lines involving the cell line that had low Annexin V-positive group after treatment with nutlin-3.

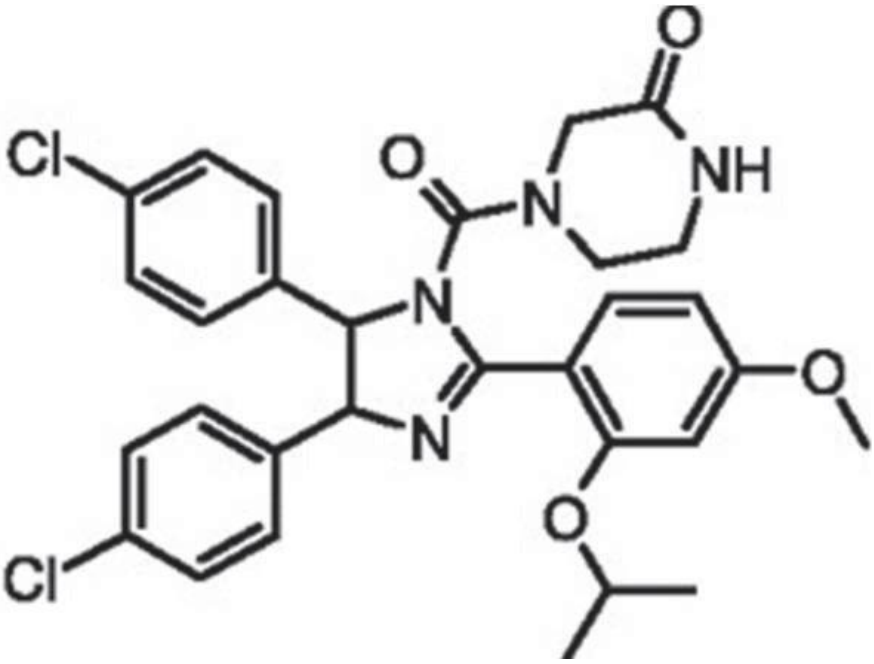

Figure 2. 1 Chemical structure of nutlin-3

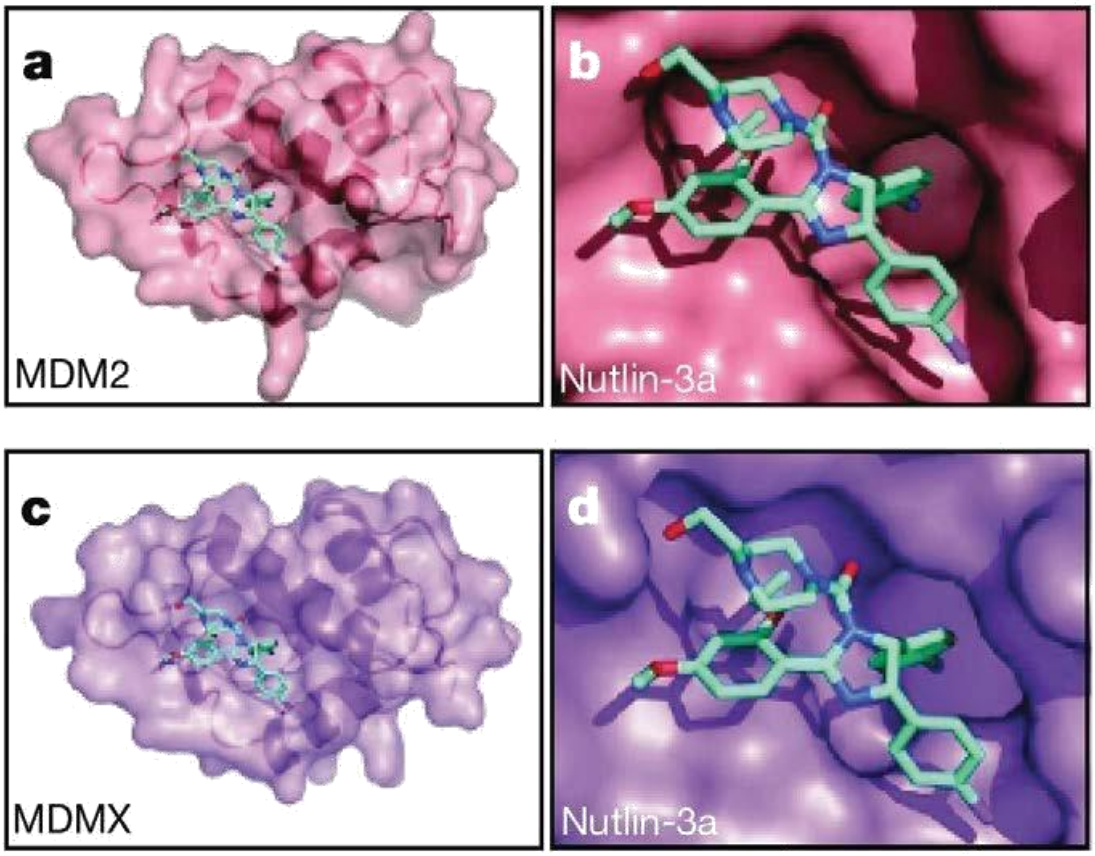

Figure 2. 2 Nutlin-3a binds to the MDM2-p53 and MDMX-p53 binding pockets

(Laurie, *et. al.*, 2006).

The authors inferred that the low apoptotic level distinguished after treatment with nutlin-3 does not cause by deficiencies in the common components of the apoptotic machinery; rather these cells may have defects in the p53-dependent apoptotic signaling process. Studies have proposed the co-relationships between high MDM2 expression and solid apoptosis response when wild-type of p53 cells are treated with nutlin-3. A noteworthy co-relation between's MDM2 expression levels and the sensitivity to nutlin-3 in wild-type of p53 cells was found in All eighteen cell lines and thirty primary samples of leukemia (Gu, *et. al.*, 2008). Nutlin-3 strongly killed All p53 wild-type cells, over-expressing MDM2. Osteosarcoma cells SJSA-1 and MHM, wild-type p53 cells with twenty-five and ten-fold MDM2 gene amplification and high MDM2 expression, had the most compelling apoptosis response among the 10 wild-type p53 cell lines tried by Annexin-V and microarray examination (Tovar, *et. al.*,2006).

LNCaP (prostate disease), RKO (colon tumor), and 22Rv1 (prostate malignancy) cells with a copy of MDM2 gene had moderate levels of apoptotic response. U20S (osteosarcoma) and HCT-

116 (colon malignancy) cell lines, without the amplification of MDM2 gene, had the most reduced apoptosis response. Accordingly, MDM2 expression in tumors might be a valuable response biomarker in the clinical facility. Though, studies for MDM2 may not specifically translate to MDMX. In fact, Hu, and co-workers reported that MDMX over expression prevents the activation of p53 by nutlin-3 (Hu, *et. al.*,2006). Moreover, some different features of nutlin-3a are worth saying: 1). Unlike radiation and conventional chemotherapy drugs, nutlin-3 initiates p53 activation in a non-genotoxic way. 2). Nutlin-3 induces apoptosis in wild-type p53 tumor cells; though, it just causes cell cycle arrest in the normal cells, which may shield normal cells from cytotoxic chemotherapies. Along these lines, nutlin-3 was proposed to behave as a chemo-protective agent (Kranz, *et. al.*,2006). 3). Various studies have recommended synergistic effects of nutlin-3 with radiations (Supiot, *et. al.*,2008) or other chemotherapeutic medications including doxorubicin and selumetinib in intense myeloid leukemia cells (Carter, *et. al.*,2010; Zhang, *et. al.*,2010),topotecan in retinoblastoma cells (Laurie, 2006), R-roscovitine in neuroblastoma cells (Ribas, *et. al.*,2006), chlorambucil, fludarabine, dasatinib, doxorubicin, in chronic lymphocytic leukemia cells (Coll-Mulet, *et. al.*,2006; Kojima, *et. al.*,2006; Secchiero, *et. al.*,2006; Zauli, *et. al.*,2011), actinomycin D, vincristine, etoposide, doxorubicin, in Ewing sarcoma cells, and bortezomib (in breast, myeloma, thyroid, colon carcinoma cells and prostate carcinomas) (Saha, *et. al.*,2010; Ooi, *et. al.*,2009).

2.1.5 In vivo antitumor effect of nutlin-3a

In vivo, nutlin-3a monotherapy exhibited anti-tumor adequacy in preclinical models of human retinoblastoma, osteosarcoma,KSHV lymphoma, prostate disease, and neuroblastoma with wild-type p53 (Vassilev, *et. al.*,2004; Laurie, *et. al.*,2006; Tovar, *et. al.*,2006; Vassilev, 2004; Sarek, *et. al.*,2007; Sarek, *et. al.*,2005). Vassilev, *et. al. (*2004) initially reported the in vivo mechanism of nutlin-3 in naked mice bearing subcutaneous human osteosarcoma xenograft (SJSA-1). Nutlin-3 (po. 200mg/kg BID for 3 weeks) was very much endured without creating significant weight loss or any gross variations from the norm, upon necropsy at the end of the treatment. Contrasted with the vehicle control group, nutlin-3 treatment brought about 90% inhibition of the tumor development.

Tovar, *et. al.*, directed in vivo investigation of nutlin-3a in naked mice bearing LNCaP (prostate malignancy), SJSA-1 (osteosarcoma), 22Rv1 (prostate growth), MHM (osteosarcoma), and T29 (colon disease) tumors (Tovar, *et. al.,* 2006). SJSA-1-bearing mice were treated with an oral

dosage of 50mg/kg, 100mg/kg or 200mg/kg nutlin-3a twice a day for 3 weeks. Treatment with Nutlin-3a conditionally suppressed the SJSA-1 tumor development, with considerable tumor shrinkage saw in the 200mg/kg treatment group. The 200 mg/kg oral nutlin-3a twice a day regimen was additionally effective in MHM (3 weeks of treatment), 22Rv1 (2 weeks treatment) and LNCaP (2 weeks of treatment) models with aggregate inhibition of tumor development greater than 98%. In p53 mutant HT29 xenograft, nutlin-3a did not lessen the tumor size.

This information demonstrated a sensible correlation between tumor responses in vitro and in vivo. Like the report from Vassile, *et. al.*,no weight reduction or critical obsessive changes were seen during the study. Laurie, *et. al.*, led the first in vivo study to evaluate the impact of nutlin-3 on retinoblastoma (Laurie, *et. al.*, 2006). Subconjunctival infusions of 1 µl nutlin-3 (170 mM) and topotecan (2 mM) both as a single and in combination were directed into every eye of tumor-bearing mice on a daily basis for about 5 days. Absolute treatment amount per eye was 85 pmol nutlin-3 and 2 nmol topotecan. Both nutlin-3 and topotecan were powerful as a single drug in the Y79-luc orthotopic model. The combination of subconjunctival topotecan and nutlin-3 brought about 82-fold tumor load lessening with no visual or systemic symptoms.

Recently Brennan*et. al.* reported a study meant to recognize better chemotherapeutic blends for the treatment of retinoblastoma in genetically designed mouse models and orthotopic xenograft models of human retinoblastoma (Brennan, *et. al.*, 2011). SCID mice bearing SJ-39 retinoblastoma tumor cells got etoposide, vincristine, carboplatin, and carboplatin /topotecan, or carboplatin (sub conj)/topotecan(sys) with nutlin-3a(OC)/topotecan(says). The nutlin-3a(OC)/topotecan(sys)- containing group indicated a much better response. Subconjunctival organizations of nutlin-3a alone or in combination with topotecan were all around endured without visual or systemic lethality.

VanMarken, *et. al.*, reported the impact of nutlin-3 on naked mice bearing the chemo-resistant, MYCN-intensified neuroblastoma (Van Maerken, T., *et. al.*, 2009). 200mg/kg oral nutlin-3 twice a day treatment lessened tumor development and metastasis in the wild-type p53 UKF-NB-3rDOX20 xenograft without bringing indications of toxicity. No effect of treatment was seen in p53 mutant UKF-NB-3rVCR10 xenograft, recommending p53 status fundamentally influences the in vivo responses to the treatment of nutlin-3.

2.1.6 Whole Body Physiologically Based Pharmacokinetic Model For Nutlin- 3A

At present, Nutlin-3a (2-piperazinone, 4-[[(4S,5R)- 4,5-bis(4-chlorophenyl)- 4,5-dihydro-2-[4

methoxy-2-(1-methylethoxy)phenyl]-1H-imidazol-1-yl]carbonyl]-) is experiencing preclinical examination as a reactivation agent for p53. While numerous malignancies and several types of tumor express transformed types of p53 (Hollstein, *et. al.,* 1991), a subset of malignancies, and especially pediatric tumors, hold wild-type of p53 (Tweddle, *et. al.,*2003). In these cases, cancerous cells utilize different mechanisms to disrupt the activity of p53. One such system is over-expression or enhancement of the MDM2 protein. The Nutlin binds specifically to p53 to quicken its turnover and hinders transcription of downstream targets, including cell cycle and apoptotic genes (Momand, *et. al.,* 1992; Momand, *et. al.,* 2000). Disturbance of the MDM2–p53 interaction is proposed as a novel technique for treatment of several cancers that don't have mutations of p53 (Shangary, *et. al.,* 2009; Shangary, *et. al.,* 2008).

Nutlins are a class of small molecules that basically targets the p53-binding pocket of MDM2 (Vassilev, *et. al.,* 2004; Klein, *et. al.,* 2004). Treatment of numerous types of cancerous cells including leukemias (Gu, *et. al.,* 2008; Kojima, *et. al.,* 2005), rhabdomyosarcoma (Miyachi, *et. al.,* 2009), neuroblastoma (Barbieri, *et. al.,* 2006) and retinoblastoma (Elison, *et. al.,*2006) with nutlin-3a initiates p53-dependent cell cycle arrest and the death of the cell, though in normal cells, infusion of Nutlin-3a prompts cell cycle arrest without causing cell death (Vassilev, 2005). Nutlin-3a has an antitumor mechanism in a preclinical xenograft model of neuroblastoma (Van Maerken, *et. al.,*2009) and was tried in a few other preclinical models of cancers (Vassilev, *et. al.,*2004; Sarek, *et. al.,*2007).

2.2 Previous Models for P53 pathway

Hundreds of genes and their products are involved in the regulation of p53 pathway which is activated by a number of stress signals (Levine, *et. al.,* 2006; Kohn Sarek, *et. al.,* 2005). The stress signals are DNA damage, oncogene activation, heat and cold shock and others are transmitted the mediator proteins and effect on the p53 level and in turn affects the transcriptional activity by several post-translational changes. A number of positive and negative feedbacks control the core regulation of p53/MDM2 (Harris and Levine, 2005). The p53 proteins by transcriptional activation or protein-protein interaction regulate the downstream events which are mediated by a number of genes and their products. Cell cycle arrest, DNA repair, apoptosis or cellular senescence are the cellular outputs of downstream events. These downstream events also result from the interaction of other signaling pathways (Vousden and Lane (2007). Relevant to the proposed model is the p53 activation in the response to the Nutlin-3a. The level of p53 in

healthy cells remains typically low by the control of MDM2 which is an ubiquitinating protein for p53 resulting in its degradation (Haupt, *et. al.*, 1997; Kubbutat, *et. al.*, 1997). Whereas p53 is the transcriptional factor for the MDM2 gene which degrades the p53 (Barak *et. al.*, 1993). The mediator proteins are activated by DNA damage, phosphorylate p53 and MDM2 hence destabilizing the p53 level and its transcriptional activity (Volgenstein, *et. al.*, 2000). The homeostatic balance between MDM2 and p53 is disturbed which results in oscillations. The phosphorylated p53 triggers the transcription of genes which are responsible for cell cycle arrest or DNA repair and/or damage is not repaired it leads to the cellular apoptosis. If these processes are not fulfilled then the mutation is passed on to the next cell generation which is lenient for cancer development (Mayo and Donner, 2002). It is the state of fact that every solid cancer lacks functional p53; in almost half of the cancers have mutated p53 in their cells and another half of the cancers have an alteration in p53 mediators. Considering these facts several models have been proposed up till now as shown in Table 2.

Table 2-1 Overview of Models presented (2000-2014)

Authors	Stochastic Model	Deterministic Model	Positive	Negative	Mediator	Time delay	Small molecule /drugs
Bar-Or, *et. al.*, (2000)	✖	✖	✖	✓	✓	✓	✖
Lahav, *et. al.*, 2004	✓	✓	✖	✓	✖	✖	✖
Ma, *et. al.*, 2005	✓	✖	✖	✓	✖	✓	✖
Wagner, *et. al.*, 2005	✖	✖	✖	✓	✖	✓	✖
Ciliberto, *et. al.*, 2005	✓	✓	✓	✓	✖	✖	✖
Wee Aguda 2006	✖	✖	✓	✓	✖	✖	✖
Geva Zetursky 2006	✓	✓	✖	✓	✖	✖	✖
Tyson, *et. al.*, 2006	✖	✖	✓	✓	✖	✓	✖
RatcitsChak *et al.* 2007	✖	✖	✓	✓	✖	✓	✖
Zhang, *et. al.*, 2007	✖	✖	✓	✓	✖	✖	✖
Puzynski *et al.* 2008	✓	✓	✓	✓	✖	✖	✖
Cai and Yuan 2009	✓	✖	✖	✓	✖	✓	✖
Hunziker, *et. al.*, 2010	✓	✓	✖	✓	✖	✖	✓
Zhang, *et. al.*, 2011	✓	✖	✖	✓	✖	✖	✖
Tuzynski, *et. al.*, 2013	✓	✖	✖	✓	✖	✖	✖
Puzynski, *et. al.*, 2014	✓	✖	✓	✓	✖	✖	✓

2.2.1 Time Delay Models

Persistent Oscillations in p53 and MDM2 level are produced by the DNA damage and these oscillations are verified by Bar-Or, *et. al.,* (2000). The negative feedback loop between p53 and MDM2 is coupled with time delay assumed that p53 induces MDM2 via an intermediary (Bar-Or, *et. al.,* 2000).

A number of mathematical models have been proposed to explain p53 and MDM2 oscillations. A persistent solution of constant amplitude and period and the sign of independent limit cycle are obtained by undamped oscillations. When the negative feedback loop is combined with the time delay and/or coexisting positive feedback loop then a persistent solution of amplitude and period is obtained (Tyson, 2006; Rateitschak and Wolkenhauer, 2007). The existence of stable oscillations in response to DNA damage was assured by Tyson and his co-workers in analyzing four different positive feedback loops in coupled with one negative feedback loop of p53 and MDM2. Two-time delays associated with MDM2 translation and transcription was explicitly introduced by Ma, *et. al.,* (2005) and Wagner, *et. al.,* (2005).

Some of the effects of intrinsic noise produced by p53-MDM2 and MDMX interactions were analyzed by Cai and Yuan (2009). In that model MDM2 mRNA was produced by some time delay. Their model has ubiquitinated and deubiquitination states of proteins, rather than just assuming all ubiquitinated proteins are degraded.

2.2.2 Stochastic and Deterministic Models

Persistent Oscillations in p53 and MDM2 level are produced by the DNA damage and these oscillations are verified by Bar-Or, *et. al.,* (2000) and then (in single cells) Lahav, *et. al.,* (2004) and Geva-Zatorsky, *et. al.,* (2006). Degradation of p53 by MDM2 is rescued by blocking MDM2 nuclear entry considering one of the three positive feedback loops (Ciliberto, *et. al.*, 2005). In spite of this feedback loop is mediated by PTEN, PIP3, and Akt, the authors neither introduced any time delay to the regulation nor considered these intermediaries explicitly. P53 blocks nuclear entry of MDM2 without any time delay in a way that MDM2 first retain itself in cytoplasm then takes entry into the nucleus which is not experimentally observed and substantially changes the dynamics of the pathway. These feedback loops have been studied by Wee and Aguda (2006); they demonstrated the existence and robustness of bi-stability in p53-Akt regulation. Zhang, *et. al.,* (2007) studied three other positive feedback loops. The oscillations appear as a result of positive feedback loops are due to the narrow gaps of

parameters. Zhang, *et. al.,* (2007) demonstrated that as MDM2 degradation constant changes, the system passes from the stable point to the limit cycle and we can relate this phenomenon to DNA damage. However, modest change of p53 synthesis rate caused by p53 transfection also produce these oscillations result (in Ciliberto, *et. al.,* 2005 model a 10% change). In other words, the behavior of the non-transfected cell is different from transfected cells only if one assuming the models proposed by Geva-Zatorsky, *et. al.,* (2006). Puzynski, *et. al.,* (2008) presented the single cell stochastic model is based on the negative feedback involving p53 and MDM2 and on the positive one mediated by PTEN, PIP3, and Akt. However, in contrast to the models considered by Zhang, *et. al.,* (2007), in their model the oscillations are due solely to the negative feedback and time delay. Puszynski, *et. al.,* (2008) advanced a complex stochastic model of p53 behavior geared toward displaying how stochastic outcomes cause variability of cellular destiny in a bistable version. Their model consists of a cytoplasmic compartment and a nuclear compartment, although p53 is not in their cytoplasmic compartment. Further to the inhibition loop of MDM2 and p53, they consist of a superb events involving a sequence of occasions that lead to MDM2 being sequestered within the cytoplasm in which it is able to now not degrade p53. Hunziker, *et. al.,* 2010 proposed a simple model for the negative feedback loop. This was a simple model showing flexibility against any kind of stress signal activating the p53 signaling pathway. Hunziker, *et. al.,* 2010 model also provide a framework for predicting differences in p53 response to different stresses and single nucleotide polymorphism. Motivated by the above considerations, Zhang, *et. al.,* 2011studied the connection between the p53 signaling pathway and the DNA damage. The model was explicitly describing the generation and DNA damage repair process by deciding the cell fate from life to death. P53 pulses are the result of p53-MDM2 and ATM-P53-Wip1 negative feedback loop but the gene-switching mechanism is activated when p53-PTEN-Akt-MDM2 positive feedback loop becomes dominant. Hence a sequential predominance of distinct feedback loops may elicit multiple-phase dynamical behaviors. Tuzynski, *et. al.,* (2013) came to the conclusion that in cancerous cells the inhibitors of p53 (MDM2/MDMX) are over expressed. Furthermore, they investigate the role of stochasticity in determining system behavior. They have found that stochasticity is able to affect system behavior profoundly.

2.2.3 p53/MDM2/Nutlin

Above mentioned all the models have been focused on the positive and negative feedbacks of a p53 signaling pathway. A major innovation has been reported by a number of the scientist in the

last few years which are focusing on the introduction of a small molecule drug with fewer side effects to the p53 signaling pathway. This may be considered as an artificial stress to trigger p53 signaling pathway. Before the introduction of a small molecule drug, there must be a series of drugs that must be targeted to the p53/MDM2 core regulation. In the present study, we are more focused to propose a model that can break the p53-MDM2 interaction. Selection of such small molecule is quite difficult as the pharmacodynamics of the proposed drug is hard to find at once. It requires an experimental data for investigation. By considering the complexity of the model we have to divide the task into two module first is to determine the simplest way to model p53-MDM2 signaling pathway, second is to integrate the determined pathway with the small molecular dynamic. As we discussed earlier that the model presented by Hunziker, *et. al.*, 2010 is suitable for all of the stresses i.e its response to different stresses can be determined experimentally so we chose this model for our first module. The other module is for the small molecule dynamics the model which supports this module was given by Pyzynski, *et. al.*, 2014.

3. Chapter 3: Methodology

Cancer is one of the leading causes of death. The tumor suppressor protein, p53, plays a significant role in the DNA repair mechanism, senescence, cell cycle regulation and apoptosis (Teodoro Jose G, Sara K Evans, and Michael R Green, 2007). The p53 signaling pathway is one of the promising and efficient currently known strategies for combating this disease (Levine Arnold J, and Moshe Oren, 2009). It stimulates several pathways to maintain the normal activity of the cell function which is affected by cellular stress and DNA damage (Graeber Thomas G, *et. al.*, 1994). Whenever a cell is endangered, p53 is there to protect it from cellular stress, UV light, and hypoxia. That's why p53 is to be called the Guardian of Genome (Lane David P., 1992).

There are numerous genes which are transcriptionally regulated by p53 depending on the cell type and stress (Zhao Renbin, *et. al.*, 2000). Along with the transcriptional activity, it has an additional role in independent transcription apoptosis (MiharaMotohiro, *et. al.*, 2003).

MDM2, which is regulated by p53, degrades the level of p53 by the proteasome (Bond Gareth , *et. al.*, 2005). MDM2 production is also stimulated by p53, forming a feedback loop (Barak, *et. al.*, 1993). Auto-ubiquitination of MDM2 marks itself for degradation by the proteasome (Fang, *et. al.*, 2000).

Numerous small molecules were reported as a repressor for the core regulation of p53-MDM2 (Vassilev, *et. al.*, 2004). Nutlin-3 is a small molecule inactivation for retinoblastoma and p53-independent effects i.e. with MDM2. The expression of MDM2 is high in hypoxic stress which is considered independent of p53 (Secchiero, *et. al.*, 2011). Moreover, Nutlin-3a has the binding capacity to many other proteins other than MDM2 for functional apoptosis (Ha, *et. al.*, 2011). The small molecule Nutlin binds with MDM2 and represses its binding with p53. The degradation of MDM2 by small interfering RNA (SiRNA) is independent of the natural cell development and gene specific, which in many cases cannot be achieved due to various experiment related factors (Puszyński, *et. al.*, 2012). Inhibition through Nutlin inefficient than SiRNA, so that why we used Nutlin.However, this binding affinity cannot change the auto-ubiquitination effect of MDM2 (Barakat, *et. al.*,2006). A mathematical model of p53 dynamics is needed, for the efficacy of our approach.

3.1 p53 Pathway Modeling

Mathematical modeling is playing a key role in the study of biological pathways. In the past, there are numerous models developed for thep53 signaling pathway. These models are based on different approaches, such as ordinary differential equation both time delay based and stochastic based (Geva, *et. al.,* 2006).

Every modeling approach has its merits and demerits, such as time delay models deal with real cell proteins because these are not produced immediately in response to the promoter. The time taken by transcription and translation is also modeled with time delay directly. In a stochastic system, the effects of proteins levels are quantized instead of immediate results; the systems are more intensive computationally for stochastic models.

p53 induces the transcription factor of MDM2 and activates MDM2 mRNA. The production rate of MDM2 depends on the weighted average rate of past p53 levels. A model of three linked module was developed to investigate the pulse generation in p53 due to the breaks in DNA strands, which are stimulating DNA repair, ATM activation and a feedback loop between MDM2 and p53 (Ma, *et. al.,*2005). p53is also activated by inducing an input signal to check the effect of CHK2, ATM and WIP1 on p53 behavior (Batchelor, *et. al.,*2008).

For the tumor suppressor p53 (Hunziker, *et. al.,* 2010) presented a model in which they investigated the negative feedback loop for p53 and MDM2. They also measured the dynamics of p53 and MDM2 and found undamped oscillations. The effect of different stresses was measured by a negative feedback loop involving p53, MDM2, and its inhibitor to examine the model output, which showed spiky oscillations at a different level of p53 (Paul, *et. al.,*2010). This particular model is simple enough for an oriented control analysis and dosage design, however, at the same time, it comprehensively covers the various aspects of p53 pathway dynamics. The process of modeling the pathways for p53 revival is represented by the following Figure 3.1

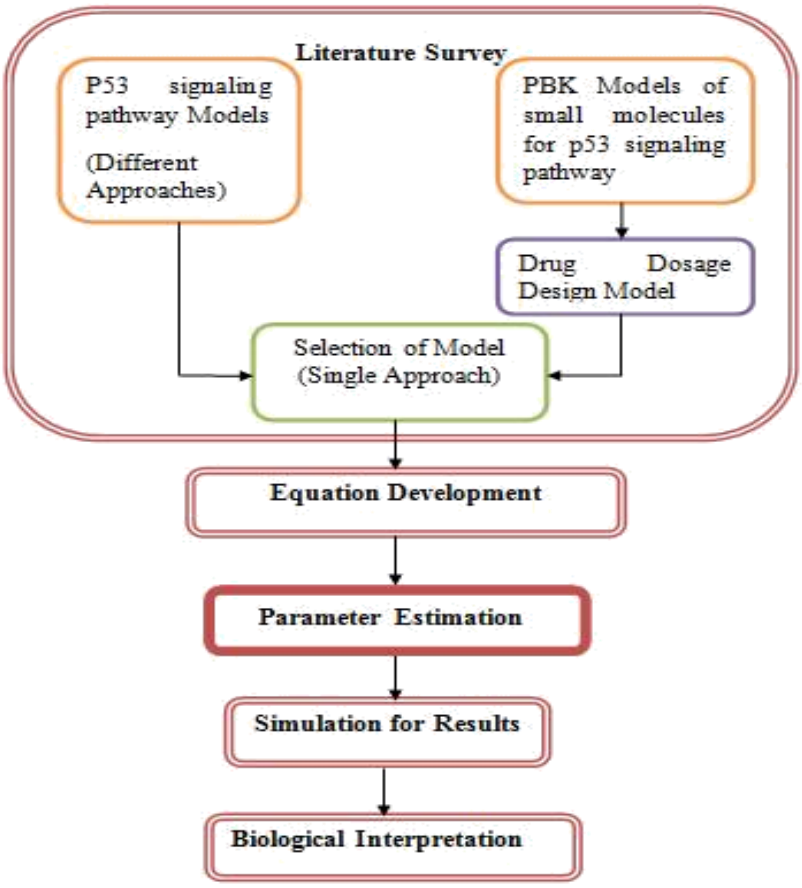

Figure 3. 1 Schemetic Diagram

Having chosen the p53 dynamics model a suitable model for introducing the PBK dynamics of Nutlin is looked into.

Drug development process is too costly and laborious as it takes millions of dollars and several years to synthesize a new drug. There is a strong need to accelerate the development process, minimize the cost and improve its accuracy (Puszynski, *et. al.,* 2008). The design of proper drug dosage is also a very critical problem. As in this case, not only p53 dynamics are important but also PK/PD properties of the proposed drug also play a crucial role. Recently, a lot of work has been done on Physiological Based Kinetics (PBK) modeling of various drugs. Hence, a strategy could be evolved to design drug dosage by integrated p53 pathway model with the PBK model of a cancer drug. In the past, it seemed difficult due to the lack of requisite computing power, but now as per advancement in technology and computational power it helps to use the *in-silico* models and consequently improve the development process. PBK model proposed by (Zhang, *et. al.*, 2011) opts due to its complete parameterization.

In our research, we have integrated the Hunziker model (Paul, *et. al.*, 2010) and Puszynski model (Puszynski, *et. al.*, 2014). In Hunziker's model p53 dynamics are emulated without delay. Both positive feedback of p53-MDM2 mRNA and negative feedback loop of p53-MDM2 are included. In Puszynski model PBK dynamics of Nutlin are given. However, p53 dynamics are described via compartmental model which would be too complicated to employ for sensitivity or bifurcation studies. In our integrated model as shown in "Figure 3.2," we added a new term in the Hunziker model for the clinical trial drug Nutlin 3a to investigate its proper dosage and p53 response.

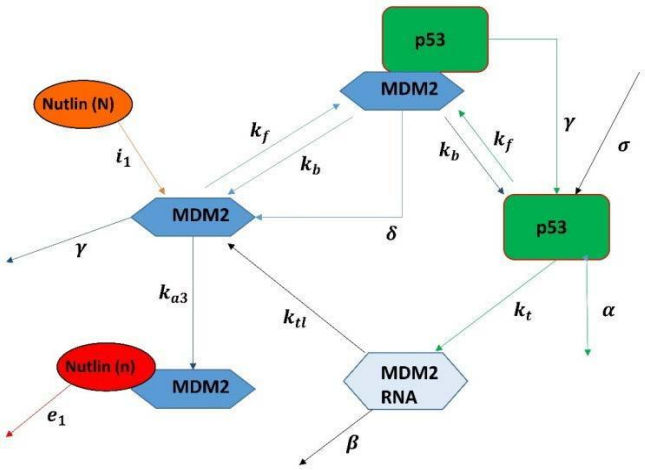

Figure 3. 2 An integrated model of Nutlin PBK dynamics and p53 pathway dynamics.

In Figure 3.2 the model represents the segments and interactions. MDM2 Transcription of and interpretation to the MDM2 protein are shown by the rate constants k_t and k_{tl}, individually. The rate of degradation of the MDM2 mRNA is shown by β. Rate of complex development and rate of complex separation is shown by k_f and kb. p53 is produced at a steady rate σ. The rate of degradation of p53 when it is MDM2-intervened is shown by by δ, and when it is MDM2-independant it is represented at the rate α. At last, we expect that the MDM2 degradation rate, γ, is free of whether it is bound to p53 or not. k_{a3} is the rate at which free MDM2 available for the nutlin while e_1 is the elimination rate of nutlin bound MDM2 from the cell. I_1 is the rate of nutlin binde to MDM2.

3.2 Mathematical Modeling of p53 Pathway

In Hunziker model (Paul, *et. al.*,2010) they focus on the following four concentrations nuclear-p53, p; MDM2, m; MDM2 mRNA, mm; and thep53-MDM2 complex, c. The differential equations of the model by their dynamics are as follows.

$$\frac{dp}{dt} = \sigma - \alpha p - k_f pm + k_b c + \gamma c \quad (3.1)$$

$$\frac{dm_m}{dt} = k_t p^2 - \beta m_m \quad (3.2)$$

$$\frac{dm}{dt} = k_{tl} m_m - k_f pm + k_b c + \delta c - \gamma m - \mathrm{ka}_3\,\mathrm{NUT\ m} \quad (3.3)$$

$$\frac{dc}{dt} = k_f pm - k_b c - \delta c - \gamma c \quad (3.4)$$

3.2.1 PBK Modeling of Nutlin

To simulate *Invivo*Nutlin treatments, Pszynsky*et. al.*, exploited the pharmacokinetics data for oral delivery in mice to compute the extra-cellular Nutlin concentration. it's miles essential to remark that a sustaintial binding of Nutlin to plasma proteins has been validated in order that the free Nutlin presence in plasma is best a small fraction of the overall Nutlin awareness. The binding data were suited to the equilibrium equation (Zhang, *et. al.*, 1998).

$$N_b = B_{max} \frac{K_a N}{1 + K_a N} \quad (3.5)$$

Where N_b denotes the protein-bound Nutlin concentration, B_{max} is the concentration of total plasma protein binding sites, and Ka is the equilibrium association constant. Zhang, *et. al.*, estimated $B_{max} = 2.86X10^{-4} M and K_a = 0.085X10^6 M^{-1}$. Denoting by N_{tot} the concentration of total Nutlin, we have[21]

$$N_{tot} = N + N_b \quad (3.6)$$

AndN can be expressed regardingN_{tot}, obtaining:

$$N = \frac{-(1 + K_a B_{max} - K_a N_{tot}) + \sqrt{(1 + K_a B_{max} - K_a N_{tot})^2 + 4 K_a N_{tot}}}{2K_a} \quad (3.7)$$

Protein binding is in all likely to occur additionally within the retina, and, as recommended in (Hunziker *et. al.*, 2010), we may also expect that in this tissue the binding is the identical that during plasma. therefore, assuming that (i) drug distribution occurs in a single compartment, (ii) nutlin that is eliminated is the free nutlin (iii) removal is linear, and (iv) Protein binding is in quasi-regular state, the simplest pharmacokinetic equation for Nutlin reads:

$$\frac{dN_{tot}}{dt} = p_{oral} Dose(t) - \delta_2 N(N_{tot}), N_{tot}(t_0) = 0 \quad (3.8)$$

Where Dose (t) is the drug dose rate in mg Kg^{-1} sec^{-1}), $t_0$is the starting time of liberation, the conversion of p_{total} from mg Kg^{-1} to moles is an important factor which is divided by the distribution volume, and N (N_{tot}) from (3.7). N (N_{tot} (t)) is the input in Eq (3.9) for Nutlin within the cell. Usually, in oral delivery representation, the release from gastro-enteric is assumed to be exponential so that Eq. (3.8), in case of administration of a single dose at time $t=t_0$, can be rewritten as

$$\frac{dN_{tot}}{dt} = p_{oral} D\delta_1 e^{-\delta_1(t-t_0)} - \delta_2 N(N_{tot}), N_{tot}(t_0) = 0$$

Where D represents system dose in miligram per kilogram. An easy modification of the

$$\frac{dNUT(t)}{dt} = i_1 N(t) + k_{d_3}\left(MDM_i(t) + MDM_{pi}(t) + MDM_{pni}(t)\right) - k_{a_3} NUT(t)\left(MDM(t) + MDM_p(t) + MDM_{pn}(t)\right) - e_1 NUT(t) \quad (3.9)$$

Where N represents free Nutlin extra cellularly.

Table 3-1 Parameters of p53 model

symbollic		Initial values	Units
p	P53	10	nanomole
m_m	mRNA MDM2	5	Nanomole
m	MDM2	90	nanomole
Pm	P53-MDM2 complex	90	nanomole
NUT	Nutlin absorbed	0	nanomole
N_{tot}	Total nutlin	0	nanomole
N_b	Nutlin bind to the plasma	0	nanomole
N	Free nutlin	0	nanomole
NUT_m	Nutlin bind to MDM2	0	nanomole
D	Drug dose	1000	nanomole
Sigma	MDM2 dependent deactivation/degradation of p53	1000	Nanomole/ hour
Alpha	MDM2 independent deactivation/degradation of p53	0.1	1/hour

k_t	Transcription of MDM2	0.03	1/(nanomole*hour)
k_{tl}	Translation of MDM2	1.4	1/hour
k_b	Dissociation rate of MDM2-p53	7200	1/hour
k_f	Association rate of MDM2-p53	5000	1/(nanomole*hour)
Beta	Degradation rate of MDM2 mRNA	0.6	1/hour
Gamma	MDM2 degradation/deactivation	0.2	1/hour
Delta	MDM2-dependent degradation/deactivation of p53	11	1/hour
Bmax	concentration of protein binding sites in plasma	2.86E-4	nanomole
i_1	rate of Nutlin intracellular import	0.9144	1/hour
kd_3	Nutlin{MDM2 dissociation rate	720	1/hour
ka_3	Nutlin{MDM2 association rate	0.261	1/(nanomole*hour)
e_1	rate of Nutlin cell export	18	1/hour
K_a	equlibrium association constant in plasma	85000	1/nanomole
poral	dose conversion factor for oral delivery	7.5E-4	1/hour
$delta_1$	gastro-enteric release rate constant	2.0E-4	1/hour
$Delta_2$	elimination rate constant	0.0054	1/hour

4. Chapter 4: Results and Discussion

In this work deterministic model has been investigated computationally which determines how wild-type p53 gene respond to the drug Nutlin (an agent that interferes with the MDM2-mediated p53 regulation)in tumor cells. In particular how experimental dose-response curves can be explained by gene-switching controlled by p53, i.e., the observed inter-cell variability of the cell viability under Nutlin action. In the proposed model the negative feedback loop mediated by MDM2 describes the regulation network of p53 and an intrinsic stress signal DNA damage which triggers ATM in the cytoplasm to disturb the p53 and MDM2 concentrations in the cell.

There are two ways in which ATM invokes p53 against an intrinsic stress resulting in DNA damage. i) If the damage is not very extensive, then the p53 pathway goes into oscillations (probably limit cycles) and puts into action the downstream proteins that halt the cell cycle and repairs the cell (Puszynski, *et. al.,* 2014). The oscillation frequency increases with the extent of the DNA damage, through the p53 pulses (every six hours) the ATM samples the DNA status whether it has been repaired or not. ii) If it gets fixed, then the p53 oscillations get stopped, and the cell cycle resumes its course and cell division is re-initiated. However, in the case of extensive cell damage, the p53 pursues a different course. Instead of oscillations, p53 shows a sustained response resulting in senescence or apoptosis.

Numerous malignancies and several types of tumor express transformed types of p53 (Hollstein, *et. al.,*1991), a subset of malignancies, and especially pediatric tumors, hold wild-type of p53 (Tweddle, *et. al.,* 2003). In these cases, cancerous cells utilize different mechanisms to disrupt the activity of p53. One such system is over-expression or enhancement of the MDM2 protein. One of the successful approaches to encounter the DNA damage in cancerious cells involving hyperactivity of the MDM2 is to dock a small molecule on MDM2 where p53 were supposed to dock (Figure 4.2) (Puzynski, *et. al.,* 2012). Nutlin is one of such a small molecule drug The Nutlin binds specifically to p53 to quicken its turnover and hinders transcription of downstream targets, including cell cycle and apoptotic genes (Momand, *et. al.,* 1992; Momand, *et. al.,* 2000).

Disturbance of the MDM2–p53 interaction is proposed as a novel technique for treatment of several cancers that don't have mutations of p53 (Shangary, *et. al.*, 2009; Shangary, *et. al.*, 2008). To date, the pharmacokinetics of nutlin-3a has not been accounted so for. A comprehension of the systemic disposition of nutlin-3a, and also the distribution to the targeted tissue or tumor areas, will give a significant premise to the choice of dosage criteria for preclinical models. Furthermore, since the in vitro tumor cell line sensitivities to nutlin-3a have been resolved, pharmacokinetic modeling can be utilized to decide the dosage and proper schedule, important to accomplish the properly unbound nutlin-3a concentrations at the site of the tumor. One way to deal with the information is the utilization of the whole body physiologically-based pharmacokinetic models, which depend on anatomical compartments and the flow of blood stream.

4.1 p53 Revival

The role of Nutlin in p53 pathway based on integrated PBPK model based on Huziker *et.al.*, (2010) and Puzynski *et. al.,(*2014*)* was simulated in two ways for the determination of its dosage. The first dosage strategy is devised for a sustained p53 response, while the other one is meant for inducing p53 oscillations.

4.1.1 Generation of sustained p53 response

For the production of a sustained p53 response, a two loop negative feedback strategy is employed. The outer loop comprises of the p53-MDM2 pathway. Since, our goal is to reduce the MDM2, so as to give some space to p53 for growth, we feedback the MDM2 concentration to p53 pathway after multiplying it with a constant "Kp1". Physiologically it implies that the reference dosage of Nutlin required is determined by multiplying "Kp1" with the actual concentration of MDM2. This gives the reference Nutlin dosage which should be present in the cell. However, to maintain "n (ref)" in the cell,a negative feedback loop is devised for the PBPK dynamics of Nutlin (Mengel, *et. al.*,2010). Thus the dosage given to the patient should be proportional to n(ref)-n, where "n" is the actual amount of the Nutlin present in the cell. Both the loops are shown in the accompanying "Figure 4.1".

The dosage D is given as:

$$D=Kp2(nref-n) \qquad (4.1)$$

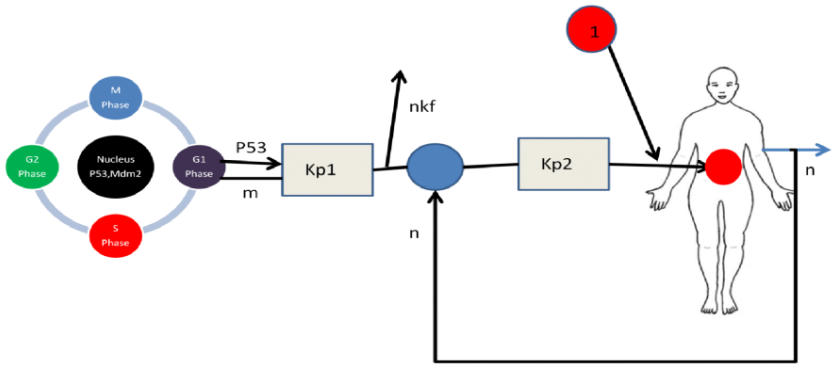

Figure 4. 1 Controlled Dosage design for Nutlin

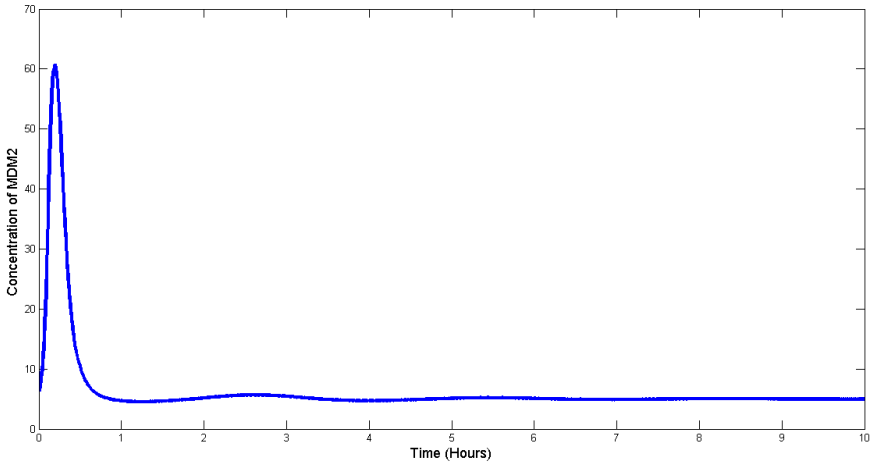

Figure 4. 2 Hyperactivity of MDM2 in abnormal cells

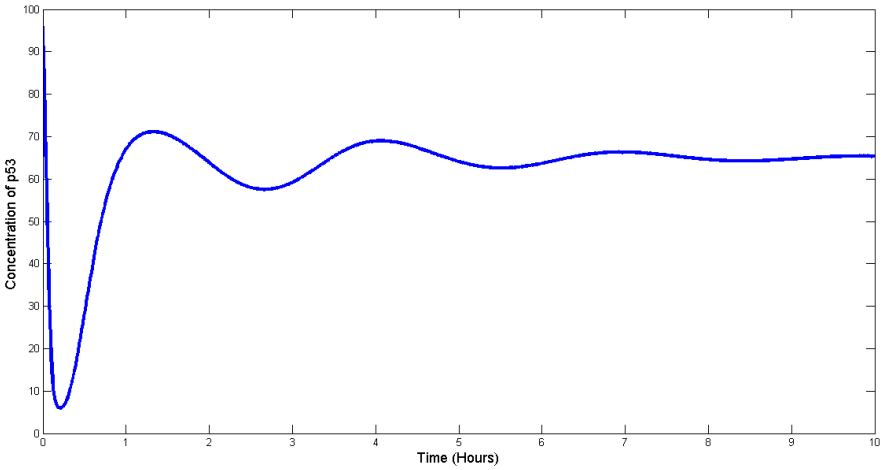

Figure 4. 3 Reduced P53 concentration due to hyperactivity of MDM2

$$\frac{dm}{dt} = k_{tl}m_m - k_f pm + k_b + \delta pm - \gamma m \quad - k_{a_3}N(t)m \qquad (4.2)$$

To understand and predict target tissue and tumor concentrations, PBPK models have been used. To search oncology Pubmed search using "physiologically based pharmacokinetic model cancer" or "physiologically based pharmacokinetic model tumor" returned 153 papers. PBPK models had been used for antibody(Baxter, *et. al.*,1995; Baxter, *et. al.*,1994; Puchalski, *et. al.*,2010; Davda, *et. al.*,2008; Kletting, *et. al.*,2009; Urva, *et. al.*,2010; Boswell, *et. al.*,2011; Friedrich, *et. al.*,2002; Kletting, *et. al.*,2010) and nanoparticles (Fallon, *et. al.*,2004; Pery, *et. al.*,2009; Yang, *et. al.*,2010; Li, *et. al.*,2010)PBPK models have been utilized to comprehend and anticipate target tissue and the tumor concentrations. In the area of oncology, a PubMed search (09/09/2016) utilizing "physiologically based pharmacokinetic cancer model" or "physiologically based pharmacokinetic tumor model" returned 153 papers. PBPK models had been utilized for radioimmunodetection and radioimmunotherapy (Zhu, *et. al.*,1998; Zhu, *et. al.*,1997), immune response (Baxter, *et. al.*,1995; . Baxter, *et. al.*,1994; Puchalski, *et. al.*,2010; Davda, *et. al.*,2008; Urva, *et. al.*,2010; Boswell, *et. al.*,2011; Friedrich, *et. al.*,2002; Kletting, *et. al.*,2010; Fang, *et. al.*,2008; Ferl, *et. al.*,2006; Ferl, *et. al.*,2005),imaging operators (Barboriak, *et. al.*,2008; Mescam, *et. al.*,2007; Armitage, *et. al.*,2005), liposomal (Qin, *et. al.*,2007), and small molecules, for example, topotecan (Shah, *et. al.*,2011), docetaxel (Hudachek, *et. al.*,2011; Bradshaw-Pierce, *et. al.*,2008), doxorubicin (Li, *et. al.*,2003), capecitabine (Tsukamoto, *et. al.*,2001; Tsukamoto, *et. al.*,2001), temozolomide (Gallo, *et. al.*,2004; Zhou, *et. al.*,2007), genistein (Zager, *et. al.*,2007; Schlosser, *et. al.*,2006,gefitinib (Wang, *et. al.*,2008; Wang, *et. al.*,2011; Zhou, *et. al.*,2011), 17-AAG (Xu, *et. al.*,2003), moxifloxacin (Edginton, *et. al.*,2009), and methotrexate (Li, *et. al.*,2002; Devineni, *et. al.*,1996).

In PBPK model an important parameter is Partition coefficient. Numerous methods for determining the partition coefficient values have been applied which are as follows: 1.Estimation using PBPK model. 2. Estimation using non-compartmental modeling. A whole body PBPK model for topotecan was proposed by Shah,*et. al..*,(2011). In their model for each tissue, the binding ratio to obtain a tissue to plasma partition coefficient (estimated from non-compartmental modeling) for the tissue to the ratio in plasma with the fixed values 3. *In vitro* measurements(Shah, *et. al*, 2011). A PBPK model for docetaxel was reported by Bradshaw-Pierce, *et. al.*,(2008). In the model partition coefficient for tumor was estimated along with the model development. When the PBPK model is established by keeping the model structure the

concentration from one species to another is scaled through physiological information. Assumption for partition coefficients between species is kept in mind for model estimation. (Xu, *et. al.*, 2003; Clewell, *et. al.*,2001).

In preclinical drug development, understanding drug disposition is critical. Zhang,*et. al..*, (2011) used physiologically-based pharmacokinetic (PBPK) modeling the disposition of nutlin-3a in mice is done for drug characterization. *In vitro* studies were done for plasma binding and compartment partitioning coeffitient estimation. Tissue concentrations of nutlin-3a in plasma, and other tissues were determined after intravenous (10 and 20 mg/kg) and oral (50, 100, and 200 mg/kg) dosage.The PBPK model developed for nutlin 3a was applicable for all PK data that have nonlinear binding to murine plasma proteins, with the unbound drug concentration of 0.7 to 11.8%. Within 2 h characterized Nutlin-3a was absorbed to plasma with peak value and two way elimination process is observed. the plasma and tissue disposition of nutlin-3a is successfully described in the final PBPK model.High bioavailability, rapid attainment of steady state along with the little accumulation of drug is suggested by the simulations when the given drug is administered once or twice daily at dosages up to 400 mg/kg. Simulations of unbound tissue concentrations were performed to determine appropriate dosing regimens for preclinical models of several pediatric malignancies. A PBPK model was successfully established to describe the disposition of nutlin-3a in plasma and tissues of interest for pediatric malignancies.

The effect of Nutlin on the p53 pathway is simulated by taking p53 dynamics from Hunziker model (2010), and Nutlin PBPK from Pszynski (2014), reintegrated into MDM2 rate equation via the term sink ka_3*n, where D= K_{p2}*(n_{ref}-n), K_p is the proportional constant. It is observed that Kp=100 acts as a damper for these stress based oscillations. Depending upon the parameter values given in table 4.1. The results showed that the p53 gets sustained within 50 hours, and there are no visible oscillations in the p53 response. The figure 4.4, 4.5 illustrates that with the addition of drug up to 1000 nanomoles of nutlin 3a, the hyperactivity (higher concentrations, 60 molecules) of MDM2 was high and p53 concentration was low within 30 minutes. As soon as drug molecules become bioavailable (binding with the target protein MDM2) the increased level of MDM2 start declining with the reasonable increase in p53 level. p53 gradually increases to normal rate and P53 level becomes sustained as the maximum drug bind with the MDM2 after 50 hoursto restore its function. which shows that when the drug is introduced in the cancerous cell with the hyper active MDM2 it triggers p53 to reduce its activity.

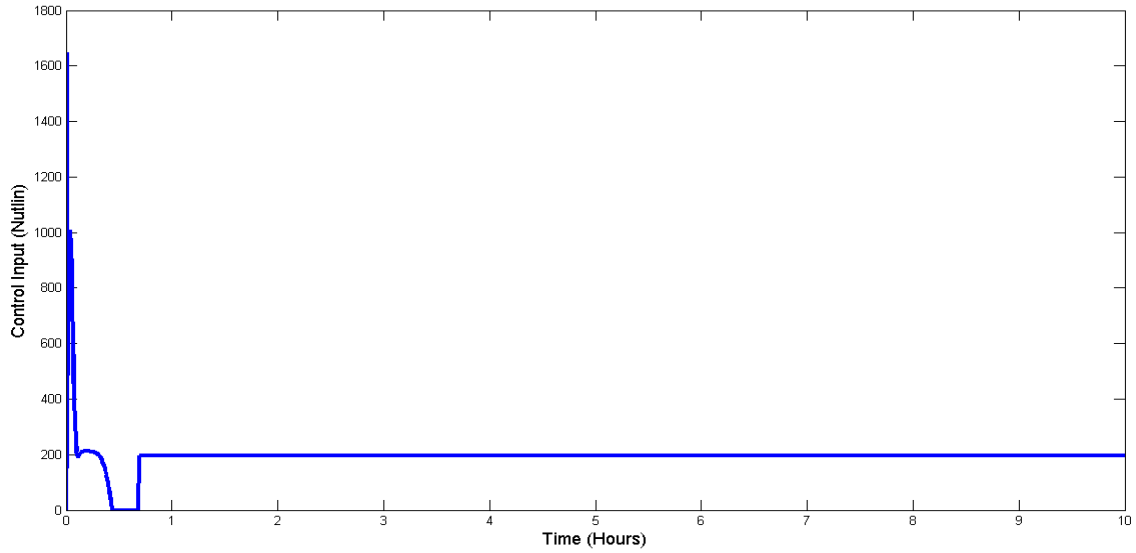

Figure 4. 4 Controlled Drug Dosage response for Nutlin

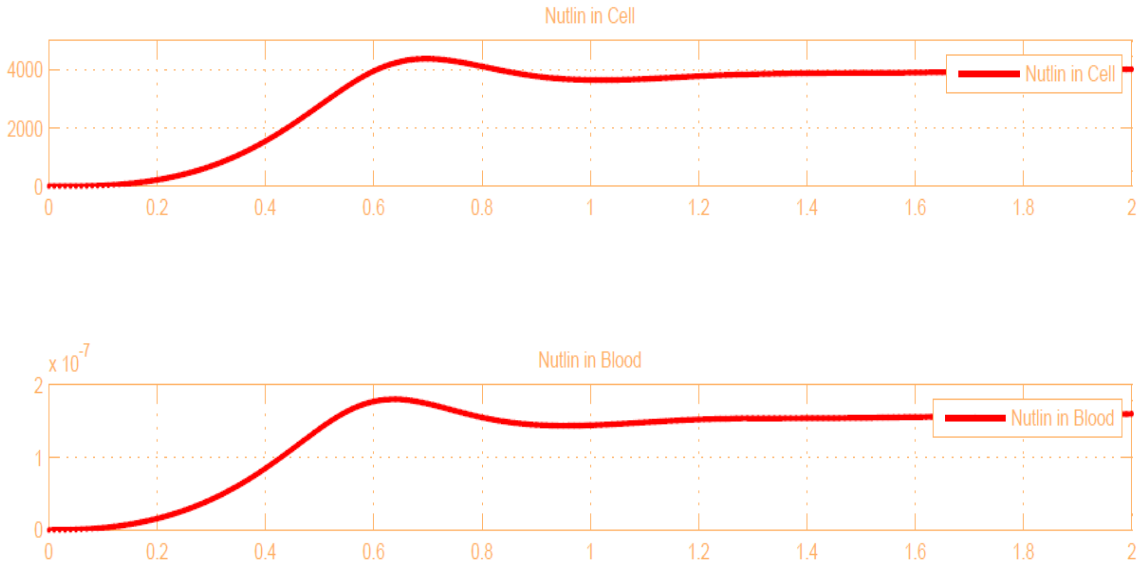

Figure 4. 5 Nutlin Disposed in Blood and Tissue

4.1.2 Inducing p53 Oscillations

For less significant damage to DNA, the p53 ought to oscillate with a constant period.From papers by (Vera, *et. al.*,2011; Kim, *et. al.*, 2013; Puszyński, *et. al.*, 2012), it is seen that whenever

there is stress on p53 pathway, p53 exhibits sustained oscillations which somehow helps in cell repairing.

According to Hunziker, 2010, when DNA damage stress is inflicted, it causes natural MDM2 decay rate to increase and the MDM2 dependant p53 decay rate to decrease.Consequently, simulations in Xpp were done to see the effect of these parameters change. The following trials were performed. Total time observed is 1000 hrs, simulations did not decay in this period unless otherwise stated.The sensitivity analysis in table 4.1 shows which parameters most affect the p53 level in our model. Integrating the information of which parameters are affected by different stresses provides predictions about which stresses will affect the p53 level the most. It has been found that changes in γ result in large changes in peak p53 levels and this change is hard to find at average p53 levels, once oscillations are set.

Table 4-1 Stability values for MDM2 decay rate (γ)

MDM2 decay rate (γ)	**p53 mean value in Molar (M)**	**peak to peak**	**Period/hours**
0.2	17M	2M	4
0.3	22.5M	24M	3.6
0.38	~ 26 M	45M	3.7

The simulations remain stable for the values of MDM2 decay rate (γ) at 0.2, 0.3,0.38. when the values are given with higher MDM2 decay rate (γ) there was unstability in the MDM2 based p53 decay rate (δ) as shown in the table 4.

Table 4-2 Effect of MDM2 decay rate (γ) on (δ) at 0.2

MDM2 based p53 decay rate (δ)	**p53 mean value in Molar (M)**	**peak to peak**	**Period/hours**
11	17 M	2 M	4
8	19.5 M		Oscillatory decays in 800
5	25 M	23 M	~4
4	30 M	30 M	~4

Table 4-3 Restoration of (γ), (δ) to its Default

Delta (δ)	Gamma (γ)	p53 mean value in Molar (M)	peak to peak (p-p)	Period/hours
8	0.3	28 M	~37M	4
5	0.38	Case failed	Case failed	Case failed

Apparently, the oscillations (mean and p-p) both increased with the decrease in δ and increase in γ, simulating increased p53 oscillations under the stress caused by DNA damage. It is interesting to note that, despite nearly 30 fold increase in p53 oscillations, the period of oscillation remains constant at ~4 hrs under all conditions. It clearly suggests a Limit Cycle.

Found that,it is not possible to induce oscillations using proportional feedback. Instead, it is proposed that the effect of the Nutlin may be simulated by increasing the value of MDM2 deactivation constant gamma. According to (Paul Steven M, *et. al.*,2010), gamma is the most sensitive parameter in the whole model too.

From (Puszynski, *et. al.*, 2014), it is clear that the p53 pathway oscillates with a constant period (6 hrs) under stress, which could be a limit cycle. Indicating that the equilibrium point should be a Hopf Bifurcation Point. Presumably, it will be a stable limit cycle. Finding Hopf Bifurcation in p53 by changing gamma (γ)

p53 is activated in response to events compromising the genetic integrity of a cell (Ciliberto *et. al.*, 2005). Increased functioning of p53 does not relate to the genetic damage its for cell sycle arrest as preferably p53 halts cell devision. That is why it fluctuates in oscillatory manner. These oscillations are the results of either both positive and negative feedbacks or by a single negative feedback loop. Negative and positive feedbacks in the p53 signaling pathway are not for determining the oscillatory response to genetic damage. The model developed is based upon th feedbacks in the p53-MDM2 network. In the present model the system under investigation is responsive to DNA damage i.e. moving from a stable steady state into a region of stable limit cycles. An all-or-none response to damage is guaranteed by large amplitude oscillations in the model. The system moves back to a stable steady state with low p53 activity when the damage is repaired with the oscillations of p53. The model reproduces experimental data in quantitative

detail. We suggest new experiments for dissecting the contributions of negative and positive feedbacks to the generation of oscillations. The models are very similar to Hunziker (2010), apparently, mention that stress causes oscillations of 6 hour period in p53. Puszynski *et. al.* (2014) has given a linear 3rd order model of the p53. It is evident from the work that p53 is required to show either a sustained level response or oscillations for the case of the DNA damage as could be seen in a cancerous cell (Puszyński, *et.al.,* 2008). However, it is considered that p53 is absent in 50% of the reported cancers (see in Literature Review section). This is sometimes attributed to the hyperactivation of the MDM2, E3 ligase that is supposed to restrict p53 production through negative feedback. MDM2 makes a bond with p53 and causes its ubiquitination.

4.2 Conclusions

Bringing the clinical applicability of nutlin in mind the simulations in the present study is suggesting that in large drug administration can produce remarkable effects on cell viability.At low doses, dose-splitting generally worsens the response. Of course, simulations of a wider set of patterns of fractionated delivery would be more valuable because these indications are preliminary. A complete study would be possible if the toxicity of different drug doses were evaluated. However, the recently proposed role of Nutlin as a "neutralizer" of chemoresistance to other antitumor drugs might open a new way for the clinical use of this agent.

It shows that by devising proper amount of Nutlin sustained response could be induced in the p53 pathway. The dosage remains within appropriate bounds. However, it is seen in Zhang, *et. al.,* (2011) that mere application of Nutlin cannot achieve p53 limit cycles as for this purpose the breaking of the p53-MDM2 complex is required.

Experiments to determine the route of elimination of nutlin-3a is not performed in this study. To extrapolate the PBPK model by modeling the elimination mechanistically is not enough to define PBPK models in other species. The unbound fration of nutlin 3a in plasma and in blood was supposed to have the same value in the model, this could be resolved in future. Simulations have been performed on the dosage values that are different from already used in model development (i.e. 400mg/kg). there were some non linear absorption and elimination processes observed at higher dosage values although a non linear characterization is still there to find the concentration in plasma for unknown non linear dosage. The dosage higher than the given rate was able to make the predictions inaccurate.

Pharmacokinetic studies of nutlin-3a and development of a PBPK model was done to design nutlin-3a dosing regimens for preclinical models data of cancers. There were still some limitations of the studies for extrapolating cytotoxicity data *in vitro*. This analysis provides a starting point for further pharmacokinetic/pharmacodynamic studies in tumor cells.

5. Chapter 5: References

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