Multi-scale based software for effective analysis of anticancer drug efficacy in a computer simulated target environment

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**NANYANG
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**Multi-scale Based Software for Effective Analysis
of Anticancer Drug Efficacy in a Computer
Simulated Target Environment**

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2019

Multi-scale Based Software for Effective Analysis of Anticancer Drug Efficacy in a Computer Simulated Target Environment

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A thesis submitted to the Nanyang Technological University in partial
fulfillment of the requirement for the degree of Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Muniraj Vivek Sheraton

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I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Dr. Peter M.A. Slood

Authorship Attribution Statement

This thesis contains material from 3 papers submitted to peer-reviewed journals where I was the first author or co-first author.

Chapter 3 is submitted as “*Emergence of Spatio-Temporal Variations in Chemotherapeutic Drug Efficacy: an In-Vitro and In-Silico 3D Tumour Spheroid Study*” by M.V. Sheraton, G.G.Y. Chiew, V. Melnikov, E.Y. Tan, K.Q. Luo, N. Verma, and P.M.A. Slood.

The contributions are as follows,

- P.M.A.S, M.V.S and N.V conceived the idea
- G.G.Y.C designed and performed all experiments and analysed the data
- M.V.S and V.M conducted all simulations
- M.V.S wrote the manuscript
- G.G.Y.C, K.Q.L, E.Y.T, N.V and P.M.A.S. helped revising the manuscript
- G.G.Y.C and M.V.S contributed equally to the manuscript.

Chapter 4 is submitted as “*Shear Stress Enables Quantitative Increase in Invadopodia Structure Formations Through Enhanced Tks5 Phosphorylation*” by M.V. Sheraton, S. Ma, S. Lim, K.Q. Luo and P.M.A. Slood

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- M.V.S conducted all simulations

- M.V.S wrote the manuscript
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- M.S and M.V.S contributed equally to the manuscript.

Chapter 5 is submitted as “*Effects of BRCA, P53 and RB on Ductal Carcinoma In-Situ to Invasive Ductal Carcinoma Transition – an In-Silico Analysis*” by M.V. Sheraton, S. Ma and P.M.A. Slood.

The contributions are as follows,

- P.M.A.S and M.V.S conceived the idea
- M.V.S conducted all simulations and wrote the manuscript
- M.S and P.M.A.S helped revising the manuscript

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List of abbreviations

Abbreviation	Definition
ABM	Agent based model
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
CA	Cellular automata
CPM	Cellular potts model
CTCs	Circulating tumor cells
DCIS	Ductal carcinoma in-situ
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
FDM	Finite difference method
FEM	Finite element method
FPP	Focal point plasticity
FRET	Fluorescence resonance energy transfer
FVM	Finite volume method
FVM	Finite volume
GGH	Glazier-Graner-Hogeweg
HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia-inducible factor
IBM	Immersed boundary method
IDC	Intraductal carcinoma
LBM	Lattice Boltzmann method
LPP	Lipoma preferred partner
MnSOD	Manganese superoxide dismutase
NADH	Nicotinamide adenine dinucleotide
NER	Nucleotide excision repair
PK	Pharmacokinetics
PODXL	Podocalyxin-like 1
ROS	Reactive oxygen species
SS	Shear stress
TKS5	Tyrosine kinase 5
VEGF	Vascular endothelial growth factor

Summary

Efficacy of chemotherapeutic cancer treatment varies from patient to patient. This variability can be attributed to the differences in the biological characteristics of the cancer cells. The effects of the biological characteristics on chemotherapy outcomes are widely studied. In most cases, such as in drug testing, it is assumed that a drug working on a biological cell type will continue to work with the same efficacy on patients having the same cell type. However, even in patients having phenotypically and genotypically identical cancer cells, the treatment efficacies vary between each other. These deviations arise from the cell-level biophysical characteristic variations in the cells that make up the tumor microenvironment. In addition to affecting the treatment outcomes, the microenvironment also alters the evasive capability of cancer cells. The tumor microenvironment therefore acts as a modifier of chemotherapeutic effects and enabler of chemotherapy evasion of the cancer cells. To completely understand the effects of microenvironment on chemotherapy outcomes, it is necessary to analyse the cell-level and tissue level biophysical evasion mechanisms of the cancer cells. These mechanisms aid in drug effect evasion and/or promote metastases. In this thesis, a simulation framework is developed for numerical modelling and simulation of interactions between cancerous tumor cells and their microenvironment. Three computational and experimental studies are included. They include, study of the, effects of chemotherapy on homogenous tumor population, influence of tumor microenvironment on the development of invadopodia structures and the transformation of benign tumor to a malignant tumor.

The mechanisms of action and efficacy of chemotherapy drugs, at cell population levels are well studied in literature. However, the localized spatio-temporal effects of the drugs are less well understood. The emergence of spatially preferential drug efficacy of cisplatin and paclitaxel resulting from variations in mechanisms of cell-drug interactions is explored. Using lab-grown 3D spheroids of HeLa-C3 cells and mechanistic model simulations, it is shown that cell repair probability, intracellular drug concentration and cell's mitosis phase interact to determine the outcomes of drug actions on a local cell population. In spheroids treated with

cisplatin, the drug induced apoptosis is found to be scattered throughout the volume of the spheroids. The efficacy of cisplatin is dependent on the stochastic cell repair probability. In contrast, the effect of paclitaxel is found to be preferentially localized along the periphery of the spheroids. The preferential action of paclitaxel can be attributed to the cell characteristics of the peripheral population. Combinatorial treatments of cisplatin and paclitaxel result in varying levels of cell apoptosis in the spheroids based on the scheduling strategy. Treatments initiated with paclitaxel are found to be more efficacious than its counterpart due to the cascading of spatial effects of the drugs. Short time drug alternation strategies produce largely similar treatment outcomes irrespective of the drug ordering.

Invadopodia of tumor cells have been sufficiently documented for their role in tumor progression and distant metastasis. Invadopodia formation is found to promote extravasation of circulating tumor cells, which has been verified by different experiment models. The influence of microenvironment on invadopodia formation of circulating tumor cells is largely unknown. The fluidic shear stress, which is an important physiological factor in the microenvironment of circulating tumor cells, is investigated for its effects on invadopodia formation. By utilizing a microfluidic system, shear stress is applied on tumor cells. Shear stress is found to promote invadopodia formation of tumor cells. The mechanism study using biological experiments and Glazier-Graner-Hogeweg method-based numerical model simulations shows that shear stress-generated reactive oxygen species is able to activate tyrosine kinase 5 and enhance invadopodia formation. This study provides insights on the invasiveness of metastatic cells and has important implications for cancer prognosis and therapy development.

Ductal carcinoma in-situ (DCIS) presents a risk of transformation to malignant intraductal carcinoma (IDC) of the breast. Three tumor suppressor genes RB, BRCA1 and TP53 are critical in curtailing the progress of DCIS to IDC. The complex transition process from DCIS to IDC involves acquisition of intracellular genomic aberrations and consequent changes in phenotypic characteristics and protein expression level of the cells. There is a lack

of proper understanding of the spatiotemporal dynamics associated with breach of epithelial basement membrane and subsequent invasion of stromal tissues during the transition. Therefore, the emergence of invasive behavior in benign tumor, emanating from altered expression levels of the three critical genes is explored in this thesis. A multiscale mechanistic model is used to unravel the phenotypical and biophysical dynamics promoting the invasive nature of DCIS. Ductal morphologies including comedo, hyperplasia and DCIS evolve spontaneously from the interplay between the gene activity parameters in the simulations. The model elucidates the cause and effect relationship between cell-level biological signaling and tissue-level biophysical response in the ductal microenvironment. The model predicts that BRCA1 mutations will act as a facilitator for DCIS to IDC transitions while mutations in RB act as initiator of such transitions.

Overall, the three model studies highlight the importance of tumor microenvironment in determining the treatment outcomes and cancer progression. Tumor microenvironment drives the non-linear response of tumor cells to chemotherapy. The studies indicate a heterogenous response to chemotherapy from a genetically homogenous tumor population. The DCIS to IDC transformation study suggests RB as a crucial drug target candidate to prevent tumor growth and arrest further metastases. Similarly, the extravasation study proposes ROS as an external environmental factor promoting cancer cell extravasation and therefore, consequently, a critical drug candidate. Thus, this thesis successfully identifies significant biophysical and chemical targets in the tumor microenvironment landscape which enable evasion and reduction of chemotherapeutic efficacy in cancer treatment.

Chapter 1. Introduction

Cells in human body are regulated by multiple active and passive processes. Here, active processes refer to the regulatory processes initiated from within the cells and passive processes denote the extracellular processes keeping the cells functioning normal. The major goals of these regulatory processes include, control of cell proliferation rate, restriction of unfavourable morphological changes and prevention of errant cell signalling [1, 2]. In short, the regulatory processes aim to establish cell homeostasis [1]. In multiple ways, events such as cell proliferation and programmed cell death affect the homeostasis of tissue systems.

Cell proliferation is initiated with the uptake of nutrients from the surrounding plasma for metabolism. During metabolism, energy is extracted from nutrients such as glucose, glutamine and amino acids. For instance, during glycolysis, glucose is generally broken down into ATP (adenosine triphosphate), pyruvic acid and NADH (nicotinamide adenine dinucleotide) [3]. ATP complex is the chemical energy provider, which when expended, is converted to ADP (adenosine Diphosphate or AMP (Adenosine monophosphate)). After required energy for cell survival is obtained, the cells use the excess nutrients and energy to increase their cell mass. Increased cell mass consequently results in volume increase of the cell. When the cell mass approximately doubles, the cell divides into two daughter cells. The process of cell division is referred to as mitosis. Thus, cell proliferation involves cell survival, growth and mitosis.

The cell level events associated with proliferation are termed collectively as cell cycle events. Cell cycle is split into two major phases, interphase and mitotic phase [4]. It is during the interphase that cell growth occurs. Interphase can be further broken down into G1, S and G2 phases. In short, G1 phase is marked by physical growth of cell and its organelles, S phase involves duplication of DNA and G2 phase is characterized by the reorganization of cell organelles to properly initiate mitosis. Cell proliferation leads to continual increase in

population of the cells as long as nutrients are available. Hence, this proliferation alone is incapable of establishing homeostasis. To avoid cell population runoff, the cell proliferation rate should be countered with cell removal to maintain equilibrium at cell level, tissue level and organ level.

Cell removal in human body is introduced via programmed cell death termed as apoptosis. Apoptosis is derived from Greek words (*apo + ptosis*) meaning falling away. Cell apoptosis is triggered by a cascade of proteases, whose family is collectively referred to as *caspases*. Caspase activation initiates the amplification of proteolytic cascade and cleaving of major proteins and nuclear lamins in the cell [5]. The activated caspases also cleave and consequently activate the DNA degrading enzyme, referred to as DNase, which breaks down the cell DNA. Thus, the family of caspases enable proper disposal of dead cells without damaging surrounding tissues. Cell proliferation and apoptosis thus enable replacement of damaged old cells with nascent cells. It is important here to distinguish between programmed cell death, *apoptosis*, and cell death due to noxious stimuli such as bacterial infection, oxygen deprivation known as *necrosis* [6, 7]. If there is shortage of nutrients (such as oxygen, glucose) or presence of toxins, a cell's growth is arrested. Due to ATP depletion, the cell's metabolism is disrupted. This results in premature death of the cell and spill out of cell contents such as organelles, cellular proteins and DNA material. Such, uncontrolled cell lysis can cause damage to neighbour cells. A sustained repetition of cell cycle should therefore eliminate cell necrosis and ensure proper health of tissue and organs.

For maintaining proper progression of cell cycle, external triggers play a very crucial role. These triggers include biophysical and chemical stimulus such as growth factors, microenvironmental crowding and cytotoxic effects from immune response. Growth factors target and boost the proliferation of a specific group of cells. For instance, vascular endothelial growth factor (VEGF) [8], erythropoietin [9] and human epidermal growth factor receptor 2

(HER2) [10] promote the growth of vascular cells, red blood cells and breast cells respectively. These growth factors are secreted by organs and are present in the local environment of the target cells. This local environment is termed as microenvironment. Microenvironment encompasses the local microscopic region composed of cells, extracellular matrix, proteins, growth factors and other solutes. In this microenvironment, cells establish cell-cell, cell-matrix tactile contacts. Cell to cell contacts establish physical signalling known as *juxtacrine* signalling [11]. Such signalling enables the cells to sense the crowdedness in their microenvironment. Over-crowding of cells restricts cell proliferation. In addition, immune response from T-cells in these hypoxic microenvironments generate cytotoxic effects [12], which is detrimental to cell survival. Thus, intracellular and extracellular mechanisms drive the emergence of cell proliferation and apoptosis and help maintain homeostasis. The effects of intracellular and extracellular mechanisms on cell cycle are not mutually exclusive. The cells and extracellular factors influence each other to initiate signalling cascades which regulate cell proliferation. For instance, in overcrowded conditions, the local oxygen concentration is depleted due to overconsumption by the cells leading to condition termed as hypoxia. This hypoxic condition triggers accumulation of Hypoxia-Inducible Factor (HIF) in the microenvironment [13]. HIF promotes the growth of endothelial cells from existing blood vessels. Their proliferation enables formation and splitting of new blood vessels from the old ones to the hypoxic zone. This process of new blood vessel formation is known as angiogenesis [14]. It ensures enhanced supply of nutrients to the microenvironment and elimination of hypoxia in the region. Consequently, this results in proliferation of the previously growth-arrested cells in this region.

Cell proliferation alone is insufficient for the blood vessels to grow towards the hypoxic regions. To properly reach the site of action the endothelial cells must proliferate towards the direction of hypoxic zone. To achieve this, in addition to proliferation and apoptosis, cells are

capable of migration. The exoskeletal filaments such as actin present inside the cell help them stretch the membrane unidirectionally and relax. This directional stretching-relaxation cycle generates cell motility. Cell motility can be characterized into two parts, (i) random motility and (ii) signal-oriented motility. As the name suggests, random motility involves movement of cells in random directions like random-walk of a molecule. Random motility can be considered as motility emanating solely from intracellular processes. In contrast, signal-oriented motility involves movement driven by cues from the environment. These signals can be in the form of chemical cues (chemotaxis), temperature variations (thermotaxis) [15], stiffness fluctuations (durotaxis) [16] and adhesion gradients (haptotaxis) [17]. In case of angiogenesis, chemotaxis is the key driver enabling directional growth of blood vessels to the affected sites. Similarly, in wound healing processes [18], fibroblasts and mesenchymal cells mobilize and migrate based on haptotactic gradients towards injury sites.

Establishing homeostasis is dependent on the interplay of intracellular and extracellular factors influencing cell proliferation, apoptosis and motility. These factors are intertwined in such a way that any perturbations at cell level will evoke response at tissue level through chemical and physical signalling and vice versa. However, there are circumstances in which the equilibrium may be disrupted. These disruptions can arise from abrupt or gradual accumulative changes in the cell's environment. A sudden fracture or organ severance can be considered as an abrupt change in cell's environment. In such cases homeostasis is thrown off balance in a short period of time and different constituents of the body (immune system, blood cells etc) respond and attempt to restore the balance. In case of accumulated changes, minor disruptions incapable of significantly affecting homeostasis accumulate over time. When these accumulated changes reach a tipping point, they can successfully offset homeostasis and warrant a response from the body. One example would be the degeneration of neurons in Parkinson's disease [19, 20]. In Parkinson's, mitochondrial homeostasis is disrupted by

accumulation of proteins in neurons. As such, early stage small-scale accumulations can be considered benign since they mostly do not affect normal functioning of the organs or its local environment. As accumulation proceeds, multiple body sites will be affected and can prove to be fatal. To generalise, almost all diseases, including ageing, can be considered as disruptors of homeostasis. The severity of a disease is dependent on the extent to which it can disrupt the system's homeostasis. One such life-threatening disease which significantly interrupts local and global equilibrium is cancer.

Cancer is characterized by uncontrolled proliferation and evasion of apoptosis by the cancer cells. Cancer is a disease condition where the disruptions are initiated at cell level, these disruption act as precursors for an avalanche of disruptions at tissue level resulting in catastrophic organ failures. There have been around 17 million cancer diagnoses in the year 2018 alone with 9 million deaths from the disease worldwide [21]. About one sixth of the deaths around the globe can be attributed to cancer. Smoking tobacco is one of the largest known risk factor accounting for 22% of cancer deaths and together with alcohol, increase the risk of cancer by a maximum of 5 folds [22]. Lung cancer and breast cancer are the two most common cancers worldwide. There is one-eighth of a chance that a woman will get diagnosed with breast cancer during her lifetime. It is important to note that breast cancer also affects men, although not as frequently as women. Unlike Parkinson's or other organ specific diseases, cancer is not a local tissue-specific disease. Cancerous cells can spread to different parts of the body resulting multiple organ failures. To understand the growth and spread of cancer, the progress of cancer can be demarked by three different phases as explained in the forthcoming sections.

1.1 Phases of cancer progression

The process of cancer progression can be simplified into three distinct phases;

- Primary site growth
- Angiogenesis and intravasation
- Extravasation and metastasis

1.1.1 Primary site growth

First, a single cell or group of cells in a tissue acquire genetic mutation(s). These mutations accumulate over time to significantly alter the cell cycle of the mutated cells. Mutations of cells can arise from multiple pathways; they can originate inherently or be acquired from environmental perturbations. Inherent mutations arise from random DNA copy errors or hereditary defects. Majority of these copy errors occur during mitosis and accumulate during proliferation as critical DNA errors. Environmental perturbations result from human-ambience interaction events such as ultraviolet or X-ray radiation exposure, chemical ingestion and radioactive material contacts. All such mutation-inducing or cancer-causing chemicals and substances can be collectively termed as carcinogens [23].

The extent of development of cancer initiating mutations through inherent and acquired mutations vary vastly for different types of cancers. For instance, pancreatic cancer has been shown to predominantly occur due to DNA copy errors in the cells. Around 82% of pancreatic cancer cases have been shown to arise from inherent mutations and the remaining 18% from environmental triggers [24]. However, environmental perturbations play a key role in initiation of cancerous mutations in lung cancer. About 65% of cancerous mutations arise from environmental effects such as smoking [24]. Once the critical mutation(s) set in, the cells carrying the mutation(s) begin to proliferate and form a local population of cells with different degrees of mutations. This local population of cells is referred to as a malignant tumor. It is classified malignant, since it is capable of creating life-threatening events given sufficient time

to evolve and survive. Thus, even at early stages of tumor growth, due to accumulation of different mutations in the cells, the tumor is inherently heterogenous [25-27].

DNA mutations promote faster growth of the tumor cells. The mutations reprogram metabolic pathways of the cells enhancing their capability to survive and divide even in nutrient deficient conditions. Specifically, *Warburg Effect* [28] enhances the energy extraction rate of cancer cells. Normal cells take up glucose and break it down to energy (ATP) and carbon dioxide (CO₂) or lactate through a process called aerobic glycolysis. CO₂ is produced upon complete oxidation of glucose molecule in the mitochondria. However, in fast proliferating cancer cells, the metabolism of the cell favors partial oxidation of glucose to lactate forgoing complete oxidation to CO₂. Hence, cancer cells have a faster uptake rate of glucose than normal cells. This process of rapid intake of glucose followed by incomplete oxidation is called Warburg Effect. In short, cancer cells favor rapid ATP generation at the cost of efficient oxidation.

Cancer cells circumvent programmed cell death in addition to their increased metabolic uptake. The cells evade apoptosis through various ways such as dysregulating the expression levels of pro- and antiapoptotic proteins [29], reducing expression level of caspases, impairing cell receptor signaling and acquiring p53 alterations [30]. Antiapoptotic proteins involve the Bcl-2 family proteins [31, 32] which are downregulated in cancer cells. Genetic mutations have been found to appear in the p53 genes, which are the regulators of apoptosis pathways. Additionally, to sidestep apoptosis triggers from external environments cancer cells are known to exhibit impaired cell receptors on their membranes. Their fast proliferation and survival capability help them outcompete and replace local populations in a tissue. At this stage of tumor growth, the microenvironment is composed of normal cells, cancerous tumor cells, extracellular matrix (ECM) and ambient diffusing solutes. Tumors at this stage lack

vasculature such as dedicated blood vessels for tumor nourishment. Hence, they can be termed as avascular tumors.

Tumor cells in avascular tumors lack proper supply of nutrients. Due to this shortage of nutrient supply, some cells in the tumor begin to undergo autophagy. Autophagy is the process of self-devouring of dysfunctional cell organelles for degradation or recycle. However, cancer cells hijack this process to prolong cell survival by removing inessential cells organelles and conserving much needed energy for survival [33]. Further depletion in oxygen or other nutrients results in necrosis of cancer cells. Thus, the cells present at the core of the avascular tumor structures are dead cells. The lysis of cells at the core leads to spilling of cellular proteins to the microenvironment causing change in pH values in surrounding tissues. At this stage, the growth of avascular tumor is saturated, further spread and proliferation of cancer cells is arrested.

Figure 1.1: Summary of cancerous tumor growth at the primary site phenomena analysed in this thesis. (a) avascular mode of tumor development and (b) Angiogenesis and intravastation in vascularized tumor growth.

1.1.2 Angiogenesis and intravasation

The next stage in cancer evolution is the acquisition of extra nutrients from ambience to sustain rapid cell proliferation and survival. Cells in avascular tumor are devoid of oxygen and other nutrients due to absence of fresh supply of solute from blood stream. To enable transport of new solute to the primary tumor site, cancer cells tap into angiogenesis. Since, hypoxia is rampant at the core of tumor, it induces overexpression of HIF [34] and VEGF [35]. Both HIF and VEGF are powerful angiogenesis promoters [36]. VEGF family of factors have been long implicated for their significant role in the production of new blood vessels (neovascularization) to the primary tumor site. The tumor cells also come in physical contact with the surrounding endothelial cells and bind to their receptors on cell membrane. This contact activates the proliferation activity of the endothelial cells, which after sufficient proliferation produce hollow blood vessels [37]. These vessels are stabilized by other factors such as angiotensin and act as conduit for fresh nutrient supply. The previously undernourished primary tumor gets copious amounts of nutrients to revitalize the tumor population and produce additional cells. The size of tumor progressively increases with uncontrolled cell proliferation until the tumor reaches a second critical mass. Once the critical mass is attained the tumor growth saturates. The neovasculature generally does not develop into a well-developed structure due to absence of multiple necessary factors to ensure proper vessel growth and vessel end termination. Hence, the current microenvironment consists of majority of tumor cells, few normal cells, proteins and cell organelles from lysed cells, nutrient solutes and irregularly formed blood vessels. This stage is characterized by a saturated vascularized cancerous tumor. The next step in tumor evolution after vascularization is the process of intravasation [38].

Intravasation involves the migration of cancer cells through the endothelial layer of blood vessel and entering the blood stream as depicted in figure 1.1. Over-expression of VEGF has been implicated in formation of leaky neovasculature [39, 40]. This enhances the

intravasation rate of cancer cells. Cancer cells have also been found to modify their membrane properties and structure to squeeze [41] themselves through tight endothelial cell junctions [42]. The process of intravasation has also been shown to begin at early stages of tumor development. There have been two different models proposed for understanding the intravasation process; (i) intravasation through vasculature lining the outer layer of tumor mass and (ii) intravasation through vasculature terminating or passing through the tumor. Considering the evidences of hypoxic necrotic core and upregulation of EGFR inside the tumor, significant intravasation of cancer cells through internal vascular architecture is possible [43, 44].

1.1.3 Extravasation and metastasis

The final stage of cancer evolution is the formation of secondary tumor sites at distant parts of the human body. The cancer cells that have intravasated into the blood stream circulate through the whole human body. These cells are simply termed as circulating tumor cells (CTCs). They experience heavy shear during their movement through the circulatory system. The shear stress experienced by the CTCs are capable of killing them [45]. The maximum average wall shear stress in aorta is around 1 N/m^2 [46]. Red blood cells can handle such high shear stress levels. However, microtubules or actin filaments of normal cells can rupture or suffer structural damage when exposed to these conditions. Therefore, cancer cells also suffer the same fate when exposed to fluid shear. However, a few of these cancer cells can evade or survive through the shear stress exposure. Studies have shown that there is a negative correlation between a cancer patient's survival and the fraction of CTCs in their blood stream. During their survival in the circulatory systems, the CTCs undergo multiple physical and biological changes which makes them hardier and more aggressive.

EpCAM marker is used to segregate and enrich CTCs from patient blood stream [47]. EpCAM is a protein found on the cell membranes of epithelial cells. In few cases, cancer cells can undergo epithelial to mesenchymal transition (EMT), which is the process by which epithelial cells lose their adhesive property and acquire invasive migratory properties. CTCs that have undergone EMT lose EpCAM from their surface hence making it impossible to detect by normal means. Additionally, the cytoskeleton of the CTCs has been found to be more flexible than normal cells. CTCs have been shown to morph from spherical to cylindrical structures when entering microcapillaries. This behavior arises as a direct result of mutations that prevent actin polymerization [48], thereby reducing cell rigidity. During their circulation through the body, the CTCs encounter vessel spots where they get arrested or become stationary. These stationary CTCs begin the process of extravasation.

Extravasation involves intrusion of cancer cells from the surface of blood vessel to an organ tissue. Extravasation can be considered as a counterpart to intravasation, however biologically the two processes are significantly different. Extravasation involves recruitment of blood cells such as platelets and myeloid cells by the cancer cells to promote the process [49-51]. For instance, the CTCs express podoplanin which induces the expression of TGF- β [52]. Expressed TGF- β enables invasive mesenchyme-like phenotype [53]. A characteristic change in the extravasating cancer cell's morphology is the formation of invadopodia [54]. They are thorn like structures forming due to polymerization of actin filaments on the cell surface membrane. They aid in penetration and extrusion of cells through the endothelial cell junctions. After the cells reach the tissue layer of the new organ they start proliferating again, resulting in the formation of a secondary colony. The CTCs can settle at different organs in the body subsequently forming multiple tumor sites. This entire process of cancer cell migration from parent site to formation of secondary site is called metastases. In particular, the process

of formation of secondary tumor sites at farther distance from the primary site in the body is called distal metastases. A graphical summary of this process is shown in figure 1.2.

Cancer cells in the secondary tumor sites have been shown to be phenotypically different compared to cells in the primary site. In most types of cancers, metastases cancer is classified as stage 4 and the patients are shifted to palliative care due to lack of chemotherapeutic drugs capable of treating both the primary and secondary sites at the same time.

Figure 1.2: Stages of metastasis. (a) Circulating cancer cells in blood vessel, (b) extravasation of a vessel-trapped cancer cell and (c) development of secondary tumor site at a distant organ.

1.2 Chemotherapy and cancer cell survival

The evolutionary nature of cancer makes it a complex system. Mutations start at cell level; tumors develop on tissue level and metastasize at organ level. Hence, cancer is an emerging

multiscale system. Clinical treatment of such a complex system requires strategies which should take into account the physiology of the patient, the pharmacodynamics and pharmacokinetics of the drugs and the evolutionary nature of individual cells. This thesis explores the various cellular mechanisms which influence the chemotherapeutic outcomes and aid cancer cells in evading negative environmental constraints. Owing to intra and inter-tumoral heterogeneity of cells in different tumors and drastically different biological makeup of different cancer types (such as lung, breast, liver cancer etc.), it is unlikely to generalize any phenotypic expressions, chemotherapeutic responses or evolutionary behaviors to all cancers. The works presented in this thesis are therefore restricted to two types of cancers, cervical and breast cancer. To thoroughly estimate the efficacy of any chemotherapeutic drug on tumors, it is imperative that tumor microenvironment should be considered alongside the drug reaction mechanisms. Additionally, it is not possible to assess and elucidate on the impact of certain cell level activities on the drug efficacy solely through experiments. Hence, mathematical models are used in conjunction with laboratory *in-vitro* experiments to unravel the interactions between drug mechanisms and cell activities.

1.3 Evolution of cancer

What are the intracellular and environmental factors aiding in evasion and manipulation of chemotherapy effects by cancer cells? – this is the key question sought out to be explored in this thesis. This research question is broken down into three sub-parts or sub-queries. They are, *what are the factors affecting spatio-temporal variations in drug efficacy?* *how does the cell morphology transform in extravasating cancer cells?* and *how do malignant cells in-situ transform to metastatic carcinoma cells?* The last two subqueries are concerned with the evasion capability of cancer cells and the first subquery seeks to explore the inherent chemotherapy manipulation strategies associated with cancer cells. As previously described, the biophysical and chemical mechanisms governing these events are analyzed using a

combination of experimental and mechanistic model studies. Mechanistic models are implemented using numerical simulations capable of handling the solute transport and intracellular processes occurring within the tumor microenvironment. A summary of the mass transfer models, and numerical methods are presented in chapter 2. The core research question – *factors affecting efficacy and evasion of chemotherapy by cancer cells* cannot be addressed in a single unified computational model or experimental study. Therefore, the thesis comprises of three different model simulations addressing the core question. Chapter 3 addresses the efficacy variation in multicellular spheroids, while, Chapter 4 and 5 deal with the evasion strategies used by the cancer cells. More specifically, a mechanistic model of two major commercially available drugs, *cisplatin* and *paclitaxel*, is developed and their localized spatio-temporal efficacies on cervical cancer cells are discussed in chapter 3. The pharmacokinetic and pharmacodynamic parameters of these drugs are also incorporated in the mechanistic model to include the transport limitations in the microenvironment. Chapter 4 delves on the cell-level evasion mechanism of cancer cells through formation of invadopodia. It aims to unravel the factors promoting the formation of invadopodia in cancer cells during extravasation. Extending from this understanding on the aggressive transformation of cancer cells, chapter 5 explores the tissue/tumor level evasion mechanism of breast cancer cells constrained within the ducts.

From the computational modelling point of view, due to the multiscale and multiphysics nature of these studies, two different computational models are used. Glazier-Graner-Hogeweg model-based simulations are used in the evasion strategy studies (chapter 4 and 5) and a hybrid cell level model is used in the drug efficacy study (chapter 3). The final chapter 6, provides an overview of the current works and the possible future models that can be built atop the discussed works.

Chapter 2. Tumor Simulation Framework

Cancer progression is driven by cascade of intracellular events triggered by external factors and gene-level interactions with those factors [55, 56]. For example, consider the rapid proliferation rate of cancer cells in a tumor. The cells uptake nutrients from the surrounding, increase in mass, undergo mitosis and finally divide into daughter cells. The cells at the initial stage of tumor growth repeat this pattern of growth until there is shortage of nutrients. The next generation of nutrient starved cancer cells either undergo autophagy for extended survival or secrete factors that trigger angiogenesis. There are multiple interconnected events that occur during this nutrient deprived proliferation phase. First, the cells deplete the nutrient source and create a hypoxic region. Then, the hypoxic environment induces secretion of factors such as HIF [13] that act as precursor for angiogenesis. Finally, genesis of new vessels to the tumor site enhances the proliferation of tumor cells. Physiological factors such as the distance of the tumor from the existing vasculature, size of the tumor, extent of hypoxia determine future progression of tumor growth. In this event cascade, hypoxia is the environmental factor that triggers the direct and indirect gene-level responses from individual cells. Hence, using a holistic approach in understanding tumor growth may not yield deep insight into the individual actors and dynamics driving the complex system operating at multiple spatial and temporal scales.

2.1 Tumor microenvironment and mass transfer

An efficient way to handle a cancerous tumor system is to segregate the various physico-chemical triggers and their responses emanating from individual cells and the abiotic components present in vicinity of these cells. If we consider the subject of tumor cell proliferation, the nutrients, hormonal factors and chemotherapy drug solutes can be considered as the ambient abiotic components of the cancer cells. These components work in tandem to influence the progress of tumor cell proliferation and act as constraints that restrict overall

tumor population evolution. Due to the spatially varying nature of these components the tumor cell population will evolve in a non-linear fashion with respect to time and space.

Table 2.1 The components, their characteristics and evolutionary triggers.

Components	Characteristics	Triggers
Cancer cells	Movement Nutrient uptake Protein secretion Cell growth	Hypoxia Necrosis
Microenvironment	Solute concentration Viscosity Diffusivity	Volume displacement Biotic degradation

Experimental observation of the intertwined actions and responses generated by the cancer cell-microenvironment system is adversely restricted by the multi-scale multi-component nature of the system. A practical way to experimentally study such systems is to reduce the number of variables in the system and observe them in a controlled environment. Such compromises often result in inadequate description of the system, leading to conclusions that lack the significant insight into the processes or deviate from events occurring in a real-life setup. For instance, a 2D cell culture grown in a petri dish in the absence of external fluid transport, will lack the solutes and hormones promoting tumor growth. Hence, complete replication of the complex tumor system is not experimentally feasible. One way to handle all the biological actors, physical constraints and chemical solute transport mechanisms associated with the complex system (listed in table 2.1) is through mathematical modelling. The trigger-response dynamics in the tumor microenvironment can be explored through numerical model

simulations without any compromises arising out of experimental limitations. These models are only limited by the time and memory required to solve them computationally. To develop and execute well-defined numerical simulations, a computational framework capable of supporting all required physical and biological simulation modules is required. In case of tumor growth simulations, the modules should include partial differential equation solvers, cell behavior and mass transport model simulators. In general, mass transport in avascular tumors involve solving Fick's law of diffusion, passive diffusive equations and other partial differential equations used to define solute kinetics such as uptake, biotic and abiotic degradation, etc. For tumors with intra-tumoral vasculature, solvers of Navier-Stokes equation or Lattice-Boltzmann Method based fluid dynamic solvers are required. The biological simulation modules should incorporate models capable of handling cell level and tissue level dynamics such as cell motility, volume expansion, cell membrane deformations etc. These physical and biological modules have to be validated against experimental or analytical evidence from similar biological problems. In addition, the numerical simulation module should exhibit sufficient levels of numerical stability and accuracy required by the biophysical model.

Figure 2.1: Schematic summary of computational methods used in the thesis.

2.2 Tumor simulation models

The physical and biological phenomenon driving cancerous tumor growth can be modelled and simulated using computational modelling. The cells in a tumor can be modelled as a collection of mass points or as an individual entity [57] of varying characteristics such as mass, motility, secretion capabilities etc. If they are modelled as the latter then additional attributes such as cellular adhesion, cell membrane stiffness, geometry of the cells and inter-cell communication pathways need to be defined. Major trade-off between defining cells as a cluster or individual entities would be the ability to generate phenotypically heterogenous mixed populations in the simulation domain. In case of mass points modeling it will not be possible to simulate cell-level interactions between the heterogenous nature. This limitation does not mean mass point modelling is an inferior approach. Tissue level dynamics, where microscopic heterogeneities can be safely excluded, can be efficiently modelled using mass points rather than individual entities due to their nature of being computationally less resource intensive. Early cancer simulation models [58, 59] modelled cells as random-walk particles moving without influence

from their peers. Other early models [60, 61] assumed cell proliferation rate as fixed mathematical functions irrespective of the duration of growth. Due to their limited ability to address for tumor microenvironmental parameters, these models failed to capture the real-life progression of tumor growth. Hence, in addition to cell-cell interactions inside the tumor, cancerous cells should also be modelled to interact with their surroundings. The surrounding encompasses the nutrients, drugs and other extracellular matrices. Due to their spatially distributed nature, the surrounding is generally modelled as fields obeying laws of mass transport and fluid dynamics. Reaction-diffusion models [62-64] have become dominant models for quantifying avascular tumor growth since the early 2000s. In these models, nutrient uptake had been modelled akin to a chemical reaction in which the nutrient is oxidized to a subsequent product at the site of the cell or mass points. The mass of the cell was assumed to increase proportional to the uptake rate. In this thesis, mass point model is used in chapter 3, where the model development is described in detail

In case of simulations where cells are modelled as single entities; methods such as Cellular Automata, agent-based models, Cellular Potts Models and Glazier-Graner-Hogeweg (GGH) method-based models are commonly used. Cellular automata [65, 66] and agent-based models [67, 68] can be classified as non-energy-based methods since the cells in the simulation are not constrained by physically governed energy equations. In most cases, these methods consider cells as a simple physical entity occupying a defined position in space, for instance a hard sphere occupying a lattice point. Hence, these numerical simulations can produce biologically irrelevant outcomes such as impossible tissue morphologies or cell adhesions. To overcome this limitation, Cellular Potts Model [69, 70] and GGH method-based models [71] introduce cells that are bound by definitive energy-based evolution rules. These rules restrict the morphological, physiological and temporal evolutions of the cells within their biological limits. Due to the introduction of the energy constraints governing the cells, these models tend

be more memory intensive and computationally demanding than their counterparts. In this thesis, GGH model is predominantly used for cell behavior simulations. Overview and methods of implementation of GGH model constraints are discussed in detail in chapters 4 and 5. A schematic summary of different methods used in the simulation is presented in the figure 2.1.

2.3 Overview of cell simulation models

Modelling of avascular tumors or tumors devoid of vasculature at cell-level begun as 2D lattice-based simulation models. Modelling of cell level models begun with development of cellular automata (CA) models. DÜchting [72] developed a CA model to simulate individual cancer cells as points occupying a single lattice site in a 2D lattice domain. It focusses primarily on cell proliferation and cell death. This model is devoid of any cell microenvironment interactions such as, nutrient uptake or cell adhesions. Even though the model is simple and computationally less intensive, it is capable of simulating the competition of growth between normal cells and cancer cells in a tumor. DÜchting et al. [73, 74] further improved this model, by incorporating, nutrient diffusion in 3D lattice domain of the CA model and nutrient-based cell proliferation. This improved model still lacks the capability to mimic the intracellular dynamics and intercellular adhesions. An additional limitation of these models is the presence of square-shaped cells introduced by the use of regularly structured grid space. This limitation can be ignored if the morphology of the cells is assumed to dynamically change within this square-shaped constraint. To overcome this shape effect, Kansal et al [66] developed a CA model with an adaptive grid lattice. They introduced Delaunay lattice [75] in their CA model to define the tumor and its surrounding space. The grid space is represented by polyhedral lattice sites on which the individual cells reside. This configuration is closer to the morphology of a cell and representative of the structural packing within a tumor. All the above-mentioned models are limited to handling avascular tumors.

Vasculature plays a crucial role in late stage tumor progression and metastasis. To model the vasculature, a separate cell type for vessel cells is a basic requirement. The terminal point of the vasculature should also act as a source of nutrient transport within the tumor. Patel et al [76] modelled the vessel cells along with the nutrient source terminals in their CA model. They considered four different cell types namely, normal, cancerous, blood vessel and dead cells. The inclusion of dead cells in the model enables the formation of necrotic core within the tumor, where nutrient supply is minimal. They analysed the influence of micro vessel density on the transport of cell metabolism by-products. The simulations produced clinically relevant spatial tumor morphologies and predicted the rise in acidity within tumors exhibiting low vascular densities.

CA models act as a great tool for modelling individual cancer cells and simulating the tumor microenvironment. However, they lack necessary biophysical principles to adequately define cell-cell interactions occurring within a tumor site. These biophysical activities include cell motility, spatial growth of individual cells, cell lysis, cell migration and cell-cell adhesion. A cell restricted to occupy only one lattice site in a CA domain does not replicate the exact biological setting, since the membrane of the cells fluctuate or twitch with time. Accumulation of cell membrane fluctuations leads to rise of cell motility and spatial proliferation. Therefore, researchers began coupling continuum-based mass transfer models with Agent-based models to accommodate for solute and enzyme kinetics. These models are referred to as hybrid models of tumor growth [77], which are governed by a combination of physical laws and algorithmic grid-update rules. Hybrid models incorporate the spatial allocation of cell positions. There are two subclasses of the hybrid models namely, lattice-free models [78-81] and multicompartment cell models [82-85]. They deal with the spatial allocation of cell positions in two different ways.

Lattice-free models or off-lattice models, as the name suggests, considers the tumor space as a continuous medium rather than as discrete lattice sites. The cells in the model can move around freely without any artificial grid boundaries and interact with other cells freely. In these models cells are modelled as spherical or other geometrically similar entities [86]. The cells are often assumed as solid rigid structures without deformability. This assumption ensures that two cells do not overlap on a same point in space. It also introduces the concept of space dependant mitosis, where, upon mitosis, a daughter cell pushes its neighbours to occupy the nearest space available. Simulation frameworks such as PhysiCell [87], CellSim3D [88] and IBCell [89] provide tools necessary for development of such off-lattice models. In essence, these off-lattice hybrid models function similar to Agent-based models, with each agent representing a single cell [90]. The agent attributes can be modified to model physically and biologically different cells such as extracellular matrices, immune cells or normal cells [91]. Immersed boundary method-based (IBM) model is a subclass of off-lattice model of tumor development introduced by ReGalle et al [92]. They model cell cytoplasm as incompressible viscous fluid with elastic membranes. Thus, the morphology of the cells in the model evolve based on the defined fluid dynamic principles. The model simulations were used to explore the biomechanical properties of the cells. Dillon et al. [93] developed a similar IBM based model to examine formation of various morphologies in ductal carcinoma. Galle et al. [78] modelled the cell contact mediated growth inhibition and anoikis using spherical cells. For cell-cell contact modelling, they proposed the use of two deformed spheres to indicate contact or adhesion establishment between these cells. However, in reality, such deformed spheres do not represent cell adhesions. Rather, the cells can freely deform and establish multiple points of contact between the cells. In addition, the displacement of neighbour cells for daughter cell position allocation is impractical. Biologically such cell divisions will not occur due to the external physical pressure exerted by the neighbours on the daughter cell during division. Also,

off-lattice models suffer a computational disadvantage of the need for interpolation of solute values at cell sites.

Multicompartment cell models were developed to serve as an alternative to CA models. They retain the discretized lattice form from CA and introduce physical laws that are to be obeyed throughout the lattice. The cells in these numerical models occupy multiple lattice sites and physical constraints are applied to each of the lattice sites. This enables separating a single cell into individual compartments which have different biomechanical properties. Robertson et al. developed cells capable of responding to external stimuli in the microenvironment. They modelled the cells as nine individual entities or compartments interacting with each other and surrounding. These entities are interconnected therefore polarised and respond individually to external stimuli. Simulation frameworks such as CompuCell3D [94] provide tools necessary for development of multicompartment models (GGH models). As previously discussed, GGH models are energy-governed models. The energy constraints prevent formation of biologically impractical cell morphologies in the simulations. The constraints also prevent onset of mitosis in cells residing in crowded environment. Dharma et al. [95] simulated the adhesion between cells and ECM to predict the transition from benign to invasive behaviour in breast cancer. Unlike off-lattice methods, the cells in their model could establish multipoint adhesion with the matrix and between themselves. Edalgo et al. [96] developed a similar adhesion model for metastatic cell migration, by modelling cancer cells as multicompartmental structures and ECM numerically modelled by partial differential equations. GGH models are also suitable for modelling mechanical or phenotypical changes occurring within a cell. The mechanical property of the cells such as stiffness can be modelled in GGH by imposing adhesion energy constraints on the individual compartments or pixels of a cell. This model property was utilized by Chowkwale et al. [97] to simulate EMT transition in tumor microenvironment. They analysed the effects of EMT-derived activated fibroblasts (which exhibit increased stiffness compared

normal fibroblasts) on tumor cell activity. This study demonstrates GGH model's ability in not only replicating tumor cell activity but also capture the biophysical and biological interactions occurring in the surrounding space. All the above studies ascertain the fact that energy-based GGH approach outperforms CA, simple ABM and off-lattice methods in mimicking the tumor microenvironment.

Owing to ease of handling multicompartmental cells and various crucial biophysical parameters, GGH model is chosen for model studies described in chapters 4 and 5. Chapter 3 introduces a model for chemotherapeutic response of multicellular spheroids. This model deals with interactions between the cells and drug solutes. Cell membrane fluctuations or a cell's morphological changes are not crucial for capturing the drug kinetics and dynamics. In other words, tissue level dynamics are crucial in this model, while cell level interactions and morphological changes associated with a single cell can be safely excluded from the model. Hence, a lattice/grid-based hybrid model is developed in chapter 3 to model the cell-drug interactions, which excludes the computationally intensive cell morphology formulations. Finite volume method is used for external field simulations in this thesis.

2.4 External field modelling

The mass transport of external fields such as drug molecules or nutrient solutes is modelled using finite volume method in this thesis. In general, mass transport and fluid dynamic models are solved computationally by three major numerical methods – finite difference method (FDM), finite element method (FEM) and finite volume method (FVM). The purpose of any numerical discretization method is to transform the set of differential equations into its algebraic form. FDM is the oldest method employed to obtain numerical solution of the external solute fields and its strength lies in its simplicity. The applicability of FDM is limited to structured grids and it fails to handle complex irregular geometries. Whereas, FEM and FVM can handle curvilinear domains that allow these methods to handle unstructured grids required

for the given geometry such an irregular shaped tumor. Finite element discretization divides up the region into a number of smaller regions (finite elements) and is based on a piecewise approximation of the solution. Finite volume (FV) discretization is based on an integral form of the governing equations, with the values of the conserved variables averaged across the volume [98]. For instance, the governing equation of steady diffusion and its integral form is given by

$$\text{div} (D \text{ grad } \phi) + S_\phi = 0, \quad (2.1)$$

and

$$\int_{CV} \text{div} (D \text{ grad } \phi) dV + \int_{CV} S_\phi dV = \int_A n \cdot (D \text{ grad } \phi) dA + \int_{CV} S_\phi dV = 0 \quad (2.2)$$

Here, ϕ is the field variable, which is the solute concentration, D is the diffusivity, S_ϕ represents the source term of the solute, CV , V and A are respectively the control volume, volume and area of the discretised finite volume cell shown in figure 2.2 (a).

In FVM, the computational domain is discretized into finite volumes (or cells) and the equations are solved for each cell [99]. Flux conservation is implemented in FVM and therefore any flux input to a particular cell should equal its output. This approach of conservation of variables averaged over the cell makes FVM a suitable choice for solving fluid dynamic and mass transfer problems. FVM allows for having variable number of neighbours which can result in an unstructured grid arrangement for complex geometries. All dependent variables defined share the same control volume and is known as co-located or non-variable staggered arrangement [100]. A major advantage of FVM is its capability to discretize equations directly on the physical space without the need for transforming it to the computational system unlike FEM. This allows direct discretization of the biological cell domain to an FVM domain and vice versa. FVM can therefore be used in a coupled fashion with GGH lattice domains or mass point grids. The nutrient uptake and diffusion can be solved on FVM domain and readily be

interpreted for cell metabolism and growth. For instance, consider the finite volume approximation for a one-dimensional steady state diffusion problem. Equation (2.1) can be simplified for a one-dimensional steady state diffusion case as,

$$\frac{d}{dx} \left(D \frac{dC_g}{dx} \right) + S_g = 0, \quad (2.3)$$

where, C_g is the concentration of glutamine (growth solute), D is the diffusion co-efficient of glutamine and S_g is the uptake rate of cells (source term). The value of C_g at the boundaries have to specified as boundary conditions for the domain. The FVM procedure for solving the equation (2.3) involves three steps; grid generation, discretization and numerical approximation of the solution to the discretised equations.

2.4.1 Grid generation

The first step of the solution involves division of the computational domain into discrete control volumes. Each control volume represents a cell where the variables are conserved. Nodal points are selected within the domain such that each nodal point is surrounded by a cell. The physical boundaries and the control volume boundaries coincide with each other by arranging the control volumes along the boundaries of the domain as shown in figure 2.2 (b). For any given node 2, the left and right adjacent nodes are denoted as 1 and 3 for a 1D domain. L and R represent the left and right faces of the control volume respectively. In the 1D domain, δx_{12} and δx_{23} denote the distance of node 2 from the nodes 1 and 3 respectively. Similarly, δx_{L2} and δx_{R2} are given the distances from L and R faces to the node 2. The width of the control volume Δx is given by δx_{LR} . This Δx can be set to the size of a single biological cell or the total width of cluster of cells used in the mass points simulation model.

2.4.2 Discretization

In this step, a discretised equation is obtained at the nodal point 2 by integrating the governing equation over the control volume. Equation (2.3) takes the form of equation (2.4) for the control volume.

$$\int_{\Delta V} \frac{d}{dx} \left(D \frac{dC_g}{dx} \right) dV + \int_{\Delta V} S_g dV = \left(DA \frac{dC_g}{dx} \right)_L - \left(DA \frac{dC_g}{dx} \right)_R + \bar{S}_g \Delta V = 0 \quad (2.4)$$

Figure 2.2: Finite volume discretization of simulation grid. (a) A 3D finite volume cell with its faces and cell centres defined and (b) one-dimensional discretization of finite volume grid.

The terms A , ΔV and $\bar{S}_g$ represent the cross-sectional area, volume and averaged uptake over the control volume. From the equation, it can be seen that the generation of diffusive flux is given by the difference between the flux entering the left face and the flux leaving the right

face. This establishes a balance equation for flux over the control volume. The properties such as C_g and D are generally defined at the nodal points and a linear approximation is used to calculate the interface values and gradients. This approach is known as central differencing scheme and is a logical way to linearly interpolate the values to right and left faces. The linearly interpolated values for diffusion co-efficient at the right and left faces are given by,

$$D_R = \frac{D_3 + D_2}{2}, \quad (2.5)$$

and

$$D_L = \frac{D_1 + D_2}{2} \quad (2.6)$$

Diffusive flux at the right and left faces are calculated by,

$$\left(DA \frac{dC_g}{dx} \right)_R = D_R A_R \left(\frac{C_{g3} - C_{g2}}{\delta x_{23}} \right), \quad (2.7)$$

and

$$\left(DA \frac{dC_g}{dx} \right)_L = D_L A_L \left(\frac{C_{g2} - C_{g1}}{\delta x_{12}} \right) \quad (2.8)$$

In practical cases, the uptake term can be a function of a dependent variable and can be linearly approximated as,

$$\overline{S_g} \Delta V = (S_1 + S_2 C_{g2}) \quad (2.9)$$

With the above defined terms, equation (2.4) becomes,

$$D_R A_R \left(\frac{C_{g3} - C_{g2}}{\delta x_{23}} \right) - D_L A_L \left(\frac{C_{g2} - C_{g1}}{\delta x_{12}} \right) + (S_1 + S_2 C_{g2}) = 0 \quad (2.10)$$

By re-arranging, we get

$$\left(A_R \frac{D_R}{\delta x_{23}} + A_L \frac{D_L}{\delta x_{12}} - S_2 \right) C_{g_2} = \left(A_L \frac{D_L}{\delta x_{12}} \right) C_{g_1} + \left(A_R \frac{D_R}{\delta x_{23}} \right) C_{g_3} + S_1 \quad (2.11)$$

The above equation can be re-written with the corresponding co-efficients as,

$$a_P \phi_P = a_W \phi_W + a_E \phi_E + S_1, \quad (2.12)$$

where, ϕ_P , ϕ_W and ϕ_E represent the flux values at the cell center, west face and east face of the control volume and

a_P	$A_R \frac{D_R}{\delta x_{23}} + A_L \frac{D_L}{\delta x_{12}} - S_2$
a_W	$A_L \frac{D_L}{\delta x_{12}}$
a_E	$A_R \frac{D_R}{\delta x_{23}}$

2.4.3 Solution to the discretised equations

The formation of discretised equation at the nodal points is a key step to find the solution.

Boundary conditions are incorporated into the control volumes that are adjacent to the boundaries. This results in a set of linear algebraic equations that are solved by a suitable matrix solution method to obtain the distribution of flux.

Chapter 3. Emergence of Spatio-Temporal Variations in Chemotherapeutic Drug Efficacy: an *In-Vitro* and *In-Silico* 3D Tumour Spheroid Study

3.1 Introduction

Multicellular tumour spheroid models have become an invaluable *in-vitro* method for gaining insight into the interaction between tumour cells and their microenvironment. 3D spheroid models have been able to replicate the features of *in-vivo* tumour, such as activation of pathways upon cell-cell interactions[101], presence of concentration gradients within the tumour, and heterogenous microenvironment which can alter pharmacokinetics (PK) of the chemotherapeutic drug[102]. These systems provide a clear advantage over the classical monolayer or 2D culture systems which do not resemble the *in-vivo* conditions of tumour growth. Moreover, 2D cultures exhibit exponential cell proliferation and altered cell morphologies, thus preventing them from becoming reliable drug testing setup. Co-culture 3D spheroids can capture the cell-cell interactions, elevated levels of protein expression and physiological conditions [103, 104]present *in-vivo*, thus presenting themselves as a suitable candidate for drug screening and post-screening analysis.

Patra et al. [105] designed a high throughput microfluidic device capable of forming multiple (5000 numbers) uniformly sized spheroids in a semi-autonomous fashion. By combining the device with flow cytometry, it was possible to conduct drug testing and analysis on the formed 3D spheroids. Such devices could expedite drug screening and be a useful tool for tissue in chip studies. Sectioning of a drug treated spheroid could reveal the internal architecture [106] and changes in morphology of the cells due to drug effects. This could also aid in understanding the PK of the drugs and its effects on cell cytology. However, sectioning and staining of spheroids is a time-consuming process. There are methods [107, 108] proposed in literature, which help to overcome such tedious process. Anand et al. [107] proposed a real-

time FRET-based cell viability monitoring system for detecting apoptosis in a 3D spheroid. Using confocal microscopy, it should be possible to observe in real time the cells in the different stages of their life cycles at different layers inside the spheroid.

There exist a few concerns regarding, the use of 3D spheroid models for therapeutic screening: (1) size reproducibility of the spheroids, (2) factors (cell density, ECM) that affect mass transport of drugs, and (3) time spent in single sample testing or analysis. It has been reported that small variations in spheroid sizes could lead to large variations in the sensitivity of tumour cells to photodynamic treatment [109] and anti-cancer drug treatment [110]. West et al. [109] showed that the treatment sensitivity of the cells decreased by approximately 12 folds with a five-fold increase in spheroid size. This clearly shows that characteristics of spheroid systems are not scalable or directly extendable from a system of different sizes. Spheroids of larger sizes have also been shown [110] to be resistant to drug treatment. Therefore, if a drug test were to be carried out on a spheroid and proven successful, it does not necessarily imply that it works on a larger *in-vivo* tumour at the same dosage. In the same study, it was also observed that large spheroids were populated with more apoptotic cells than viable cells. This observation indicates that there are variations in the spatial distributions of different cell types and cell packing density within the spheroid. These variations could alter the drug diffusion kinetics and the uptake rate at different layers within the spheroid leading to a localized effect, which would otherwise be absent in the homogeneously packed small-sized spheroids. Further, spheroid systems that use alginate or agarose coatings [111-113] could lead to changes in transport phenomena and cytotoxicity within the spheroid due to imperfections in coating or non-uniform drying of the agarose material.

Spheroid models for drug screening are known to be tedious and time-consuming. Friedrich et al. [114] proposed that the time taken to run the complete protocol for a proper spheroid-based drug screening will be around 170 hours. The post screening analysis must be

carried out for the next 14 days in succession. Also, analysis must be carried out for different drug dosages, spheroids of different sizes, support material of different stiffness, and combinations of all the above in case of combinatorial drug tests. It is well known that drug concentration is not the only free parameter in the spheroid model, factors such as mass transport within the tumour, size of the tumour and local density of viable cells, can also influence the drug efficacy. Because of this inherent complexity, it is hard to separate and study the major contributors of drug efficacy and their impacts on the tumour environment through a single experiment or multiple experiments. In some cases, it is not even possible to tailor the experimental setups needed for drug testing with adequate accuracy, using existing 3D spheroid culture techniques, such as setups needed for studying the effects of variation in intra-tumoral mass transfer on the drug efficacy. One way to handle such complexities associated with interactions of the cells, variations in local densities and drug's mass transfer is through the use of *in-silico* models. If sufficient data on diffusion parameters, lethal drug concentrations and drug mechanisms are available, all major factors controlling tumour-drug evolution can be included into a computational model and simulated to predict the outcome of drug screening experiments,

Cellular automata (CA)-based models have been used to simulate 3D tumour growth [83, 115]. These models include the interaction between the tumour cells and their micro-environment. Alarcon et al.[115] proposed a cellular automata and finite difference-based hybrid model to describe the evolution of nutrient concentration. The cells were modelled to proliferate based on nutrient uptake. Jiang et al. [83] developed a multi timescale-based model combining lattice Monte Carlo and CA-based methods for predicting the avascular tumour growth. The predictions from such models resemble closely the growth of multicellular spheroids without any drug testing parameters. Other models such as cancer stem cell driven cell growth models [116, 117] and tumour shrinkage model [65] have also been developed for

simulating the tumour growth. These models have shown that the growth of multicellular spheroid can be captured using simpler reaction kinetics and force balances. However, numerical models, that are formulated based on experimental evidence or clinical data should be able to capture much more than just tumor growth. This aspect is currently inadequately addressed in existing literature.

In this study, we develop an experimental data driven numerical model capable of simulating drug response in spheroids. We interpret the drug chemotherapeutic mechanism to physically and stochastically definable parameters that can be directly implemented in the model. These parameters can be related to experimental observations and also can be quantified. The developed model enables us to analyse the spatially localized effects of the drug and the heterogeneity associated with a drug treated spheroid. By simulating multicellular spheroids, it should be possible to explore complex microenvironment settings, which would be otherwise not feasible to study using experiments. We consider the effects of two commercially available chemotherapy drugs, namely, *paclitaxel* and *cisplatin* on the spheroids of liver cancer HeLa cell line. We perform experiments of growth dynamics and drug testing on the 3D HeLa spheroids. Using the experimental data, the model is tuned for the cell proliferation rate and changes in cellular oxidative stress levels and cell cycles arising from drug actions. The model simulations are validated against the spatial effects caused by the drugs used in the experiments. The model is further used for exploring temporal effects of combinatorial treatments on the simulated and experimental spheroids.

3.2 Material and Methods

3.2.1 Cell Culture

HeLa-C3 cells were generated by transfecting HeLa cells with the C3 plasmid encoding for a FRET-based sensor caspase 3 (Luo). HeLa-C3 cells were maintained in Dulbecco's modified Eagle medium (DMEM, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine

serum (FBS, Hyclone, USA) and 1% penicillin/streptomycin (PS, Thermo Fisher Scientific). The spheroid cultures were maintained in DMEM with 20% FBS and 1% PS.

3.2.2 3D HeLa-C3 Spheroid Cell Proliferation Assay

The 3D co-culture was performed as previously described (Chiew). Non-adhesive round bottom 96-well plates (Sigma Aldrich, USA) were first pre-coated with 1% Pluronic-F127 (Sigma Aldrich) before seeding HeLa-C3 cells. The plates were then centrifuged at 1,000 g for 5 min in the Sorvall Legend XTR Centrifuge (Thermo Fisher Scientific) and left to incubate at 37°C and 5% CO₂. The medium was changed every day to ensure maximal oxygen and nutrient penetration into the spheroid. Prestobblue (Invitrogen) was added to the cells for each day and the absorbance was measured 2 h post addition of reagent. The radius of the spheroids were measured from 10 spheroid samples.

3.2.3 Chemotherapeutic Response with HeLa-C3 Spheroids

200 nM of paclitaxel and 10 µM of cisplatin were added to the spheroids 24 h after the seeding of 1,500 HeLa-C3 cells. The concentration of paclitaxel and cisplatin exposed to the spheroids were assessed every 24 h. Prestobblue was added to the cells and the absorbance at 570 nm and 600 nm were measured with the SpectraMax M5 Microplate Reader (Molecular Device) 2 h post addition of the reagent. For each individual well, the absorbance at 570 nm was subtracted by the absorbance at 600 nm to obtain sample readings before subtracting the average value of the control well.

G.G.Y.C designed and performed all the experiments and analysed the experimental data used in this study.

3.3 Model description

3.3.1 Tumour cell proliferation and apoptosis

We use a 151x151x151 3D lattice simulation domain in which each lattice site can be occupied by fixed mass of cells. At the start of the simulation, $t=0$, the domain is filled with a crucial

growth nutrient, glutamine [118], of concentration, C_g and diffusion coefficient, D_g . A cluster of cells is placed at the centre of the lattice domain. The cells consume glutamine at their lattice point at a rate r_c , which is dependent on its mass M_c , metabolic maintenance coefficient 'm', specific growth rate ' K_{cg} ', half saturation coefficient 'S' and yield coefficient 'Y' as described in equation (3.1). Time variation of glutamine concentration at different lattice points is modelled by the reaction-diffusion equation (equation (3.2)). In the corresponding experiments, we replace the spent glutamine each day with fresh glutamine from new medium. Therefore, glutamine is available in excess throughout the duration of the experiments. In a similar fashion, glutamine solute is modelled as a constant source boundary on all sides of the simulation domain. The open source finite volume method-based solver, FiPy [119] (3.1.1), is used to simulate the steady state reaction-diffusion of glutamine within the domain. The simulation time taken to reach steady state concentration is assumed to be one hour, after which cells proliferate, die or move. The cells uptake the surrounding glutamine and increase in mass after expending a part of the uptake proportional to the constant cell metabolic maintenance factor (m), governed by the Monod equations (3.1) and (3.3). It is assumed that the cell mass at a lattice site divides when the mass doubles its initial value ($M_{c,ini}$). At the instance after division, the daughter cell is placed at a random location within the radius of 2.5 lattice sites from the mother cell. If no space is available within the 2.5 lattice radius, then the cell mass will not divide. If the concentration of glutamine decreases to a level below the prescribed threshold ' C_{gmin} ', the cell can neither grow nor satisfy the metabolic maintenance cost, then the cell is assumed to undergo apoptotic stress damage ' d_t '. The stress evolves with time (equations (3.4) and (3.5)) until it reaches the critical apoptotic stress value ($d_{apoptosis}$). Cells having cumulative stress damage $d(t)$ higher than $d_{apoptosis}$ are declared as dead cells (equation (3.6)), which continue to occupy the same lattice site for four hours. After four hours (clearing time, c_t) the dead cells are cleared from the system, by allowing the cells directly above the

empty sites to move and occupy the space. This replicates the experimental and clinical observation of temporal shrinkage in tumour size.

$$r_c(C_g, M_c) = \frac{\left(\frac{K_{cg}}{Y} + m\right) M_c C_g}{S + C_g} \quad (3.1)$$

$$\frac{\partial C_g}{\partial t} = D_g \left(\frac{\partial^2 C_g}{\partial x^2} + \frac{\partial^2 C_g}{\partial y^2} + \frac{\partial^2 C_g}{\partial z^2} \right) - r_c \quad (3.2)$$

$$\frac{dM_c}{dt} = M_c * K_{cg} * \frac{C_g}{S + C_g} \quad (3.3)$$

$$d(t) = \sum_t d_t \quad (3.4)$$

$$d_t = \begin{cases} 1, & \text{when } C_g < C_{gmin} \\ 0, & \text{when } C_g \geq C_{gmin} \end{cases} \quad (3.5)$$

$$M_c = 0, \quad \text{when } d(t) > d_{apoptosis} \quad (3.6)$$

3.3.2 Effects of cisplatin on cells

Cisplatin is primarily considered to mediate cell death by targeting fast proliferating cells. It damages cellular DNA by cross linking the DNA of the cells to form DNA-cisplatin adducts (Dasari, siddik). The nucleotide excision repair (NER) pathway is activated in order to remove these adducts; however, upon failure of repair, cell apoptosis is triggered in mitotic cells and the dividing cells undergo cell death. Yet, recent studies have indicated that the cytotoxicity of cisplatin is not only limited to replicating cells. Cisplatin may elicit cell death independently of DNA damage via oxidative stress [120, 121] at three primary locations [122]. The first is the plasma membrane, where NOX induces reactive oxygen species (ROS) [123] which in turn trigger Fas aggregation [124] and influence the activity of membrane channels such as Ca^{2+} channels [123, 125]. Engagement and clustering of Fas receptor mediates caspase activation, while Ca^{2+} influx changes electrolyte capacities of the cells [123], which ultimately leads to

cell apoptosis. Second, cytoplasm produces cellular superoxide formation through the interaction of cisplatin with nuclear DNA, thereby activating an important regulator of cell proliferation, differentiation and survival, the MAPK pathway [122, 126]. The final and most important location is the mitochondrion, which generates free radical during oxidative phosphorylation and is one of the most important source of endogenous ROS [122]. Treatment of cisplatin in NER deficient cells displayed similar mitochondrial ROS generation as NER proficient cells, demonstrating cisplatin-induced mitochondrial ROS production regardless of the ability of cells to repair cisplatin-induced nuclear DNA damage [120]. At high doses of 10 μ M or more, loss of mitochondrial membrane permeabilization, activation of BAK and caspases [127] are triggered by superoxide formation induced by cisplatin [120, 124], which is effectively blocked by antioxidants MnSOD and glutathione [124, 128] and reactive oxygen species (ROS) scavengers [127].

$$\frac{\partial C_{cis}}{\partial t} = D_{cis} \left(\frac{\partial^2 C_{cis}}{\partial x^2} + \frac{\partial^2 C_{cis}}{\partial y^2} + \frac{\partial^2 C_{cis}}{\partial z^2} \right) - K_{intra} \delta_{cell} (C_{cis} - C_{intracis}) \quad (3.7)$$

$$\frac{\partial C_{intracis}}{\partial t} = K_{intra} \delta_{cell} (C_{cis} - C_{intracis}) - K_{bonding} \delta_{cell} C_{intracis} - K_{deg} \delta_{cell} C_{intracis} \quad (3.8)$$

$$\frac{\partial C_{adduct}}{\partial t} = K_{bonding} C_{intracis} \quad (3.9)$$

$$\delta_{cell} = \begin{cases} 0, & \text{where } M_c = 0 \\ 1, & \text{where } M_c > 0 \end{cases} \quad (3.10)$$

$$C_{adduct} = \begin{cases} 0, & \text{if } P_{rep}(t) < R \\ C_{adduct}, & \text{if } P_{rep}(t) \geq R \end{cases} \quad (3.11)$$

$$C_l = C_{cismax} \left(\frac{1}{A + B e^{-t_g}} \right) \\ \text{here, } t_g = (t_m - t_{cg}) \quad (3.12)$$

$$M_c = 0, \quad \text{when } C_{adduct} > C_l \quad (3.13)$$

The effects of cisplatin on the cells are numerically modelled by assuming that cancer cells experiencing oxidative stress suffer DNA damage upon treatment with cisplatin. DNA damage builds-up during the course of the treatment. Therefore, ROS formation in the model correlates with the concentration of drug used or the quantity of drug uptake by the cells. The drug uptake is modelled by equations (3.7) – (3.10), where A and B are constants. To model the uptake of cisplatin more accurately, cisplatin concentration is split into two parts, cisplatin concentration outside the cell or extracellular concentration (C_{cis}), with diffusion coefficient (D_{cis}) and concentration inside the cell or intracellular concentration ($C_{intracis}$) with passive diffusion rate constant (K_{intra}). The intracellular cisplatin degrades based on the degradation rate constant (K_{deg}). The rate of formation of DNA adducts (C_{adduct}) is assumed to be directly proportional to the intracellular concentration with a rate constant ($K_{bonding}$). The cells attempt to remove excess ROS via their endogenous antioxidant defence mechanism *in-vivo* and *in-vitro*. In accordance with these experimental observations, in the model, the cells eliminate the adducts formed based on the probability ‘R’ and $P_{rep}(t)$ as shown in equation (3.11). The cells in the model are assumed to repair themselves with equal probability ‘R’. Cells exposed to cisplatin respond differently based on their exposure times to the drug. Therefore, an apparent lethal concentration C_l is calculated based on equation (3.12), which is dependent on the cell’s cumulative generational age t_{cg} , maximum generational age for mutation t_m , and the age-independent maximum adduct concentration C_{cismax} as shown in equation (3.12). Cumulative generational age is the total existing time of the cell’s generation. The cumulative age is divided equally between the daughter cells at the time of division. In the model, we assume no mutation of the cells and therefore do not consider the effects of acquired drug resistance to cell lysis. Therefore, we include the maximum generational age parameter t_m , which imposes maximum probability of cell lysis for a long-lasting cell generation capable of mutating. This sets an

artificial time boundary after which none of cells are allowed to exist in the simulation and accumulate mutations. If the adduct concentration C_{adduct} is larger than the permissible lethal adduct concentration C_l , after all possible cell repair processes, the cells undergoes apoptosis. However, if the cell is able to overcome cisplatin cytotoxicity, *in-vivo* or *in-vitro*, with its NER pathway, and reduce ROS through its antioxidant defence, the cell mass divides with the oxidative stress level lesser than the lethal threshold. This phenomenon is modelled in such a way that both the daughter cell and the parent cell do not inherit any DNA damage, that is, C_{adduct} and C_{cis} are reset.

3.3.3 Effects of paclitaxel on cells

Cells uptake paclitaxel from their surroundings through passive diffusion [129]. Paclitaxel uptake is modelled by equations (3.14) – (3.16). Paclitaxel is separated into three different components based on its localization [130], concentration outside the cell C_{tax} , concentration inside the cell $C_{intratax}$ and concentration of paclitaxel bound to intra-cellular components C_{bound} . A part of extracellular paclitaxel binds to the proteins in the medium, which is quantified by the third term in equation (3.14), where, k_b is the rate constant for protein binding, B_m and K_p are the Michaelis-Menten constants for protein binding. The drug molecules remaining after protein binding move from the medium into the cells through passive diffusion with clearance c_l . Similar to the extracellular protein binding, the intracellular paclitaxel binds to saturable and non-saturable sites [ref] of the cellular components as quantified by the second and third terms in equation (3.15) respectively, where B_{mc} and K_{pc} are the Michaelis-Menten constants for cellular component binding and N_s is the rate constant for binding to non-saturable sites. The bound paclitaxel (C_{bound}) then acts on the cells in their G2/M phase by stabilising the microtubules. This causes mitotic arrest of the cells at the spindle checkpoint [131], resulting in cell apoptosis. There are reports [132, 133] on the formation of multipolar spindles in the cells post paclitaxel treatment. It is important to note that paclitaxel

is found to affect only the cells that have undergone mitosis in the presence of drug and the cells that are in their interphase during the drug treatment. Similar to the progressive, concentration based, lethal effects of cisplatin on the cells, the activity of paclitaxel is dependent on the intracellular concentration levels of the drug [134]. To mimic these mechanisms of actions, in our simulation model, paclitaxel is assumed to affect only the parent cells that have divided in the presence of the drug. This assumption is applicable through the generation of the cells, meaning, the daughter cells from a cell division will be affected by the drug only if they have been exhibited their G2/M phase in the presence of the drug. This assumption makes sure that the effects of the drug do not carry over to the future generations after cell division as observed by (Gascoigne). Cells in the simulation whose mass is higher than 1.5 times the initial mass of cell ($M_{c,ini}$) are tagged as cells in their G2/M phase (G_{2cell}). Cell apoptosis is modelled in a stochastic fashion in accordance with experimental observations (Gascoigne). Cells in the simulations are chosen for apoptosis only if their bound paclitaxel concentration is above lethal paclitaxel concentration ($C_{leathal}$) and the chosen cells are declared dead if the probability of cell survival ($P_{sur}(t)$) is lower than the fixed cell apoptosis probability (R_d).

$$\frac{\partial C_{tax}}{\partial t} = D_{tax} \left(\frac{\partial^2 C_{tax}}{\partial x^2} + \frac{\partial^2 C_{tax}}{\partial y^2} + \frac{\partial^2 C_{tax}}{\partial z^2} \right) - c_l \delta_{cell} (C_{tax} - C_{intratax}) - k_b * \frac{B_m}{K_p + C_{tax}} * C_{tax} \quad (3.14)$$

$$\frac{\partial C_{intratax}}{\partial t} = c_l \delta_{cell} (C_{tax} - C_{intratax}) - k_b * \frac{B_{mc}}{K_{pc} + C_{intratax}} * C_{intratax} - N_s * C_{intratax} \quad (3.15)$$

$$\frac{\partial C_{bound}}{\partial t} = k_b * \frac{B_{mc}}{K_{pc} + C_{intratax}} * C_{intratax} + N_s * C_{intratax} \quad (3.16)$$

$$G_{2cell} = \begin{cases} 0, & \text{where } M_c < 1.5 * M_{c,ini} \\ 1, & \text{where } M_c \geq 1.5 * M_{c,ini} \end{cases} \quad (3.17)$$

$$M_c = \left\{ \begin{array}{l} 0, \text{ if } C_{bound} \geq C_{lethal}, P_{sur}(t) < R_d \text{ and } G_{2cell} = 1 \\ M_c, \text{ if } C_{bound} < C_{lethal} \end{array} \right\} \quad (3.18)$$

3.4 Results and discussions

3.4.1 HeLa-C3 cell proliferation profiles

Experimental quantification and validation of simulation profiles for proliferation of HeLa-C3 cells and increases in spheroid diameter are prerequisites for subsequent drug efficacy estimations. Hence, experiments were carried out using three different initial cell seedings, 1000, 1500 and 2000 cells and their proliferation rates are summarized in figure 3.1a. For cell seedings 1000 and 1500, the experimental results and simulated values are numerically similar to each other at different time points within acceptable error margins (approximately $\pm 5\%$). However, for initial cell seeding of 2000 cells, the mismatch of cell viability between the experiments and simulations is high. This is due to the fixed domain size introduced in the simulations, which artificially constrains the available free glutamine in the simulated medium. In case of experiments, glutamine is an excess solute. Due to the limited availability of glutamine for a large simulated tumor, the cell growth saturates. This numerical artefact is well reflected in the simulated diameters of the spheroids (Fig. 3.1b), where the diameter increase is linear at the start of simulation and saturates as the simulation progresses. Therefore, to avoid any artificial growth saturation effects, simulations and experiments with 1500 cell seedings are used in all drug and parameter estimation studies.

Figure 3.1: Cell proliferation in absence of drug treatment. Comparison of experimental data and simulation results for (a) normalized cell viability of HeLa-C3 cells, (b) spheroid diameter, (c) shape evolution of spheroids and (d) sectioned spheroids at different time points. Green spots in the experimental panel of (c) indicate live cells and cyan spots indicate dead cells.

The shape variations in tumor growth is summarized in figure 3.1 (c). Spheroids retain their spherical shapes with little localized distortions throughout the observation duration in both simulation and experiment. The green spots in the experiments panel, figure 3.1 (c), indicate live cells and cyan spots indicate cells undergoing apoptosis. The cyan spots are seen to increase at the core of the spheroids as time progresses suggesting the formation of necrotic core. The simulated spheroids in figure 3.1 (c) also exhibit a similar color profile to that of the experiments. However, because of variations in hue and translucency of simulated and experimental spheroids, sectioning of spheroids is necessary to properly visualize the formation of necrotic core in the spheroids. Sectioned and stained spheroids are shown in figure 3.1 (d) experiments panel. There are empty spots (white color) present in the sectioned spheroids in both the experiments and simulations. These spots indicate the absence of live cells at those positions, indicating improper packing of cells within the tumor. This improper packing can be a result of slow cell migration/motility to occupy a lysed cell's spot in the experiments and an outcome of the discrete clearing time c_t used in the simulations. In the sectioned simulation contours, the cells undergoing apoptosis, because of value interpolation, will have a value ranging from 3 ng to 0 ng color bars. The necrotic cells in the experimental images are indicated by white arrows. The dotted circle in the experimental and simulated sectioned images indicates the extent of necrotic zone in the spheroids. Necrotic core forms as a result of diffusion limitation of glutamine resulting from increased spheroid sizes at later stages of growth.

3.4.2 Spatial effects of cisplatin on HeLa-C3 spheroids

An initial cell seeding of 1500 cells was selected as the starting configuration for all simulations incorporating drug treatment. To avoid any undesirable artifacts arising from drug depletion due to cell uptake, cisplatin used in the experiments was replaced at the end of every day. The differential response of cells to various cisplatin concentrations is simulated using the

numerical model for cisplatin described in the model description. To avoid overfitting of the numerical model, most of the parameters used in the cisplatin simulations were adapted from experimental values in the literature. Only two values, lethal adduct concentration C_l and cell repair probability R , were free (adjustable) parameters in the simulation. These values were tuned through trial and error process with multiple simulation runs, to obtain the optimum parameter values that represent the experimental results. The comparison of experimental and simulated cell viability values is summarized in figure 3.2 (a). Cell viability values for cisplatin treated spheroids was always lower than the control values in both experiments and simulations. The simulated values of normalized cell viability are found to correspond to the experimental values at all time points as shown in figure 3.2 (a). To further understand the spatial variations of cell viability, the concentration contours of normalized adduct concentration are plotted at different time points as shown in figure 3.2 (b). The spatial adduct concentration is found to be randomly distributed throughout the spheroid at all time points. Peak adduct concentrations (red color) are observed at random locations within the spheroids and do not exhibit any localization effects.

Figure 3.2: Cell proliferation under cisplatin drug treatment. (a) Comparison of experimental data and simulation results for normalized cell viability of HeLa-C3 cells at different time

points, (b) Contours plots of adduct concentration in the cells at the centre slice of simulated spheroid at different time points and (c) Comparison of experimental data and simulation results for spatial activity of cisplatin at different time points. Green spots in the experimental panel of (c) indicate live cells, cyan spots indicate dead cells due to apoptosis alone and red spots indicate dead cells from apoptosis triggered by cisplatin. Green spots in the simulation panel of (c) indicate live cells and red spots indicate dead cells.

Three different fluorescence indicators were used in experiments to differentiate the effect of cisplatin and cell apoptosis in spheroids. The green spots in the experiments panel of figure 3.2 (c) indicate the live cells, cyan spots indicate apoptotic cells and red spots indicate cells affected by cisplatin. In figure 3.2 (c), from the experimental panel it is evident that drug activity increases with time progress as indicated by increase in red spots. Results summarized in the simulation panel are found to exhibit multiple dead cells (red color) at random locations within the spheroid. This observation is further confirmed by the microscopy images with color-coded cells in the spheroids as shown in the experiments panel of figure 3.2 (c). Red dots (cells affected by cisplatin) are spotted all over the volume of spheroids indicating the spatially randomized effect of cisplatin. From these observations it is clear that cisplatin effects are not localized to specific cell populations in the spheroid. It is also known that cisplatin's toxicity is more effective at higher concentrations, this means that cells exposed to higher cisplatin concentration should be affected earlier than those residing in low concentrations. From the simulations it is observed that the drug penetrates evenly throughout the volume of the tumor. This should mean that intracellular drug concentration and consequently the adduct concentration must be almost uniform throughout the spheroid, but the simulations do not conform to this logic, adduct concentration is not uniformly distributed within the spheroid. As seen in figure 3.2 (b) the distribution of adduct concentration within the spheroids is randomly distributed. Based on equations (3.7) – (3.9), the adduct concentration (C_{adduct}) is dependent

on the diffusivity of cisplatin, passive diffusion rate constant (K_{intra}) and degradation rate (K_{deg}). All these values are constants and should therefore result in uniform concentration profile development in the domain. A stochastic parameter that can drastically alter the distribution profile is the cell repair probability (R) listed in equation (3.11), which is an inherent property of the cell. This property enables efflux of any adduct formed post cell repair, thereby altering the distribution dynamics throughout the spheroid. Any spatially localized segregation of this property will result in preferential action or inaction of cisplatin on this local population. In fact, cell repair has been largely implicated in cisplatin drug resistance development [135-138]. In contrast to drug resistance development, cell repair property has been exploited to increase efficacy of cisplatin treatment in recent studies [136, 139, 140]. Cell repair property has also been implicated in pathological complete response of triple-negative breast cancers [141]. In cell populations without any abnormal cell repair characteristics, cisplatin will not exhibit any structurally preferential effects over the spheroid volume.

3.4.3 Spatial effects of paclitaxel on HeLa-C3 spheroids

4 same cell seeding of 1500 cells used in cisplatin simulations and experiments was selected as the starting configuration for taxol drug treatment. Taxol used in the experiments was replaced at the end of every day to avoid any artificial artefacts. Similarly the boundaries of the domain are fixed with constant concentration boundary conditions in the simulations,. Cell cycle is indirectly modelled in paclitaxel simulations by considering the cell's mass. Any growing cell whose mass is higher than 1.5 times its initial mass is considered to be in G2/M phase. Cell cycle phase is considered in the numerical model due to the mechanistic requirement of paclitaxel's activity as explained in the model description. All parameters used in the paclitaxel model simulations were adapted from experimental values in the literature except for two values lethal paclitaxel concentration C_{lethal} and the fixed cell apoptosis probability (R_d). Cell apoptosis probability is the same for all cells in the simulations.

Therefore, all the cells exhibit same levels of resistance to paclitaxel activity. The two values were tuned through trial and error process with multiple simulation runs, to obtain the optimum parameter values that represent the experimental results. The comparison of experimental and simulated cell viability values is shown in figure 3.3 (a). Cell viability values for the paclitaxel treated spheroids in experiments and simulations are found to be within their each other's variations and lower than the control values at all time points. Between cisplatin (figure 3.2 (a)) and paclitaxel (figure 3.3 (a)), paclitaxel is more effective with much lower cell viability values than cisplatin. To examine the spatial variations of cell viability, the concentration contours of normalized bound paclitaxel concentration are plotted at different time points as shown in figure 3b. From the contour plots it can be observed that as time progresses the fraction of cells with drug bound to cellular components decreases. This is because of the fast drug-induced apoptosis permeated by paclitaxel, as evident from the viability plots. Compared to cisplatin's adduct concentration (Fig. 3.2 (b)), paclitaxel's cell bound concentration appears to be more homogeneously distributed, barring a couple of hotspots at each time point. If paclitaxel's efficacy is solely dependent on its cell bound concentration, then it should induce apoptosis in all cells in the spheroid irrespective of their positions. However, the experiments and simulations panel of figure 3.3 (c) show that most of the cells along the periphery of the spheroid are affected. The experiments panel visually distinguishes the localization effect of paclitaxel and nutrient deficient cell apoptosis. Specifically, on day 4 a cyan core can be observed at the centre of the spheroid surrounded by concentric layers of red cells affected by paclitaxel. Similar to experimental observations, simulation images also show preferential action of paclitaxel along the periphery of the spheroids. To understand the reason behind such spatially preferential action of paclitaxel, the mechanism of paclitaxel action should be carefully considered. As discussed in the model description, paclitaxel only affects the cells that have previously undergone mitosis and are in G2/M phase of cell cycle. Cells that are

present along exterior layers of the spheroids have a high likelihood of undergoing multiple cell divisions due to ready availability of glutamine in the surrounding. This increased frequency of cell mitosis at the periphery will result in high probability cell apoptotic induction by paclitaxel. Thus, the efficacy of paclitaxel is affected by the nutrient concentration available to local population. The response of cells to paclitaxel is, therefore, not only dependent on the quantity of drugs bound to the cells, but also on their mitotic history and current cell cycle.

To compare and contrast the spatial effects of cisplatin and paclitaxel on the cells in the spheroid, the simulated dead to live cell ratio should be quantified. The spatial domain is binned into twelve areas. These areas occupy the space between concentric circles with the increase in radius by one grid. Ratio of dead cells to live cells present within these binned areas is plotted as a heatmap at different timepoints as shown in figure 3.3 (d). For uniform representation, empty areas, (where, $\text{dead/live} = 0/0$) a value of 0.0 is used. At all timepoints, cisplatin is found to exhibit a homogenous dead to live cell distribution compared to paclitaxel. In both cisplatin and paclitaxel, the core of the spheroid (bin 1) is found to have a value of 0.0 indicating absence of dead cells, suggesting absence of necrotic core until day 4 because of decreased cell viability in the drug treated spheroids compared to control. From experimental data and simulation results we know that paclitaxel preferentially affects the cells in the periphery. This is once again confirmed by the appearance of dark patches at day 2 along zones 8,9 and 10 in figure 3.3 (d) for paclitaxel. In addition to the local segregation of paclitaxel effect, a remarkable pattern occurs in paclitaxel treated spheroids with progress of time. This pattern is the inward shifting of high efficacy zones (dark color) as the simulation progresses. Paclitaxel shows maximum efficacy in zones 8,9 and 10 on day 2, in zones 6,7,8 on day 3 and in zones 5 and 6 on day 4. These results show that paclitaxel affects the spheroid in a concentric manner with progress of time. Such a pattern emerges from the interplay of newly exposed layer of cells to the incoming nutrients and the binding action of paclitaxel. Excess glutamine promotes cell

proliferation, while bound paclitaxel increases the probability of apoptosis induction in such frequently dividing cells.

3.4.4 Temporal effects of drug scheduling on HeLa-C3 spheroids:

The unique spatial effects of cisplatin and paclitaxel assert that each drug has its own preferential sites of action where its efficacy is high. If these two drugs are used in a combinatorial manner and if the overlap of their high efficacy zones are minimized, then the outcomes from such combinations should surmount the efficacy of individual drug treatments. In fact, such combinatorial treatments are quite common for treating carcinoma patients. However, most of their combinatorial effects have been attributed to synergistic effects arising from the action of drugs in tandem. In addition to these synergistic effects, spatial effects must play a crucial part in increased efficacy of combinatorial treatments. For instance, consider an alternating paclitaxel-cisplatin treatment schedule. In this treatment schedule, initially the cells are treated with paclitaxel for a fixed duration followed by cisplatin treatment for the same fixed duration and the cycle is repeated. According to our findings, paclitaxel should first affect the outer layers of the tumor. Then, a lag sets in for inward paclitaxel effect propagation, since the cells in the layer which is in immediate proximity should take their time for proliferation. Introduction of cisplatin during this lag interval will help eliminate other cells in the tumor and also give the required time for cell proliferation. Subsequent paclitaxel treatment should result in immediate elimination of the penultimate layer, thereby making the combinatorial process highly efficient. Therefore, to analyse these temporal effects, in addition to the studies on spatial effects of the drug on HeLa-C3 spheroids, the temporal effect of combinatorial drug treatment was studied experimentally and numerically.

Figure 3.3: Cell proliferation under paclitaxel (taxol) drug treatment. (a) Comparison of experimental data and simulation results for normalized cell viability of HeLa-C3 cells at different time points, (b) Contours plots of bound paclitaxel concentration in the cells at the centre slice of simulated spheroid at different time points, (c) Comparison of experimental data and simulation results for spatial activity of cisplatin at different time points and (d) Heat map of ratio of dead to live cells found at different areas of spheroids treated with cisplatin and paclitaxel at different time points. Green spots in the experimental panel of (c) indicate live cells, cyan spots indicate dead cells due to apoptosis alone and red spots indicate dead cells from apoptosis triggered by cisplatin. Green spots in the simulation panel of (c) indicate live cells and red spots indicate dead cells. The color bar in (d) indicates the dead to live cell ratio.

The results from these studies are summarized in figure 3.4. Two different schemes of drug scheduling were implemented, alternating treatment of cisplatin and paclitaxel for (i) 24 hours and (ii) 12 hours. The schemes are further split into two strategies, (a) treatment initialized with paclitaxel and (b) treatment initialized with cisplatin. In figure 3.4 (a) and 3.4 (b) the tags, cisplatin/taxol(paclitaxel) indicate the treatment strategy and the time points indicate the scheduling. For example, a 24-hour scheme initialized with taxol (paclitaxel) will be as follows; day 1 – paclitaxel treatment, day 2 – cisplatin treatment (with paclitaxel removed) and day 3 – paclitaxel treatment (with cisplatin removed). The removal of the treated drug at the end of a treatment cycle ensures that treated drug effects are not carried over to the next cycle.

For the spheroid treatment initialized with paclitaxel, the cell apoptosis effects on day 2 are predominantly found along the periphery compared to the volume-spread effects of treatment initialized cisplatin as seen in figure 3.4 (a) (panel 48 h). After alternating the treatment on day 2, both cisplatin initialized, and paclitaxel initialized treatments are found to exhibit identical spatial effects (panel 72 h). This shows that irrespective of the initialization

strategy, the spatial effects should converge for alternating drug treatments of same duration. However, this result should not be generalized to the overall efficacy of the treatment strategy or the cell viability quantification. The results of cell viability are summarized in figure 3.4 (c). The experimental data and simulation results show that the average cell viability at the end of treatment (day 4) is lower for paclitaxel initialized treatment strategies than cisplatin initialized treatments. Thus, although the spatial effects converge with progress of treatment schedule, the efficacy of the treatments is dependant on the order of scheduling. The importance of schedule ordering arises from the fact that on day 1 most of the cells in the periphery are in their G2/M phase due to abundance of glutamine solute. This local segregation of G2/M phase cells aids in expediting the action of paclitaxel.

Figure 3.4: Summary of cell proliferation behaviours for different drug scheduling strategies. Experimental data/microscopy images of spheroids subjected to different treatment combinations at different time points for (a) 24-hour alternate scheduling and (b) 12-hour alternate scheduling, (c) Comparison of experimental data and simulation results for normalized cell viability of HeLa-C3 cells at different time points for 12-hour and 24-hour alternate treatment. The tags cisplatin and taxol in (a) and (b) indicate the initialisation drug or the drug used at the start of the scheduling after which the other drug is alternated and the cycle is repeated at different time points.

In contrast to the outcomes of 24-hour scheduling, 12-hour scheduling produces uniform spatial effects and cell viabilities irrespective of cisplatin or paclitaxel initialization as shown in figure 3.4 (b) and 3.4 (c). An additional phenomenon that occurs in the 12-hour scheduling is the drastic reduction in size of the spheroid with progress of time. From the viability plots, we observe that 12-hour scheduling is more efficacious than 24-hour treatment scheduling. Hence, the size decrease can be attributed to the expedited cell elimination produced by the 12-hour combinatorial treatment. The end points (day 4) of the simulated and experimental viability values for 24-hour treatment scheduling, are found to be in a close range, however the model overestimates the effect of cisplatin on day 3. Similarly, the 12-hour model simulated viability values follow a trend similar to the 12-hour experimental values. However, they quantitatively differ from each other, with the simulations underestimating the efficacy of the combinatorial treatment. This could arise from the missing numerical parameters for defining any synergistic effects that arise from such short alternation (12h) durations. Although, 12-hours scheduling produces highly efficacious treatment strategy, use of such scheduling strategy for clinical purposes could be restricted by toxicity and practicality considerations.

Table 3.1. Summary of model simulation parameters

Parameter	Notation	Value	Units
Concentration of glutamine (maximum)	C_g	2.0	mM
Diffusivity of glutamine	D_g	7.6×10^{-10}	$\text{m}^2 \text{s}^{-1}$
Initial mass of cell	$M_{c,ini}$	3.3×10^{-12}	kg
Metabolic maintenance coefficient	m	2.0×10^{-8}	s^{-1}
Max Specific growth rate	K_{cg}	8.75×10^{-6}	s^{-1}
Half saturation coefficient	S	0.1	mol m^{-3}
Yield coefficient	Y	100	
Critical apoptotic stress value	$d_{apoptosis}$	8 - 14	h
Threshold concentration of glutamine	C_{gmin}	0.6	mM
Cell clearing time	c_t	4	h
Maximum concentration of cisplatin	C_{cis}	10.0	μM
Diffusivity of cisplatin	D_{cis}	8.2×10^{-10}	$\text{m}^2 \text{s}^{-1}$
Diffusivity of cisplatin (Intra-spheroidal)	D_{cis}	1.78×10^{-11}	$\text{m}^2 \text{s}^{-1}$
Cisplatin degradation rate constant	K_{deg}	1×10^{-4}	s^{-1}
Cisplatin bonding rate constant	$K_{bonding}$	3×10^{-3}	s^{-1}
Maximum generational age	t_m	8	days
Maximum age-independent adduct concentration	C_{cismax}	2.8×10^{-3}	μM
Passive diffusion rate constant of cisplatin	K_{intra}	20.96	h^{-1}
Concentration of paclitaxel (maximum)	C_{tax}	200.0	nM
Diffusivity of paclitaxel (Intra-spheroidal)	D_{tax}	9.25×10^{-12}	$\text{m}^2 \text{s}^{-1}$
Diffusivity of paclitaxel	D_{tax}	4.26×10^{-10}	$\text{m}^2 \text{s}^{-1}$
Paclitaxel drug clearance	c_l	1.91×10^{-1}	s^{-1}
Lethal paclitaxel concentration	C_{lethal}	1.5×10^{-3}	nM
Drug binding rate constant of paclitaxel	k_b	3.0×10^{-6}	s^{-1}
Drug binding rate constant of paclitaxel to unsaturable sites	N_s	4.1×10^{-5}	s^{-1}
Michaelis-Menten constant for extracellular protein binding	B_m	3.94×10^{-3}	mol m^{-3}
Michaelis-Menten constant for cellular component binding	B_{mc}	5.92×10^{-2}	mol m^{-3}
Half saturation coefficient for extracellular protein binding	K_p	7.81×10^{-4}	mol m^{-3}
Half saturation coefficient for cellular component binding	K_{pc}	4.93×10^{-6}	mol m^{-3}

3.5 Conclusions

In this study, we examined the drug response of HeLa-C3 spheroids to the combinatorial administration of two commercially available drugs—cisplatin and paclitaxel. Our mechanistic model was validated by experimental findings that demonstrate the spatio-temporal effects of these drugs. From our studies, it is conclusive that it is the innate nature of paclitaxel to affect the cells along the periphery of the spheroid. In contrast, the capability of cisplatin was to influence the entire volume of the spheroid limited by the cell DNA repair mechanism. Although, there are multiple studies in literature which analyse the effects of various drug combinations, there is a lack of clear understanding of the localized spatial effects of multiple alternative drug scheduling. In our study, combinatorial scheduling (24 h) of the two drugs manifested in drastically different treatment outcomes for different drug ordering. Treatments (24 h) initiated with paclitaxel were found to be more effective than their counterpart due to the concentric inward penetrating effect of paclitaxel. The numerical model enabled us to quantify the effect of such spatial drug activity levels by providing a microscopic picture of the individual cells, their cycle phase, drug uptake, DNA damage and other parameters which cannot be observed in real time using laboratory experiments. Although, this numerical model was successfully validated using experiments on spheroids, the model was found to underpredict the treatment outcomes for alternation periods less than 24 hours. Such limitations arise from the presence of unknowns such as synergistic effects, which are needed to completely mimic the short-term temporal drug efficacies. Nevertheless, the developed model was capable of capturing single drug-cell interactions and 24-hour drug alternation treatments to qualify and quantify the combinatorial drug efficacy. Missing parameters such as hysteresis loops in PK of the drugs and synergy variables could be included in future models to improve the prediction capability of the simulations. Such in-silico models can be used as a test bed for predicting the drug treatment efficacies within practically acceptable accuracies. Improving the

model to accommodate for human physiological and tumour micro-environmental parameters will pave way for precise patient specific chemotherapy.

Chapter 4. Shear Stress Enables Quantitative Increase in Invadopodia Structure Formations Through Enhanced Tks5 Phosphorylation

4.1 Introduction

Metastasis has been the cause of majority of fatalities in cancer patients [142]. About 90% of cancer-related deaths have been known to occur from metastatic complications [143]. The spread of cancer from the primary tumor site to distant parts of the body generally involves two crucial steps [142]. The first step is the transport of tumor cells from the primary site through the blood vessels. During this transport, circulating tumour cells (CTCs) are subjected to multiple physical and biological perturbations. These perturbations include fluid shear stress, cytotoxicity of immune cells and anoikis. Only a minor fraction of the CTCs overcome these perturbations, while others are eliminated from circulation. The perturbations induce phenotypical changes in surviving cells that make them more invasive. CTCs have been shown to express different apoptotic suppressor proteins such as PD-L1 [144], survivin [145] and HER2 epidermal growth factors [146]. In addition to variations in extracellular protein expression levels, the CTCs also undergo changes in their intracellular survival pathways. Afu et al. showed that fluidic shear kills tumor cells by generating cellular reactive oxygen species and the antioxidant enzyme manganese superoxide dismutase (MnSOD) protects the cells during circulation by disseminating ROS [147]. The cells with high level of MnSOD were also found to be resistant to chemotherapeutic treatment using doxorubicin. Ma et al. [148] further elucidated the complicated role of MnSOD in cellular response to shear stress. Fluidic shear stress was found to generate mitochondrial superoxide which is able to induce cell apoptosis. MnSOD protects circulating tumor cells by converting superoxide to hydrogen peroxide which is less toxic to cells. Moreover, the hydrogen peroxide can diffuse through mitochondrial membrane into cytosol to activate ERK1/2 and FAK, hence stimulate tumor cell migration and adhesion. The second step of metastasis is the extravasation of CTCs into a distant organ tissue

to form secondary sites. In most cases, the cancer cells at the secondary sites are genotypically and phenotypically different from their parent sites [25, 26]. Intertumor heterogeneity arises from the mutations acquired by the CTCs during their circulatory phase. Intertumor heterogeneity further complicates the adjuvant chemotherapy administered to a patient. Due to genotypical and phenotypical variations between the primary and secondary tumor sites, the sites may not respond to the same treatment strategy [149]. The CTCs extravasate to the distant organ tissue through active and passive processes [150]. The CTCs come in close proximity of distant organ tissues resulting from their arrest in the vasculature of the distant organ. This is a passive process. However, the CTCs move from the blood vessels into the stroma occurs in an active manner. CTCs that have survived harshness of the circulatory system have to be aggressive enough to penetrate through the basal membrane to reach the distant organ tissue. CTCs that successfully penetrate basal membrane have been shown to possess both biological and biophysical aggressiveness. Recruitment of platelets and subsequent recruitment of granulocytes by the platelets from the blood stream by the trapped CTCs can be considered as biological aggressiveness. The CTCs achieve the recruitment process by exploiting the chemotactic nature of platelets. The recruited platelets and granulocytes enhance the endothelial cell permeability and provide a safe cover for the tumor cells from immune system [50]. The next crucial step in extravasation of the CTCs is the restructuring of cell morphology to physically penetrate through the permeable endothelial cell membrane. A prominent structure that aids in the physical penetration of CTCs is the invadopodia. They are long tentacle-like projections that emanate from the cell membrane of the cells. Largely composed of actin filaments, these elongated projections are essential for CTCs' cell membrane extension into the extravascular stroma [151-153]. Invadopodia are one of the major contributors to the biophysical aggressiveness of CTCs. Formation of invadopodia can therefore be considered as a crucial milestone in CTCs metastasis journey.

The role of invadopodia in extravasation has been well established in literature [154]. In general, chemical factors such as p27^{kip1} [155], podocalyxin-like 1 (PODXL) [156] and lipoma preferred partner (LPP) [157] are proposed as the major influencers for invadopodia formation and tumor invasiveness. PODXL and LPP knockdown have shown to result in reduced invadopodia formation. All these factors have been shown to be major drivers of the stability of invadopodia in CTCs. In addition, these factors are all intracellular in nature and the extracellular factors (physical or biological), that regulate these factors are not well studied. In short, the physical precursor events that enable rapid formation and stabilization of invadopodia is still unknown. Numerous studies have focussed on the phenotypical and genotypical changes undergone by CTCs during circulation. However, the contribution of ambient fluid stresses, experienced by these CTCs, to the invasiveness of invadopodia structures remains unclear. In this paper, we hypothesize that hemodynamic shear stress promotes formation of invadopodia in CTCs. To test this hypothesis, we subject breast cancer MDA-MB-231 cells to varying shear stress levels similar to the study by Afu et al. [147]. These shear subjected cells are used to unravel the mechanisms underlying the mechanochemical precursors of invadopodia formation. We also develop a numerical model based on Glazier-Graner-Hogeweg method for simulating invadopodia formation in CTCs. The numerical model is further used to gain insight into the effect of physical, cell-level molecular-complex aggregates on the invasiveness of CTCs. Combining *in-vitro* experiments and *in-silico* modelling, we quantify and demonstrate the influence of hemodynamic shear stress on invadopodia formation.

4.2 Model description

The GGH method-based [70, 71, 158] model developed in the study was implemented using the open-source “CompuCell3D v3.7.9” framework [159, 160]. A 2D cancer cell was modelled as a collection of pixels occupying discrete lattice points in the two-dimensional GGH domain.

The cell in the domain was initialized with a volume of V_{cell} in all the simulations. The cells were modelled to be motile. Parts of the cell can move around the simulation domain through fluctuations on their membrane. These fluctuations were modelled to be governed by the GGH Temperature term or fluctuation amplitude term (T_m). In the simulations, cell's pixels were chosen at random and copied to a randomly chose neighbour lattice site, this action is termed as pixel-copy attempt. The success or failure of such copy attempts were determined by the Hamiltonian described in equation (4.1).

$$P\left(\sigma(\vec{l}) \rightarrow \sigma(\vec{l}')$$

Here, the energy arising from pixel copy event is ΔH , $\vec{l}'$ and $\vec{l}$ are the pixels contained in the cell σ in the simulation. In short, equation (4.1) minimizes the energy of the system with the progress of simulation. The larger the value of T_m , less energetically stable the system becomes and faster the membrane fluctuations occur in the simulation. Cell volume was constrained to the target-volume V_T as dictated by equation (4.2).

$$E_V = \sum_{\sigma} \lambda (V_{\text{cell}}(\sigma) - V_T(\sigma))^2 \quad (4.2)$$

$$\Delta E_v = E'_v - E_v \quad (4.3)$$

Here, λ is the volume potential, ΔE_v is the change in energy from energy associate with volume change during the copy attempt from E_v and E'_v . A connectivity constraint ΔE_p was introduced as a penalty parameter to ensure that the pixels of the cell were held throughout the simulations [94].

The actin-network inside the cell was modelled as an inverse binary field ‘ $f_a(\vec{i})$ ’ as shown in equation (4.4). The lattice points outside the cell were assumed to be devoid of actin polymers, hence the actin field was set to one at those lattice points.

$$f_a(\vec{i}) = \begin{cases} 0, & \vec{i} \in \sigma \text{ and } \alpha = 1 \\ 1, & \vec{i} \notin \sigma \end{cases} \quad (4.4)$$

Here, α denotes the lattice positions where actin polymerization had occurred. We developed a Lennard-Jones potential based model to determine the vectors of membrane-anchored actin-cortactin complex precursors. At the start of simulation, the membrane-anchored complexes ‘ N_m ’ were assumed to be randomly distributed throughout the cell membrane. The complexes were then allowed to perform random walk along the membrane. The random walk of precursor complexes were biased towards left half of the disc to better represent the *in-vivo*, vessel-oriented directional development of invadopodia. During the random walk, the complexes began to cluster based on the change in Leonard-Jones interaction potential (E_{LJ} and ΔE_{LJ}) defined in equations (4.5) and (4.6).

$$E_{LJ} = \sum_{N_m} \left(\left(\frac{r_m}{d_m} \right)^{12} - 2 \left(\frac{r_m}{d_m} \right)^6 \right) \epsilon \quad (4.5)$$

$$\Delta E_{LJ} = E'_{LJ} - E_{LJ} \quad (4.6)$$

$$P(m(\vec{j}) \rightarrow m(\vec{j}')) = \begin{cases} \left[\exp \left(-\frac{\Delta E_{LJ}}{T_{LJ}} \right) \right], & \Delta E_{LJ} > 0 \\ 1, & \Delta E_{LJ} \leq 0 \end{cases} \quad (4.7)$$

The probability of an actin-complex molecule’s movement from position $\vec{j}$ to $\vec{j}'$ is dictated by equation (4.7). Here, r_m is the distance of minimum potential between the

membrane-anchored complexes, d_m is the distance between the membrane-anchored complexes and ϵ is the minimum potential. The Leonard-Jones model was numerically solved at the start of every simulation to obtain the positions of the molecular complexes on the cell membrane. Once their positions were obtained, vectors starting from the center of mass of the cell passing through these positions were calculated.

$$\overrightarrow{r_{actin}} = \overrightarrow{X_{com}} + k \cdot \vec{j} \quad (4.8)$$

Here, k is a scalar, $\overrightarrow{X_{com}}$ is the vector position of centre of mass of the cell and $\overrightarrow{r_{actin}}$ is the vector of polymerised actin network through the cell. α is a subspace of the vector $\overrightarrow{r_{actin}}$.

To simulate the development of invadopodia from the cell membrane, a protrusion energy was introduced in the GGH model. This, potential was implemented similar to the chemotaxis potential of GGH model. Equation (4.9) and equation (4.10) describe the protrusion energy and protrusion energy change used in the model simulations respectively.

$$E_{pp} = \lambda_{pp} \left(f_a(\vec{i}) - f_a(\vec{i}') \right) \quad (4.9)$$

$$\Delta E_{pp} = E'_{pp} - E_{pp} \quad (4.10)$$

Here, λ_{pp} is the protrusion potential, E'_{pp} and E_{pp} are the protrusion energies during the copy attempt. Energy changes arising from volume change, protrusion formation and penalty factor were combined to obtain the Hamiltonian change ΔH of associated with the system as shown in equation (4.11).

$$\Delta H = \Delta E_v + \Delta E_{pp} + \Delta E_p \quad (4.11)$$

Pixel-copy attempts as many as the total number of lattice sites are carried out at every Monte-Carlo time step (MCS). The numerical simulations were carried out for a total of 1000 mcs.

4.3 Materials and Methods

4.3.1 Cell culture

Breast cancer cell line MDA-MB-231 was provided by Professor Xiaofeng Le, when he was working in the Department of Experimental Therapeutics at The M.D. Anderson Cancer Center, University of Texas. 231-C3 cell is engineered MDA-MB-231 cells which stably express CDP-DEVD-YFP proteins [161, 162]. 231-C3 cell has stable green fluorescence. All cells were cultured in DMEM (Thermo Fisher Scientific, USA) supplemented with 10% FBS (HyClone, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific, USA).

4.3.2 The microfluidic circulatory system

The microfluidic circulatory system was previously described in detail [148, 163, 164]. Briefly, a peristaltic pump (Ismatec, Germany) was used to generate pulsatile shear flow. Tumor cells were suspended in culture medium and circulated in a micro-tubing of 500 μm in diameter and 1.5m in length. SS was calculated based on Poiseuille's equation [165]: $\tau=4Q\eta/\pi R^3$, in which τ is the shear stress in dyne/cm², Q is the flow rate in cm³/s, η is the fluid dynamic viscosity (0.012 dynes s/cm²), and R is the tubing radius (250 μm). Control cells were seeded in 96-well plates coated with a solution of 1% Pluronic f127 (Invitrogen, USA).

4.3.3 Immunoprecipitation

Immunoprecipitation was performed using the Pierce Classic IP Kit (Thermo Fisher Scientific, USA). Details were described previously [163]. Briefly, the MDA-MB-231 cells were lysed using the IP Lysis/Wash Buffer with protease inhibitors (1:100). After pre-clearing, the lysate was incubated with primary anti-TKS5 antibodies at 4 °C for 24 h. The mixture was then loaded into the spin column containing pre-washed Protein A/G Agarose and incubated for 1 h with gentle end-over-end mixing. The agarose resin was washed with IP Lysis/Wash Buffer and conditioning buffer. Then, Elution Buffer was added to the column, and after 10-min incubation at room temperature, the column was centrifuged to collect the eluate. The isolated TKS5 protein was then used in Western analysis. Blot was probed with either rabbit anti-TKS5

antibody (Cell Signaling, USA) or rabbit anti-phosphotyrosine antibody, and then anti-rabbit secondary antibody.

4.3.4 2-D invadopodia assay

Add 100 µl of gelatin solution (0.2 mg/ml in PBS) into the 8-well chamber (Ibidi, Germany) and incubate under 37 °C for 2 h. The circulated MDA-MB-231 cells were seeded onto the 8-well chamber at 1000 cells/well after removing the gelatin solution. After 24-h incubation, the cells were fixed with 4% paraformaldehyde and stained with Phalloidin-rhodamine (Thermo Fisher Scientific, USA) to detect invadopodia. The number of invadopodia was counted and averaged as number/cell in each group and normalized to control group. The efficiency of the invadopodia detection using this assay was verified by immunofluorescence. In short, the fixed cells were probed with rabbit anti-Cortactin antibody (Cell Signaling, USA) and subsequently FITC labeled anti-rabbit secondary antibody, before staining with Phalloidin-rhodamine. Images were taken to evaluate the co-localization of the two fluorescence.

M.S designed and performed all experiments and analysed the experimental data used in this study.

4.4 Results and discussions

The invadopodia formation process is summarized in figure 4.1 (a). Shear stress affects the CTCs in circulation by either inducing apoptosis or promoting phenotypical and genotypical changes in the surviving cells. At the cellular level, invadopodia development happens in three simplified steps. Unstable actin-cortactin precursor complexes comprising of the major molecules, cofilin, N-WASp, Arp 2/3, actin and cortactin develop in the cytoplasm of the cells [166]. In the next step, the unstable precursor complex is stabilised by the binding of Tks5 molecules to the complex. Finally, Tks5 mediates the anchoring of the stable complex to cell membrane by coupling with the PI(3,4)P2 present on the cell membrane. The colocalizations of actin and cortactin indicating the presence of invadopodia formations in the experiments is shown in figure 4.1 (b). Any quantitative fluctuations in invadopodia formation in the cells

subjected external shear stress should arise from variations in the interplay of these steps involved in invadopodia formation.

Figure 4.1: Schematic representing the formation of invadopodia in CTCs and cellular level activities involved in formation of these structures.

Any effect of mechanochemical transductions of shear stress on a cell's invasiveness should reflect in quantitative changes in the number of invadopodia formations observed. To elucidate on the effect of shear stress on the formation of invadopodia structures, we adopted a widely used 2-D invadopodia assay to detect and quantify invadopodia formation, in which actin was stained and the actin enriched dot-like structures were identified as invadopodia. To examine the efficiency of this assay, we seeded MDA-MB-231 cells on gelatin-coated slides and stained actin, cortactin and nucleus. The microscopy images of these stained cells are

shown in figure 4.2 (a), in which actin dot structures co-localized well with cortactin. The cells circulated under various levels of shear stress were then subjected to the assay to quantify the invadopodia formations in the cells. The stained images are presented in figure 4.2 (b). From these images, it is clear that stable actin structures increase with increasing shear stress. To quantify this observation, a 2D-Invadopodia assay was used to count the exact number of invadopodia formed in the shear experienced cells. The variations in invadopodia count with respect to shear stress is shown in figure 4.2 (d). A significant increase in the number of protrusions with increasing levels of shear stress can be observed. Cells subjected to shear stress equivalent to hemodynamical shear (SS15) are found to exhibit about 2.5 times more protrusions than the cells devoid of any shear stress exposure. This observation suggests that shear stress promotes formation of invadopodia. The observed trend of protrusion increase cells with shear stress is qualitatively similar to the increase in ROS expression levels of shear-subjected MDA-MB-231 cells[147]. Hence, elevated ROS levels in shear-subjected cells can be hypothesised as promoters of invadopodia formation. A further examination of the influence of elevated ROS levels on invadopodia formation would require experiments on ROS suppressed shear-subjected cells. Therefore, two antioxidants, N-acetylcysteine (NAC) and propyl gallate (PG) were used to decrease the shear stress-elevated ROS level in the SS15 cells. The nucleus and actin of these cells were stained, and the corresponding microscopy images are shown in figure 4.2 (b). The microscopy images of antioxidant subjected cells show less stabilized actin structures than their counterparts. This visual observation is further confirmed by the quantitative results from the 2D-Invadopodia assay of antioxidant-subjected cells (fig. 4.2e). The protrusion count in antioxidant-subjected cells are significantly lower than the cells with elevated ROS. The protrusion counts in the antioxidant-subjected cells were however marginally higher than the protrusion counts of the cells devoid of shear exposure. Thus, the effect of antioxidants clearly suppresses the shear stress-enhanced invadopodia formation.

Figure 4.2: Experimental data for invadopodia formations in shear stress subjected cells. (a) Stained single cell microscopy images depicting colocalization of actin and cortactin, panel (b) shows actin enriched structures in cells subjected to different shear stress levels, panel (c) shows actin enriched structures in cells subjected to different shear stress levels and treated with antioxidants, (d) variations in count of invadopodia structures formed at different shear

stress levels and (e) variations in count of invadopodia structures formed at different shear stress levels treated with antioxidants.

To narrow down on the mechanism(s) or event(s) that actuate the shear stress promoted formation of invadopodia, numerical simulations were carried out using the developed GGH method-based model. Three major variational parameters were incorporated in the model. They include, r_m , λ_{pp} and N_m . These three model parameters can be related to three different physico-chemical processes occurring in the cell. They are,

1. The interaction range between precursor complexes governed by r_m
2. Actin network polymerisation rate characterized by λ_{pp}
3. Number of membrane-bound precursor complexes in a cell denoted by N_m

Simulations were carried out with a range of values for the three variational parameters. The initial shape of the cell in the simulation was assumed to be circular (disk-like). The simulations are able to capture the development of protrusions from the cell membrane as shown in figure 4.3. Multiple configurations of invadopodia formations were obtained from the simulations (figures 4.3 (a) and 4.3 (c)). These configurations are very similar to invadopodia structures observed in other experimental studies [167, 168]. Figures 4.3 (b) and 4.3 (d) show the corresponding actin network polymerization for invadopodia structures formed in figures 4.3 (a) and 4.3 (c). Although the simulations are capable of capturing the development of protrusions, the cells tend to exhibit unrealistic shape distortions at large parameteric values. Hence, it is important to restrict the parameteric values to recreate realistic protrusion development and restrict large morphological deviations.

Figure 4.3: Simulation results showing the development of protrusions from cells for different parametric values at various time steps. Lattice points occupied by the cell (red color) in the simulations with parametric values (a) $N_m = 10$, $r_m = 4$ and $\lambda_m = 10$ and (c) $N_m = 70$, $r_m = 4$ and $\lambda_m = 100$. Lattice points occupied by the actin-network field inside the cell (blue color) (b) and (d) in the simulations with the same parametric values as (a) and (c) respectively

To quantify the morphological deviations, a volume-distortion factor V_f based on the number of cell lattice points lying outside the initial cell configuration is introduced in the study as shown in equation (4.12).

$$V_f = \frac{V_e}{V_i} \quad (4.12)$$

Here, V_i indicates the number of lattice points within the initial configuration of the cell and V_e indicates the number of lattice points lying outside the initial cell configuration. The phase-diagram for volume-distortion at different parameteric values is shown in figure 4.4 (a). It can be seen that for parametric values of $N_m > 90$ and $\lambda_m > 100$, V_f values are larger than 0.25. This means that at these parametric values, more than 25 % of the cell volume at the end of simulations lie outside the initial configuration. These simulations are omitted from further consideration in the results summary. The change in landscape of the total number of invadopodia formations N_p at different values of λ_m is shown in figure 4.4 (b), (c) and (d). It can be observed that the trend of invadopodia formations remains similar at different values of λ_m . This means that the rate of actin polymerization does not significantly affect the number of protrusions formed irrespective of the other two parameters. In addition, it can be seen that the number of protrusions does not significantly vary at different values of r_m , given a fixed value of N_m . This means that the quantity of protrusions formed is predominantly influenced by the number of stabilised membrane-bound complexes.

Figure 4.4: Simulation results and experimental data showing the influence of membrane bound actin-cortactin complexes on invadopodia formation. (a) Phase-diagram showing the variations of volume distortion factor for different parametric values. Surface plot showing the variation of number of invadopodia formed for different values of N_m and r_m with fixed λ_m value of (b) (c) (d) (e) IP: anti-TKS5 Wb: anti-TKS5 (f) IP: anti-TKS5 Wb: anti-phosphotyrosine

1, (c) 10 and (d) 100. Western blot analysis of Tks5 precipitated from antioxidant treated, shear stress subjected cells using (e) anti-Tks5 antibody and (f) anti-phosphotyrosine antibody.

From the simulations, it can be concluded that increase in stable membrane-bound complexes will result in increased formation of protrusions from the cell membrane. Anchoring of stabilized actin-cortactin complexes to the membrane through Tks5 binding is the biological equivalent of the N_m parameter. Therefore, experiments were carried out to analyse the effect of shear stress elevated ROS levels on the qualitative variations in stabilised membrane-bound actin-cortactin complexes. Irrespective of shear levels experienced, the cells in experiments are found to express similar levels of Tks5 as shown in figure 4.4 (e). This observation suggests that Tks5 is not overexpressed in MDA-MB-231 cells subjected to hemodynamical shear stress. Following this observation, the search for mechanism behind the shear stress induced invadopodia formations can be narrowed down to the formation of stable membrane-anchored actin-cortactin complexes. A major event during the anchoring process is the phosphorylation of Tks5 in the actin-cortactin complex. Western blot analysis of Tks5 extract with phosphotyrosine antibody shows varying levels of Tks5 phosphorylation in control, SS15 cells and antioxidant treated SS15 cells as observed in figure 4.4 (f). The Tks5 phosphorylation levels are maximum for the shear stress (SS15) subjected cells and minimum for static MDA-MB-231 cells. Since shear stress subjected cells possess higher levels of phosphorylated Tks5, the probability of invadopodia formation in these cells tends to be higher than their counterparts. From the above experimental data and simulation results, the pathway for formation of large number of invadopodia structures in shear stress subjected cells can be summarized in four temporally consecutive steps. They include, shear stress promotion of ROS expression in the cells, ROS enhanced phosphorylation of Tks5, stabilization and anchoring of actin-cortactin complexes to the cell membrane by phosphorylated Tks5 and consequent formation of multiple invadopodia structures.

4.5 Conclusions

In this study, we examined the role of shear stress in heightened invasiveness of CTCs during extravasation. The GGH method-based model simulations suggest a dominant role of membrane-anchored actin-cortactin complexes in the rapid development of invadopodia. This observation is further confirmed by the experimental data. To avoid any unwarranted conclusions from the model artifacts of the simulation, results used in the simulation study are restricted to permissible levels of cell volume distortions. The numerical simulations aided in elimination of physico-chemical parameters such as actin polymerization rate and cell membrane complex aggregation from experimental considerations for the test of enhanced invadopodia formation. The simulation aided experimental design has helped identify Tks5 phosphorylation as a major driver in the rapid development of invadopodia from cells. Consequently, the elevated invasive behaviour of shear stress subjected cells can therefore be traced back to the Tks5 phosphorylation cycle. The choice of shear stress value used in the experiments (SS15) closely resembles the shear stress experienced by CTCs during their journey through the circulatory system. Therefore, the results presented in the study can be directly interpreted to a clinical setting, even though the developed simulation model cannot be completely translated to real world dynamics. The simulation model can be further improved by incorporating chemotaxis to predict the directional development of invadopodia. Since Tks5 phosphorylation has been identified as the crucial step in the invadopodia development pathway, it can act as a new therapeutic target to suppress CTCs' invasiveness.

Chapter 5. Effects of BRCA, P53 and RB on Ductal Carcinoma In-Situ to Invasive Ductal Carcinoma Transition – an In-Silico Analysis

5.1 Introduction

Mutations in the intraductal epithelial cells of the mammary gland result in unrestrained proliferation of the cells resulting in ductal carcinoma in situ (DCIS) [169]. This condition is classified as a non-invasive lesion. These epithelial cells are confined within the lumen and therefore are restrained from spreading outside the duct to the surrounding tissues. Further progression of DCIS and disruption of the basal membrane results in malignant condition known as invasive ductal carcinoma (IDC) [170]. After the onset of IDC, the cancer cells invade other parts of the breast tissue and turn metastatic. Transformation from benign DCIS to life threatening IDC has long been a subject of clinical research [171-173]. Studies have suggested that DCIS is a precursor for IDC and on average 40% of patients with DCIS subsequently develop IDC [174]. Luminal B1 tumors [175] have shown faster progression from DCIS to IDC than luminal A, triple negative and HER2 type tumors. HER2 positive tumors are found to transit from DCIS to IDC slower by staying in the DCIS state longer. Though, specific pathways and biomarkers for this transformation are yet to be discovered. Logullo et al. [176] analyzed epithelial to mesenchymal transition (EMT) markers for their association with DCIS to IDC transformation and found that c-met and TGF β 1 had positive association with the tumor transformation. However, most EMT biomarkers did not yield significant prognosis value. One important characteristic associated with IDC is the presence of intra-tumor morphological and genetic heterogeneity. “Evolutionary bottleneck” was suggested as an outcome of progression from DCIS to IDC by Cowell et al. [170], to explain the genetic heterogeneity observed in IDCs. Therefore, the transformation is a complex process involving multiple mutations resulting in a highly heterogenous tumor microenvironment. p53 overexpression [177] has been observed in both DCIS and IDC, with the overexpression

leading to lower mitotic index and apoptotic index in luminal cell phenotype and the opposite in stem cell phenotype. There are evidences [178, 179] suggesting that DCIS lesions and IDC tumors have deactivated retinoblastoma gene, ie., loss of Rb function and that the Rb pathway is a likely regulator of the transformations. Rb has also been shown to be a deciding factor in the recurrence of DCIS [179] through overexpression of p16ink4a. Mutations in the *BRCA* genes (the tumor suppressor gene widely associated with breast cancer) and the gene encoding p53 (*TP53*) have been found to occur together in both DCIS and IDC tumors [180]. Kumar et al. [181] studied the combined effect of the defects of these three major tumor suppressors genes, *RB*, *BRCA1* and *TP53*. They observed that simultaneous deactivation of these pathways resulted in formation of highly metastatic invasive breast cancer tumors in their mouse models and concluded that the pathways have a combinatorial effect on the progress and evolution of the tumor growth. The resulting tumors were found to have heterogenous morphology suggesting a product of “Evolutionary bottleneck” similar to studies of Cowell et al [170]. Thus, alterations in these three pathways would result in formation of highly invasive tumors and could possibly act as precursors in DCIS to IDC transformations.

Histological staining of tissue sections is traditionally used to study DCIS-IDC transformation, yet it is rather difficult to understand the complexities associated with the disease and it is less feasible to design experiments to track the progress of tumor evolution. Computational modelling of biological cells has become a useful tool in predicting the outcome of tumor growth, angiogenesis, and tissue morphologies in-silico [182, 183]. Multiscale agent based models have been accepted widely due to their versatility in handling multiple cell phenotypes and therefore tissue heterogeneity [184, 185]. Qiao et al. [186] developed an agent based model to simulate the proliferation of multiple myeloma tumor cells and analyzed the effect of drugs on the population of osteoclasts and osteoblasts. They modelled cells as agents and drugs as apoptosis inducers. The cells were modelled to undergo apoptosis at variable rates

based on the quantity of drugs they are in contact with. The effect of tyrosine inhibitor kinases (TKIs) on brain cancer was studied using a similar multiscale model by Sun et al [187]. They incorporated EGFR signaling in their model using partial differential equations to simulate various phenotypes observed in the tumor. The developmental stages of DCIS have also been simulated using agent based modeling. The model developed by Macklin et al. [188] was able to provide insight into the formation of necrotic core and calcification regions. Though these models can quantify the tumor proliferation and apoptosis, they lack proper energy based realistic cell allocations from mitosis or cell motility. These mesoscale interactions determine the spatial distribution and localized effects associated with the position of tumor cells. A better way of modelling the cells in an energy optimistic way with cell motility while retaining the individual characteristics of cells is through cellular potts modelling. Boghaert et al. [189] used CPM to simulate the progression of DCIS growth. Due to the inherent energy minimization principle of CPM, they were able to simulate four different forms of DCIS morphologies, namely micropapillary, cribriform, solid and comedo. These morphologies would not have evolved in other agent-based models due to the absence of localized energy interactions. However, the model was unable to predict behind the transition from DCIS to IDC and explain the reasons for the observed phenotypic heterogeneity in the clinical IDC sections. In this study, we use CPM to develop a model to elucidate on the transition from DCIS and IDC stage. We consider the three tumor suppressor pathways involving p53, Rb and BRCA to simulate the phenotypic variations associated with the underlying genotypic changes. We make valid assumptions based on available literature data to interpret the effects of genotypic changes into physical model parameters. By combining phenotypic changes, genotypic influences on cell proliferation and energy minimization principles we model the intraductal luminal epithelial cells and myoepithelial cells to elaborate the mechanism of DCIS to IDC transformation and the associated morphological heterogeneity.

5.2 Model description

Two basic cell types, epithelial cells and myoepithelial cells were modeled. The former cell type forms the inner layer and the latter stays along the periphery as shown in figure 5.1. Individual cells were modelled as collection of pixels on the simulation grid. The Glazier-Graner-Hogeweg (GGH) method-based model was implemented using an open-source software framework called CompuCell3D v3.7.5. The developed method is an extension of the model developed by Boghaert et al. [189] to simulate DCIS. The cells in the simulation are free to deform based on the energy constraints listed in equation (5.1) at each monte-carlo step (mcs). The cells deform at pixel level at each mcs [159]. The probability of pixel copy is defined by equation (5.1), where, $\sigma(\vec{i})$ denotes the pixel or lattice point occupied by a cell σ , $\vec{i}'$ represents the new lattice point where the pixel copy attempt is supposed occur, ΔH is the change in energy of the system and T_m is the temperature or fluctuation amplitude of the system. The pixel-level deformations, on a longer time scale, constitute the motility of the cells. Volume constraints were imposed on the cells to ensure that the cells stay within permissible levels of volume increase or decrease using equation (5.2). The term E_v denotes the volume energy of the cell, V_{cell} denotes the total number of pixels, V_T is the target volume and λ_v denotes the volume potential (similar to a spring constant) of the cell. A surface area constraint was introduced in the model to prevent the cells from evolving into biologically unreasonable shapes (Equation (5.3)). The terms E_s , S_{cell} , S_T and λ_s denote the surface energy, total number of pixels on the surface (perimeter in 2D), target surface area and the surface potential respectively. In addition, to restrict the cells from disintegrating and assuming fractured morphologies, a penalty function E_p was imposed [94]. This parameter retains individual cells as a single entity. The focal point plasticity (FPP) of the cell and contact energies between the cells are dictated by the equations (5.4) and (5.5). Here, l_{ij} is the distance between cells at positions i and j , L_{ij} is the target distance between the cells, and λ_{ij} is the FPP potential. We

used the physically equivalent values as suggested by Boghaert et al. [189], to simulate the cells' attraction-repulsion potential and adhesion forces. The energy arising from a pixel copy attempt ' H ' is therefore given by equation (5.6). We assume the cells to be spherical with a diameter of 15 μm .

$$P\left(\sigma(\vec{l}) \rightarrow \sigma(\vec{l}')$$

$$E_v = \sum_{\sigma} \lambda_v (V_{cell}(\sigma) - V_T(\sigma))^2 \quad (5.2)$$

$$E_s = \sum_{\sigma} \lambda_s (S_{cell}(\sigma) - S_T(\sigma))^2 \quad (5.3)$$

$$E_c = \sum_{i,j} J(\tau_{\sigma(i)}, \tau_{\sigma(j)}) (1 - \delta_{\sigma(i), \sigma(j)}) \quad (5.4)$$

$$E_f = \sum_{i,j} \lambda_{i,j} (l_{i,j} - L_{i,j})^2 \quad (5.5)$$

$$H = E_v + E_s + E_c + E_f \quad (5.6)$$

Each cell has three major biological parameters associated with it, the DNA damage level, oxidative stress, and proliferation potential. All these biological parameters were modelled as non-dimensional continuous variables from 0 to 1.0. Equation (5.6) describes the change in DNA damage level within the cells. Cells accumulate DNA damage ' d ' in a stochastic manner with a probability f_{dd} [190] as shown in equation (5.7). A_B indicates the expression level of BRCA. The proliferation potential ' p ' was assumed to decrease with increase in number of epithelial neighbors, since a crowded microenvironment would result in

less nutrient available for cell growth. To calculate the number of neighbors, we used a neighbor order of 1.5 times the cell radius for epithelial (N_e) and myoepithelial cell neighbors (N_m). Also, the proliferation potential was modelled to increase with DNA damage accumulated in the cancer cells. The increased proliferation potential of a DNA damaged cell can be considered as an inherent property of a mutated cell with higher survivability capacity [191]. Proliferation potential was calculated using equation (5.8). Where, β , k_{os} , C_{Rb} and O represent specific growth rate, oxidative stress coefficient, activity of Rb and oxidative stress respectively. The oxidative stress within the cells was assumed to increase from crowding of cell neighbors (decrease in nutrient availability) and the cell type of these neighbors [192]. Epithelial contribution to oxidative stress was considered to be negligible for a cell with fewer than 5 neighbors. The oxidative stress contribution from myoepithelial neighbors was also considered to reduce with increase in number of neighbors as dictated by equation (5.9). This assumption is based on the ready availability nutrient for epithelial cells that are located near myoepithelial cells, as observed by Norton et al. [67]. In equation (5.9), the constants ω_1 and ω_2 are the weighting fractions for oxidative stress contribution from the two types of neighbor cells, W is the maximum contribution to oxidative stress from the neighbors and ' a ' stress generation rate coefficient.

The gene activity and protein expression levels control the cell cycle by indirectly controlling the three major cell survivability parameters. *RB* functions as a tumor suppressor gene by being a negative regulator of cell proliferation. Cells which have suffered DNA damage or accumulated oxidative stress are arrested in their G1 phase by the activity of RB [193]. In our model, this gene activity is modelled by considering Rb (Rb protein or pRb) as a regulator of cell proliferation potential. Thus, in the simulation, Rb reduces and eventually prevents the proliferation of cells that have accumulated DNA damage and oxidative stress as

defined in equation (5.8). The extent of Rb effect is dependent on its activity potential in the cell ' C_{Rb} '.

In normal cells, the DNA damage must be limited to avoid run-off cell proliferations. Proliferation reduction in DNA damaged cells is handled by BRCA which is involved in DNA repair [194]. In cases of breast cancer, the inactivation of *BRCA* genes have been the major reason for DNA damage and accumulation of multiple mutations. We therefore modelled BRCA activity ' A_B ' as a modifier of the DNA damage levels in the cells as shown in equation (5.7).

If the DNA damage or oxidative stress in cells exceed a critical level, then apoptosis is initiated based on the lethal cell DNA damage level (d_l) and probability dictated by the effectivity of p53 (C_{p53}) [195] respectively. Cells with apoptosis index ' I_A ' values larger than maximum cell apoptotic index value ' I_{Ac} ' are marked for removal from the simulation domain. These cells are removed if they continue to express I_A values greater than I_{Ac} for more than ' N_A ' simulation time steps. Similarly, in case of mitosis, cells divide in simulation if the mitosis index ' I_M ' (equation (5.11)) is higher than the maximum cell mitotic index value ' I_{Mc} '

$$d = f_{dd} [1 - A_B] \quad (5.7)$$

$$\frac{dp}{dt} = \beta(1 - k_{os} C_{Rb} O + C_{Rb} d), \text{ with } \max(p) = 1.0 \quad (5.8)$$

$$\frac{dO}{dt} = \begin{cases} W[\omega_1 e^{-aN_m} + \omega_2(1 - e^{-a(N_e - N_{max})})], & \text{if } N_e \geq N_{max} \\ W[\omega_1 e^{-aN_m}], & \text{else} \end{cases}, \text{ with } \max(O) = 1,0 \quad (59)$$

$$I_A = O * C_{p53} \quad (5.10)$$

$$I_M = p * C_{p53} \quad (5.11)$$

The cells were allowed to evolve based on the constraints dictated by equation (5.6) throughout the simulation. The calculations based on activity level equations (5.7) – (5.11) were computed for every 100 mcs. This cycle ensures that the cells relax for sufficient time after their growth, thereby, assuring minimal energy state of the system. Each 100 mcs corresponds to 0.25 hours real time. The simulations were carried out for a maximum of 100000 mcs (250 hours) or until the maximum cell count in the simulations reached 1000, whichever event occurred earlier.

5.3 Results and Discussions

The model simulations with maximum levels (1.0) of A_B , C_{p53} and C_{Rb} must result in a normal ductal structure. Maximum levels of activity should prevent apoptosis evasion or proliferation runoff of the cells. These structures should possess a single layer of myoepithelial cells enclosing one or two layers of epithelial cell with minimal distortions to ductal morphology. Ensuring that the model establishes and maintains the above-mentioned structure throughout the simulation duration is the first step in verifying if the model assumptions represent *in-vivo* dynamics. As hypothesized, the simulations carried out with maximum expression levels are found to produce benign normal ductal structures as shown in figure 5.1 (a). The structure is found to be in a dynamic equilibrium. Meaning, the aging cells are removed with simulation progress and are replaced by new daughter cells. This cycle repeats itself throughout the entire simulation duration. The variation in formation of new cells and live epithelial cell count during the course of simulation for this case are shown in figure 5.1 (b) and 5.1 (c) respectively. The intertwined effects of apoptosis and mitosis can be seen to regulate the ductal level cell population homeostasis. Thus, the simulations capture the *in-vivo* co-operative effect of BRCA, RB and P53 on the regulation of ductal development and functioning. A cell-field plot of oxidative stress levels is presented in figure 5.1 (d). Oxidative stress levels are found to vary throughout the entire structure suggesting the absence of localized overcrowding in these

structures. The plots also capture the elimination of any cells that have accumulated critical oxidative stress. The major fixed parameters used in the study, W and β play a crucial role in establishing this mitosis and apoptosis balance. The value of W was chosen in such a way that in the absence of DNA damage and oxidative stress, on average the cells divide around every 17 hours. This proliferation duration is similar to those reported in experimental studies [196, 197]. β -value is an analog of W -value for oxidative stress, both these values were quantitatively set to be equal. Hence, the dynamic equilibrium established between the proliferation-apoptosis cycle is driven by the perturbations arising from the oxidative contributions of the neighboring cells. Figure 5.1 € shows the normalized duration since the last cell division, which closely follows the trend of oxidative stress levels with the cells. On an average all the cells in the simulation were replaced every 8500 mcs (21.25 hours). This novel dynamic replacement behavior is more realistic than previous DCIS models in literature [67, 189]. These models used a stochastic way of apoptosis, where in a cell in the simulation domain was picked randomly at fixed intervals and removed based on probability outcomes. These methods introduced a model artifact of ‘irreplaceable cells’ which are never removed from the simulation domain. Our approach eliminates these cells and closely mimics the *in-vivo* mechanism of replacement of damaged aging cells with new cells.

Figure 5.1: Model simulation results for normal ductal structure formation at maximum (1.0) A_B , C_{p53} and C_{Rb} values. (a) Panel showing spatial changes in ductal structure (white color and red color, denote the epithelial and myoepithelial cells respectively) at 0, 50000 and 100000 mcs, (b) temporal variations in number of new cell formations, (c) temporal variations in total

epithelial cell count, panel showing spatial variations of (d) oxidative stress and (e) normalized cell age at 0, 50000 and 100000 mcs.

The values of A_B , C_{p53} and C_{Rb} were varied from 0.25 to 1.0 in increments of 0.25 in a combined manner in the simulations. Figure 5.2 (a) shows the number of cases with less than 125 surviving cells at the end of simulations. These cases, in general, produce a ductal configuration very similar to the normal state, that is, a single layer of myoepithelial cells binding fewer than three layers of epithelial cells. The x-axis in figure 5.2 (a) denotes the cases where the values of A_B , C_{p53} and C_{Rb} were kept maximum (1.0). As observed, simulations with maximum values of C_{Rb} tend to produce most controlled growth of epithelial cells. Even though Rb was not modelled as a direct influencer of apoptosis, it is found to be a major driver of cell aging control. This simulation result also correlates with other experimental observations where lower Rb expression levels have been implicated with risk of ipsilateral breast event (IBE) [198] and DCIS to IDC transformations [178, 179]. It should be noted that there are other cases where even with maximum expression of Rb, the cells proliferated in an uncontrolled manner. This means that C_{Rb} is not the sole controller of apoptosis-mitosis equilibrium. To further unravel the intertwined effects of A_B and C_{p53} , 2D density plots of cell counts for various fixed values of C_{Rb} are used as shown in figure 5.2 (b), 5.2 (c) and 5.2 (d). It can be observed that for fixed values of $C_{Rb} = 0.5$ and 0.75 , reduced cell population levels are seen for C_{p53} value of 0.25 , irrespective of the A_B value. These values are lower than their counterparts with C_{p53} values 0.5 and 0.75 . This outcome is unexpected since a reduced C_{p53} value would mean more chances of cell survival as dictated by equation (5.10). Although counterintuitive, this observation can be attributed to the decreased levels of both apoptotic and mitotic indices of these cells (I_A and I_M) at minimum C_{p53} value. This results in a pathway-race between proliferation and apoptosis. Hence, for fixed values of C_{Rb} at 0.5 and 0.75 , effect of apoptosis

is more pronounced in simulations with C_{Rb} value of 0.25 than simulations with C_{Rb} values greater than 0.25. The above observations do not hold true for simulations with fixed C_{Rb} and C_{p53} values of 1.0 and 0.5 respectively. In these cases, the total live cell population is found to be lower than simulations with fixed C_{Rb} of 1.0 and C_{p53} values of other than 0.5. Interestingly, simulations with C_{p53} value of 0.5 are the only simulations with more than 150 cells at maximum C_{Rb} expression levels. Thus, the system produces non-linear response to C_{p53} effectivity levels and the outcomes are dependent on the interplay of all three parameters (C_{p53} , C_{Rb} and A_B).

Figure 5.2: Variation of cell count for different parametric values. (a) Number of cases with cell count less than 125 and maximum parameter values (1.0) Total cell count at the end of simulations for different values of C_{p53} and A_B and fixed value C_{Rb} of (b) 0.25, (c) 0.5 and (d) 0.75. The circle size indicates the number of cells, with maximum cell count (1000) corresponding to the maximum circle diameter.

Heightened levels of cell proliferation and survival alone cannot be considered as indicators of invasive transformations. Elevated proliferation and cell survival are characteristics of both DCIS and IDC tumors. To be characterized invasive, the epithelial cell populations should proliferate enough to fill the space between the ducts and penetrate the myoepithelial layer to invade surrounding tissues. The simulation results were examined for morphological differences arising from variations in the activity parameters. Four unique ductal structures were obtained from the parametric simulation studies. The structures are shown in figure 5.3 (a) - 5.3 (d). They can be classified as (1) normal ductal configuration, (2) ductal hyperplasia, (3) solid or comedo and (4) invasive ductal configuration. The spatial development of these configurations is shown in figure 5.3 (a) - 5.3 (d) respectively. In ductal hyperplasia structures (fig. 5.3 (b)), layers of epithelial cells are found to extend from the myoepithelial wall towards the ductal core. Such structures are generally categorized as benign *in-vivo*. However, they are considered as risk factors for breast cancer development. Further uncontrolled proliferation of cells in hyperplasia structures will result in formation of solid or comedo structures such as the one shown in figure 5.3 (c). These structures are categorized as high-grade DCIS. If left untreated these structures can progress to invasive ductal carcinoma. Figure 5.3 (d) shows the invasive ductal carcinoma formed in simulation. The major difference between solid and invasive structures is the presence of cells that have penetrated the myoepithelial layer. Invasive ductal carcinoma (IDC) structures are malignant in nature, after penetration, the IDC cells invade the local tissue and establish a population there. In most simulation cases, the penetration of cells is aided by the intraductal pressure or energy build up. This energy build up makes the myoepithelial chain unstable resulting in local adhesion failures and epithelial cell break away.

Figure 5.3: Ductal structures formed during the simulations. (a) Formation of normal duct structure with parameter values of $A_B = 1$, $C_{p53} = 1$ and $C_{Rb} = 1$, (b) formation of ductal hyperplasia structure with parameter values of $A_B = 1$, $C_{p53} = 0.25$ and $C_{Rb} = 0.75$, (c)

formation of solid structure with parameter values of $A_B = 1$, $C_{p53} = 0.25$ and $C_{Rb} = 0.5$ and (d) formation of invasive ductal carcinoma structure with parameter values of $A_B = 0.25$, $C_{p53} = 0.5$ and $C_{Rb} = 0.75$. The white spots indicate epithelial cells and red spots indicate myoepithelial cells.

Multiple structures can form for a single value of A_B , C_{p53} and C_{Rb} parameter in the parametric combinations. To better understand the contribution of each of these values towards formation of different ductal structures, figure 5.4 summarizes the number of instances of formation of various structures at different expression levels. Conclusively, all variational combinations are found to predominantly produce IDC structure. This means DCIS to IDC transformations are possible from all variations in C_{p53} and C_{Rb} . From simulation data, for the case of variations in A_B values, IDC transformations are possible only if coupled with variation in expression levels of other parameters. This observation suggests a promotional role for BRCA in IDC transformations rather than as an initiator. C_{Rb} is found to produce most of the normal ductal structures in the simulation at the maximum expression level. Therefore, Rb should act as a major restraint in DCIS to IDC transformations. In addition, minimum level $C_{Rb}(0.25)$ is found to solely produce IDC structures. This shows the crucial effect of C_{Rb} reduction on formation of IDC structures. The non-linear nature of the responses evoked from C_{p53} variations is further evident in figure 5.4. p53 effectivity level of 0.5 is found to produce solely of IDC structures. The number of structures produced at this effectivity level is even higher than the number of structures produced at a lower effectivity level of 0.25. As previously discussed, this phenomenon can be attributed to the pathway-race condition between apoptosis and cell proliferation.

Figure 5.4: Formation of various ductal structures for different values of A_B , C_{p53} and C_{Rb} . The color legend indicates various activity or effectivity levels of the simulation parameters.

5.4 Conclusions

The developed GGH model for simulation of intraductal carcinoma has captured the dynamics of development of various ductal structures. These structures include normal duct, ductal hyperplasia, comedo and invasive ductal carcinoma structure. Cells in the simulations were replaced periodically by new cells similar to *in-vivo* tissue cell population homeostasis. DCIS to IDC transformation is found to be initiated through variations in expression levels and effectivity of Rb and p53 respectively. The model simulations suggest a promoter role for BRCA in progression from DCIS to IDC. The model can be further developed to include myoepithelial cell flexibility. Inclusion of cell flexibility will help predict cell intravasation occurring during the process of intraductal overcrowding.

6. Conclusions and future works

Three novel research works have been presented in this thesis. All these works have dwelled on the common phenomenon of cancer progression in human body, disruption of homeostasis. In other words, the evolutionary behaviour of cancerous system at different phases of progression, subjected to external perturbations, has been explored in the studies. A unified simulation framework comprising of multiple modules for cell behaviour and physical field simulations has been developed in the thesis. The modules consist of GGH method-based model simulator (CompuCell3D), Finite volume solver (FiPy), grid-based cell simulator, Lennard Jones potential and simple numerical model solvers. From this unified simulation framework, chapter 3 employs grid-based cell simulator and FiPy, in a coupled manner to predict the spatio-temporal interactions between biological cells and their surroundings. In contrast, chapter 4 analyses the response of a single cell to its intracellular and membranal changes predominantly using CompuCell3d and Lennard Jones potential solver. Thus, the simulation framework is adequate for capturing tissue level events and the morphological changes occurring on a cell's surface. Chapter 5 introduces a GGH model for combining energy minimization principles with biological regulatory cycles and solves the model using CompuCell3D and simple numerical model solver. Thus, appropriate tools from the framework are used to understand and explore the perturbation and their effects on cancerous system at different spatial scales.

The three models discussed in the thesis examined three different biophysical phenomena occurring during the growth of a cancerous tumor. These phenomena are linked together by their effect on chemotherapeutic efficacy. The model simulating the intratumoral spatial efficacy of the drugs, cisplatin and paclitaxel, unravels the changes in the chemotherapeutic outcomes originating within a homogenous tumor mass. It successfully

analyses the heterogeneous mechanistic effects of the drugs on the local population of a tumor. Similar to this model, the DCIS model simulates the entire mass of a tumor and considers homogeneous cell populations. But, unlike the previous model, this model investigates the tipping point in benign to malignant transformation of tumors. The model elucidates on the genetic aberrations capable of altering the progression of tumor growth and consequently modifying the efficacy of any chemotherapeutic treatment. Thus, although the first two models simulate on the same scale (tumor-level), they simulate two different phenomena emanating from tumor – microenvironment interactions. The model on invadopodia formation simulates a single cancer cell to understand the effects of tumor microenvironment on the acquisition of invasive nature in circulating cancer cells. In general, after the development of invadopodia, the circulating cell extravasates to a new site in the body. This results in complete evasion of targeted chemotherapy by the cells extravasated to the new sites.

The developed models are not restricted to providing a high-level understanding of the biophysical interactions promoting chemotherapeutic efficacy manipulation. They also identify crucial biological and chemical drug targets capable of enhancing efficiency of chemotherapy treatment. The first study identifies proper alternating drug scheduling strategy to eliminate the tumor mass in minimal treatment duration. This model suggests the use of drug combinations based on the shape and size of tumor at different time points to attain maximum efficacy. The model on DCIS transformation identifies RB as an initiator of aggressive DCIS proliferation and consequent metastases to the surrounding tissue. Targeting the activity of RB in cancer cells will promote proper tumor growth suppression and prevent chemotherapy evasion of cancer cells. Invasiveness of cancer cells is also studied in the invadopodia model. The model results suggest that TKS5 is crucial for enhanced formation of invadopodia by circulating tumor cells. Thus, TKS5 suppressors/inhibitors will act as deterrent of aggressive cancer cell metastases. Overall, the developed models have made significant contribution to the

understanding of chemotherapeutic effects at a local population level and control of evasion strategies used by cancer cells for their life cycle progression.

There are a few limitations of the models described in this thesis. The drug scheduling model lacks a numerical formulation to define drug transportation due to convection. Convective transport is crucial in vascular tumors which grow aggressively in nature. DCIS model is currently simulated in a 2D domain, transition to 3D is necessary for extensive validation against experimental evidence. Although, 2D model should suffice for analysing tumor positioned a center of ducts, tumors marginalized toward the ductal orifice will exhibit significantly different dynamics based on nutrient presence.

In the future, the single cell invadopodia model can be coupled with the DCIS model to create a multiscale model to understand the intravasation process. Such a model can contribute to the understanding of the process of the breast cancer cells metastasizing to lung tissues. It will also help in estimating the rate of successful intravasation in sparse young tumors compared to aggressively growing tumors. This quantification can then be used to provide patient-specific therapy suggestions to oncologists. The standalone DCIS model can be used to analyze the mutation status and expression levels of BRCA, Rb and p53, to predict the disease progression and survival of patients. Further incorporation of drug scheduling model with DCIS model will enable virtual-testing of drugs in a DCIS – IDC setting. Drugs that directly target BRCA, Rb and p53 or inhibit the related pathways have been approved for breast cancer treatment, such as Palbociclib, Ribociclib and Olaparib. Based on the mutations and expression data of the genes, the developed model can help clinicians determine the proper usage of related drugs, including dosage and drug combinations.

7. References

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