Mathematical Modeling of Therapies for MCF7 Breast Cancer Cells

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In Genetics, Bioinformatics and Computational Biology

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Abstract

Estrogen receptor (ER)-positive breast cancer is responsive to a number of targeted therapies used clinically. Unfortunately, the continuous application of any targeted therapy often results in resistance to the therapy. Our ultimate goal is to use mathematical modelling to optimize alternating therapies that not only decrease proliferation but also stave off resistance. Toward this end, we measured levels of key proteins and proliferation over a 7-day time course in ER-positive MCF7 breast cancer cells. Treatments included endocrine therapy, either estrogen deprivation, which mimics the effects of an aromatase inhibitor, or fulvestrant, an ER degrader. These data were used to calibrate a mathematical model based on key interactions between ER signaling and the cell cycle. We show that the calibrated model is capable of predicting the combination treatment of fulvestrant and estrogen deprivation. Further, we show that we can add a new drug, palbociclib, to the model by measuring only two key proteins, c-Myc and hyperphosphorylated RB1, and adjusting only parameters associated with the drug. The model is then able to predict the combination treatment of estrogen deprivation and palbociclib. Then we added the dynamics of estrogen concentration in the medium into the model and extended the short-term model to a long-term model. The long-term model can simulate various mono- or combination treatments at different doses over 28 days. In addition to palbociclib, we add another Cdk4/6 inhibitor to the model, abemaciclib, which can induce apoptosis at high concentrations. Then the model can match the effects of abemaciclib treatment at two different doses and also capture the apoptosis effects induced by abemaciclib. After calibrating the model to these different treatment conditions, we used the model to explore the synergism among these different treatments. The mathematical model predicts a significant synergism between palbociclib or abemaciclib in combination with fulvestrant. And the predicted synergisms are verified by experiments. This critical synergism between these Cdk4/6 inhibitors and endocrine therapy could reflect the reason that Cdk4/6 inhibitors achieve pronounced success in clinic trails. Lastly, we used protein biomarkers (cyclinD1, cyclinE1, Cdk4, Cdk6 and Cdk2) and palbociclib dose-response proliferation assays to
assess the difference between mono- and alternating therapy after 10 weeks of treatments. But neither the protein levels nor palbociclib dose-response show significant differences after 10 weeks of treatment. Therefore, we cannot conclude that alternating therapy delays palbociclib resistance compared with palbociclib mono-treatment after 10 weeks. Longer term experiments or other methods will be needed to uncover any difference. However, in this research we showed that a mechanism-based mathematical model is able to simulate and predict various effects of clinically-used treatments on ER-positive breast cancer cells at different time scales. And this mathematical model has the potential to explore ideas for potential drug treatments, optimize protocols that limit proliferation, and determine the drugs, doses, and alternating schedule for long term experiments.
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General Audience Abstract

Estrogen receptors are proteins found inside breast cancer cells that are activated by the hormone estrogen. Estrogen-receptor positive breast cancer is the most common type of breast cancer and accounts for about 70% of breast cancer tumors. Endocrine therapy, which inhibits estrogen receptor signaling, and Cyclin-dependent kinase 4 and 6 (Cdk4/6) inhibitors are the preferred first-line therapy for patients with estrogen receptor-positive cancers. We built a mathematical model of MCF7 cells (an estrogen receptor-positive breast cancer cell line) in response to these standard first-line therapies. This mathematical model can capture the experimentally observed protein and cell proliferation changes in response to various treatment conditions, including different drug combinations, different doses, and different treatment durations up to 28 days. The model can then be used to look for more effective treatment possibilities. In particular, our mathematical model predicted a strong synergism between Cdk4/6 inhibitors and endocrine therapy, which could allow significant reductions in drug dosage while producing the same effect. This synergism was verified by experiments. In addition to treatment methods where one drug or combination of several drugs is used continuously, we consider alternating among various therapies in a fixed cycle. The mathematical model can help us determine which drugs and which doses might be most appropriate. Since an alternating therapy doesn’t inhibit one particular target non-stop, the hope is that alternating therapies can delay the onset of drug resistance, where the drug becomes less effective or stops working completely. Unfortunately, an initial 10-week experiment to test for differences in resistance to a mono-therapy versus an alternating therapy did not show a significant difference, pointing to the need for longer experiments to see if alternating therapies can actually make a difference in resistance. Mathematical models will be important for determining the drugs, doses, and time intervals to be used in these experiments, as figuring out the best options by trial and error in such long-term experiments is not practical.
Dedication

To my parents.
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I would like to express my heartfelt gratitude to my collaborator Prof. Ayesha Shajaban-Haq at Georgetown University. All the experiment data were generated by her lab and this dissertation would not be possible without their support. Prof. Ayesha always raised valuable thoughts on the experimental and model results, and provoked me to think about the clinical significance of the mathematical modelling results. This not only enriched my knowledge of oncology, but also taught me how to a model can make tangible contributions to clinical treatments. I would like to thank her group members, Diane Demas, Isabel Conde and Yassi Fallah; the experience of working with them is precious to me.
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I would like to thank my colleagues from the GBCB program and friends I met in Blacksburg. I would like to express my special thanks to Ms. Dennie Munson, who gave me great help and support.

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Attribution

Chapter 2 of the dissertation is published research article. I presented the published work ‘Mathematical modelling of breast cancer cells in response to endocrine therapy and Cdk4/6 inhibition’, which is shown in Journal of the Royal Society Interface at https://royalsocietypublishing.org/doi/10.1098/rsif.2020.0339. Prof. Baumann from Department of Electrical and Computer Engineering at Virginia Tech co-authored this paper. He conceived of the study, oversaw the modelling and helped draft the manuscript. Prof. Shajahan-Haq from Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center co-authored this paper. She conceived of the experiments, oversaw the molecular laboratory work and critically revised the manuscript. Prof. Shajahan-Haq’s group members Diane M. Demas and Isabel P. Conde co-authored this paper. They carried out the molecular laboratory work and critically revised the manuscript.
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## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AF</td>
<td>Transactivation Function</td>
</tr>
<tr>
<td>AI</td>
<td>Aromatase Inhibitor</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>AP1</td>
<td>Activator Protein 1</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia-Telangiectasia Mutated</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>B-cell lymphoma-extra large</td>
</tr>
<tr>
<td>BCAR</td>
<td>Breast Cancer anti-Estrogen Resistance Protein 1</td>
</tr>
<tr>
<td>BIK</td>
<td>BCL-2 Interacting Killer</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast Cancer Gene 1</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent Kinase</td>
</tr>
<tr>
<td>CHK</td>
<td>Checkpoint Kinase</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>DYRK</td>
<td>Dual Specificity Tyrosine Phosphorylation Regulated Kinase</td>
</tr>
<tr>
<td>E2</td>
<td>Estrogen</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>ERCC</td>
<td>Excision Repair Cross-Complementation Group</td>
</tr>
<tr>
<td>ERE</td>
<td>Estrogen Response Elements</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-Regulated Kinase</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast Growth Factor Receptor</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead Box</td>
</tr>
<tr>
<td>GSK</td>
<td>Glycogen Synthase Kinase</td>
</tr>
<tr>
<td>HER2</td>
<td>Human Epidermal Growth Factor Receptor 2</td>
</tr>
<tr>
<td>HIPK</td>
<td>Homeodomain Interacting Protein Kinase</td>
</tr>
<tr>
<td>HR</td>
<td>Hormone receptor</td>
</tr>
<tr>
<td>IC_{50}</td>
<td>Half Maximal Inhibitory Concentration</td>
</tr>
<tr>
<td>ICI</td>
<td>ICI 182780/Faslodex/Fulvestrant</td>
</tr>
<tr>
<td>IGFR</td>
<td>Insulin-like growth factor receptor</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon Regulatory Factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Name</td>
</tr>
<tr>
<td>---------</td>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>mTOR</td>
<td>mechanistic Target of Rapamycin</td>
</tr>
<tr>
<td>Miz</td>
<td>Myc-interactin Zn Finger</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch Repair</td>
</tr>
<tr>
<td>NCOA</td>
<td>Nuclear Receptor Co-Activator</td>
</tr>
<tr>
<td>NEIL</td>
<td>Nei Like DNA Glycosylase</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa B</td>
</tr>
<tr>
<td>PDK</td>
<td>Phosphoinositide-dependent Kinase</td>
</tr>
<tr>
<td>PELP1</td>
<td>Proline, Glutamate and Leucine Rich Protein 1</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression-Free Survival</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-Kinase</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>Phosphatidylinositol 4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone Receptor</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin Homolog</td>
</tr>
<tr>
<td>RBL</td>
<td>Retinoblastoma-Like Protein</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular Endothelial Growth Factor Receptor</td>
</tr>
<tr>
<td>XBP</td>
<td>X-box-binding Protein</td>
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Chapter 1. Background and Introduction

1.1 Breast cancer and subtypes

Cancer is an important public health problem as it is the second leading cause of death in the United States [1]. Breast cancer is a commonly diagnosed cancer among women and there were approximately 268,600 new cases of female breast cancer in 2019 in United States [1]. Despite advances in treatment options, in United States it is estimated that 41,760 women died as a result of breast cancer in 2019, and it is the second leading cause of death among women after lung cancer [1].

Based on gene expression analysis and immunohistochemistry markers, breast cancer can be classified into different molecular subtypes. Distinct molecular portraits of breast tumors differ markedly in respect of therapeutic treatment responsiveness [2]. There are four main molecular subtypes that are distinguished using clinical evaluation of biomarkers for the estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and Ki67, which is an indicator of actively dividing cells. Hormone receptor positive (HR+) breast cancer denotes cells with ER, PR, or both. The Luminal A subtype is the most common, with breast cancer cells that are HR+ and HER2 negative (HER2-). In addition to being HR+, the Luminal B subtype is characterized by being positive for either HER2 or Ki67. The Luminal A subtype has low levels of Ki67, indicating slower cell growth, and is associated with the best prognosis. The Luminal B subtype tends to grow faster and the prognosis is slightly worse than the Luminal A subtype [1]. The Basal-like subtype, which is also called the triple-negative subtype, is HR- and HER2-. It is commonly found in patients with the BRCA1 (Breast Cancer Gene 1) mutation and it has a poorer prognosis than other subtypes as there is a lack of efficient treatments and specific molecular targets for this aggressive subtype. The last subtype is called HER2-enchriched and is HR- and HER2+. This subtype grows faster than the Luminal subtypes and used to have a worse prognosis. But widespread targeted therapies aimed at inhibiting HER2 have substantially improved the therapeutic outcomes [3].

1.2 ER+ breast cancer and treatments

ER+ (Estrogen Receptor Positive) breast cancer, which belongs to the Luminal subtype, is present in approximately 70% to 80% of all breast cancers [4]. There are two major ERs, ERα and ERβ.
ER+ breast cancer commonly denotes breast cancer cells expressing ERα. ERβ is less well characterized [5]. Estrogens are the regulators of ER+ breast cancer cell growth. They are steroid hormones, with the most potent estrogen being 17β-estradiol (E2) [6]. The main effect of E2 is growth maintenance and reproduction through the regulation of various processes, including cell proliferation and survival [7, 8]. E2 exerts its effects by binding to nuclear receptor ERα [9]. ERα is frequently overexpressed in breast cancer cells, and its gene is the major oncogene responsible for E2-induced enhancement of cell proliferation [10]. In the classical signaling mechanism, E2 binds to ERα (E2:ERα) in the cytoplasm, producing conformational changes that result in homodimerization, translocation to the nucleus, and binding to estrogen response elements (ERE) in the promoter region of estrogen-responsive genes to regulate transcription [10]. In addition to the classical mechanism, there are three other ways ERα can exert its gene regulation activity. First, E2:ERα can bind to other transcription factors by protein-protein interaction in the nucleus [6]. Second, ERα not bound to E2 can be phosphorylated by a sequence of signaling events from receptor tyrosine kinases (RTKs). Growth factors, such as insulin-like growth factor (IGF) and epidermal growth factor (EGF), can activate RTKs, which in turn can activate extracellular signal-regulated kinase (ERK) and protein kinase B (AKT). After that, ERα itself can be phosphorylated by these serine/threonine kinases leading to estrogen-independent activation of ERα [6]. Third, estrogen binding to membrane-bound ERα results in assembly of a protein complex that activates protein kinase cascades leading to transcription factor activation [10].

Because ERα signaling is central to ER+ breast cancer cell proliferation and reproduction, endocrine therapy, which interferes with ERα signaling, is widely used to treat the ERα+ clinical subtype [11, 12]. Its use has resulted in dramatic improvements in long-term survival rates. Endocrine therapies are targeted therapies that can improve survival while avoiding the toxicity of traditional chemotherapies. They act to decrease ERα signaling in a variety of ways. First, by depriving ERα of its ligand E2 (e.g., inhibiting E2 synthesis using an aromatase inhibitor (AI)). AIs, like letrozole or anastrozole, are used in postmenopausal women for the treatment of breast cancer [13]. AIs lower the E2 level by inhibiting aromatase, which is an enzyme responsible for a key step in the synthesis of E2 [14]. Second, by competitively inhibiting the binding of E2 to ERα (the mode of action of tamoxifen). Third, by increasing the degradation of ERα via the proteasome (the mode of action of ICI 182780 (ICI; Faslodex/Fulvestrant)). The steroidal anti-estrogen ICI is
used in postmenopausal women. It competitively blocks E2 binding to the ERα and causes a proteasome-dependent degradation of the receptor [15]. Inhibition of ERα signaling halts cell proliferation by arresting the cell cycle at the G1 phase [16]. It has substantially improved in the treatment of ERα+ breast cancer and is widely used [12].

In addition to endocrine therapy, which has been the standard of care for patients with ERα+ cancer since 1970s, selective cyclin-dependent kinase 4 and 6 (Cdk4/6) inhibitors have become significant for the treatment of ER+/HER2- breast cancer [17]. Cdk4/6 inhibitors are notable for delaying cancer progression and improving survival in the Luminal A subtype. Adding these inhibitors to endocrine therapy has become the first-line choice of treatment based on the significant improvement in survival outcomes from clinical trials [17]. Currently, there are three Cdk4/6 inhibitors approved by the FDA (Food and Drug Administration): palbociclib (Pfizer), ribociclib (Novartis and Astex Pharmaceuticals) and Abemaciclib (Eli Lilly). In 2015, the FDA initially approved palbociclib in combination with the AI letrozole based on clinical trials showing substantially improved progression-free survival (PFS) in the palbociclib combined with letrozole arm compared to the letrozole and placebo arms [17]. In 2017, the FDA approved ribociclib in combination with AIs for ER+/HER2- breast cancers. Abemaciclib was also approved in 2017 for ER+/HER2- breast cancer treatment. Several clinical trials have proved the efficacy of the Cdk4/6 inhibitors in improving PFS in combination with AIs and ICI. These combinations have become the first line of therapies for ER+/HER2- breast cancer [17]. Moreover, according to the MONARCH 1 phase II abemaciclib single-agent clinical study, abemaciclib has been approved as a monotherapy in ER+/HER2- breast cancer patients who have progressed on prior endocrine therapy and chemotherapy [18]. These three Cdk4/6 inhibitors, with low IC50 (half maximal inhibitory concentration) values in nanomolar range, have brought remarkable improvements and became pivotal in ER+/HER2- breast cancer patient treatment as endocrine therapy.

1.3 Resistance in ERα positive breast cancer treatment

Although endocrine therapy and Cdk4/6 inhibitors substantially improved treatment outcomes for many ER+/HER2- breast cancer patients, not all patients have satisfactory responses to these two classes of targeted therapies. Acquired and intrinsic resistance are critical problems of these targeted therapies. Even in the ERα+ subtype, about 30% of patients exhibit intrinsic resistance to endocrine therapies, which means that these patients fail to respond to the initial therapy. Gene
signatures associated with intrinsic resistance to endocrine therapy have been reported [19]. ESR1 (estrogen receptor 1) gene mutation can cause intrinsic resistance, although it is rare [20]. Disruption of the mismatch repair (MMR) signaling pathway can also lead to intrinsic resistance. Defects of the MutL gene complex deactivate ATM (Ataxia-Telangiectasia Mutated) and CHK2 (Checkpoint kinase 2), cell cycle checkpoint proteins, causing loss of the capability to suppress Cdk4 activity when using endocrine therapy [20]. Loss of CETN2 (Centrin 2), NEIL2 (Nei Like DNA Glycosylase 2) and ERCC1 (Excision Repair Cross-Complementation Group 1) genes has been experimentally validated to contribute to intrinsic endocrine resistance in ERα+ breast cancer cell lines and ERα+ patient-derived xenograft models [21]. Non-functional cytochrome P450 2D6 is unable to convert tamoxifen to its metabolite, which induces intrinsic resistance to tamoxifen [10].

In addition to intrinsic resistance to endocrine therapies, 30% to 40% of ERα+ breast cancer tumors acquire resistance after long time exposure to endocrine therapy, so there is an initial response to endocrine therapy but relapse eventually occurs [22]. There are many mechanisms proposed to contribute to acquired resistance: loss of ERα and mutations of the ESR1 gene [22]; estrogen-independent activation of ERα [10]; increased activator protein 1 (AP1) [10]; increased NF-κB (Nuclear Factor Kappa B) [23]; overexpression and increased phosphorylation of NCOA3 (Nuclear receptor co-activator 3) [10]; deregulation of PELP1(Proline, Glutamate and Leucine Rich Protein 1) expression and localization [24]; increased EGFR [10]; increased HER2 [10]; increased IGFR [10]; activating mutations in PIK3CA (Phosphatidylinositol 4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha) [10]; loss or methylation of PTEN (Phosphatase and Tensin Homolog) [10]; upregulation of c-Myc [10]; upregulation cyclinD [10]; upregulation of cyclinE [10]; downregulation of IRF1 (Interferon Regulatory Factor 1) [10]; overexpression of XBP1 (X-box-binding Protein 1) [10]; overexpression of the Src family of tyrosine kinases [25]; overexpression of BCAR1,3 (Breast Cancer anti-Estrogen Resistance Protein) [26, 27]; loss of function of p21 (Cyclin-Dependent Kinase Inhibitor 1A) [28]; loss of function of p27 (Cyclin-Dependent Kinase Inhibitor 1B) [28]; inactivation of protein RB (Retinoblastoma protein) [28]; upregulation of Bcl-xL (B-cell lymphoma extra-large) [28]; decreased BIK (BCL2 (B-Cell Lymphoma 2) Interacting Killer) [28]; amplification of FGFR (Fibroblast Growth Factor Receptor) [28]; amplification of VEGFR (Vascular Endothelial Growth Factor Receptor) [28];
overexpression of cyclinA/Cdk2 [29]; activation of autophagy [30]; increased sensitivity to E2 [31]; and more.

Like endocrine therapy resistance, intrinsic and acquired resistance to inhibitors of Cdk4/6 are also universal issue. Moreover, because Cdk4/6 inhibitors are widely used in first-line treatment, management of resistance to Cdk4/6 inhibitors has emerged as a critical clinical need. About 20% of ER+/HER2- breast cancers are intrinsically resistant to Cdk4/6 inhibitors [17]. Because RB-like protein family members are the only known substrates of Cdk4/6, including RB, RBL1/p107 (Retinoblastoma-Like Protein 1) and RBL2/p130 (Retinoblastoma-Like Protein 2), loss of function of RB is a key reason for intrinsic resistance [17]. Another reason for intrinsic resistance is FAT1 loss-of-function, which causes Cdk6 overexpression [32]. Growth factor receptor amplification or mutation, such as FGFR2 or IGFR, can also induce intrinsic resistance to Cdk4/6 inhibitors [33]. RAS and AKT1 mutations have also been identified as contributors to intrinsic resistance [33].

Similar to endocrine therapy resistance, there are many mechanisms claimed to contribute to the acquired resistance to Cdk4/6 inhibitors: loss of function of RB due to mutation or deletion, as in intrinsic resistance [17]; amplification of Cdk6 [34, 35]; suppression of TGFβ (Transforming Growth Factor Beta) via miR-432-5p microRNA [36]; overexpression of cyclinE [34-37]; activation of PDK1 (Phosphoinositide-dependent Kinase 1) [38]; activation P13K (Phosphoinositide 3-Kinase)/AKT/mTOR (mechanistic Target of Rapamycin) pathway [34, 38]; activation of MAPK (Mitogen-Activated Protein Kinase) [39]; amplification or mutation of FGFR [40, 41]; and more.

For endocrine therapies or Cdk4/6 inhibition, long-term application can often result in resistance to these therapies [28, 42, 43]. The resistance mechanisms, as mentioned above, are varied and in many cases not well understood. They can include protein overexpression, epigenetic changes, gene mutation, gene amplification and deletion [10, 14, 34, 42, 44]. While targeted therapies are important methods for breast cancer treatment, eventually cancer cells become resistant and grow again, which makes the advantage of targeted therapies only temporary for many patients. Investigating the drug resistance mechanisms and ultimately preventing resistance is a universal challenge in applying targeted therapies.
1.4 Mathematical modeling for treatment design

Mathematical modeling has been used to inform therapy design and treatment decisions, especially in chemotherapy and radiotherapy [45,46]. Sometimes the mathematical model identifies therapy protocols that are non-intuitive and would likely never be found by experimental approaches, as a mathematical model can explore a huge number of possibilities. One example is schedules derived from a mathematical model that significantly improved efficacy and led to survival [46]. In [45], evolutionary principles were applied in a model to account for resistance and showed that adaptive treatment was more effective than maximum-tolerated dose treatment for prostate cancer. The research in these references considers pharmacokinetics and drug interaction effects without modeling the mechanism of these drugs. Systematic application of a mathematical model that integrates molecular cell biology and drug pharmacology can help us understand and explore different drug therapy effects on cancers [47-49]. More importantly, testing these various drug effects is likely to be experimentally impractical based on the huge number of variations in combinations, dosing and timing of these drugs. Mathematical modeling of dynamics of cancer cell drug responses can be used to investigate better treatment regimens, in terms of choice of drugs, combinations and schedules of dose frequencies. Although in clinical studies finding optimal dosing frequencies will benefit the treatments [50, 51], it is impractical and unethical to test different kinds of combination and dosing strategies in pre-clinical and clinical settings. A mathematical model can systematically search through hundreds of thousands of possible scenarios to determine the most effective treatment possibilities to test experimentally. Note that what we mean by effectiveness might be patient specific and may include maximally prolonging patient lifespan, administering drugs at low doses, limiting side effects, reducing toxicity, and adding drug holidays. A mathematical model is useful for fulfilling such multi-target optimization tasks. Furthermore, a mathematical model can easily look for synergism among a variety of drugs and suggest the best functional proteins or molecules to target in a signaling network. Overall, incorporating experimental and clinical data into a mechanism-based mathematical model can enable it to predict the responses to many different treatments, results that cannot be efficiently tested one by one through experiments [52].
1.5 Research objectives and innovation

Optimizing a drug treatment protocol for a specific cancer patient can also be realized by mathematical models in terms of battling the emergence of resistance, minimizing tumor cell proliferation or controlling the toxicity of the drugs [53-54]. Although mathematical models have been applied in various types of cancers [55-58], there are no such mechanism-based mathematical models for ERα+ breast cancer treatment. The aims of this study are to build a mechanism-based mathematical model for the response of MCF7 cells (ER+/HER2- Luminal A subtype breast cancer cell line) to the standard first-line therapies used in this clinical subtype.

While there have been a number of previous mathematical models dealing with ERα signaling and its impact on the cell cycle [61-63], these models differ from this study in that they are phenomenological and not experimentally calibrated or validated. There are also a number of mathematical models that describe the G1-S cell cycle transition in single cells [63-68]. Most of these models investigate the bistable switch governing the commitment to proliferation at the restriction point [64-68]. While our model is based on a similar structure, our aim is to capture the behavior of an asynchronous population of cells, creating an average model, so we do not expect our parameterization to create a bistable switch or be valid for single cells. The novelty proposed here is the use of a mathematical model to capture the experimentally observed protein and proliferation changes of MCF7 cells in response to different treatment conditions and combinations. The strategy is to model the kinetics of signaling networks targeted by endocrine therapies and Cdk4/6 inhibitors. Subsequently the mathematical model is used to simulate and predict proliferation in response to alternating therapy. The mathematical model can be applied to optimize the period and sequence of therapies to meet specified objectives and constraints (e.g., control a resistance biomarker below a certain fold increase in response to therapy). The final goal of this study is testing the hypothesis that an alternating therapy protocol, which applies endocrine therapy and a Cdk4/6 inhibitor sequentially for rationally determined time periods will avoid or delay the development of resistance. Although a continuous therapy may delay resistance by increasing dose or adding additional drugs, the increase in toxicity will limit the ability to increase dose or combinations and cause more potential risk to older and fragile patients [69, 70]. Therefore, we want to find a less toxic protocol by minimizing the doses and drug overlap periods while controlling proliferation at the same time. A mechanistic mathematical model becomes necessary
because it enables application of optimization algorithms to find effective alternating therapy protocols. To optimize alternating therapies by experiment may lead to an infeasible number of experiments because of the number of different treatment combinations, sequences and periods that can be used in a protocol. Limited experimental resources are more effective if they are used to validate an optimized treatment strategy that has been determined by searching through millions of possibilities. Consequently, a mathematical model based on the well-established first-line treatments of ERα+ breast cancer provides the possibility of benefiting the clinical treatment of this most common subtype of breast cancers.

We tested the MCF7 cell response to different treatments by cell culture. This study used E2 deprivation (–E2) as a surrogate for treatment with aromatase inhibitors, which have proven as effective as tamoxifen [71], and ICI as another representative endocrine therapy. Since tamoxifen produces both agonist and antagonist effects [72, 73] it was not used as a treatment in this study. The study used two kinds of Cdk4/6 inhibitors, palbociclib (PD0332991) and abemaciclib (LY2835219). In Chapter 2, we model the short-term (7 days) protein and proliferation changes in response to endocrine therapies and palbociclib. In Chapter 3, we extend the short-term ODE model to handle longer time scales (weeks) in response to different continuous therapies. Moreover, another Cdk4/6 inhibitor abemaciclib is added to the model. After that, the mathematical model is used to explore the different synergisms among the treatments considered, which showed a strong, unexpected synergy between two drugs. In Chapter 4, we used the long-term mathematical model to propose an alternating therapy experiment (drug selection, dose, alternating period) to ensure the cells will not be overcrowded during or at the end of the experiment. Finally, we experimentally test whether the proposed alternating therapy can delay the development of resistance. In Chapter 5, we provide a short discussion and summary of our study.
Chapter 2. Short-term mathematical model on MCF7 cells in response to different therapies

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2.1 Biological justification for the model

Although endocrine treatments have been used in the clinic for a long time, there are no mechanistic mathematical models that explain the dynamic response of proteins and proliferation to these treatments. The first step, therefore, is to build a model based on known mechanisms from the literature to describe the changes in protein expression and proliferation of an E2-dependent, asynchronous population of MCF7 breast cancer cells in response to various clinically relevant therapies over a time period of 7 days.

One important class of estrogen-responsive genes regulate progression through the G1 phase of the cell cycle [10, 74]. This progression mainly depends on transcriptional expression of regulatory proteins c-Myc and cyclinD1 [74]. c-Myc responds to E2 stimulation within 15 minutes, making it one of the earliest estrogen-responding genes [75-77]. The rapid induction of c-Myc by E2:ERα is via the P2 promoter region of the c-Myc gene, which contains an atypical ERE region [75, 78]. c-Myc is a nuclear transcription factor with high-affinity for DNA-binding and its level is highly correlated with breast cancer cell proliferation [79, 80]. The c-Myc oncogene is usually overexpressed in human breast cancer cells and is an essential positive regulator of the G1-S
transition [80]. c-Myc represses transcription of p21 by interacting with Miz-1 (Myc-interactin Zn Finger Protein-1) and several other proteins at the transcriptional start site [81], transforming Miz-1 from an activator to a repressor [82, 83]. In addition, c-Myc also can transcriptionally repress p27 expression in breast cancer cells [81] by binding to the initiator element at the start site of the TATA-less p27 promoter [84]. Moreover, the box II domain of c-Myc can interact with the N-terminal DNA-binding region of FOXO3a (Forkhead Box O3) at the proximal Forkhead element on the p27 promoter. This interaction inhibits the FOXO3a-mediated activation of the p27 promoter [85].

Besides c-Myc, E2:ERα also transcriptionally up-regulates cyclinD1, whose expression is key to G1-S progression [86, 87]. ERα binds upstream (-96 and -29bp) of the cyclinD1 promoter region encompassing a cAMP (cyclic Adenosine Monophosphate) response element (CRE-D1) [86, 88]. The up-regulation is E2-dependent and the AF-1 (Transactivation Function-1), AF-2 (Transactivation Function-2) and DNA binding domains of ERα are required [86, 88]. In addition, c-Myc has been reported to transcriptionally up-regulate [89], down-regulate [90-92], or have no effect on cyclinD1 expression level [93-95]. When tested on breast cancer cells [74], induction of c-Myc failed to increase cyclinD1 expression and induction of cyclinD1 had no effect on c-Myc expression in the interval from 3 to 24 hours after induction [74]. Therefore, we model the increased transcription of cyclinD1 and c-Myc as a direct effect of E2:ERα.

CyclinD1 binds with cyclin-dependent kinases Cdk4 or Cdk6 (mostly Cdk4 in MCF7 cells [96]) to form holoenzymes and activate their kinase activity [97]. CyclinD1:Cdk4/6 phosphorylates retinoblastoma protein (RB1) to a hypophosphorylated form (RB1-p) [74]. The Cdk inhibitors p21 and p27, which can bind to the cyclinD1:Cdk4/6 complexes and inhibit their kinase activity [98], are downregulated by c-Myc to further activate the holoenzyme. Since p21 and p27 also inhibit the kinase activity of cyclinE:Cdk2, the increased sequestration of these inhibitors by cyclinD and their downregulation by c-Myc, serve to activate the cyclinE:Cdk2 complex [99]. This mechanism of cyclinE:Cdk2 activation by estrogen treatment is confirmed by several studies [100]. Finally, active cyclinE:Cdk2 hyperphosphorylates and fully inactivates RB1 [74, 80, 100], which is a key step in the G1-S phase transition [74, 75, 79, 81, 99]. The hyperphosphorylated RB1 (RB1-pp) releases E2F transcription factors that transactivate the genes required for DNA synthesis [81, 99]. All of these mechanisms have been well-established and recognized in ERα+ breast cancer cells.
There are numerous feedback loops involving E2F in the G1-S transition machinery [101]. While it is enticing to include all the well-known mechanisms around the G1-S transition, the limited data we are able to obtain does not warrant the increase in parameters, which would be practically unidentifiable.

Based on the signaling mechanisms mentioned above, the interactions of key elements governing the biological mechanism associated with the effect of E2 signaling on proliferation are shown in Figure 2.1. E2 bound to ERα enhances the production of cyclinD1 and c-Myc. CyclinD1 with its kinase partner Cdk4/6 drives RB1 to a hypophosphorylated state, RB1-p. CyclinE with its kinase partner Cdk2 drives RB1-p to a hyperphosphorylated state, RB1-pp, in which it can no longer bind and inhibit E2F, allowing E2F to drive the G1–S transition and proliferation. p21, representing both itself and p27, inactivates both cyclinD1:Cdk4/6 and cyclinE:Cdk2, but the increased production of c-Myc increases its inhibition of p21 production, helping to take the brakes off proliferation.

Figure 2.1. Wiring diagram of the biological mechanism. Solid lines with balls represent reversible binding reactions, other solid lines represent production or degradation of proteins, dashed lines represent influences with arrowheads representing enhancement and blunt heads representing inhibition. Biological mechanism consisting of the following process: (1) ERα binds it ligand E2; (2) ICI binds ERα and enhances its degradation; (3) E2:ERα enhances production of c-Myc; (4)
E2:ERα enhances production of cyclinD1; (5) c-Myc inhibits the production of p21; (6) cyclinE binds to Cdk2; (7) p21 binds to cyclinE:Cdk2 and inactivates it; (8) cyclinD1 binds to Cdk4/6; (9) p21 binds to cyclinD1:Cdk4/6 and inactivates it; (10) Palbociclib binds to Cdk4/6 and inactivates it; (11) RB1 binds to E2F inactivating it; (12) cyclinD1:Cdk4/6 phosphorylates RB1; (13) RB1-p binds to E2F; (14) cyclinE:Cdk2 phosphorylates RB1-p, preventing it from binding E2F; (15) free E2F enhances production of c-Myc; (16) free E2F enhances production of RB1; (17) free E2F drives the G1-S transition and proliferation. The species in red were measured by experiment and those in black were not. For p21, cyclinD1 and RB1, the total protein was measured. In addition, RB1-pp was measured.

The two common endocrine therapies used in this study, E2 deprivation and ERα down-regulation, decrease the transcription factor E2:ERα, down-regulate c-Myc and cyclinD1, increase p21 and p27, and inhibit cyclinD1:Cdk4/6 and cyclinE:Cdk2 kinase activity [102]. The consequence of this decreased kinase activity is reduced phosphorylation of RB1, which limits the transcriptional activity of E2F, impeding the G1-S transition and arresting the cell in a state with characteristics of quiescence [96, 102].

With regard to proliferation, in the simple situation where total RB1 and total E2F are constant, the level of hyperphosphorylated RB1, RB1-pp, should be related to the proliferation rate. But our experimental data, as well as that of others, shows that total RB1 is not constant in response to endocrine therapy. In fact, the interactions between the RB1 and E2F families of proteins is quite complicated: there are three types of pocket proteins that bind E2F (RB1, p107, and p130) and nine types of E2F (E2F1-9), not to mention the 15 phosphorylation sites on RB1, and the binding efficiencies are governed by many phosphorylation sites on the pocket proteins [103]. In addition, p130 is known to increase in response to endocrine therapy. From our experimental data it appears that proliferation closely follows the level of RB1-pp (associated with phosphorylation on S612, which is associated with proliferation [104]) and so in our simplified model we use the RB1-pp level to drive changes in proliferation.

In the mathematical model (Figure 2.2), we make some simplifying assumptions to reduce the number of species modelled so as to be more in line with the number of species measured. In particular, we do not model Cdk4/6 explicitly, but assume that all cyclinD1 not bound to p21 is
bound to Cdk4/6 and active. Thus, to model the effect of palbociclib, which inactivates cyclinD1:Cdk4/6, we allow palbociclib to bind to cyclinD1 in the model and hold it inactive. Similarly, we do not explicitly model Cdk2. In addition, we do not model E2F, but assume that the level of RB1-pp reflects the transcriptional activity of E2F. While not modelling E2F may seem a step too far, the actual biological complexity, as discussed above, is more significant than that shown in Figure 2.1. Since our ultimate objective is predicting proliferation, we settled on associating the RB1-pp level (phosphorylation on S612) with proliferation, as this matches our experimental data.

2.2 Material and methods

2.2.1 Cell culture and reagents

MCF7 cells were obtained from Tissue Culture Shared Resources at Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC. MCF7 cells depend on E2 for growth, and therefore, to enable us to control the level of E2 in the cell culture medium, cells were grown in phenol red-free improved minimal essential medium (Life Technologies, Grand Island, NY; A10488-01) with 10% charcoal-stripped calf serum (CCS) and supplemented with 10 nM E2). E2 deprivation was obtained by washing cells 24h post-plating (t = 0) with phosphate-buffered saline (PBS) and adding complete medium without E2 for the indicated times. ICI (Faslodex/Fulvestrant; ICI182,780) and palbociclib were obtained from Tocris Bioscience (Ellisville, MO). MCF7 cells were authenticated by DNA fingerprinting and tested regularly for Mycoplasma infection. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

![Figure 2.2 Wiring diagram of the mathematical model. The meaning of the lines and colors are the same as Figure 2.1. The ODE equations and table of parameter values are shown in Section 2.5.](image-url)
2.2.2 Cell proliferation assays

Cells were seeded at a density of 1-2 × 10^5 cells well⁻¹ or 4-5 × 10^4 cells well⁻¹ in 100 mm or 60 mm plates, respectively. Cells were then trypsinized, resuspended in PBS and counted using a Z1 Single Coulter Counter (Beckman Coulter, Miami, FL). Three independent experiments were done. Data were normalized to cell number at t = 0 and are presented as the mean ± s.e. from all three experiments.

2.2.3 Western blot analysis

For Western blot analysis, cells were lysed for 30 min at °C in lysis buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 1% IGEPAL CA-630, 0.1% sodium dodecyl sulfate (SDS), 1 mM Na₃VO₄, 44 µg ml⁻¹ phenylmethylsulfonyl fluoride) supplemented with Complete Mini protease inhibitor mixture tablets (Roche Applied Science). Total protein was quantified using the bicinchoninic acid assay (Pierce). Whole-cell lysate (20 µg) was resolved by SDS-polyacrylamide gel electrophoresis. The following antibodies were used: c-Myc (no. 5605), cyclinD1 (no. 2978), p21 (no. 2947) and RB1 (no. 9309) were from Cell Signaling, Danvers, MA; ESRα (MA514104) was from Invitrogen; RB1-phosphorylated on Ser612 (OAAB16108) was from Aviva Systems Biology, San Diego, CA; actin (sc-47778) was from Santa Cruz Biotechnology, Santa Cruz, CA; β-tubulin (T7816) was from Sigma, St. Louis, MO.

2.2.4 Data collection

Table 2.1 summarizes the data that were collected. Protein levels were analyzed by western blotting and cell numbers were determined using a Coulter counter. Protein levels and cell numbers were first normalized to 0 h. Protein levels in treatment conditions were then normalized to the +E2 case (control; untreated, grown with E2 in medium).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein name</th>
<th>Protein time points</th>
<th>Cell number time points</th>
</tr>
</thead>
<tbody>
<tr>
<td>+E2 (10 nM)</td>
<td>ERα, c-Myc, cyclinD1, p21, RB1-pp, total RB1</td>
<td>0 h, 4 h, 1 d, 3 d, 6 d, 7 d</td>
<td>0 h, 1 d, 3 d, 6 d, 7 d</td>
</tr>
<tr>
<td>+E2 (10 nM) + ICI</td>
<td>ERα, c-Myc, cyclinD1, p21, RB1-pp, total RB1</td>
<td>0 h, 4 h, 1 d, 3 d, 6 d, 7 d</td>
<td>0 h, 1 d, 3 d, 6 d, 7 d</td>
</tr>
<tr>
<td>–E2</td>
<td>ERα, c-Myc, cyclinD1, p21, RB1-pp, total RB1</td>
<td>0 h, 4 h, 1 d, 3 d, 6 d, 7 d</td>
<td>0 h, 1 d, 3 d, 6 d, 7 d</td>
</tr>
</tbody>
</table>
Table 2.1. Time points at which the various protein species and cell numbers were measured in response to the various treatments. +E2 (untreated, grown with E2 in medium), +E2 + ICI (treated with ICI in +E2 medium), –E2 (deprived of E2), –E2 + ICI (treated with ICI in –E2 medium), +E2 + palbo (treated with palbociclib in +E2 medium) and –E2 + palbo (treated with palbociclib in –E2 medium). Underlined time points were used for normalization; italicized for model parameter calibration; and bold for model validation.

2.2.5 Modelling estrogen deprivation

Removing E2 from cultured cells that have been growing in medium containing E2 cannot be accomplished by simply changing to a medium containing no E2 [105]. MCF7 cells growing in +E2 conditions have a much higher internal concentration of E2 than that of the medium due to non-specific binding of E2 in the cytoplasm as well as specific binding of E2 to various estrogen receptors in the cell. When the medium is changed to a –E2 medium, E2 from the cells leaches into the new medium, establishing a new E2 level that can be significant for maintaining proliferation as MCF7 cells can proliferate significantly in 10 pM of E2 [106]. In 7-day experiments, the medium was changed to treated medium on day 0 and on day 3. For treatments involving E2 deprivation, we incorporated two additional model parameters, one for the E2 concentration in the medium after day 0 and one for the concentration after day 3, to account for the fact that the E2 concentration does not go to zero and that it decreases with additional changes in the medium.

2.2.6 Parameter estimation

The degradation rates for different proteins were assigned according to half-lives found in the literature, where \( k_d = \log(2)/T_{\text{half-life}} \). The other parameters were optimized to minimize the discrepancy between the model simulation and the experimental result. The cost function was:

\[
M(p) = \sum_{i=1}^{n} \sum_{j=1}^{m} \frac{(y_{ij}^E(t_j) - y_{ij}(t_j,p))^2}{a_{ij}^2}
\]
where \( i \) indexes the state variables (proteins or cell number), \( j \) indexes the experimental time points, \( y_{ij}^{E}(t_j) \) is the experimental measurement of the \( i \)th variable at time \( t_j \), \( y_{ij}(t_j, p) \) is the simulation result of the \( i \)th measurement at time \( j \) using parameter vector \( p \), and \( \sigma_{ij} \) is the standard deviation of the measured value based on three replications. The data and time points used for parameter estimation are listed in Table 2.1. Parameter estimation was performed using MATLAB [107] and the default genetic algorithm function, \textit{ga}, in the global optimization toolbox as well as the \textit{fminsearch} function to refine the results of \textit{ga}. We broke the parameters into groups, initially tuning the parameters related to ER\( \alpha \), then tuning those related to all other proteins, and finally tuning all these parameters together. The three parameters associated with palbociclib were tuned separately, following which those associated with proliferation were tuned. Finally, all the parameters were tuned together to produce the final result.

2.2.7 Statistical analysis

The Mann-Whitney U-test was used for statistical comparisons [108]. The western blot protein data from the treatment conditions were first normalized to the control condition and then compared with 1 to test whether the treatments significantly decrease or increase the protein levels after time 0. Cell numbers after treatment were directly compared with the control numbers to test whether the treatments decreased cell proliferation.

2.3 Results

2.3.1 The proposed model structure can largely explain the experimental data

The training data for estimating the model parameters consisted of time-course measurements of the proteins in red in Figure 2.2 for the –E2 and +E2 + ICI treatment conditions. These data are shown in Figure 2.3, and it can be seen that the majority of measurements are statistically significant (asterisks), although the p21 measurements are quite noisy, and there is no real trend in the cyclinD1 data for the –E2 case. However, there are clear trends in most of the data, and it is critical that the model structure be capable of reproducing these trends. Figure 2.3a shows the experimental observations over 7 days (red) in response to –E2. The significant ER\( \alpha \) increase after treatment can be captured by the model because the half-life of E2:ER\( \alpha \) is about 3–4h [109] and the half-life of unbound ER\( \alpha \) is about 4–5h [109]. Therefore, the depletion of E2 stabilizes ER\( \alpha \) and causes the total ER\( \alpha \) level to increase. The jump in ER\( \alpha \) after 3 days is due to the medium
exchange that decreases the concentration of E2 in the medium that is leached from the cells. The small decrease in the cyclinD1 level can be captured by the model since E2:ERα is a transcription factor for cyclinD1. By contrast, c-Myc not only decreases at the beginning, but decreases abruptly at 3d and continues to decrease over the remainder of the time course. The model captures the sharp decreases due to changes in the E2 concentration levels at 0d and 3d and the gradual decrease via RB1-pp, which drives the production of c-Myc. As the cells are gradually blocked in G1, RB1-pp decreases steadily, causing a decrease in c-Myc. The noisy p21 data do appear to increase; this is captured in the model as decreasing c-Myc releases its transcriptional inhibition of p21. We should note that there are likely to be many ways in which c-Myc influences RB1-pp, such as activation of cyclinE:Cdk2 kinase activity via Cdc25A [110], but, for simplicity, we have chosen to model c-Myc’s effect only through the p21 pathway. The model can capture the reduction in RB1-pp, since this follows from the reductions in c-Myc and cyclinD1.

At first glance, it seems odd that total RB1 should decrease much more than RB1-pp and that this decrease should be associated with the observed decrease in proliferation, as less total RB1 might be expected to create free E2F and promote the G1–S transition. This decrease in total RB1 has been observed by others in response to E2 deprivation [111]. In the model, free RB1 is always much more prevalent than either of the phosphorylated forms. The level of RB1-p is controlled by the cyclinD1 level, since the influence of free RB1 is saturated in the Hill function kinetics and has little effect on the RB1-p level. In turn, the level of RB1-pp is controlled by cyclinE and not influenced significantly by the RB1 level. The model captures the changes in total RB1 through the presumed transcriptional effect of RB1-pp on RB1.

Figure 2.3b shows the experimental observations in response to treatment with ICI. The model can capture the decrease in ERα since ICI binds to ERα, displacing E2 and enhancing ERα degradation via the proteasome [15]. The reduced availability of the transcription factor E2:ERα due to ICI treatment causes changes in c-Myc, cyclinD1, p21, RB1-pp and total RB1 similar to those caused by reduced availability of E2:ERα due to E2 deprivation, and for the same reasons.
Figure 2. 3. Simulation and experimental results for protein-level changes under the different treatment conditions used for model calibration. The experimental measurements are shown in red (mean value ± s.e., n = 3) and the simulation results are shown in cyan (lowest cost simulation as a solid line and the interval containing the central 98% of the cohort simulations as a shaded region). Statistically significant changes from T = 0 are denoted by an asterisk. (a) Protein level changes after E2 deprivation over 7 days. (b) Protein-level changes after +E2 + ICI treatment over 7 days. ICI concentration is 500 nM and +E2 concentration is 10 nM. The simulation values for total cyclinD1 and total p21 are plotted to enable comparison with experiment.
2.3.2 Model calibration and the simulation cohort

To calibrate the model, we used a genetic algorithm to search for parameter values minimizing the least-squares cost function. To address the fact that the model may not be practically identifiable from the data, we identified 399 other parameter sets that provided a reasonable fit to the data to use as a simulation cohort. Any member of the cohort is a reasonable parameterization of the model and when we use the model for prediction, we simulate all 400 parameter sets. The resulting spread in the predictions enables us to see how well the data used to calibrate the model constrain the prediction. The coefficients of variation of the parameters and the variations in the cost function for the simulation cohort are plotted in supplementary material, Section 2.5.6, Figures 2.11 and 2.12.

The final results of calibrating the model to the training data are shown in Figure 2.3. The results are quite reasonable, given the simplicity of our model. The spread of results from the simulation cohort reflects both the variation in the parameters for the chosen cohort as well as the sensitivity of the model to these parameters. The least spread is exhibited by cyclinD1 and RB1-pp. Although RB1-pp is not extremely sensitive to the parameters driving it (see sensitivity analysis in the supplementary material), proliferation is very sensitive to RB1-pp level and forces its level to be heavily constrained. CyclinD1, on the other hand, is simply not very sensitive to the parameters driving it and the limited coefficients of variation of these parameters in the cohort result in a small spread.

Of course, there is always the question of whether the fit is reasonable because the model captures the essentials of the mechanism or simply because enough parameters were added to the model (44 parameters in the model and 194 independent observations in the experiments). In the following, we argue that our ability to add a new drug to the model without recalibration, and the ability of the model to predict a non-obvious effect of drug combinations, shows that the model is capturing some of the essential mechanism.

2.3.3 Adding a new drug to the model requires limited new data

If the model is capturing the basic mechanism driving protein changes, adding a new drug to the model should require only calibrating a few new parameters associated with the new drug. Such calibration should not require measuring the response of all proteins in the model to the new drug, but only a few key proteins. We illustrate this by incorporating palbociclib, which has been used
clinically in combination with endocrine therapy [17, 34], into our model. Palbociclib inhibits Cdk4/6 kinase activity, reducing RB1 phosphorylation, primarily at S780/S795 [112], and ultimately leading to reduced hyperphosphorylation of RB1 and cell cycle arrest. To calibrate the binding and unbinding parameters associated with palbociclib in our model, we measured only two proteins, c-Myc and RB1-pp (S612, associated with proliferation but not directly affected by palbociclib [104]), which should experience strong downstream reactions to the drug. Figure 2.4 shows the results of calibrating the model to 1 μM of palbociclib added to the +E2 medium. As expected, the inhibition of cyclinD1:Cdk4/6 kinase activity by palbociclib decreases the RB1-pp level. This, in turn, leads to decreased transcription of c-Myc, causing the c-Myc level to decrease.

![Figure 2.4](image)

Figure 2.4. Simulation and experiment results for protein-level changes in response to palbociclib. Simulations are shown in cyan and experimental measurements in red (mean value ± s.e., n = 3). Statistically significant changes from T = 0 are denoted by asterisks.

### 2.3.4 The proliferation results can be explained by the RB1-pp level

Proliferation is a primary endpoint for treatment decisions, so it is important for the model to be able to capture how proliferation changes with treatment. When considering treatments that produce G1 arrest, the key transition governing the execution of the cell cycle is the transition from the G1 phase to the S phase. A major determinant of the G1-S transition is the phosphorylation status of RB1 [113], but there are many other factors affecting the transition (see the discussion in the Section 2.1 under biological justification for the model). Based on our experimental data, we assume that the rate of G1-S transition, hence of proliferation, in our model of an asynchronous population of cells is governed by the level of RB1-pp according to

\[
\frac{d_{cell}}{dt} = k_{pro1} \times \left( 1 + k_{pro2} \times \frac{RB1pp^{p2}}{p_{1}^{p2} + RB1pp^{p2}} \right) \times cell \times \left( 1 - \frac{cell}{k_{carrying}} \right)
\]
The rate of cellular proliferation is modelled as proportional to the current number of cells subject to a carrying capacity constraint (logistic growth). The proportionality constant depends on the RB1-pp level via a Hill function. We expect proliferation to be near maximal at the pre-treatment RB1-pp level and, in our experimental data, proliferation essentially stops when the RB1-pp level reaches half of its normalized value, necessitating a relatively large Hill exponent of 6. The carrying capacity, assumed constant across all experiments, is used to account for the fact that in the control condition the cells approach confluence in 7 days; it has little effect on the other, slower-growing conditions. Figure 2.5 shows that the model does a good job of matching the experimental proliferation results for the untreated (+E2), deprived (–E2), ICI-treated and palbociclib-treated cases on which the model was calibrated. This helps validate our claim that proliferation in these cases can be modelled using the RB1-pp (S612) level.

![Graphs showing model simulations and experimental measurements](image)

Figure 2. 5. Model simulations and experimental measurements of the normalized cell numbers under different treatment conditions. Experimental counts are in red (mean value ± s.e., n = 3) and simulations are in cyan. ICI concentration is 500 nM, palbociclib concentration is 1 µM and E2 concentration is 10 nM. Cell numbers are normalized to T = 0 value. Statistically significant changes from the E2 control are denoted by asterisks.

### 2.3.5 The model can predict the effect of combination treatments

To partially validate the model, we test its ability to predict the effect of combination therapies on protein levels and cell proliferation. Figure 2.6 shows how the model simulations of combining E2
deprivation and ICI compare to the experimental results. With the exception of cyclinD1, the predictions match the experimental results for the six measured protein species well. By reducing the supply of E2 and the ERα level simultaneously, larger changes in the protein levels are observed than with either monotherapy, as would be expected.

Although one might argue that predicting a combination therapy will have greater effect than either therapy by itself is not surprising, there is one surprising prediction made by the model, namely that ERα for the combination decreases below the level for ICI treatment alone. Since E2 deprivation alone causes a significant increase in ERα and ICI alone causes a significant decrease, the prediction of the ERα level for the combination therapy is not clear cut. Only a quantitative model can resolve the outcome. The reason the model predicts a stronger decrease in ERα level is that the scarcity of E2 allows more ICI to bind to ERα, causing greater degradation than occurs when ICI competes with E2 for binding to ERα.

The combination of E2 deprivation and palbociclib attacks a key G1 kinase, cyclinD1:Cdk4/6, by both reducing the level of cyclinD1 and inactivating Cdk4/6. As expected, the combination reduces the level of c-Myc and RB1-pp to a greater extent than either monotherapy. The predictions of the model are compared with experimental protein measurements in Figure 2.7a. The agreement between measured and predicted c-Myc is good, but the simulation misses the low level of RB1-pp at the final two time points. Since proliferation in the model essentially stops when the normalized RB1-pp level falls below 0.5, it is still possible to capture the proliferation well while somewhat overestimating the RB1-pp level.
Figure 2. 6. Prediction simulations for protein-level changes in response to combination –E2 + ICI therapy. ICI concentration is 500 nM. Experimental results are in red (mean value ± s.e., n = 3), simulations are in blue and monotherapy experimental results are in cyan. Statistically significant changes from T = 0 are denoted by asterisks.

Figure 2.7b shows that the model also predicts the decreased proliferation in response to the combination therapies quite well. This follows from the fact that RB1-pp decreases more in response to the combinations than to either monotherapy.

2.3.6 Local sensitivity analysis of protein levels and proliferation

A local sensitivity analysis is used to check if the output of our model is very sensitive to certain parameters. The sensitivity coefficient for a given model output and a given parameter is the percent change in the model output divided by the percent change in the parameter value (see Section 2.5.5). To calculate this coefficient, we changed the parameter by ±5% from its best-fit value, so the sensitivity coefficient is not unduly local. In Figure 2.8, we plot the sensitivity coefficients for the most sensitive outputs in our model, c-Myc, total RB1 and cell number, to each model parameter at the day 7 time point. The sensitivity coefficients of the other proteins are shown in the supplementary material and are of the order of 4 or less, indicating relatively low sensitivity.
Figure 2. Comparison of model predictions with experiment for combination therapies. Experimental data (red), simulations (blue) and monotherapy experimental results (cyan). (a) Prediction of c-Myc and RB1-pp levels in response to –E2 + palbo combination treatment. (b) Prediction of proliferation in response to –E2 + ICI and –E2 + palbo combination treatments. Asterisks denote statistically significant changes from T = 0 for proteins, and from the E2 control for proliferation.

The most significant sensitivity for c-Myc occurs for the ICI treatment case and involves parameters related to the basal translation of cyclinE and p21 and the phosphorylation of RB1-p. This is not surprising as these parameters regulate the level of RB1-pp, which helps drive the c-Myc level in our model. Total RB1 is most sensitive for the –E2 + ICI case, and the parameters producing the greatest sensitivity involve basal transcription of cyclinE and c-Myc, the downregulation of p21 by c-Myc, phosphorylation of RB1-p by cyclinE and the dephosphorylation of RB1-pp. These parameters all converge on the level of RB1-pp.

Cell number is most sensitive in the ICI and palbociclib treatment cases and its greatest sensitivity is to parameters involving basal transcription of cyclinE and its phosphorylation of RB1-p. Again, these parameters drive the level of RB1-pp, which in turn drives proliferation. The fact that cell proliferation is essentially modelled as exponential accounts for the large sensitivity.
Figure 2. 8. Local sensitivity analysis of the effect on c-Myc level, total RB1 level and cell number of each model parameter. Each of the parameters is changed by ± 5% and the sensitivity at day 7 for each treatment is plotted.

2.3.7 The model can be used to explore the effect of sequential therapies

While the initial response of patients to a mono- or combination therapy is often promising, in many cases, resistance to continuous therapy eventually arises (e.g., palbociclib [34], letrozole [14, 44] and ICI [114]). The resistance mechanisms are varied, and in many cases not well understood, but can include protein overexpression, epigenetic changes, gene mutation and gene amplification or deletion [10, 14, 34, 42, 44]. Our idea to address some of these mechanisms is straightforward: alternate various therapies, perhaps including drug holidays, in a fixed cycle to significantly suppress proliferation of tumor cells, but not target one particular mechanism non-stop to avoid selecting for resistance mechanisms. The hope is that such an approach can hold off, or at least delay, the onset of resistance.

Optimizing sequential therapies under various restrictions is likely to be impractical experimentally because of the large number of variations in dosing and timing of multiple therapies that must be considered. On the other hand, a reliable mathematical model may be able to sort through the huge number of possibilities to find particularly promising therapy protocols that can be tested experimentally.
Figure 2.9. Model simulations of possible alternating therapies compared with a continuous monotherapy with an added resistance mechanism. (a) Continuous palbociclib therapy with and without the resistance mechanism are compared. (b) Continuous palbociclib therapy is compared with alternating of E2 deprivation and palbociclib therapies. (c) Continuous palbociclib therapy is compared with alternation of ICI and palbociclib therapies. Dose: E2 (10 nM), ICI (500 nM), palbociclib (1 µM). The simulations used the lowest cost parameter set, and all simulations in (b) and (c) include the resistance mechanism.

To illustrate the basic idea with our first model, we consider resistance to palbociclib, which is frequently associated with the overexpression of Cdk6, which increases over time with continuous palbociclib therapy [35, 36]. Presumably, the increasing Cdk6 eventually binds enough palbociclib to free up cyclinD1:Cdk4/6 to phosphorylate RB1 and move the cell toward proliferation. In our first model, we did not include Cdk4/6 explicitly but rather assumed that all free cyclinD1 was complexed with Cdk4/6 and, therefore, active. Palbociclib inhibits this activity in the model by binding to cyclinD1 and holding it inactive. We mimic the resistance effect of increased Cdk6 sequestering palbociclib by slowly increasing the level of cyclinD1 transcription under palbociclib therapy and allowing it to slowly decrease when palbociclib is withdrawn. So palbociclib is sequestered by cyclinD1, rather than Cdk6, in the model. Figure 2.9a shows how adding this resistance mechanism changes the predicted results of monotherapy, with resistance emerging after 30 days.

Figure 2.9b compares a continuous therapy of palbociclib with a therapy that alternates E2 deprivation with palbociclib for one-week periods in a repeating cycle. While the cyclinD1 and RB1-pp levels slowly increase under the alternating therapy, the onset of resistance is effectively delayed. Figure 2.9c shows a similar effect when treatment with ICI and palbociclib are alternated with one-week periods.

2.4 Discussion

In Chapter 2, we created a relatively simple mathematical model to capture the effects of ERα signaling on key cell cycle proteins governing progression through the G1–S checkpoint in breast cancer cells. The model was calibrated using experimental monotherapy data and was capable of predicting the experimental data from combination therapies. We further showed that it was
relatively easy to add in the effect of a new drug that impinged on this checkpoint without having to measure the response of all the proteins in the model to the new drug or to recalibrate the previously determined parameters of the model. Finally, we linked the level of hyperphosphorylated RB1 (S612) to the proliferation rate and showed that this linkage could capture the proliferation observed in our experimental data.

Clearly, our model is a highly simplified version of the actual system. We assume that the behavior of a population of cells can be adequately modelled by allowing the average levels of species in the population to interact according to a simplified version of the mechanism existing in individual cells. While there are theoretical objections to such a model (the interaction rate between A and B in a population is not necessarily proportional to the average concentration of A multiplied by the average concentration of B), this approach has produced useful models in other contexts [56]. One consequence of this approach is that our model, unlike the model of a single cell, will not exhibit a bistable switch governing the G1–S transition [64], as a population will not completely stop cycling even though individual cells will. Probably, the fact we are trying to model the behavior of a large population of asynchronous cells allows the model to be simpler than if we were trying to model the intricacies of an individual cell.

Our ultimate goal is to use the model to derive alternating therapies that may stave off the development of resistance often associated with continuously applied therapies [115, 42]. This may be a particularly appropriate approach in Luminal breast cancer, where resistance to therapy develops slowly and is often reversible if therapy is discontinued before resistance becomes permanent. In many cancers, pre-existing resistant cells or a high likelihood of critical mutations may lead to a rapid expansion of resistant clones in response to targeted therapy. The long latency associated with resistance to estrogen therapy in breast cancer patients argues against pre-existing resistant cells. Also, MCF7 cells take many months to develop permanent resistance to estrogen therapy or Palbociclib, again arguing against pre-existing resistant cells. Luminal breast cancer also does not often develop new critical mutations, as evidenced by the fact that driver mutations do not significantly change between primary and metastatic disease (ESR1 mutation and CYP19A1 amplification are the exceptions in a small fraction of cases), and that de novo metastatic patients initially respond well to estrogen therapy [116-118].
Clearly, pausing treatment to reverse resistance is not an acceptable option in most cases, but alternating treatment to continually hold down proliferation while not constantly attacking the same target may hold promise. We illustrated some possibilities along this line, but clearly additional experimental confirmation is required. Confirmation that the model can accurately predict proliferation over longer time scales and in response to switching therapies will be the subject of next two Chapters, as will confirmation that cells subjected to alternating therapies are truly less resistant than cells subjected to continuous therapy.

We should also note that the treatments we are currently considering all impinge on the G1–S transition. Thus, a resistance mechanism that completely eradicates the G1–S checkpoint, such as loss of RB1, RB1 (a rare event see [119, 120]), is likely to be unaffected by our approach. Drugs that arrest the cell cycle at a different point, for example G2, or that target pathways resulting in cell death will be necessary to address such cases. Drugs that induce significant cell death will also be necessary if we want to provide a drug holiday. While the holiday may help prevent the development of resistance, it will also allow significant proliferation and holding down the net proliferation will require a drug that can kill cells [121]. Finally, we realize that successful results in vitro are potentially a long way off from success in animal models or human tumors, but it is the place to start for mechanism-based modelling.

2.5 Supporting materials

2.5.1 Model variables and parameters

<table>
<thead>
<tr>
<th>Variable name</th>
<th>Description</th>
<th>Initial value</th>
<th>Half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) ER</td>
<td>Estrogen receptor α</td>
<td>0.002μM</td>
<td>4-5h [109]</td>
</tr>
<tr>
<td>(2) E2ER</td>
<td>Estrogen bound estrogen receptor alpha</td>
<td>0.068μM</td>
<td>3-4h [109]</td>
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<tr>
<td>(3) ICIER</td>
<td>ICI 182,780 bound estrogen receptor</td>
<td>0μM</td>
<td>&lt; 3-4h [109]</td>
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<tr>
<td>(4) cyclinD1</td>
<td>Protein cyclinD1</td>
<td>0.183μM</td>
<td>0.4h [122]</td>
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<td>(5) cyclinD1p21</td>
<td>p21 bound cyclinD1 protein</td>
<td>0.219μM</td>
<td>-</td>
</tr>
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<td>(6) cyclinD1palbo</td>
<td>palbociclib bound cyclinD1 protein</td>
<td>0μM</td>
<td>-</td>
</tr>
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<td>(7) cMyc</td>
<td>Protein c-Myc</td>
<td>6.535μM</td>
<td>0.333h [123]</td>
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<tr>
<td>(8) p21</td>
<td>Protein p21</td>
<td>0.102μM</td>
<td>0.33-1h [124]</td>
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<td>(9) cyclinE</td>
<td>Protein cyclinE</td>
<td>0.306μM</td>
<td>0.5h [125]</td>
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<td>(10) cyclinEp21</td>
<td>p21 bound cyclinE protein</td>
<td>0.274μM</td>
<td>-</td>
</tr>
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<td>(11) RB1</td>
<td>Retinoblastoma protein</td>
<td>76.857μM</td>
<td>2-3h [126]</td>
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<tr>
<td>(12) RB1p</td>
<td>Hypophosphorylated RB1 (RB1-p)</td>
<td>0.481μM</td>
<td>2-3h [126]</td>
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<td>(13) RB1pp</td>
<td>Hyperphosphorylated RB1 (RB1-pp)</td>
<td>7.306μM</td>
<td>&gt;4h [126]</td>
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<tr>
<td>(14) cell</td>
<td>Cell number</td>
<td>1a.u.</td>
<td>a.u. arbitrary units</td>
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### Table 2.2. Model variable description.

<table>
<thead>
<tr>
<th>Parameter name</th>
<th>Description</th>
<th>Value</th>
<th>Fixed or calibrated</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) $k_{ER}$</td>
<td>Translation rate of ERα</td>
<td>0.02μM/hour</td>
<td>Calibrated</td>
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<tr>
<td>(2) $k_{d_{ER}}$</td>
<td>Degradation rate of ERα</td>
<td>0.10/hour</td>
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<td>(3) $k_{E2ER}$</td>
<td>Degradation rate of E2ER</td>
<td>0.30/hour</td>
<td>Fixed</td>
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<tr>
<td>(4) $k_{b_{E2ER}}$</td>
<td>Binding rate between E2 and ERα</td>
<td>4266.27/(hour×μM)</td>
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<td>(5) $k_{ub_{E2ER}}$</td>
<td>Unbinding rate between E2 and ERα</td>
<td>1.0/hour</td>
<td>Fixed</td>
</tr>
<tr>
<td>(6) $k_{b_{ICIER}}$</td>
<td>Binding rate between ICI and ERα</td>
<td>206.80/(hour×μM)</td>
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<td>(7) $k_{ub_{ICIER}}$</td>
<td>Unbinding rate between ICI and ERα</td>
<td>1.0/hour</td>
<td>Fixed</td>
</tr>
<tr>
<td>(8) $k_{d_{ICIER}}$</td>
<td>Degradation rate of ICIER</td>
<td>0.52/hour</td>
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<td>Translation rate of cyclinD1</td>
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<td>(10) $k_{d_{cycinD1}}$</td>
<td>Degradation rate of cyclinD1</td>
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<td>(11) $k_{cycinD1E2ER}$</td>
<td>Increased cyclinD1 translation by E2ER</td>
<td>11.57</td>
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<td>(12) $p_{cycinD1E2ER1}$</td>
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<td>(16) $k_{ub_{cycinD1pala}}$</td>
<td>Binding rate between cyclinD1 and palbociclib</td>
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<td>(18) $k_{eMyc}$</td>
<td>Translation rate of c-Myc</td>
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<td>(20) $k_{cMycE2ER}$</td>
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<td>14.18</td>
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<td>(28) $p_{p21cMyc1}$</td>
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<td>Degradation rate of cyclinE</td>
<td>1.39/hour</td>
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<td>(32) $k_{ub_{cycinE21}}$</td>
<td>Binding rate between cyclinE and p21</td>
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<td>(33) $k_{ub_{cycinE21}}$</td>
<td>Unbinding rate between cyclinE and p21</td>
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<td>Translation rate of RB1</td>
<td>3.27μM/hour</td>
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<td>(35) $k_{d_{RB1}}$</td>
<td>Degradation rate of RB1</td>
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<td>(38) $p_{RB1RB1pp2}$</td>
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<td>(39) $k_{RB1cycinD1}$</td>
<td>Phosphorylation rate of RB1 by cyclinD1</td>
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<td>(40) $k_{RB1pdephe}$</td>
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<td>Dephosphorylation rate of RB1-p</td>
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<tr>
<td>(42) $k_{RB1pCycinE}$</td>
<td>Phosphorylation rate of RB1-p by cyclinE</td>
<td>5.37/hour</td>
<td>Calibrated</td>
</tr>
<tr>
<td>(43) $k_{RB1ppdephe}$</td>
<td>Dephosphorylation rate of RB1-pp</td>
<td>9.27μM/hour</td>
<td>Calibrated</td>
</tr>
</tbody>
</table>
Table 2. 3. Model parameter description.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$kd_{RB1pp}$</td>
<td>Degradation rate of RB1-pp</td>
<td>0.05/hour</td>
</tr>
<tr>
<td>$p_{cyc</td>
<td>linD1RB1_1}$</td>
<td>Parameter 1 of RB1 phosphorylation by cyclinD1</td>
</tr>
<tr>
<td>$p_{cyc</td>
<td>linD1RB1_2}$</td>
<td>Parameter 2 of RB1 phosphorylation by cyclinD1</td>
</tr>
<tr>
<td>$p_{RB1pdepho_1}$</td>
<td>Parameter 1 of RB1-p dephosphorylation</td>
<td>0.68μM</td>
</tr>
<tr>
<td>$p_{RB1pdepho_2}$</td>
<td>Parameter 2 of RB1-p dephosphorylation</td>
<td>7</td>
</tr>
<tr>
<td>$p_{cyc</td>
<td>linERB1p_1}$</td>
<td>Parameter 1 of RB1-p phosphorylation by cyclinE</td>
</tr>
<tr>
<td>$p_{cyc</td>
<td>linERB1p_2}$</td>
<td>Parameter 2 of RB1-p phosphorylation by cyclinE</td>
</tr>
<tr>
<td>$p_{RB1ppdepho_1}$</td>
<td>Parameter 1 of RB1-pp dephosphorylation</td>
<td>59.85μM</td>
</tr>
<tr>
<td>$p_{RB1ppdepho_2}$</td>
<td>Parameter 2 of RB1-pp dephosphorylation</td>
<td>2.11</td>
</tr>
<tr>
<td>$k_{pro}$</td>
<td>Basal proliferation rate</td>
<td>$1.2 \times 10^{-4}$/hour</td>
</tr>
<tr>
<td>$p_{proRB1pp_1}$</td>
<td>Proliferation rate increased by RB1-pp</td>
<td>6.01μM</td>
</tr>
<tr>
<td>$p_{proRB1pp_2}$</td>
<td>Parameter 1 of proliferation rate increased by RB1-pp</td>
<td>266.30</td>
</tr>
<tr>
<td>$k_{carying}$</td>
<td>Carrying capacity</td>
<td>37.39a.u.</td>
</tr>
<tr>
<td>$E2_{dep1}$</td>
<td>Estrogen concentration in E2 deprivation media 0 to 3 days</td>
<td>$9.67 \times 10^{-5}$μM</td>
</tr>
<tr>
<td>$E2_{dep2}$</td>
<td>Estrogen concentration in E2 deprivation media 3 to 7 days</td>
<td>$2.30 \times 10^{-5}$μM</td>
</tr>
<tr>
<td>$E2$</td>
<td>Estrogen concentration</td>
<td>0.01μM</td>
</tr>
<tr>
<td>$ICI$</td>
<td>ICI 182,780 concentration</td>
<td>0.5μM</td>
</tr>
<tr>
<td>palbo</td>
<td>Palbociclib concentration</td>
<td>1μM</td>
</tr>
</tbody>
</table>

The culture media, including any drugs, is changed at T = 0 and T = 3d for the experiments (E2 control, E2 deprivation, +E2 + ICI and –E2 + ICI, +E2 + palbo and –E2 + palbo), so the longest period without resupplying the drugs is 4 days. In the model, the drug concentration is assumed to be constant throughout the experiment. There is no data we are aware of for the half-life of either palbociclib or fulvestrant in our in-vitro culture conditions. Most data are for the plasma half-life or terminal half-life in-vivo, which are not applicable to our case as they are determined primarily by processing in the liver and excretion through the kidneys. There is some data for the in-vitro stability of these drugs in human plasma, which is somewhat akin to our conditions. Palbociclib shows less than 5% degradation in human plasma at room temperature over 3 days [127]. Fulvestrant shows no degradation in human plasma at room temperature over 7 hours [128]. Based on this data, we believe the half-life in our system is sufficiently long that assuming a constant level of drug is a reasonable approximation.

2.5.2 Model Equations

\[
\frac{dER}{dt} = k_{ER} - kd_{ER} \times ER
\]

\[
-kb_{E2ER} \times E2 \times ER + kub_{E2ER} \times E2ER
\]
\[-kb_{ICIER} \times ICI \times ER + kub_{ICIER} \times ICI\] (3)

1) Translation and degradation of ERα

2) Binding and unbinding between ERα and E2

3) Binding and unbinding between ERα and ICI 182,780

\[
\frac{dE2ER}{dt} = -kd_{E2ER} \times E2ER + kb_{E2ER} \times E2 \times ER - kub_{E2ER} \times E2ER
\] (4)

4) Degradation of E2ER

5) Binding and unbinding between ERα and E2

\[
\frac{dICIER}{dt} = kb_{ICIER} \times ICI \times ER - kub_{ICIER} \times ICIER - kd_{ICIER} \times ICI
\] (6)

6) Binding and unbinding between ICI 182,780 and ERα

7) Degradation of ICIER

\[
\frac{dcyclinD1}{dt} = -kd_{cyclinD1} \times cyclinD1 + k_{cyclinD1} \times \left(1 + k_{cyclinD1E2ER} \times \frac{E2ER_{p}cyclinD1E2ER_{2}}{P_{cyclinD1E2ER_{1}} + P_{cyclinD1E2ER_{2}} + E2ER_{p} cyclinD1E2ER_{2}}\right)
\] (9)

\[-kb_{cyclinD1p21} \times cyclinD1 \times p21 + kub_{cyclinD1p21} \times cyclinD1p21\] (10)

\[-kb_{cyclinD1pa} \times cyclinD1 \times palbo + kub_{cyclinD1pa} \times cyclinD1pa\] (11)

8) Degradation of cyclinD1

9) Basal translation of cyclinD1 and the increased translation by E2ER

(10) Binding and unbinding between cyclinD1 and p21

(11) Binding and unbinding between cyclinD1 and palbociclib

\[
\frac{dcyclinD1p21}{dt} = -kd_{cyclinD1} \times cyclinD1p21 + kb_{cyclinD1p21} \times cyclinD1 \times p21 - kub_{cyclinD1p21} \times cyclinD1p21
\] (12)

(12) Degradation of p21 bound cyclinD1
(13) Binding and unbinding between cyclinD1 and p21
\[
\frac{dc_{\text{cyclinD1palbo}}}{dt} = -kd_{\text{cyclinD1}} \times \text{cyclinD1palbo} + kb_{\text{cyclinD1palbo}} \times \text{cyclinD1} \times \text{palbo} - kub_{\text{cyclinD1palbo}} \times \text{cyclinD1palbo}
\]

(14) Degradation of palbociclib bound cyclinD1

(15) Binding and unbinding between cyclinD1 and palbociclib
\[
\frac{dc_{\text{Myc}}}{dt} = -kd_{\text{cMyc}} \times \text{cMyc} + k_{\text{cMyc}} \times (1 + k_{\text{cMyc\text{E2ER}}}) \times \frac{E2ER_{p\text{Myc\text{E2ER}_{1}}}}{p_{\text{Myc\text{E2ER}_{1}}} + E2ER_{p\text{Myc\text{E2ER}_{2}}}} + k_{\text{cMycRB1pp}} \times \frac{RB1pp_{p\text{MycRB1pp_{1}}}}{p_{\text{MycRB1pp_{1}}} + RB1pp_{p\text{MycRB1pp_{2}}}}
\]

(16) Degradation of c-Myc

(17) Basal translation of c-Myc and the increased translation by E2ER

(18) Increased translation of c-Myc by RB1-pp
\[
\frac{dp_{21}}{dt} = -kd_{p_{21}} \times p_{21} + k_{p_{21}} \times \frac{p_{p_{21cMyc_{1}}} p_{p_{21cMyc_{2}}}}{p_{p_{21cMyc_{1}}} + e_{\text{Myc}} p_{p_{21cMyc_{2}}}} - kb_{\text{cyclinD1p_{21}}} \times \text{cyclinD1} \times p_{21} + kub_{\text{cyclinD1p_{21}}} \times \text{cyclinD1p_{21}} - kb_{\text{cyclinEp_{21}}} \times \text{cyclinE} \times p_{21} + kub_{\text{cyclinEp_{21}}} \times \text{cyclinEp_{21}}
\]

(19) Degradation of p21

(20) Basal translation and the inhibition of translation by c-Myc

(21) Binding and unbinding between cyclinD1 and p21

(22) Binding and unbinding between cyclinE and p21
\[
\frac{dc_{\text{cyclinE}}}{dt} = k_{\text{cyclinE}} - kd_{\text{cyclinE}} \times \text{cyclinE} - kb_{\text{cyclinEp_{21}}} \times \text{cyclinE} \times p_{21} + kub_{\text{cyclinEp_{21}}} \times \text{cyclinEp_{21}}
\]

(23) Translation and degradation of cyclinE

(24) Binding and unbinding between cyclinE and p21
\[
\frac{dcyclinEp_{21}}{dt} = -kd_{cyclinE} \times cyclinEp_{21} \\
+kb_{cyclinEp_{21}} \times cyclinE \times p21 - kub_{cyclinEp_{21}} \times cyclinEp_{21} \tag{25}
\]

(25) Degradation of p21 bound cyclinE

\[
\frac{dRB1}{dt} = k_{RB1} - kd_{RB1} \times RB1 \\
+kr_{RB1RB1pp} \times \frac{RB1_{pp}^{PRB1RB1pp_2}}{PRB1_{RB1pp_2} + RB1_{pp}^{PRB1RB1pp_2}} \tag{27}
\]

(27) Degradation of RB1 and basal translation

\[
-k_{RB1cyclinD1} \times cyclinD1 \times \frac{RB1^{p\text{cyclinD1RB1}_{12}}}{p_{\text{cyccilinD1RB1}_{12}} + RB1^{p\text{cyclinD1RB1}_{12}}} \\
+kr_{RB1p\text{depho}} \times \frac{RB1_{pp}^{PRB1p2}}{PRB1_{p2} + RB1_{pp}^{PRB1p2}} \tag{28}
\]

(28) Increased translation by E2F, modeled as proportional to RB1 pp

\[
-k_{RB1pcyclinE} \times cyclinE \times \frac{RB1_{pp}^{p\text{cyclinERB1p2}}}{p_{\text{cyclinERB1p2}} + RB1_{pp}^{p\text{cyclinERB1p2}}} \\
+kr_{RB1pp\text{depho}} \times \frac{RB1_{pp}^{PRB1pp_2}}{PRB1_{pp_2} + RB1_{pp}^{PRB1pp_2}} \tag{29}
\]

(29) Phosphorylation of RB1 by cyclinD1

\[
-k_{RB1pcyclinE} \times cyclinE \times \frac{RB1_{pp}^{p\text{cyclinERB1p2}}}{p_{\text{cyclinERB1p2}} + RB1_{pp}^{p\text{cyclinERB1p2}}} \\
+kr_{RB1pp\text{depho}} \times \frac{RB1_{pp}^{PRB1pp_2}}{PRB1_{pp_2} + RB1_{pp}^{PRB1pp_2}} \tag{30}
\]

(30) Dephosphorylation of RB1-p

\[
-k_{RB1pcyclinE} \times cyclinE \times \frac{RB1_{pp}^{p\text{cyclinERB1p2}}}{p_{\text{cyclinERB1p2}} + RB1_{pp}^{p\text{cyclinERB1p2}}} \\
+kr_{RB1pp\text{depho}} \times \frac{RB1_{pp}^{PRB1pp_2}}{PRB1_{pp_2} + RB1_{pp}^{PRB1pp_2}} \tag{31}
\]

(31) Degradation of RB1-p

\[
-k_{RB1pcyclinE} \times cyclinE \times \frac{RB1_{pp}^{p\text{cyclinERB1p2}}}{p_{\text{cyclinERB1p2}} + RB1_{pp}^{p\text{cyclinERB1p2}}} \\
+kr_{RB1pp\text{depho}} \times \frac{RB1_{pp}^{PRB1pp_2}}{PRB1_{pp_2} + RB1_{pp}^{PRB1pp_2}} \tag{32}
\]

(32) Phosphorylation of RB1 by cyclinD1

\[
-k_{RB1pcyclinE} \times cyclinE \times \frac{RB1_{pp}^{p\text{cyclinERB1p2}}}{p_{\text{cyclinERB1p2}} + RB1_{pp}^{p\text{cyclinERB1p2}}} \\
+kr_{RB1pp\text{depho}} \times \frac{RB1_{pp}^{PRB1pp_2}}{PRB1_{pp_2} + RB1_{pp}^{PRB1pp_2}} \tag{33}
\]

(33) Dephosphorylation of RB1-p

\[
-k_{RB1pcyclinE} \times cyclinE \times \frac{RB1_{pp}^{p\text{cyclinERB1p2}}}{p_{\text{cyclinERB1p2}} + RB1_{pp}^{p\text{cyclinERB1p2}}} \\
+kr_{RB1pp\text{depho}} \times \frac{RB1_{pp}^{PRB1pp_2}}{PRB1_{pp_2} + RB1_{pp}^{PRB1pp_2}} \tag{34}
\]

(34) Phosphorylation of RB1-p by cyclinE

\[
-k_{RB1pcyclinE} \times cyclinE \times \frac{RB1_{pp}^{p\text{cyclinERB1p2}}}{p_{\text{cyclinERB1p2}} + RB1_{pp}^{p\text{cyclinERB1p2}}} \\
+kr_{RB1pp\text{depho}} \times \frac{RB1_{pp}^{PRB1pp_2}}{PRB1_{pp_2} + RB1_{pp}^{PRB1pp_2}} \tag{35}
\]

(35) Dephosphorylation of RB1-pp
\[
\frac{dRB_{1pp}}{dt} = -kd_{RB_{1pp}} \times RB_{1pp} \quad (36)
\]
\[
+k_{RB_{1pcyclinE}} \times cyclinE \times \frac{RB_{1pp}^{p_{cyclinERB_{1p2}}}}{p_{cyclinERB_{1p1}}^{p_{cyclinERB_{1p2}}} + RB_{1pp}^{p_{cyclinERB_{1p2}}}} \quad (37)
\]
\[
-k_{RB_{1ppdepho}} \times \frac{RB_{1pp}^{p_{RB_{1pp2}}} + RB_{1pp}^{p_{RB_{1pp2}}}}{p_{RB_{1pp1}}^{p_{RB_{1pp2}}} + RB_{1pp}^{p_{RB_{1pp2}}}} \quad (38)
\]

(36) Degradation of RB1-pp

(37) Phosphorylation of RB1-p by cyclinE

(38) Dephosphorylation of RB1-pp

\[
\frac{dcell}{dt} = k_{pro} \times (1 + k_{proRB_{1pp}} \times \frac{RB_{1pp}^{p_{proRB_{1pp2}}}}{p_{proRB_{1pp1}}^{p_{proRB_{1pp2}}} + RB_{1pp}^{p_{proRB_{1pp2}}}}) \times cell \times (1 - \frac{cell}{k_{carrying}}) \quad (39)
\]

(39) Basal proliferation and the increased proliferation by RB1-pp

To model resistance, we added the following equation:

\[
\frac{dres}{dt} = par_{1res} \times palbo - par_{2res} \times res \quad (40)
\]

and added one term to the cyclinD1 equation:

\[
\frac{dcyclinD1}{dt} = -kd_{cyclinD1} \times cyclinD1 \quad (8)
\]
\[
+k_{cyclinD1} \times \left(1 + k_{cyclinD1E2ER} \times \frac{E2ER^{p_{cyclinD1E2ER2}}}{p_{cyclinD1E2ER1}^{p_{cyclinD1E2ER2}} + E2ER^{p_{cyclinD1E2ER2}}}ight) \quad (9)
\]
\[
-k_{b_{cyclinD1p21}} \times cyclinD1 \times p21 + k_{ub_{cyclinD1p21}} \times cyclinD1p21 \quad (10)
\]
\[
-k_{b_{cyclinD1palbo}} \times cyclinD1 \times palbo + k_{ub_{cyclinD1palbo}} \times cyclinD1palbo \quad (11)
\]
\[
+par_{3res} \times \frac{res^{par_{5res}}}{par_{4res}^{res^{par_{5res}} + res^{par_{5res}}}} \quad (41)
\]

\[par_{1res} = 1e^{-4}, par_{2res} = 1e^{-3}, par_{3res} = 0.819, par_{4res} = 0.06, par_{5res} = 4.87\]

2.5.3 Model Summary

The mathematical model contains 14 ordinary differential equations (ODEs) and has 62 parameters. It is implemented in MATLAB (MathWorks, Inc., Massachusetts, United States). The synthesis, degradation, phosphorylation, dephosphorylation, association and dissociation reactions are modeled by mass action laws and Hill functions. ICI 182,780 and palbociclib effects are modeled
by competitive binding to ERα [129] or cyclinD1, representing cyclinD1:Cdk4/6 [60]. The ODEs are solved numerically by the ode23tb function. RB1 has an important role in the G1-S transition and it is inactivated by phosphorylation [130]. A total of over 15 phosphorylation sites are found on RB1 (i.e., T5, S249, S252, T356, T373, S567, S608, S612, S780, S788, S795, S807, S811, T821, T826) [103]. In late G1, cyclinE:Cdk2 kinase mediates the phosphorylation of RB1 on S612 and T373 relieving RB1’s repression of E2F activity [103, 104]. As cyclinD1:Cdk4/6 kinases hypophosphorylate RB1 in G1 and cyclinE:Cdk2 kinase hyperphosphorylates RB1-p in late G1 [131], we chose one form of phosphorylated RB1 (S612) to represent the phosphorylation status of RB1 by cyclinE:Cdk2 kinase.

2.5.4 Data Normalization

Protein levels were measured by Western blotting at time points 0h, 4h, 1d, 3d, 6d and 7d for the E2 deprivation, +E2 +ICI and −E2 +ICI conditions, and at 0h, 1d, 3d, 6d and 7d for the +E2 +palbo and −E2 +palbo conditions. The blots for the treatment conditions are normalized to actin and β-tubulin, to account for loading variation, normalized to time 0 h, to account for antibody affinity variations, and normalized to the (normalized) E2 control values for each species at each time point, to account for variations in the expression levels of species in the cells used for a given experiment. Since the control cells are not subject to treatment perturbations (they continue to be grown in E2), the asynchronous population would ideally have constant expression levels of the various molecules. This is not the case in our experiments as there are frequently transients during the first few time points, perhaps due to the shock of reattachment upon plating before time 0. Furthermore, after 3 days the control cells begin to approach confluence, while the cells under treatment conditions do not begin to approach confluence until 7 days. At confluence, paracrine signaling and contact inhibition suppress proliferation via cell cycle arrest [132]. To avoid the confounding effect of confluence on the control cells, we assume the expression levels of the species in the control cells after 3 days are the same as their levels at the 3d time point and use the 3d levels for normalization of the future time points of the treated cells. This approach allows our normalization to deal with the initial transients in expression levels while not confounding the normalization of time points after 3 days with the effects of confluence on the control cells.
2.5.5 Local sensitivity analysis

Local sensitivity analysis concerns the sensitivity of a model output to changes in the input parameters [133]. The local sensitivities of the seven measured output variables are calculated with respect to the parameters at day 7 time point [134].

\[
s_{ijk} = \frac{\partial \log(X_{ij})}{\partial \log(P_k)} = \frac{\partial X_{ij}}{\partial P_k} \times \frac{P_k}{X_{ij}}
\]

where \( s_{ijk} \) is the local sensitivity value, which is the derivative of output \( X_{ij} \) with respect to parameter \( P_k \) multiplied by the ratio \( P_k / X_{ij} \). It represents the relative change in the model output induced by a small relative change in a parameter. In the equation, \( i \) indexes the outputs (protein level or cell number), \( j \) indexes the time points, and \( k \) indexes the parameters.

\( s_{ijk} \) is approximated by the second-order central finite difference. Each parameter is individually varied by \( \pm 5\% \) of its value. Therefore,

\[
s_{ijk} \approx \frac{X_{ij}(P_k + 5\% \times P_k) - X_{ij}(P_k - 5\% \times P_k)}{10\% \times P_k} = \frac{X_{ij}(P_k + 5\% \times P_k) - X_{ij}(P_k - 5\% \times P_k)}{10\% \times X_{ij}(P_k)}
\]

All parameters that were varied to fit the data were selected for local sensitivity analysis, while the fixed parameters were excluded. The 44 parameters, from top to bottom in Figure 2.8 and Figure 2.11, are: \( k_{ER}, kb_{E2ER}, kb_{ICIER}, kd_{ICIER}, k_{cyclinD1}, k_{cyclinD1E2ER}, p_{cyclinD1E2ER1}, p_{cyclinD1E2ER2}, kb_{cyclinD1p21}, kb_{cyclinD1p1}, k_{cMyc}, k_{cMycE2ER}, p_{cMycE2ER1}, p_{cMycE2ER2}, k_{cMycRB1pp}, \)
$p_{CyclinB1pp}$, $p_{p21}$, $p_{p21cMyc}$, $k_{cyclinD1}$, $k_{cyclinEp21}$, $k_{RB1}$, $k_{RB1pp}$, $p_{RB1RB1pp}$, $p_{RB1pdepho}$, $p_{RB1cyclinE}$, $k_{RB1pdepho}$, $p_{cyclinD1RB1}$, $p_{RB1ppdepho}$, $p_{RB1ppdepho}$, $k_{pro}$, $k_{proRB1pp}$, $k_{carrying}$, $E_{dep1}$, $E_{dep2}$

2.5.6 Variation of the cohort parameters and plots of the individual trajectories

Figure 2.11. Local sensitivity of total cyclinD1, total ER$\alpha$, total p21 and RB1-pp. Sensitivity of each protein at the 7d time point with respect to all variable parameters.
Figure 2. 12. Coefficients of variation and histogram of cost values for parameter sets in the simulation cohort. Ordering of parameters corresponds to that in the sensitivity plots.

Figure 2. 13. All cohort trajectories plotted with experimental protein data that was used to fit the model. (a) –E2 condition. (b) +E2 + ICI condition.
Figure 2. 14. All cohort trajectories plotted with experimental proliferation data that was used to fit the model.

Figure 2. 15. All cohort trajectories plotted with experimental protein data used to fit the parameters involving palbociclib in the model.
Figure 2. 16. All cohort trajectory predictions for the –E2 + ICI case plotted with experimental protein validation data.

Figure 2. 17. All cohort trajectory predictions plotted with the experimental validation data. (a) Protein predictions for the –E2 + palbo case. (b) Proliferation predictions for the –E2 + ICI and –E2 + palbo cases.

2.5.7 Synergy analysis of the combination treatments

Figure 2.18 shows the synergy scores of the combination treatments –E2 + ICI and –E2 + palbo for proliferation at different time points. The HSA and Bliss synergy scores are calculated using the R package SynergyFinder [135]. The score, in percent, is equal to the percent inhibition of the combination therapy minus the expected inhibition. For HSA the expected inhibition is the highest percent inhibition of the two monotherapies, and for Bliss the expected inhibition is the sum of the percent inhibition of each monotherapy minus the product of the percent inhibitions of the two therapies. Thus, a positive score represents a synergistic effect. There is no significant synergy, and some antagonism indicated in the experimental results for the –E2 + ICI combination therapy. The simulations show some synergy for this case because they slightly under-predict the true proliferation of the combination. For the case of the –E2 + palbo combination, there is some indication of synergy in the experimental results and slightly less in the simulation results due to the slight over-prediction of proliferation for the combination in the simulations. A synergistic
effect between –E2 and palbociclib is consistent with the use of palbociclib in conjunction with endocrine therapies in the clinic [136].

Figure 2. 18. HAS and Bliss cell proliferation synergy scores of –E2 + ICI and –E2 + palbo treatments. The scores of the experimental results are shown with solid lines (mean value ± s.d., n = 3). The scores of the simulation results are shown dashed lines (mean value ± s.d., n = 400).
Chapter 3. Long-term mathematical model for MCF7 cells: response to therapies and drug synergies

3.1 Extend short-term model to long-term model

In Chapter 2, we built a mechanistic ODE model that allows us to investigate the effect of different treatments that target components in the model. The model can be used to predict changes in protein levels and proliferation in response to combination treatment conditions. The ability of the model to predict combination treatments and to incorporate palbociclib provides evidence that the model captures key aspects of the system, which makes it reasonable and to extend this short-term model to longer time scales. Therefore, one of the ideas of Chapter 3 is to extend the short-time scale (days) model to a longer-time scale (weeks). The goal is a model that can accurately predict the effect of alternating therapies over long time scales to enable the design of long-term experiments and the development of optimized protocols that can be tested experimentally. Moreover, extending the simulation time of the short-term model may not produce accurate results because some effects important in short-term may not carry over to the long-term. We need to check if the model that it can handle long-term continuous therapy and determine any changes needed to handle alternating therapies.

Figure 3.1. Directly extend short-term model simulation to long-term for E2 deprivation case.
Even for continuous therapy, the simulation result from directly extending the short-term model to a longer time scale may cause the simulation to diverge from experiment. In Figure 3.1, the blue dashed line is the simulation result for E2 deprivation from the short-term model by extending the simulation time to 21 days. The short-term simulation result shows a continuing proliferation, but the experimental result shows that the cell number becomes nearly constant after 7 days, illustrating the need to revise the model. The potential cause of this mismatch might be from the E2 deprivation procedure, which is conducted by exchanging the +E2 medium with 5% charcoal stripped calf serum (CCS) and phenol-red free media [137, 138]. The E2 level in CCS is routinely measured to be less than 4 pM [137], equating to 0.2 pM E2 in 5% CCS media [139]. However, the E2 in the cell, which is at a significantly higher concentration than that in the previous medium, can diffuse back into the medium and cause an increase in the E2 concentration. While the concentration of E2 might be low, its effect might not be negligible because 10 pM can cause significant proliferation of MCF7 cells [140]. As shown in Figure 3.2 left, even in 1 pM estrogen concentration media, MCF7 cells can grow about seven times at 1 week. As the medium is replaced as the experiment proceeds, the E2 level will continue to drop. In a typical one-week experiment, we change the medium at time zero and at 3 days, counting the cells on day 7. In the right plot of Figure 3.2, an extra media change was inserted at 3 hours. We can see that for both initial conditions (cell number plated), the extra media change, which further decrease the residual E2 level, significantly decreases the overall MCF7 proliferation at 1 week.

Figure 3. 2. Effect of E2 concentration on MCF7 proliferation.
While the changes in E2 concentration with each successive medium change might not be critically important for continuous –E2 therapy, these changes will be important when we consider alternating therapies. For example, if we alternate +E2 + palbo with –E2 therapy, then after the transition from +E2 to –E2 medium there will be excess E2 in the medium that will cause significant undesired growth during the first week. This issue drove us to model the dynamics of E2 concentration more accurately.

In the short-term model in Chapter 2, we modeled the E2 concentration after deprivation simply as constant levels that changed with each medium change. But this simple method requires extra parameters for the E2 level in the cell at each successive medium change. In the worst case of a 28 day –E2 simulation, we may need 8 new parameters for the medium changes. So, we need to model the effect dynamically. From the data, the initial growth is period is short and the cells nearly stop growing later on. Furthermore, other than estrogen receptors, there exist nonspecific bindings between estrogen and other elements inside the cell [141]. Therefore, when we deprive the medium of estrogen, a new balance between the estrogen levels inside and outside the cell must be achieved. It is likely that the growth after deprivation results from this new, smaller level of estrogen. Thus, modeling the E2 dynamics during and after the deprivation becomes important and modeling the E2 level may be needed to capture the effect of alternating therapy.

3.2 Material and methods

The cell culture and reagents, cell proliferation assays, western blot analysis, parameter estimation and statistical analysis is the same as Chapter 2.

3.3 Dynamic modelling estrogen deprivation

In the diffusion of E2 between cell and media, the total amount (# number) of E2 diffuse back and forth should be constant, which means the amount of E2 left from the cell should be equal to the amount of E2 come into the media and vice versa. The rate of number of E2 in the cell change cause by diffusion is:

\[
\frac{dE2_{\text{cell}}}{dt} = -k_{\text{diff}} \times ([E2]_{\text{cell}} - [E2]_{\text{media}}) \times N \times S_{\text{cell}}
\]  

(1)

where \(E2_{\text{cell}}\) is the total number (#) of E2 in the cells, \(k_{\text{diff}}\) is a diffusion rate of cell and \(-k_{\text{diff}} \times ([E2]_{\text{cell}} - [E2]_{\text{media}})\) has unit #/(m²×t), \([E2]_{\text{cell}}\) is the E2 concentration in the cell,
$[E2]_{\text{media}}$ is the E2 concentration in the media, $N$ is the total cell number and $S_{1\text{cell}}$ is the surface area of a single cell.

Because $E2_{\#\text{cell}} = [E2]_{\text{cell}} \times V\text{ol}_{\text{cells}}$, where $V\text{ol}_{\text{cells}}$ is the total volume of MCF7 cells, then equation (1) becomes:

$$\frac{d([E2]_{\text{cell}} \times V\text{ol}_{\text{cells}})}{dt} = -k_{\text{diff}} \times ( [E2]_{\text{cell}} - [E2]_{\text{media}} ) \times N \times S_{1\text{cell}}$$

(2)

$$\frac{d[E2]_{\text{cell}}}{dt} \times V\text{ol}_{\text{cells}} + \frac{dV\text{ol}_{\text{cells}}}{dt} \times [E2]_{\text{cell}} = -k_{\text{diff}} \times ([E2]_{\text{cell}} - [E2]_{\text{media}}) \times N \times S_{1\text{cell}}$$

(3)

then,

$$\frac{d[E2]_{\text{cell}}}{dt} \times V\text{ol}_{\text{cells}} = -k_{\text{diff}} \times ([E2]_{\text{cell}} - [E2]_{\text{media}}) \times N \times S_{1\text{cell}} - \frac{dV\text{ol}_{\text{cells}}}{dt} \times [E2]_{\text{cell}}$$

(4)

$$\frac{d[E2]_{\text{cell}}}{dt} = -k_{\text{diff}} \times ([E2]_{\text{cell}} - [E2]_{\text{media}}) \times N \times S_{1\text{cell}} - \frac{dV\text{ol}_{\text{cells}}}{dt} \times \frac{[E2]_{\text{cell}}}{V\text{ol}_{\text{cells}}} \times \frac{1}{V\text{ol}_{\text{cells}}}$$

(5)

$$\frac{d[E2]_{\text{cell}}}{dt} = -k_{\text{diff}} \times \frac{V\text{ol}_{\text{cell}}}{V\text{ol}_{\text{cell}}} \times ([E2]_{\text{cell}} - [E2]_{\text{media}}) - \frac{dV\text{ol}_{\text{cells}}}{dt} \times [E2]_{\text{cell}} \times \frac{1}{V\text{ol}_{\text{cells}}}$$

(6)

$$\frac{-k_{\text{diff}} \times S_{1\text{cell}} \times ([E2]_{\text{cell}} - [E2]_{\text{media}})}{V\text{ol}_{\text{cell}}}$$

is the rate change related to diffusion. $\frac{dV\text{ol}_{\text{cells}}}{dt} \times [E2]_{\text{cell}} \times \frac{1}{V\text{ol}_{\text{cells}}}$ is the rate change related to volume variations of total cells (dilution). The total amount E2 diffuses in or out from the cell is $\frac{-k_{\text{diff}} \times S_{1\text{cell}} \times N \times V\text{ol}_{\text{cell}}}{V\text{ol}_{\text{cell}}} \times ([E2]_{\text{cell}} - [E2]_{\text{media}})$. The total number of E2 molecules diffusing in or out from the media is $k_{\text{diff media}} \times V\text{ol}_{\text{media}} \times ([E2]_{\text{cell}} - [E2]_{\text{media}})$.

$k_{\text{diff media}}$ is a rate with unit 1/t and we need an expression for $k_{\text{diff media}}$. As mentioned above, the number of E2 molecules diffusing out of the cell must equal the number of E2 molecules coming in to the medium. Suppose $[E2]_{\text{cell}} > [E2]_{\text{media}}$ or vice versa, we can get

$$\frac{k_{\text{diff}} \times S_{1\text{cell}} \times N \times V\text{ol}_{\text{cell}}}{V\text{ol}_{\text{cell}}} ([E2]_{\text{cell}} - [E2]_{\text{media}}) = k_{\text{diff media}} \times V\text{ol}_{\text{media}} ([E2]_{\text{cell}} - [E2]_{\text{media}})$$

(7)

Then

$$k_{\text{diff media}} = \frac{k_{\text{diff}} \times S_{1\text{cell}} \times N}{V\text{ol}_{\text{media}}}$$

(9)
If we give set $k'_{\text{diff}} = \frac{k_{\text{diff}} \times S_{\text{cell}}}{\text{Vol}_{\text{cell}}}$, then equation (7) becomes

$$\frac{d[E2]_{\text{cell}}}{dt} = -k'_{\text{diff}} \times ([E2]_{\text{cell}} - [E2]_{\text{media}}) - \frac{dV_{\text{cells}}}{dt} \times [E2]_{\text{cell}} \times \frac{1}{V_{\text{cell}} \times \text{Vol}_{\text{cells}}} \quad (10)$$

Then equation (9) becomes

$$k'_{\text{diff, media}} = \frac{k'_{\text{diff}} \times \text{Vol}_{\text{cell}} \times N}{\text{Vol}_{\text{media}}} \quad (11)$$

If we suppose the volume of the culture media doesn’t change (no dilution), then the rate of E2 concentration changes in the media becomes

$$\frac{d[E2]_{\text{media}}}{dt} = \frac{k'_{\text{diff}} \times \text{Vol}_{\text{cell}} \times N}{\text{Vol}_{\text{media}}} \times ([E2]_{\text{cell}} - [E2]_{\text{media}}) \quad (12)$$

Next, we need an expression for $\frac{dV_{\text{cells}}}{dt}$ in equation (10). Suppose there exist alive cells and apoptotic cells in the total population of cells, then $N = N_{\text{alive}} + N_{\text{apoptosis}}$ and

$$\frac{dN}{dt} = \frac{dN_{\text{alive}}}{dt} + \frac{dN_{\text{apoptosis}}}{dt} \quad (13)$$

Then

$$\frac{dN_{\text{alive}}}{dt} \times \text{Vol}_{1_{\text{cell}}} = \left(\frac{dN_{\text{alive}}}{dt} + \frac{dN_{\text{apoptosis}}}{dt}\right) \times \text{Vol}_{1_{\text{cell}}} \quad (14)$$

$$\frac{dV_{\text{cells}}}{dt} = \left(\frac{dN_{\text{alive}}}{dt} + \frac{dN_{\text{apoptosis}}}{dt}\right) \times \text{Vol}_{1_{\text{cell}}} \quad (15)$$

Then

$$\frac{dV_{\text{cells}}}{dt} \times \frac{1}{\text{Vol}_{\text{cells}}} = \left(\frac{dN_{\text{alive}}}{dt} + \frac{dN_{\text{apoptosis}}}{dt}\right) \times \text{Vol}_{1_{\text{cell}}} \times \frac{1}{\text{Vol}_{\text{cells}}} \quad (16)$$

$$\frac{dV_{\text{cells}}}{dt} \times \frac{1}{\text{Vol}_{\text{cells}}} = \frac{\left(\frac{dN_{\text{alive}}}{dt} + \frac{dN_{\text{apoptosis}}}{dt}\right)}{N} \quad (17)$$

Then equation (10) becomes

$$\frac{d[E2]_{\text{cell}}}{dt} = -k'_{\text{diff}} \times ([E2]_{\text{cell}} - [E2]_{\text{media}}) - \frac{\left(\frac{dN_{\text{alive}}}{dt} + \frac{dN_{\text{apoptosis}}}{dt}\right)}{N} \times [E2]_{\text{cell}} \quad (18)$$

Then equations (18) and (12) can be used to model the E2 dynamics during and after the deprivation. Each time the medium is changed to one without E2, the value $[E2]_{\text{media}}$ is set to zero.
3.4 Adding an apoptosis term in the model

In order to model the decrease of cell numbers that happens in long time treatments, we added an apoptosis rate in the equations. Like Chapter 2, breast cancer cell proliferation is proportional to current cell number and the proportionality constant depends on the RB1-pp level via a Hill function. Similar to the proliferation, the rate of cellular apoptosis is modelled as proportional to the current number of cells. There is a basal death rate not affected by Cdk4/6 inhibitors, which represents the many other factors affecting apoptosis, as well as a term dependent on the abemaciclib concentration.

3.5 Data collection

The parameters are calibrated on the data including all experiment data used Chapter 2 (including the data used for predication in Chapter 2), experiment data from Figure 3.3, Figure 3.5, Figure 3.6 and Figure 4.1.

3.6 Result

3.6.1 Model simulation of long-term proliferation results in response to different mono- and combination therapies

Figure 3.3 shows that the model can simulate long-term experimental proliferation results in response to different therapies. The E2 control condition only has seven days of data in order to avoid cell confluence happen when cell cultured longer than 21 days. Statistically significant changes from the E2 control are denoted by asterisks. Because the E2 control data are limited to one-week, different treatment results longer than 1 week without an asterisk don’t imply insignificance but rather no data for comparison. In the –E2 condition in Figure 3.3, we can see that cells proliferate during the first seven days and then stop growing, which is the same as Figure 3.1. But by re-calibrating the parameters and adding the E2 dynamic equations to the model, unlike the blue dash line in Figure 3.1, which directly extends the short-term model simulation to a longer-term and fails to match the experimental data, the long-term model simulation result is consistent with the –E2 experimental result by lowering the E2 concentration at each media change. Moreover, as mentioned in Section 3.1, even 10 pM of E2 can cause significant growth in 3 weeks. The model can also accurately simulate the responses to various doses of ICI and palbociclib given as monotherapies as well as different combination therapies. The cell numbers decrease under
long-term –E2 + palbo treatment because the RB1-pp level is driven extremely low, causing the proliferation rate to be lower than the apoptosis rate. The simulation result can fit these data by adding an apoptosis rate in the equations, which is modelled as proportional to the current number of cells (see Section 3.6).

Figure 3.3. Long-term model simulation and experimental measurements of the normalized cell numbers under different treatment condition. Experimental results are in red (mean value ± s.e., n = 3) and simulations are in cyan. Cell numbers are normalized to T = 0 value. Statistically significant changes from the +E2 control are denoted by asterisks. +E2 control condition data is limited to one-week due to confluence, different treatment results longer than 1 week without an asterisk have no data to compare.

At the same time capturing the short- and long-term proliferation results, the model can roughly capture the protein level changes data used in Chapter 3 (see Section 3.6).

3.6.2 Adding abemaciclib to the model

In Section 2.3.3, we added palbociclib to the model, an inhibitor of Cdk4/6 that is used in conjunction with endocrine therapies to provide a more durable inhibition of proliferation [136].
Palbociclib is the pyrido [2, 3-d] pyridopyrimidine approved in the United States in 2015 that reversibly inhibits Cdk4/6 kinase activity [142]. It binds in the ATP binding pocket between the N-terminal and C-terminal domains of the kinase and separates cyclinD1:Cdk4/6 complexes [143, 144]. It inhibits cyclinD1/Cdk4, cyclinD3/Cdk4 and cyclinD2/Cdk6 kinase activities and is ineffective on cylinE2/Cdk2, cyclinA/Cdk2, and cyclinB/Cdk1 activities [62]. Although palbociclib can induce apoptosis in xenograft animal models, the drug’s effect is cytostatic (G1 arrest) in cell culture systems [62]. As mentioned above, palbociclib has been used as standard treatment with this Luminal A subtype breast cancer patients in conjunction with endocrine therapies (aromatase inhibitor or ICI) to provide a more durable inhibition of tumor proliferation [12, 61, 62].

In addition to palbociclib, we now consider one of the other Cdk4/6 inhibitors, abemaciclib (LY2835219). Abemaciclib is a 2-anilino-2, 4-pyrimidine-[5-benzimidazole] derivative [62]. Unlike palbociclib, it has been reported to be effective as a single-agent [18, 145, 146]. It inhibits cyclinD1/Cdk4 and cyclinD1/Cdk6 kinase activities at low nanomolar concentrations [62]. And it can also decrease cyclinB1/Cdk1, cyclinE/Cdk2, cyclinH/Cdk7 and cyclinK/T1/Cdk9 kinase activities at higher micromolar concentrations [62, 147]. Moreover, other than Cdk4/6, abemaciclib is able to inhibit DYRK (Dual Specificity Tyrosine Phosphorylation Regulated Kinase)/HIPK (Homeodomain Interacting Protein Kinase) [148] and GSK3α/β (Glycogen Synthase Kinase-3 α/β), activating the Wnt signaling pathway [149]. Most importantly, in contrast to palbociclib, which only induces cell cycle arrest in the G1 phase with little or no apoptosis, abemaciclib can arrest the cell in both the G1 and G2 phases of the cell cycle and elicit apoptosis at doses greater than 0.3 µM, which might be caused by Cdk1/2 inhibition [79]. Based on the signaling mechanism of abemaciclib, we add the key interactions between abemaciclib and Cdk4/6 and Cdk2. And we explicitly add the variable Cdk4/6 to the model. The wiring diagram of the mathematical model after adding abemaciclib is shown in Figure 3.4. Abemaciclib can bind to Cdk4/6 and Cdk2 (represented by cyclinE), and it can also induce apoptosis.

We assume that the major effect of inhibiting ERα signaling is to stop proliferation by arresting the cell in G1 [16]. So E2 deprivation and ICI treatment will not increase the apoptosis rate. Similarly, palbociclib is cytostatic and will not cause cell death [62]. As shown in Figure 3.3, this
basal death rate accounts for the cell number decrease in the –E2 + palbo condition. In addition to the basal death rate, the apoptosis rate depends on the abemaciclib level via a Hill.

Figure 3.4. Wiring diagram of the mathematical model after adding abemaciclib. The meaning of the lines and colors are the same as Figure 2.1.

As in Section 2.3.3 about adding palbociclib to the model, we measured only c-Myc and RB1-pp to calibrate the binding and unbinding parameters associated with abemaciclib in the model. Figure 3.5 shows the results of calibrating the model to 500 nM abemaciclib added to the +E2 medium. The inhibition of cyclinD1:Cdk4/6 and cyclinE:Cdk2 kinase activity by abemaciclib decreases the RB1-pp level, which in turn cause the c-Myc level to decrease.
Figure 3.5. Simulation and experimental results for protein-levels changes in response to abemaciclib. Simulations are shown in blue and experimental measurements in red (mean value ± s.e., n = 3). Statistically significant changes from T = 0 are denoted by asterisks.

Figure 3.6 shows that model can also fit the experimental proliferation results for the +E2 + abema (300nM) and +E2 + abema (500nM) cases on which the model was calibrated. This again support that proliferation in these cases can be modelled using the RB1-pp (S612) level.

Figure 3.6. Model simulations and experimental measures of the normalized cell numbers under different abemaciclib treatment conditions. Experimental counts are in red (mean value ± s.e., n = 3) and simulations are in blue. E2 concentration is 10 nM. Cell numbers are normalized to T = 0 value. Statistically significant changes from the +E2 control are denoted by asterisks. +E2 control condition data is limited to one-week due to confluence, different treatment results longer than 1 week without an asterisk have no data to compare.
Because abemaciclib induces concentration-dependent apoptosis of breast cancer cells in addition to G1 arrest, abemaciclib should be more versatile than palbociclib at blocking cell proliferation. We used the Apotracker probe to detect apoptotic cells in culture. Annexin V, used in a standard assay for apoptosis, binds to phosphatidylserine, which is a marker of apoptosis normally restricted to the intracellular leaflet but appearing on the outer leaflet when apoptosis occurs because of the remodeling of the plasma membrane caused by the apoptosis. Since Annexin V can exhibit high background staining caused by its Ca\(^{2+}\) dependence, we used the highly stable fluorogenic peptide Apotracker that binds to apoptotic cells in a Ca\(^{2+}\) independent manner [150]. It has been demonstrated that Apotracker selectively stains apoptotic cells in vitro and can be used for quantification of apoptosis induced by drug treatment [150].

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Figure 3. 7. Simulation and experiment result of apoptosis in MCF7 cells in response to abemaciclib treatment, measured by Apotracker. (a) Flow cytometry plots for Apotracker (x-axis) and Helix NP NIR nucleic acid stain (y-axis) at T = 0. (b) Flow cytometry plots at T = 1 day, 3 day, 6 day and 7 day on +E2 control condition. (c) Flow cytometry plots at T = 1 day, 3 day, 6 day
and 7 day on +E2 + abema (500nM) condition. (d) Model simulation result of the normalized apoptosis ratio.

Figure 3.7 shows the flow cytometry plots for Apotracker. The x-axis is the stain of Apotracker, the same as Annexin V, which is compete for binding to phosphatidylserine with Annexin V [150]. This strong preference may be favored by the electrostatic interactions between the negatively-charged groups in phosphatidylserine and the positively-charged residues of Apotracker [150]. The y-axis is Helix NP which is impermeant to live cells and so can discriminate between live and dead cells. The lower-left quadrant represents the live cell percentage, the upper-left quadrant represents the dead cell percentage, the lower-right quadrant represents the early apoptotic cell percentage, and the upper-right quadrant represents the late apoptotic cell percentage [151]. We define the percentage of apoptotic cells as the sum of the percentages of early and late apoptotic cells. Figure 3.7a shows the percentages of all cell states (alive, dead, early apoptosis and late apoptosis) at T = 0. Figures 3.7b-c show how the cell states changes from 1 day to 7 days in the +E2 control and +E2 + abema (500nM) conditions. We can see that 500nM abemaciclib treatment increases the apoptotic cell percentage from day 1 through 7 compared to the +E2 control condition. Figure 3.7d plots the simulation results for the apoptotic percentage for the +E2 + abema (500nM) condition normalized to the +E2 control condition. Because there is only one replication of the experimental result, we didn’t try to match the data quantitatively, but qualitatively show that adding the death rate caused by abemaciclib will increase the apoptosis percentage. In the ODE model, the apoptotic cells represent the sum of early and late apoptotic cells (see Section 3.6).

3.6.3 The model can predict the synergy of various combinations

Cancer cells depend on several molecular mechanisms for proliferation or survival, so people often use drug combinations to simultaneously target these key molecular mechanisms to more effectively stop proliferation, reduce cell number, or delay or overcome resistance [152]. One of the most important questions in drug combinations is whether there is a synergism between the drugs. Testing for synergism experimentally can be time consuming as there are many possible variations in combinations and doses.

When the combined effect of two drugs is enhanced compared to the effect predicted by their individual potencies, it is said that the combination is synergistic [153]. A synergistic interaction
between drugs may allow to dramatically lower doses of the individual drugs when used in combination as opposed to individually. It may benefit patients by reducing toxicity and adverse effects. In order to reduce the labor and resource intensive procedure of identification of drug synergy, systematic computational approaches to calculate and predict synergy have been used [154].

Figure 3.8. Illustration of the isobologram. (a) Measurement of mono- or combination-drug treatment effects. (b) Illustration of an isobole. (c) Evaluation of different interaction types between drugs by isoboles. Black isoboles: independent relationship; yellow isobole: antagonistic relationship; gray isobole: additive relationship; dashed purple isobole: subadditive relationship; solid purple isobole: superadditive relationship.

A common way to quantitatively assess the interaction relationship between two drugs is by isobologram, which is a graph of isoboles, lines of constant effect, proposed by Loewe in 1953 [153]. Figure 3.8 shows an illustration of the idea of an isobologram. The isobologram is based on drug effect measurements made for a large number of different doses for the drugs given individually and in combination. Figure 3.8a illustrates an ideal sampling scheme, where each axis represents the dose for a specified drug and each point represents the doses used in a given measurement. Each red filled circle is a measurement of mono-drug effect from drug 1 and each green square is a measurement of mono-drug effect from drug 2. Each blue pentagon is a measurement of the combination effect from the specified doses of drug 1 and drug 2. By drug effect we mean a change in some measurable attribute of interest. It might be the percent of apoptotic cells in a culture, the cellular death or proliferation rate, a reduction in tumor size, the
DNA replication rate, the toxicity, or some other physiological index such as heart rate or blood pressure. Any measurable metric of interest can be used to defined an isobologram.

After the measuring the effect at each dosage point of the isobologram, we can draw the isoboles, which are lines joining the points of equal measured effect. So, an isobole is a contour line of equal drug treatment effect. Figure 3.8b shows an example isobole, where the effect of the different drug treatments on the isobole have the same value. The relationship between the two drugs can be defined from the shape of the isoboles. The two black isoboles in Figure 3.8c show an independent relationship between drug 1 and drug 2. When we combine these two drugs, cells will only respond to the most effective single drug (drug 1 or drug 2, depending on which has a dominant effect) [155]. If drug 1 has the most potency, then adding drug 2 doesn’t increase or decrease the overall effect and vice versa. The yellow line in Figure 3.8c illustrates an antagonistic relationship between drug 1 and drug 2, meaning that adding drug 2 with drug 1 forces the dose of drug 1 to be increased in order to have the same effect and vice versa. The gray line in Figure 3.8c shows an additive relationship between drug 1 and drug 2, meaning that the combination effect of the two drugs is consistent with their individual potencies. Put another way, if \( d \) is the dose of a drug and \( D \) is the dose of that drug producing the specified effect as a monotherapy, then an additive effect means that changing the normalized dose of drug 1, \( d1/D1 \), requires an equal and opposite change in the normalized dose of drug 2, \( d2/D2 \), in order to preserve the specified effect. Then, additive (linear) isobole can be used to distinguish between superadditive and subadditive combinations [153]. If the position of an isobole is lower than the gray additive isobole, the solid purple isobole in Figure 3.8c, it is called superadditive [156]. We define the superadditive effect as synergistic because adding a small amount of drug 2 can significantly decrease the amount of drug 1 need to achieve the same effect as monotherapy with drug 1 and vice versa. If the position of the isobole is higher than the gray additive isobole, the dashed purple isobole in Figure 3.8c, it is called subadditive [156]. In the subadditive situation, decreasing the dose of drug 1 by adding drug 2, while keeping the same effect, requires a larger amount of drug 2 compared with the additive relationship, and an even larger amount compared with the superadditive (synergistic) relationship.

Now that our model is calibrated for four different mono-therapies (E2 deprivation, +E2 + ICI, +E2 + palbo, and +E2 + abema) at different doses, we can use it to simulate combination effects and predict the relationships between combinations of therapies. Because growth rate is the critical
effect that we want to analyze using different drug treatments, the metric we used in our isobologram is the normalized cell number.

Figure 3.9 shows the simulated isobologram for normalized cell number between ICI and E2. The value on each isobole is the normalized cell number simulated at 17 days. Compared with Figure 3.8, we can see that the relationship between ICI and E2 deprivation is additive. This is reasonable because both ICI and E2 deprivation target the estrogen signaling pathway and their effects are similar, to decrease the E2:ER transcription factor level without any other targets in the model. There are only binding and unbinding reactions between E2 and ER, and ICI and ER, so the level of E2:ER will linearly decrease after increasing ICI or decreasing E2. Therefore, the effects of ICI and E2 deprivation as mono and combination therapies are the same, to linearly decrease E2:ER level.
Figure 3.10. Simulated isobolegram between palbociclib and E2 at different ranges. The value on each isobole is the normalized cell number simulated at 17 days.

Figure 3.10 shows the simulated isobologram on normalized cell number between palbociclib and E2. The value on each isobole is the normalized cell number simulated at 17 days. The left plot of Figure 3.10 shows a coarse grid of palbociclib and E2 which is at high concentration levels. The right plot of Figure 3.10 shows a refined grid of palbociclib and E2 at low concentration. Compared with Figure 3.8, we can see that the relationship between palbociclib and E2 deprivation will be independent when E2 level is high, which means a lower E2 level doesn’t decrease palbociclib dose when aiming for the same degree of cell proliferation inhibition. At lower levels of E2, we can see from the right plot of Figure 3.10 that there will be a mildly subadditive effect between palbociclib and E2 deprivation.

Figure 3.11 shows the simulated isobologram on normalized cell number between abemaciclib and E2. The value on each isobole is the normalized cell number simulated at 17 days. The relationship between abemaciclib and E2 is similar as palbociclib with E2, which is independent at high levels of E2 and additive or synergistic at low level of E2.
Figure 3. 11. Simulated isobologram between abemaciclib and E2 at different ranges. The value on each isobole is the normalized cell number simulated at 17 days.

Figure 3. 12. Simulated isobologram between abemaciclib and palbociclib at different ranges. The value on each isobole is the normalized cell number simulated at 17 days.

Figure 3.12 shows the simulated isobologram on normalized cell number between abemaciclib and palbociclib. The value on each isobole is the normalized cell number simulated at 17 days. The relationship between abemaciclib and palbociclib is additive. Additivity is reasonable because abemaciclib and palbociclib both target the Cdk4/6 activity with a binding-unbinding reaction.
Figure 3.13. Simulated isobologram between ICI and palbociclib for two different concentration ranges and the experimental test results. (a) The value on each isobole is the normalized cell number simulated at 17 days. (b) Experimental results of the normalized cell number at 17 days.

Figure 3.13a shows the simulated isobologram between ICI and palbociclib for two different concentration ranges. From this plot we can see that there is a significant synergism between ICI and palbociclib, which means if we keep the same inhibition effect, adding a little bit of palbociclib will drastically decrease ICI dose and vice versa. It also means that if we keep ICI concentration constant, adding a small amount of palbociclib will significantly decrease the normalized cell number. This huge synergism between ICI and palbociclib is a prediction of the model and needs to be tested experimentally. Therefore, we did a 17 day proliferation experiment and the results are shown in Figure 3.13b. From this plot we can see that adding 50 nM palbociclib to 200 nM ICI dramatically decreases the proliferation from over 40 times to about 10 times. Adding 100 nM palbociclib to 200 nM ICI further decreases the proliferation and nearly stops the proliferation. So the model-predicted synergism is confirmed by the experimental results. What we want to emphasize is that we only calibrated the model parameters on ICI and palbociclib monotherapies, which means we didn’t use any +E2 + palbo + ICI combination treatment experimental data to calibrate the model. We believe the reason that the model can give an experimentally consistent prediction of this significant synergism is because the structure of the model is based on the dominant signaling pathways of the system. This result also illustrates the advantage of a mechanistic model. In our mechanistic model, we include ICI’s effects on E2:ER, E2:ER’s effects on cyclinD1, and palbo’s effects on Cdk4/6. Therefore, the activity of the cyclinD1:Cdk4/6 kinase is attacked from both the cyclinD1 and Cdk4/6 directions to create this dramatic synergism. This may be the reason that palbociclib in combination with endocrine therapies achieved substantial
improvement in survival outcomes in clinical trials and quickly became the first-line choice of treatment of ER+ breast cancer [17]. While usefulness of palbociclib in combination with endocrine therapies might be argued from a knowledge of the pathways alone, only a mathematical model is capable of predicting that there will be a dramatic synergy. This synergy is in contrast to the combination of ICI and E2 deprivation, whose mechanism overlap, both target E2:ER, and produce an additive but not synergistic response.

Figure 3. 14. Simulated isobologram between ICI and abemaciclib for different concentration ranges and the experimental test results. (a) The value on each isobole is the normalized cell number simulated at 17 days. (b) Experimental results of the normalized cell numbers at 17 days.

Figure 3.14a shows the simulated isobologram between ICI and abemaciclib for different concentration ranges. From this plot we can see that there is also a significant synergism between ICI and abemaciclib as was the case with ICI and palbociclib. As previously, we did a 17 day experiment to confirm the predicted result, as shown in Figure 3.14b. The explanation for the synergism between abemaciclib and ICI is similar to that for palbocilib and ICI. Abemaciclib, like palbociclib, inhibits Cdk4/6 kinase activity from a complementary direction to that from endocrine therapies to create the dramatic synergism with ICI.

3.7 Discussion

In Chapter 3, we first extended the short-time scale (days) model to a longer-time scale (weeks) to enable simulation of long-term treatment effects that are not visible in the short term. In order to extend the model, we added E2 dynamics into the model to account for the media change effect. After that, we also added another Cdk4/6 inhibitor, abemaciclib, into the model, which not only
inhibits the kinase activity but also increases apoptosis. Then we can use the model to simulate not only the proliferation of cells but also the death of cells. Finally, we used the model to explore synergism among the different treatments included in the model and verified the significant synergisms predicted between ICI and palbociclib or abemaciclib. This significant synergism between these Cdk4/6 inhibitors and endocrine therapy can be predicted and explained by our mechanistic mathematical model, which includes their corresponding pathways. A small amount of Cdk4/6 inhibitor can diminish the remaining Cdk4/6 activities targeted by endocrine therapy and this effect can be reflected by the mathematical model. The combination of Cdk4/6 inhibitors and endocrine therapy creates noteworthy synergism, which could be the reason that Cdk4/6 inhibitors achieved great success in the clinic from the mechanistic side. Most importantly, we illustrated that the mechanistic mathematical model can be utilized to predict and optimize a drug treatment protocol that can be successfully validated experimentally. Mathematical modeling can potentially complement current experimental and clinical research, possibly providing nonintuitive drug treatment protocols and benefiting the future study of cancer biology.

3.8 Supporting materials

3.8.1 Supplement plots for protein

In Chapter 3, we extend the short-term model to a long-term model and simulate the proliferation results. We don’t have long-term experimental results for protein level changes in response to various mono- or combination treatments. But we would like to make the model roughly match the 7-day protein level changes shown in Chapter 2. So, the long-term model is calibrated using the protein data in addition to the proliferation data and the simulation results are shown from Figure 3.15 to Figure 3.19. The figures show that the simulation results for these protein level changes do not match the experimental results as well as in Chapter 2. For explaining these results, it is important to clarify what can be expected of a model of the type we propose. The behaviour of protein levels in cells in response to therapeutic perturbations is the result of thousands, if not millions, of interactions among DNA, RNA and proteins. It is clearly impossible at present to model this level of complexity, and so of necessity we use a vastly simplified model to attempt to capture the key effects of therapy. It means that the simple model is unlikely to provide an excellent match to the protein data if we also want to match all the various mono-, combination and alternating proliferation data. Therefore, the protein plots here are different from the Chapter 2
protein plots. To explain how simplification of the model may impact matching the data, we know that E2:ERα is not only a transcription factor of cyclinD1, an effect included in our wiring diagram, but also a transcription factor of cyclinE1 [10], an effect not included in our model as we don’t have a measurement of cyclinE1. Exclusion of the E2:ERα transcriptional effect on cyclinE1 in the model could lead the model to increase the inhibition effect on cyclinD1. Because now the decrease of kinase activity by –E2 or ICI treatment all depends on the decrease of cyclinD1, instead of a decrease of both cyclinD1 and cyclinE1. It can potentially make an over reduction of cyclinD1 when we use the simplified model to match the experimental data of endocrine treatment. This effect is seen in Figure 3.15, Figure 3.16 and Figure 3.17 where the model used a large change in total cyclinD1 that is not supported by the data.

Another issue is that we only use RB1-pp to control the proliferation, but there are many other factors affecting the G1 to S phase transition that impact the proliferation. Furthermore, in RB1-pp itself there are over 15 phosphorylation sites and we only use the S612 phosphorylation site [102]. Therefore, if we only use RB1-pp changes to match proliferation data under various treatments, it is easy to create some discrepancy between the simulation and experiment results on RB1-pp level, which is shown on Figure 3.18. And the mismatch of RB1-pp can cause a mismatch of c-Myc, as its level is impact by RB1-pp.

The last implication for the model is that many reaction rates used in the model account for numerous unmodelled effects and because there is no corresponding rate in the literature. For example, we use the decay rate of Cdk4/6 for all the complexes bound with Cdk4/6, such as cyclinD1:Cdk4/6. The half-life of cyclinD1 is 0.4 hour and of Cdk4/6 is 5 hours, but we suppose the binding of cyclinD1 to Cdk4/6 would not shorten the Cdk4/6 half-life over 10 times so we keep its half-life unchanged.
Figure 3. 15. Simulation and experimental results for protein-level changes under –E2. The experimental measurements are shown in red (mean value ± s.e., n = 3) and the simulation results are shown in blue. Statistically significant changes from T = 0 are denoted by an asterisk.

Figure 3. 16. Simulation and experimental results for protein-level changes under +E2 + ICI treatment. The experimental measurements are shown in red (mean value ± s.e., n = 3) and the simulation results are shown in blue. Statistically significant changes from T = 0 are denoted by an asterisk. ICI concentration is 500 nM and +E2 concentration is 10 nM.
Figure 3. 17. Simulations for protein-level changes in response to combination –E2 + ICI therapy. ICI concentration is 500 nM. Experimental results are in red (mean value ± s.e., n = 3), simulations are in blue. Statistically significant changes from T = 0 are denoted by asterisks.

Figure 3. 18. Simulations for of c-Myc and RB1-pp levels in response to +E2 + palbo combination treatment. Asterisks denote statistically significant changes from T = 0.
Figure 3. 19. Simulations for of c-Myc and RB1-pp levels in response to –E2 + palbo combination treatment. Asterisks denote statistically significant changes from T = 0.

![Image showing western blot data for +E2 + abema treatment.

Abemaciclib concentration is 500 nM.

3.8.2 Model variables and parameters

<table>
<thead>
<tr>
<th>Variable name</th>
<th>Description</th>
<th>Initial value</th>
<th>Half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) E2media</td>
<td>E2 concentration in the media</td>
<td>9.9nM</td>
<td>-</td>
</tr>
<tr>
<td>(2) E2cell</td>
<td>E2 concentration in the cell</td>
<td>9.9nM</td>
<td>-</td>
</tr>
<tr>
<td>(3) ER</td>
<td>Estrogen receptor α</td>
<td>6.5nM</td>
<td>4-5h [109]</td>
</tr>
<tr>
<td>(4) E2ER</td>
<td>Estrogen bound estrogen receptor alpha</td>
<td>1003.3nM</td>
<td>3-4h [109]</td>
</tr>
<tr>
<td>(5) E2NSB</td>
<td>Estrogen bound non-specific binding</td>
<td>6944.8nM</td>
<td>-</td>
</tr>
<tr>
<td>(6) ICIER</td>
<td>ICI 182,780 bound estrogen receptor</td>
<td>0nM</td>
<td>&lt; 3-4h [109]</td>
</tr>
<tr>
<td>(7) cyclinD1</td>
<td>Protein cyclinD1</td>
<td>6.6×10^{-6}μM</td>
<td>0.4h [122]</td>
</tr>
<tr>
<td>(8) cdk46</td>
<td>Protein Cdk4/6</td>
<td>6905.3nM</td>
<td>5h [157]</td>
</tr>
<tr>
<td>(9) cdk46palbo</td>
<td>palbociclib bound cdk4/6</td>
<td>0nM</td>
<td>-</td>
</tr>
<tr>
<td>(10) cdk46abema</td>
<td>abemaciclib bound cdk4/6</td>
<td>0nM</td>
<td>-</td>
</tr>
<tr>
<td>(11) cyclinD1cdk46</td>
<td>cyclinD1 bound cdk4/6</td>
<td>327.8nM</td>
<td>-</td>
</tr>
<tr>
<td>(12) cyclinD1cdk46p21</td>
<td>p21 bound cyclinD1:Cdk4/6</td>
<td>8.4nM</td>
<td>-</td>
</tr>
<tr>
<td>(13) cyclinD1cdk46palbo</td>
<td>palbociclib bound cyclinD1:Cdk4/6</td>
<td>0nM</td>
<td>-</td>
</tr>
<tr>
<td>(14) cyclinD1cdk46abema</td>
<td>abemaciclib bound cyclinD1:Cdk4/6</td>
<td>0nM</td>
<td>-</td>
</tr>
<tr>
<td>(15) cMyc</td>
<td>Protein c-Myc</td>
<td>13.2nM</td>
<td>0.333h [123]</td>
</tr>
<tr>
<td>(16) p21</td>
<td>Protein p21</td>
<td>0.0003nM</td>
<td>0.33-1h [124]</td>
</tr>
<tr>
<td>(17) cyclinE</td>
<td>Protein cyclinE</td>
<td>0.182nM</td>
<td>0.5h [125]</td>
</tr>
<tr>
<td>(18) cyclinEp21</td>
<td>p21 bound cyclinE protein</td>
<td>0.005nM</td>
<td>-</td>
</tr>
<tr>
<td>(19) cyclinEabema</td>
<td>abemaciclib bound cyclinE</td>
<td>0nM</td>
<td>-</td>
</tr>
<tr>
<td>(20) RB1</td>
<td>Retinoblastoma protein</td>
<td>2.28×10^{-5}nM</td>
<td>2-3h [126]</td>
</tr>
<tr>
<td>(21) RB1p</td>
<td>Hypophosphorylated RB1 (RB1-p)</td>
<td>542.7nM</td>
<td>2-3h [126]</td>
</tr>
<tr>
<td>(22) RB1pp</td>
<td>Hyperphosphorylated RB1 (RB1-pp)</td>
<td>0.556nM</td>
<td>&gt;4h [126]</td>
</tr>
</tbody>
</table>
### Table 3.1. Model variable description.

<table>
<thead>
<tr>
<th>Parameter name</th>
<th>Description</th>
<th>Value</th>
<th>Fixed or calibrated</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{diff}$</td>
<td>Diffusion rate of E2</td>
<td>4943.26/hour</td>
<td>Calibrated</td>
</tr>
<tr>
<td>$k_{bNSB}$</td>
<td>Binding rate between non-specific binding and E2</td>
<td>694.57/(hour×nM)</td>
<td>Calibrated</td>
</tr>
<tr>
<td>$k_{ubNSB}$</td>
<td>Unbinding rate between non-specific binding and E2</td>
<td>1.0/hour</td>
<td>Fixed</td>
</tr>
<tr>
<td>$Vol_{cell}$</td>
<td>Volume of MCF7 cell</td>
<td>$8\times10^5$mL</td>
<td>Fixed</td>
</tr>
<tr>
<td>$Vol_{media}$</td>
<td>Volume of media</td>
<td>10mL</td>
<td>Fixed</td>
</tr>
<tr>
<td>$k_{ER}$</td>
<td>Translation rate of ERα</td>
<td>301.65nM/hour</td>
<td>Calibrated</td>
</tr>
<tr>
<td>$k_{dER}$</td>
<td>Degradation rate of ERα</td>
<td>0.10/hour</td>
<td>Fixed</td>
</tr>
<tr>
<td>$k_{dE2ER}$</td>
<td>Degradation rate of E2ER</td>
<td>0.30/hour</td>
<td>Fixed</td>
</tr>
<tr>
<td>$k_{bE2ER}$</td>
<td>Binding rate between E2 and ERα</td>
<td>20.02/(hour×nM)</td>
<td>Calibrated</td>
</tr>
<tr>
<td>$k_{ubE2ER}$</td>
<td>Unbinding rate between E2 and ERα</td>
<td>1.0/hour</td>
<td>Fixed</td>
</tr>
<tr>
<td>$k_{bICIER}$</td>
<td>Binding rate between ICI and ERα</td>
<td>0.48/(hour×nM)</td>
<td>Calibrated</td>
</tr>
<tr>
<td>$k_{ubICIER}$</td>
<td>Unbinding rate between ICI and ERα</td>
<td>1.0/hour</td>
<td>Fixed</td>
</tr>
<tr>
<td>$k_{dICIER}$</td>
<td>Degradation rate of ICIER</td>
<td>0.52/hour</td>
<td>Calibrated</td>
</tr>
<tr>
<td>$k_{cyclinD1}$</td>
<td>Translation rate of cyclinD1</td>
<td>5.27nM/hour</td>
<td>Calibrated</td>
</tr>
<tr>
<td>$k_{dCDK}$</td>
<td>Degradation rate of cyclinD1</td>
<td>1.73/hour</td>
<td>Fixed</td>
</tr>
<tr>
<td>$P_{cyclinD1E2ER_1}$</td>
<td>Parameter 1 of cyclinD1 increased translation by E2ER</td>
<td>837.11nM</td>
<td>Calibrated</td>
</tr>
<tr>
<td>$P_{cyclinD1E2ER_2}$</td>
<td>Parameter 2 of cyclinD1 increased translation by E2ER</td>
<td>4.70</td>
<td>Calibrated</td>
</tr>
<tr>
<td>$k_{bcl cyclinD1Cdk46}$</td>
<td>Binding rate between cyclinD1 and Cdk4/6</td>
<td>8051.60</td>
<td>Calibrated</td>
</tr>
<tr>
<td>$k_{ubcl cyclinD1Cdk46}$</td>
<td>Unbinding rate between cyclinD1 and Cdk4/6</td>
<td>1/hour</td>
<td>Fixed</td>
</tr>
<tr>
<td>$k_{dCdk46}$</td>
<td>Translation rate of Cdk4/6</td>
<td>836.39nM/hour</td>
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</tr>
<tr>
<td>$k_{dCdk46}$</td>
<td>Degradation rate of Cdk4/6</td>
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<tr>
<td>$k_{bcl cyclinD1Cdk46Palbo}$</td>
<td>Binding rate between cyclinD1:Cdk4/6 and palbociclib</td>
<td>0.028/(hour×nM)</td>
<td>Calibrated</td>
</tr>
<tr>
<td>$k_{ubcl cyclinD1Cdk46Palbo}$</td>
<td>Unbinding rate between cyclinD1:Cdk4/6 and palbociclib</td>
<td>1/hour</td>
<td>Fixed</td>
</tr>
<tr>
<td>$k_{bcl cyclinD1Cdk46Abemaca}$</td>
<td>Binding rate between cyclinD1:Cdk4/6 and abemaciclib</td>
<td>0.04/(hour×nM)</td>
<td>Calibrated</td>
</tr>
<tr>
<td>$k_{ubcl cyclinD1Cdk46Abemaca}$</td>
<td>Unbinding rate between cyclinD1:Cdk4/6 and abemaciclib</td>
<td>1/hour</td>
<td>Fixed</td>
</tr>
<tr>
<td>$k_{dCdk46Palbo}$</td>
<td>Binding rate between Cdk4/6 and palbociclib</td>
<td>20.73/(hour×nM)</td>
<td>Calibrated</td>
</tr>
<tr>
<td>$k_{dCdk46Abemaca}$</td>
<td>Binding rate between Cdk4/6 and abemaciclib</td>
<td>10.00/(hour×nM)</td>
<td>Calibrated</td>
</tr>
<tr>
<td>$k_{bcl cyclinD1Cdk46P21}$</td>
<td>Binding rate between cyclinD1:Cdk4/6 and p21</td>
<td>100.21/(hour×nM)</td>
<td>Calibrated</td>
</tr>
<tr>
<td>$k_{ubcl cyclinD1Cdk46P21}$</td>
<td>Unbinding rate between cyclinD1:Cdk4/6 and p21</td>
<td>1/hour</td>
<td>Fixed</td>
</tr>
<tr>
<td>$k_{dMyc}$</td>
<td>Translation rate of c-Myc</td>
<td>4.53nM/hour</td>
<td>Calibrated</td>
</tr>
<tr>
<td>$k_{dCMyC}$</td>
<td>Degradation rate of c-Myc</td>
<td>2.31/hour</td>
<td>Fixed</td>
</tr>
<tr>
<td>$k_{dMycE2ER}$</td>
<td>Increased translation of c-Myc by E2ER</td>
<td>8.60</td>
<td>Calibrated</td>
</tr>
<tr>
<td>$P_{CMyC_1}$</td>
<td>Parameter 1 of c-Myc increased translation by E2ER</td>
<td>1332.97nM</td>
<td>Calibrated</td>
</tr>
<tr>
<td>$P_{CMyC_2}$</td>
<td>Parameter 2 of c-Myc increased translation by E2ER</td>
<td>2.74</td>
<td>Calibrated</td>
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<td>$k_{dMycRB1pp}$</td>
<td>Increased translation of c-Myc by RB1-pp</td>
<td>2608.65</td>
<td>Calibrated</td>
</tr>
<tr>
<td>$k_{dMycRB1pp_1}$</td>
<td>Parameter 1 of c-Myc increased translation by RB1-pp</td>
<td>1.14nM</td>
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</tr>
<tr>
<td>$k_{dMycRB1pp_2}$</td>
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<td>9.42</td>
<td>Calibrated</td>
</tr>
<tr>
<td>$k_{p21}$</td>
<td>Translation rate of p21</td>
<td>0.32nM/hour</td>
<td>Calibrated</td>
</tr>
</tbody>
</table>
Table 3. 2. Model parameter description.

### 3.8.3 Model equations

\[ N = N_{\text{alive}} + N_{\text{apoptosis}} \]  

(1)
(1) Total cell number equal alive cells plus apoptosis cells

\[
\frac{dN_{alive}}{dt} = k_{pro} \times (1 + k_{proRB1pp} \times \frac{RB1pp^p_{proRB1pp}}{p_{proRB1pp}^p_{proRB1pp} + RB1pp^p_{proRB1pp}^2}) \times N_{alive} \times (1 - \frac{N}{k_{carrying}})
\]  

(2)

\[
-k_{death} \times N_{alive}
\]

(3)

\[
-k_{deathabema} \times \frac{abema_{deathabema_2}^p}{p_{deathabema_1}^p + abema_{deathabema_2}^p} \times N_{alive}
\]

(4) Basal proliferation and the increased proliferation by RB1-pp

(3) Basal apoptosis

(4) Increased apoptosis by abemaciclib

\[
\frac{dN_{apoptosis}}{dt} = k_{death} \times N_{alive}
\]

(5)

\[
+k_{deathabema} \times \frac{abema_{deathabema_2}^p}{p_{deathabema_1}^p + abema_{deathabema_2}^p} \times N_{alive}
\]

(6)

\[
-k_{disappear} \times N_{apoptosis}
\]

(5) Basal apoptosis

(6) Increased apoptosis by abemaciclib

(7) Disappearance of apoptotic cells

\[
\frac{dE2_{media}}{dt} = \frac{k_{diff} \times N \times Vol_{cell}}{Vol_{media}} \times (E2_{cell} - E2_{media})
\]

(8) E2 concentration changes in media

\[
\frac{dE2_{cell}}{dt} = -k_{diff} \times (E2_{cell} - E2_{media}) - \left(\frac{\frac{dN_{alive}}{dt} + \frac{dN_{apoptosis}}{dt}}{N}\right) \times E2_{cell}
\]

(9)

\[
-kb_{E2ER} \times E2_{cell} \times ER + kub_{E2ER} \times E2ER
\]

(10)

\[
-kb_{NSB} \times E2_{cell} + kub_{NSB} \times E2NSB
\]

(11)

\[
+kd_{E2ER} \times E2ER
\]

(9) E2 concentration changes in cell

(10) Binding and unbinding between ERα and E2 in the cell

(11) Binding and unbinding between non-specific binding and E2 in the cell
(12) Degradation of E2ER

\[ \frac{dE_{ER}}{dt} = k_{ER} - kd_{ER} \times ER \]

(13)

\[-kb_{E2ER} \times E_{cell} \times ER + kub_{E2ER} \times E2ER \]

(14)

\[-kb_{ICIER} \times ICI \times ER + kub_{ICIER} \times ICIER \]

(15)

(13) Translation and degradation of ERα

(14) Binding and unbinding between ERα and E2 in the cell

(15) Binding and unbinding between ERα and ICI 182,780

\[ \frac{dE2ER}{dt} = -kd_{E2ER} \times E2ER \]

(16)

\[ +kb_{E2ER} \times E_{cell} \times ER - kub_{E2ER} \times E2ER \]

(17)

(16) Degradation of E2ER

(17) Binding and unbinding between ERα and E2 in the cell

\[ \frac{dE2NSB}{dt} = kb_{NSB} \times E_{cell} - kub_{NSB} \times E2NSB \]

(18)

(18) Binding and unbinding between non-specific binding and E2 in the cell

\[ \frac{dICIER}{dt} = kb_{ICIER} \times ICI \times ER - kub_{ICIER} \times ICIER \]

(19)

\[-kd_{ICIER} \times ICIER \]

(20)

(19) Binding and unbinding between ICI 182,780 and ERα

(20) Degradation of ICIER

\[ \frac{dcyclinD1}{dt} = -kd_{cyclinD1} \times cyclinD1 \]

(21)

\[ +k_{cyclinD1} \times \left( 1 + k_{cyclinD1E2ER} \times \frac{E2ER^{p_{cyclinD1E2ER}}}{p_{cyclinD1E2ER}^{p_{cyclinD1E2ER}} + E2ER^{p_{cyclinD1E2ER}}} \right) \]

(22)

\[-kb_{cyclinD1cdk46} \times cyclinD1 \times cdk46 + kub_{cyclinD1cdk46} \times cyclinD1cdk46 \]

(23)

\[ +kub_{cyclinD1cdk46palbo} \times cyclinD1cdk46palbo \]

(24)

\[ +kub_{cyclinD1cdk46abema} \times cyclinD1cdk46abema \]

(25)

(21) Degradation of cyclinD1

(22) Basal translation of cyclinD1 and the increased translation by E2ER
(23) Binding and unbinding between Cdk4/6 and cyclinD1
(24) Unbinding between palbociclib and cyclinD1:Cdk4/6
(25) Unbinding between abemaciclib and cyclinD1:Cdk4/6

\[
\frac{dc_{dk46}}{dt} = k_{cdk46} - kd_{cdk46} \times cdk46
\]  
(26)

\[-kb_{cyclinD1cdk46} \times cyclinD1 \times cdk46 + kub_{cyclinD1cdk46} \times cyclinD1cdk46 \]  
(27)

\[-kb_{cdk46palbo} \times cdk46 \times palbo + kub_{cdk46palbo} \times cdk46palbo \]  
(28)

\[-kb_{cdk46abema} \times cdk46 \times abema + kub_{cdk46palbo} \times cdk46abema \]  
(29)

(26) Translation and degradation of Cdk4/6
(27) Binding and unbinding between Cdk4/6 and cyclinD1
(28) Binding and unbinding between palbociclib and Cdk4/6
(29) Binding and unbinding between abemaciclib and Cdk4/6

\[
\frac{dc_{dk46palbo}}{dt} = -kd_{cdk46} \times cdk46palbo
\]  
(30)

\[+kb_{cdk46palbo} \times cdk46 \times palbo - kub_{cdk46palbo} \times cdk46palbo \]  
(31)

\[+kub_{cyclinD1cdk46palbo} \times cyclinD1cdk46palbo \]  
(32)

(30) Degradation of palbociclib bound Cdk4/6
(31) Binding and unbinding between palbociclib and Cdk4/6
(32) Unbinding between palbociclib and cyclinD1:Cdk4/6

\[
\frac{dc_{dk46abema}}{dt} = -kd_{cdk46} \times cdk46abema
\]  
(33)

\[+kb_{cdk46abema} \times cdk46 \times abema - kub_{cdk46palbo} \times cdk46abema \]  
(34)

\[+kub_{cyclinD1cdk46abema} \times cyclinD1cdk46abema \]  
(35)

(33) Degradation of abemaciclib bound Cdk4/6
(34) Binding and unbinding between abemaciclib and Cdk4/6
(35) Unbinding between abemaciclib and cyclinD1:Cdk4/6
\[
\frac{dcyclinD_1cdk46}{dt} = -kd_{cdk46} \times cyclinD1cdk46
\]  
(36) Degradation of cyclinD1:Cdk4/6

\[
+kb_{cyclinD1cdk46} \times cyclinD1 \times cdk46 - kub_{cyclinD1cdk46} \times cyclinD1cdk46
\]  
(37) Binding and unbinding between Cdk4/6 and cyclinD1

\[-kb_{cyclinD1cdk46p21} \times cyclinD1cdk46 \times p21 + kub_{cyclinD1cdk46p21} \times cyclinD1cdk46p21
\]  
(38) Binding and unbinding between p21 and cyclinD1:Cdk4/6

\[
-kb_{cyclinD1cdk46palbo} \times cyclinD1cdk46 \times palbo
\]  
(39) Binding between palbociclib and cyclinD1:Cdk4/6

\[-kb_{cyclinD1cdk46abema} \times cyclinD1cdk46 \times abema
\]  
(40) Binding between abemaciclib and cyclinD1:Cdk4/6

\[
\frac{dcyclinD1cdk46p21}{dt} = -kd_{cdk46} \times cyclinD1cdk46p21
\]  
(41) Degradation of p21 bound cyclinD1:Cdk46

\[
+kb_{cyclinD1cdk46p21} \times cyclinD1cdk46 \times p21 - kub_{cyclinD1cdk46p21} \times cyclinD1cdk46p21
\]  
(42) Binding and unbinding between p21 and cyclinD1:Cdk4/6

\[
\frac{dcyclinD1cdk46palbo}{dt} = -kd_{cdk46} \times cyclinD1cdk46palbo
\]  
(43) Degradation of palbociclib bound cyclinD1:Cdk4/6

\[
+kb_{cyclinD1cdk46palbo} \times cyclinD1cdk46 \times palbo - kub_{cdk46palbo} \times cyclinD1cdk46palbo
\]  
(44) Binding and unbinding between palbociclib and cyclinD1:Cdk4/6

\[
\frac{dcyclinD1cdk46abema}{dt} = -kd_{cdk46} \times cyclinD1cdk46abema
\]  
(45) Degradation of abemaciclib bound cyclinD1:Cdk4/6

\[
+kb_{cyclinD1cdk46abema} \times cyclinD1cdk46 \times abema - kub_{cdk46abema} \times cyclinD1cdk46abema
\]  
(46) Binding and unbinding between abemaciclib and cyclinD1:Cdk4/6
(46) Binding and unbinding between abemaciclib and cyclinD1:Cdk4/6

\[
\frac{dc\text{Myc}}{dt} = -kd_{c\text{Myc}} \times c\text{Myc} \\
+ k_{c\text{Myc}} \times (1 + k_{c\text{Myc} E2\text{ER}} \times \frac{E2\text{ER}^{p\text{cMyc} E2\text{ER}_1}}{p\text{cMyc} E2\text{ER}_1 + E2\text{ER}^{p\text{cMyc} E2\text{ER}_2}}) \times (1 + k_{c\text{Myc}RB1pp} \times \frac{RB1pp^{p\text{cMyc}RB1pp_2}}{p\text{cMyc}RB1pp_2 + RB1pp^{p\text{cMyc}RB1pp_2}}) \\
+ k_{c\text{Myc}RB1pp} \times (1 + k_{c\text{Myc}RB1pp} \times \frac{RB1pp^{p\text{cMyc}RB1pp_2}}{p\text{cMyc}RB1pp_2 + RB1pp^{p\text{cMyc}RB1pp_2}}) \\
\]

(47) Degradation of c-Myc

(48) Basal translation of c-Myc and the increased translation by E2ER

(49) Increased translation of c-Myc by RB1-pp

\[
\frac{dp_{21}}{dt} = k_{p_{21}} - kd_{p_{21}} \times p_{21} \\
+ k_{p_{21}c\text{Myc}} \times (1 + k_{p_{21}c\text{Myc}} \times \frac{p_{21}c\text{Myc}_1}{p_{21}c\text{Myc}_1 + p_{21}c\text{Myc}_2}) \\
- k_{b\text{cyclinD1Cdk46p21}} \times \text{cyclinD1Cdk46} \times p_{21} + k_{b\text{cyclinD1Cdk46p21}} \times \text{cyclinD1Cdk46p21} \\
- k_{b\text{cyclinEp21}} \times \text{cyclinE} \times p_{21} + k_{b\text{cyclinEp21}} \times \text{cyclinEp21} \\
\]

(50) Translation and degradation of p21

(51) Inhibition of translation by c-Myc

(52) Binding and unbinding between cyclinD1:Cdk4/6 and p21

(53) Binding and unbinding between cyclinE and p21

\[
\frac{dc\text{cyclinE}}{dt} = k_{c\text{cyclinE}} - kd_{c\text{cyclinE}} \times c\text{cyclinE} \\
- k_{b\text{cyclinE}\text{p21}} \times c\text{cyclinE} \times p_{21} + k_{b\text{cyclinE}\text{p21}} \times c\text{cyclinE}\text{p21} \\
- k_{b\text{cyclinEabema}} \times c\text{cyclinE} \times abema + k_{b\text{cyclinEabema}} \times c\text{cyclinEabema} \\
\]

(54) Translation and degradation of cyclinE

(55) Binding and unbinding between cyclinE and p21

(56) Binding and unbinding between cyclinE and abemaciclib
\[
\frac{d\text{cyclinEp21}}{dt} = -kd_{\text{cyclinE}} \times \text{cyclinEp21} + kb_{\text{cyclinEp21}} \times \text{cyclinE} \times p21 - kub_{\text{cyclinEp21}} \times \text{cyclinEp21}
\]  
(57) Degradation of p21 bound cyclinE

\[
\frac{d\text{cyclinEabema}}{dt} = -kd_{\text{cyclinE}} \times \text{cyclinEabema} + kb_{\text{cyclinEabema}} \times \text{cyclinE} \times \text{abema} - kub_{\text{cyclinEabema}} \times \text{cyclinEabema}
\]  
(58) Binding and unbinding between cyclinE and p21

\[
\frac{dRB1}{dt} = k_{RB1} - kd_{RB1} \times RB1 + k_{RB1RB1pp} \times \frac{RB1ppPRB1RB1pp2}{PRB1RB1pp1PPRB1RB1pp2 + RB1ppPRB1RB1pp2}
\]

\[
- k_{RB1\text{cyclinD1cdk46}} \times \text{cyclinD1cdk46} \times \frac{RB1p\text{cyclinD1cdk46RB12}}{p\text{cyclinD1cdk46RB12} + RB1p\text{cyclinD1cdk46RB12}} + k_{RB1pdepho} \times \frac{RB1ppPRB1p2}{PRB1p1PPRB1p2 + RB1ppPRB1p2}
\]

(59) Degradation of abemaciclib bound cyclinE

(60) Binding and unbinding between cyclinE and abemaciclib

\[
\frac{dRB1}{dt} = k_{RB1} - kd_{RB1} \times RB1
\]

(61) Degradation of RB1 and basal translation

(62) Increased translation by E2F, modeled as proportional to RB1-pp

(63) Phosphorylation of RB1 by cyclinD1:Cdk4/6

(64) Dephosphorylation of RB1-p

\[
\frac{dRB1p}{dt} = -kd_{RB1p} \times RB1p
\]

\[
+ k_{RB1\text{cyclinD1cdk46}} \times \text{cyclinD1cdk46} \times \frac{RB1p\text{cyclinD1cdk46RB12}}{p\text{cyclinD1cdk46RB12} + RB1p\text{cyclinD1cdk46RB12}}
\]

\[
- k_{RB1pdepho} \times \frac{RB1ppPRB1p2}{PRB1p1PPRB1p2 + RB1ppPRB1p2}
\]

(65) Dephosphorylation of RB1-p
\[-k_{RB1pcyclinE} \times cyclinE \times \frac{RB1p_{cyclinERB1p2}}{p_{cyclinERB1p2}} \times \frac{RB1p_{cyclinERB1p2} + RB1p_{cyclinERB1p2}}{p_{RB1pp2 + RB1pp_{RB1pp2}}} \]  
(68)

\[+k_{RB1ppdepheo} \times \frac{RB1pp_{RB1pp2}}{p_{RB1pp2 + RB1pp_{RB1pp2}}} \]  
(69)

(65) Degradation of RB1-p

(66) Phosphorylation of RB1 by cyclinD1:Cdk4/6

(67) Dephosphorylation of RB1-p

(68) Phosphorylation of RB1-p by cyclinE

(69) Dephosphorylation of RB1-pp

\[\frac{dRB1pp}{dt} = -kd_{RB1pp} \times RB1pp \]  
(70)

\[+k_{RB1pcyclinE} \times cyclinE \times \frac{RB1p_{cyclinERB1p2}}{p_{cyclinERB1p2}} \times \frac{RB1p_{cyclinERB1p2} + RB1p_{cyclinERB1p2}}{p_{RB1pp2 + RB1pp_{RB1pp2}}} \]  
(71)

\[-k_{RB1ppdepheo} \times \frac{RB1pp_{RB1pp2}}{p_{RB1pp2 + RB1pp_{RB1pp2}}} \]  
(72)

(70) Degradation of RB1-pp

(71) Phosphorylation of RB1-p by cyclinE

(72) Dephosphorylation of RB1-pp
Chapter 4. Long-term alternating treatment of MCF7 cells

4.1 Extend the long-term model to simulate alternating therapy

In Chapter 3, we extended the short time scale model to a longer time scale model and added a second Cdk4/6 inhibitor, abemaciclib, into the model. This enabled the model to accurately simulate long term continuous treatments and be used to explore synergism among several drug treatments. The capability of the model to simulate 3 to 4 weeks of various treatments and predict a significant synergism between Cdk4/6 inhibitors and ICI suggests confidence that the model has included essential signaling mechanisms for long-time simulations and gives us confidence to extend the simulation from continuous therapies to alternating therapies.

As we introduced in Chapter 1, although continuous or combination therapies can improve overall survival in the clinic, cancer cells often develop resistance to these continuous treatments [10, 14, 34, 44, 158]. This resistance represents a significant impediment to successful treatment and we propose a hypothesis that alternating application of various therapies and drug holidays in a repeating cycle may provide a means of delaying or preventing resistance. Researchers have shown that cancer cell populations can display a transient, reversible, drug-tolerant state to protect the cell from eradication [42, 115]. Therefore, we think that alternating different drugs may reverse a tolerant state to one drug back to a sensitive state and thereby delay or prevent the development of resistance to this drug. So, the final aim of this research is to test whether alternating therapy can indeed delay the development of resistance. As a first step, and with an eye toward using the model to optimize alternating therapies, we assess the model’s capability to simulate proliferation changes in response to alternating therapies.
Figure 4.1. Model simulations and experimental measurements of the normalized cell numbers under different alternating treatment conditions. Experimental counts are in red (mean value ± s.e., n = 3) and simulations are in blue. Cell numbers are normalized to T = 0 value. Statistically significant changes from the +E2 control are denoted by asterisks (compared to +E2 control in Figure 3.3). +E2 control condition data is limited to one-week due to confluence, alternating treatment results longer than 1 week without an asterisk means we have no data to compare.

Figure 4.1 shows the model simulation results and experimental measurements for two alternating therapies, palbociclib alternating with –E2 and palbociclib alternating with ICI. The duration of each treatment is 7 days and the total treatment period is 28 days. The left of Figure 4.1 shows palbociclib (250 nM) alternating with –E2. We can see that the model simulation is consistent with the experiment result and the cell proliferates about ninety times in 4 weeks. This growth increase is much larger than we initially expected based on our monotherapy data from Figure 3.3, where cells proliferated about eighty fold under palbociclib (250 nM) monotherapy and proliferation essentially stopped under –E2 after 1 week. The reason, discussed in Chapter 3, is the dynamics of E2. The palbociclib treatment has E2 in the medium, which is absorbed by the cells, so when the medium is changed to eliminate E2 the cellular E2 diffuses back into the medium and the resulting concentration is sufficient to drive proliferation. This long term palbociclib and –E2 alternating experiment result confirms the necessity of incorporating E2 dynamics in alternating therapies involving deprivation.

The right plot of Figure 4.1 shows palbociclib (500 nM) alternating with ICI (500 nM). We can see that the model simulation is consistent with the experimental result and the cells proliferate about twenty-five fold in 4 weeks. From Figure 3.3, we see the proliferation of the alternating therapy is consistent with the proliferation of the ICI and palbociclib monotherapies. The averaged increased ratio of palbociclib 250nM mono-treatment is about 2.95 times per week and of ICI 500nM mono-treatment is about 1.65 times per week. The increased proliferation of the alternating therapy in the right plot of Figure 4.1 can be approximated by $2.95 \times 1.65 \times 2.95 \times 1.65 \approx 24$, which is consistent with the experimental result. Therefore, when we considering alternating palbociclib with an endocrine therapy in a culture experiment, ICI would be a better choice than –E2 in terms of controlling the proliferation.
After showing that the model can simulate these two alternating therapies, we would like to check whether the model can predict the effects of other alternating treatments. Figure 4.2 shows the model prediction and experimental measurements of the normalized cell numbers under two alternating treatments. The first alternating treatment, on the left of Figure 4.2, is palbociclib (750 nM) alternating with ICI (500 nM). The duration of each treatment is 7 days and the total treatment period is 14 days. The second alternating treatment, on the right, is palbociclib (750 nM) for 6 days, followed palbociclib (750 nM) plus ICI (500 nM) for one day, followed by ICI (500 nM) for 7 days. The difference between the first and second treatment is that the second treatment adds a 1 day overlap of palbociclib 750 nM and ICI 500 nM treatments. Therefore, as shown in Figure 4.2, the total proliferation of the second alternating treatment is smaller than the first alternating treatment due to this 1 day combination treatment which has a stronger inhibition effect compared with the mono-treatments. The prediction for the second alternating treatment is smaller than the prediction for the first alternating treatment as well.

Figure 4.2. Model prediction and experimental measurements of the normalized cell numbers under different alternating treatment conditions. Experimental counts are in red (mean value ± s.e., n = 3) and simulations are in blue. Cell numbers are normalized to T = 0 value. Statistically significant changes from the +E2 control are denoted by asterisks (compared to +E2 control in Figure 3.3). +E2 control condition data is limited to one-week due to confluence, alternating treatment results longer than 1 week without an asterisk means we have no data to compare.
4.2 Test whether alternating therapy can delay or prevent the development of resistance

The last aim of the research is test whether an alternating therapy can indeed delay or prevent the development of resistance to some degree. Knowing a priori that alternating therapy will work is difficult as resistance to monotherapy develops over a long time frame and is likely a complex interaction of many factors with many possible variations. Therefore, we would like to use biomarkers from the literature to evaluate the degree of resistance. Considering the relatively short period required to develop palbociclib resistance (4 months in our experience) compared with endocrine resistance, which may take years to develop [159-161], we decided to test whether alternating therapy can delay palbociclib resistance in this research. Both the E2 deprivation and ICI treatments target the estrogen signaling pathway, so to target two different mechanisms the natural candidates for alternating therapy are E2 deprivation alternating with palbociclib and ICI alternating with palbociclib, using different drug doses and periods. However, as shown in Figure 4.1, alternating palbociclib with –E2 can generate significant cell growth because of the difficulty of quickly removing E2 from both the cells and the medium. Hence, we decided to use ICI alternating with palbociclib to better control the proliferation over a long time scale.

Figure 4.3. Procedure for long time alternating experiment to assess if there is a delay in acquiring palbociclib resistance. Palbociclib dose is 750 nM. ICI dose is 750 nM. The mono-treatment is continuous palbociclib at 750 nM. In alternating treatment, palbociclib alternates with ICI week by week. The total treatment period for both monotherapy and alternating therapy is 10 weeks.
The following figures show the preliminary results of our 10 week alternating-treatment protocol. All of these experiments involve 2 replications instead of 3 replications, and the results are not conclusive. For this reason, we didn’t use the model to simulate the proliferation data over 10 weeks. Figure 4.5 shows the procedure for the long-time alternating experiment to assess a potential delay in acquiring palbociclib resistance. Based on the mono-treatment results in Figure 3.3 and model simulations, we chose the doses of both palbociclib and ICI to be 750 nM. The total treatment period for both monotherapy and alternating therapy is 10 weeks.

![Graph](image)

Figure 4.4. Experimental measurements of the normalized cell numbers for 10 weeks of monotherapy or alternating treatment (mean value ± s.d., n = 3). Cell numbers are normalized to T = 0 value. The mono-treatment is continuous palbociclib at 750 nM. In the alternating treatment, palbociclib at 750 nM alternates with ICI at 750 nM week by week. The total treatment period for
both mono- and alternating therapy is 10 weeks. At 5 weeks (35 days), in order to avoid confluence, the cells are re-plated and the cell number is re-normalized.

Figure 4.4 shows the experimental measurements of the normalized cell numbers during 10 weeks of mono-treatment and alternating treatment. At 5 weeks (35 days), in order to avoid confluence, the cells are re-plated and the cell number is re-normalized. So, the initial values of the normalized proliferation at $T = 0$ and $T = 35$ days are both equal to 1. From Figure 4.4, we can see the experiment results provide more variance as time goes on, which is due to the exponential proliferation of the cells. Moreover, we found that the 10 week alternating experiment doesn’t inhibit cell proliferation as much as we first predicted, which forced us to replate of the cells at 5 weeks. The reason might be that the cells go through a round of division after palbociclib is replaced by ICI treatment, which is also observed in the clinic. In neoadjuvant studies, the patients who stop palbociclib before surgery displayed a rebound in Ki67 evaluated at surgery but the patients who received palbociclib immediately before surgery do not [162, 163], which indicates that abruptly stopping palbociclib might provoke cell proliferation. The reason could be the cytostatic function of palbociclib, which arrests cells at G1 phase, so that abruptly relieving its inhibition effect will induce the cells finish the cell cycle undergo at least one doubling in cell number.

We will use two strategies to evaluate the resistance-delay effect of alternating therapy at 10 weeks. First, based on the literature, proteins having a high expression level in resistant cell lines can be chosen as biomarkers. From research on the adaption and acquired resistance to Cdk4/6 inhibitors [34, 75], the expression levels of biomarkers such as RB1, cyclinE, Cdk4 and Cdk6 [75, 164, 165] can be used to evaluate the degree of resistance. Therefore, we chose cyclinD1, Cdk4, Cdk6, cylinE1 and Cdk2 as biomarkers to evaluate the difference in moving towards resistance between mono- and alternating therapies. These expression levels under mono- and alternating therapies were compared at 35 days and 70 days.
Figure 4.5 shows western blot quantifications for different biomarker protein levels for mono- and alternating therapies at 35 days and 70 days. We can see that Cdk4, Cdk6 and Cdk2 have not significantly increased after 10 weeks of palbociclib treatment. Moreover, the Cdk6 and Cdk2 levels decreased at 5 weeks under both mono- and alternating treatments and their levels come back at 10 weeks. In terms of these biomarkers, there is no significant difference between mono- and alternating therapies. We can see that cyclinD1 and cyclinE1 significantly increase under palbociclib treatment at both 5 weeks and 10 weeks. The changes in cyclinD1 level has large variations under the alternating therapy, so there is no significant difference between mono- and alternating therapy. The only difference we can see is that cyclinE1 is lower under alternating therapy at 10 weeks compared with monotherapy. However, there is no difference between the cyclinE1 level of alternating therapy and the level of mono-therapy at 5 weeks. Thus, it is hard to conclude that the cyclinE1 level is lower under alternating therapy compared with mono-therapy. The reason might be that, under alternating therapy, the cell is under palbociclib treatment during...
week 5 and under ICI treatment during week 10, so the decreased level of cyclinE1 might be due to the inhibitory effect of ICI since E2:ERα is a transcription factor of cyclinE1 [10].

In addition to the evaluation of the protein expression level under alternating and continuous treatment, we also use a proliferation assay to determine whether alternating therapy can delay the development of resistance. At the end of 5 weeks and 10 weeks, a 7-day palbociclib drug-response assay is used to compare the proliferation of these MCF7 cells after undergoing the mono- and the alternating therapy.

Figure 4. 6. Dose-response (normalized cell number change after 7 days palbociclib treatment) for cells that have undergone 35- or 70- day treatments for mono- or alternating therapies(mean value ± s.e., n = 2). (a) Normalized to vehicle. (b) Normalized to T = 0. (c) GR value. Veh: Vehicle (+E2 control).
Figure 4.6 shows the palbociclib dose response for mono- and alternating therapies. In Figure 4.6a, the proliferation rate is normalized to proliferation in vehicle, which is the usual method of normalization in biological experiments. From this plot we can see that the alternating treatment is much more sensitive to palbociclib compared with the mono-treatment at all doses of palbociclib. This would lead one to think that the alternating therapy is producing less resistant cells compared to mono-therapy. But looking at the 5-week bars, one would conclude that both alternating and mono-therapy cells are more resistant to palbociclib at 5 weeks than they are at 10 weeks. This certainly seems unreasonable.

However, when the dose-response results are normalized to $T = 0$, shown in Figure 4.6b, we can see that the proliferation of the cells from mono-therapy at 5 weeks and 10 weeks is low in the vehicle control condition (+E2 control). Because the proliferation under mono-therapy at 5 weeks and 10 weeks is already very low, and palbociclib rarely induces apoptosis, it cannot decrease the proliferation much further, which makes the cells appear less sensitive to palbociclib when normalized to vehicle. In contrast, the cells from alternating treatment have a relatively higher proliferation in vehicle, and palbociclib can inhibit the proliferation more, which makes the cells appear sensitive to palbociclib. It should be noted that for all doses, the alternating therapy cells proliferate faster than the mono-therapy cells, which makes it impossible to claim that they are more sensitive than the mono-therapy cells.

This problem of interpretation has been noticed previously and drove the development of a new metric, $GR$, that normalizes the proliferation rate under treatment to the proliferation rate of the control [166]. If control cells undergo different numbers of divisions because of a difference in proliferation rate, the dose-response values will vary dramatically [166]. The dose response is sensitive to the basal proliferation rate and $GR$ accounts for this basal proliferation rate in calculation of the dose response. The equation for $GR$ is [166]:

$$GR(c) = 2^{\log_2 \left( \frac{\text{cell count under drug treatment}}{\text{cell count at T = 0 prior to drug treatment}} \right)} - 1$$

The cell count under the drug treatment is normalized to the vehicle control cell count. $x(c)$ is the cell count in the presence of palbociclib at concentration $c$. $x_{ctrl}$ is the cell count for vehicle control. $x_0$ is the cell count at $T = 0$ prior to drug treatment. The maximum value of $GR$ is 1 and the lowest value of $GR$ is -1. $GR = 0$ means the drug treatment has a fully cytostatic response and
a negative value means the drug treatment has a cytotoxic effect [166]. Cell division rate can change as cell density changes and such variation can impact IC\textsubscript{50} 100 times or more, and therefore make the true drug treatment effect unclear [166]. GR is robust to variations in cell growth rate and quantifies the efficacy of a drug on a per-division basis, which can ensure that fast- and slow dividing cells responding equally to a drug are scored equivalently [166]. Figure 4.6c shows the GR values for the palbociclib dose response. Although the cells after 10 weeks treatment are more resistant to palbociclib compared to cells after 5 weeks treatment and nontreatment cells, we can see that there is no significant difference between the mono- and alternating therapy at 10 weeks. Therefore, although the dose response normalized to vehicle shows a difference between monono- and alternating therapy, this effect comes from the different basal cell division rates of the mono- and alternating therapy and obscures the true nature of palbociclib dose response. The cells from mono- and alternating are off of the drug and in +E2 control media for 24 hours before the palbociclib dose response experiment. We choose 7 days instead of 1 day or 3days proliferation assay to evaluate the palbociclib dose response in order to provide enough time for cell proliferation. However, the cells from the mono-therapy still have small cell division rate under vehicle condition.

From the above experiment, it is not possible to conclude that alternating therapy has delayed the onset of resistance. It is possible that we need a longer experiment that allows the monotherapy cells time to fully develop resistance and start proliferating well in order to see if the alternating treatment can delay resistance.

4.3 Discussion

In this Chapter, we show that the model can simulate two alternating therapies, which are palbociclib alternating with –E2 and palbociclib alternating with ICI. It also can predict two 14-day alternating therapies. Moreover, we used protein biomarkers and a proliferation assay to try to assess the palbociclib-resistance difference between mono- and alternating therapy after 10 weeks of treatments. However, neither of the of these two approaches shows distinct differences at 10 weeks. The reason might be that the basal growth rate after long period palbociclib treatment is low or we need an even longer time to uncover the difference.
4.4 Supporting plot

Figure 4.7. Representative western blot data for 10 weeks mono-treatment and alternating treatment.
Chapter 5. Conclusions and future perspective

This dissertation aims to build a mechanistic ODE model to capture the responses to clinically used treatments for ER+ breast cancer, which is the most common type of breast cancer and accounts for about 70% of breast cancer tumors. Chapter 5 briefly summarizes the conclusions and contributions of this research and briefly discuss the future works.

5.1 Conclusions and contributions

The major contribution of this research was to build a useful mathematical model to recapitulate and predict drug treatment effects on MCF7 ER+ breast cancer cells. The model also can be used to optimize treatment for different drug combinations. Specific contributions are as follows:

(1) We built the first mechanism-based mathematical model for this ER+ breast cancer treatment. It can simulate 7-day protein level changes in response to endocrine therapies.

(2) Because the model has a mechanistic basis, it can be easily extended to test the effect of any drug with known molecular targets in the signaling pathway. For instance, we were able to add Cdk4/6 inhibitors into the model merely by adjusting a few new parameters related to the new interactions. This was possible because the relevant targets were already included when first creating the model for endocrine therapies. The model can recapitulate the 7-day protein level changes in response to the two Cdk4/6 inhibitors, palbociclib and abemaciclib. And the model can simulate the increased apoptosis induced by abemaciclib.

(3) In addition to capturing protein level changes, the model can capture proliferation changes up to 28 days in response to various mono- or combination treatments at different doses of endocrine therapy and Cdk4/6 inhibitors. The model is also able to predict the combination treatment effect of endocrine therapies and Cdk4/6 inhibitors on proteins and proliferation at 7 days. The mathematical model predicted a significant synergism between Cdk4/6 inhibitors in combination with endocrine treatment. And the predicted synergisms were verified by experiments.

5.2 Directions of future work

In Chapter 4, we try to use protein biomarkers and a proliferation assay to test if there is a palbociclib-resistance difference between mono- and alternating therapy. However, we did not
find clear differences. Based on these indeterminate results, we see the future work as having three aspects:

(1) We propose to do a gene array on the cells from these two treatment methods and also include our palbociclib-resistant cell line. These arrays will allow us to compare the gene expression of cells from mono- and alternating treatments with the palbociclib-resistant cell line.

(2) From Figure 4.5, we can see that cyclinD1 and cyclinE1 protein levels increase under palbociclib treatment at 5 weeks and 10 weeks. We propose to do western blots on cyclinD1 and cyclinE1 after 1 week of palbociclib treatments to check whether the protein levels are gradually increased during the palbociclib treatment period or the protein levels are increased to a stable level just after 1 week palbociclib treatment.

(3) If the protein levels of cyclinD1 and cyclinE1 truly mark the beginning of resistance, their changes can be included in the model, which will facilitate the model-guided optimization of a therapeutic protocol. We will add the increase of cyclinD1 and cyclinE1 into the model to capture the proliferation results of 10-week alternating therapy in Figure 4.4. We could include a resistance mechanism related to cyclinD1 or cyclinE1 overexpression in the model in the future and derive a cost function that includes proliferation and resistance. Then we can use the model to search for protocols that minimize the cost value. The cost function to be optimized can be constructed based on cyclinD1 and cyclinE1 levels and the treatment period. An example of this kind of cost function is:

\[
\text{cost} = \text{cellnum}(t_1) + \int_{t_0}^{t_1} \max\left(0, \beta \times \left(1 - \frac{\text{cyclinE1}(t_0)}{\text{cyclinE1}(t_1)}\right)\right) dt
\]

where \text{cellnum} is the MCF7 cell number at the end time point of the treatment; \text{cyclinE1} is the cyclinE1 protein level; \beta is a weighting coefficient; and \(t_0\) and \(t_1\) represents the time period to be considered. In this cost function, cyclinE1 is used as a resistance biomarker for palbociclib and has been incorporated into the model; its expression value increases when using palbociclib. Therefore, minimizing the cost function means decreasing the cell number and simultaneously keep cyclinE1 expression at a low level to avoid the resistance.
References


