MACHINE LEARNING AND NETWORK-BASED SYSTEMS TOXICOLOGY MODELING OF CHEMOTHERAPY-INDUCED PERIPHERAL NEUROPATHY

By

Peter Bloomingdale

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Abstract

The overarching goal of my thesis work was to utilize the combination of mathematical and experimental models towards an effort to resolve chemotherapy-induced peripheral neuropathy (CIPN), one of the most common adverse effects of cancer chemotherapy. In chapter two, we have developed quantitative-structure toxicity relationship (QSTR) models using machine learning algorithms that enable the prediction of peripheral neuropathy incidence solely from a chemicals molecular structure. The QSTR models enable the prediction of clinical neurotoxicity, which could be potentially useful in early drug discovery to screen out compounds that are highly neurotoxic and identify safer drug candidates to move forward into further development. The QSTR model was used to suggest modifications to the molecular structure of bortezomib that may reduce the number of patients who develop peripheral neuropathy from bortezomib therapy. In the third chapter, we conducted a network-based comparative systems pharmacology analysis of proteasome inhibitions. The concept behind this work was to use in silico pharmacological interaction networks to elucidate the neurotoxic differences between bortezomib and carfilzomib. Our theoretical results suggested the importance of the unfolded protein response in bortezomib neurotoxicity and that the mechanisms of neurotoxicity by proteasome inhibitors closely relate to the pathogenesis of Guillain-Barré syndrome caused by the Epstein-Barr virus. In chapter four we have written a review article to introduce the concept of Boolean network modeling in systems pharmacology. Due to the lack of knowledge about parameter values that govern the cellular dynamic processes involved in peripheral nerve damage, the development of a quantitative systems pharmacology model would not be feasible. Therefore, in chapter five, we developed a Boolean network-based systems pharmacology model of intracellular signaling and gene regulation in peripheral neurons. The model was used to simulate the neurotoxic effects of bortezomib and to identify potential treatment strategies for proteasome-inhibitor induced peripheral neuropathy. A novel combinatorial
A treatment strategy was identified that consists of a TNFα inhibitor, NMDA receptor antagonist, and reactive oxygen species inhibitor. Our subsequent goals were aimed towards translating this finding with the endeavor to hopefully one-day impact human health. Initially we had proposed to use three separate agents for each of these targets, however the clinical administration of three agents to prevent the neurotoxicity of one is likely unfeasible. We then came across a synthetic cannabinoid derivative, dexanabinol, that promiscuously inhibits all three of these targets and was previously developed for its intended use to treat traumatic brain injury. We believe that this drug candidate was worth investigating due to the overlapping pharmacological activity with suggested targets from network analyses, previously established favorable safety profile in humans, notable in vitro/vivo neuroprotective properties, and rising popularity for the therapeutic potential of cannabinoids to treat CIPN. In chapter six we assessed the efficacy of dexanabinol for preventing the neurotoxic effects of bortezomib in various experimental models. Due to the limited translatability of 2D cell culture techniques, we investigated the pharmacodynamics of dexanabinol using a microphysiological model of the peripheral nerve. Bortezomib caused a reduction in electrophysiological endpoints, which were partially restored by dexanabinol. In chapter 7 we evaluated the possible interaction of dexanabinol on the anti-cancer effects of bortezomib. We observed no significant differences in tumor volume between bortezomib alone and in combination with dexanabinol in a multiple myeloma mouse model. Lastly, we are currently investigating the efficacy of dexanabinol in well-established rat model of bortezomib-induced peripheral neuropathy. We believe that positive results would warrant a clinical trial. In conclusion, the statistical and mechanistic models of peripheral neuropathy that were developed could be used to reduce the overall burden of CIPN through the design of safer chemotherapeutics and discovery of novel neuroprotective treatment strategies.
Introduction

Chemotherapy-induced Peripheral Neuropathy

Chemotherapy-induced peripheral neuropathy (CIPN) is one of the most common adverse side effects of cancer therapy. CIPN occurs from exposure to neurotoxic chemotherapeutics that damage peripheral nerves, and sensory nerves are more frequently affected than motor nerves. The symptoms of CIPN usually arise from damage to dorsal root ganglion (DRG) neurons or their axons. The extent of drug exposure in the DRG is high, relative to other components of the peripheral nerve, and is in close equilibrium with plasma due to the lack of a blood-nerve-barrier. Symptoms of CIPN often manifest as a tingling or numbness sensation in the extremities of the body, since longer nerves are more vulnerable and affected first. This phenomenon is referred to as a stocking-glove distribution or dying-back neuropathy. Patients with CIPN exhibit fatigue and muscle weakness, which decreases the overall quality of life. Simple tasks, such as buttoning clothing and tying shoe laces, are difficult for patients with CIPN. Severe symptoms of CIPN consist of burning pain and paralysis, which can be life threatening as nerves controlling autonomic processes (e.g., breathing and heart rate) become compromised. The incidence of peripheral neuropathy differs significantly across chemotherapeutic agents, but has reported to occur in approximately 30-40% of patients undergoing chemotherapy. The prevalence and severity of CIPN are dependent upon several factors relating to both drug pharmacokinetics (e.g., cumulative dose and treatment duration) and pharmacodynamics (e.g., mechanisms of toxicity and patient characteristics). A few patient-specific risk factors have been identified that are associated with the susceptibility for developing CIPN. A major risk factor is preexisting nerve damage from alcohol, diabetes, prior exposure to neurotoxic agents, physical injury, and hereditary neuropathies (e.g., Charcot-Marie-Tooth). Other risk factors include obesity, certain genetic variants, smoking, abnormal
creatinine clearance, and specific sensory changes during chemotherapy. However, an experimental diagnostic tool or in silico model to predict the development CIPN in individual patients does not exist.

There are four main drug classes associated with the development of CIPN: (1) platinum agents, (2) vinca alkaloids, (3) taxanes, and (4) proteasome inhibitors. CIPN is often reversible upon cessation of treatment; however, some patients suffer from lifelong irreversible effects. Platinum agents, exhibit a phenomenon referred to as coasting, in which CIPN symptoms may worsen for several months after the discontinuation of therapy. Microtubule inhibitors, such as paclitaxel and vincristine, can be extremely neurotoxic, with up to 100% of patients developing neuropathic symptoms at higher dose levels. Platinum chemotherapeutics, cisplatin and oxaliplatin, damage the DRG through the formation of DNA adducts that creates oxidative stress and leads to neuronal apoptosis. Bortezomib, a proteasome inhibitor, causes a painful length-dependent neuropathy that is predominately distal and sensory (Aβ, Aδ, and C nerve fibers). Approximately 37% of patients taking bortezomib develop peripheral neuropathy.

The molecular mechanisms of neurotoxicity by chemotherapeutics are multifactorial and complex. There are a wide range of cellular components and processes that are disrupted by cancer chemotherapy. Certain mechanisms of neurotoxicity may be exclusive to a specific drug class or individual agent. However, many molecular pathways of toxicity are shared across neurotoxic chemotherapeutics. In 2012, Jaggi and Singh reviewed several of the possible mechanisms involved in the pathogenesis of CIPN and neuropathic pain. These mechanisms include mitochondrial dysfunction, disruption in ion homeostasis, inflammatory processes, oxidative stress, activation of the intrinsic apoptosis pathway, MAPK pathway alterations, modulation of NMDA receptors, and several others.
The mitotoxicity hypothesis, with mitochondrial dysfunction resulting in the disruption of Ca\(^{2+}\) homeostasis, oxidative stress, and activation of intrinsic apoptosis, has emerged as a fundamental cause of CIPN, especially for distal sensory neuropathies. Mitochondria in the sensory axons of dorsal root ganglion (DRG) neurons become swollen and vacuolated when exposed to paclitaxel, oxaliplatin, and bortezomib. Opening of the mitochondrial permeability transition pore (mPTP) increases the permeability of the inner mitochondrial membrane to molecules less than 1500 Da, which results in a loss of membrane potential, mitochondrial swelling, decreased energy production, and rupture of the outer mitochondrial membrane. Rupturing of the outer mitochondrial membrane initiates apoptosis through the release of pro-apoptotic proteins. Opening of the mPTP can occur in the presence of high levels of reactive oxygen species (ROS) and Ca\(^{2+}\). The concentrations of cytoplasmic ROS and Ca\(^{2+}\) are highly regulated and modulate each other. One source for the increase in cytoplasmic Ca\(^{2+}\) is through the activation of IP3 receptors on the endoplasmic reticulum membrane, and ROS can be generated through the unfolded protein response. The activation of NMDA receptors results in the translocation of extracellular Ca\(^{2+}\) across the plasma membrane into the cell and generates ROS through NADPH oxidases. Action potentials cause large influxes of Ca\(^{2+}\) into the cell, which demands high energy consumption to restore intracellular Ca\(^{2+}\) concentrations by ATP-dependent transporters. Hence, dysfunctional mitochondria can lead to energy deficits that result in aberrant intracellular Ca\(^{2+}\) concentrations and oxidative stress.

Neuroinflammation is another major mechanism underlying the development and progression of CIPN. Secondary to initial nerve injury, resident macrophages, Schwann cells, satellite glial cells, and recruited immune cells secrete prostaglandins, chemokines, and cytokines. Components of the nerve microenvironment drive the complex communication between cells of the neuroimmune system to promote adaptive (survival and growth) and maladaptive (neuropathic pain) responses. Several of the highly neurotoxic chemotherapeutics (paclitaxel, oxaliplatin, vincristine, and bortezomib) have shown to...
increase pro-inflammatory cytokines (TNFα and IL-1β) and downregulate anti-inflammatory cytokines (IL-10) in the dorsal root ganglion and spinal cord. Pro-inflammatory cytokines (TNFα, IL-1β, IL-6) and chemokines (CCL2) have been proposed as potential biomarkers and therapeutic targets for predicting and preventing CIPN-related pain. The administration of an anti-TNFα antibody attenuated CIPN symptoms and exhibited partial neuroprotection in rodent models of bortezomib-induced peripheral neuropathy. CCL2 was significantly increased in the DRG of rats given bortezomib, and mechanical allodynia was partially reversed by an anti-CCL2 antibody. IL-1 receptor antagonism and upregulation of IL-10 both reverse paclitaxel-induced mechanical allodynia in rats. The role of the immune system in the development and severity of bortezomib-induced peripheral neuropathy is highlighted by the recent success of intravenous immunoglobulin (IVIG) for preventing bortezomib-induced peripheral neuropathy in rats and the onset of severe polyradiculoneuropathy in some patients.

The discovery and development of more effective anti-cancer agents has resulted in a decline in cancer death rates and an increase in the number of cancer survivors managing long-term adverse side effects from cancer chemotherapy. The decline in cancer death rates over the past three decades has translated to over two million fewer deaths. The number of cancer survivors is rapidly increasing and is projected to reach 26.1 million by 2040, and at least 50% of patients suffer from treatment related side effects. Therefore, there is an increase in the number of cancer survivors that will need to manage the long-term adverse side effects of cancer chemotherapy. In 2014, an American Society of Clinical Oncology panel of experts reviewed several clinical trials to assess the therapeutic potential of agents for their ability to prevent or treat CIPN. They recommended no agents for the prevention of CIPN in cancer patients treated with neurotoxic agents. Opioids, Cannabis sativa, and gabapentinoids are frequently prescribed as symptomatic treatments to treat neuropathic pain; however, they do not prevent underlying nerve damage and possess an addictive potential owing to their psychoactive
The use of *Cannabis sativa* has a long history for its use in the treatment of pain. There is an interest in the potential use of cannabinoids to treat CIPN related symptoms, such as neuropathic pain. The antinociceptive effects of cannabinoids have been established in animal models of nerve injury and neuropathic pain. In addition, several cannabinoids (WIN55,212-2, MDA7, (R)-AM1241, AM1714) have successfully reversed paclitaxel and vincristine induced mechanical allodynia in rodents. However, the main limitation for the use of cannabinoids for the treatment of CIPN is tolerance resulting from receptor desensitization, physical withdrawal, and unwanted psychoactive effects. Patients that develop severe CIPN will have to either completely discontinue chemotherapy or reduce the amount/frequency of dosing, which may compromise treatment efficacy. The National Cancer Institute sponsored 15 clinical trials to investigate the prevention and symptomatic treatment of chemotherapy-induced peripheral neuropathy. Duloxetine exhibited efficacy for the treatment of neuropathic pain in some patients. In addition to the effects on serotonin and norepinephrine in the central nervous system, recent findings suggest that the beneficial effects of duloxetine may involve the activation of peripheral delta opioid receptors. Several nutraceuticals and alternative medicine techniques have been evaluated for their potential utility in CIPN, such as vitamin E, goshajinkigan, acetyl-L-carnitine, alpha-lipoic acid, acupuncture, and electrotherapy. None of the nutraceuticals were effective and in certain cases may have worsened peripheral neuropathy (i.e., acetyl-L-carnitine for taxane-induced PN). Scrambler therapy, a form of electrotherapy, has shown promise in a recently published phase II clinical trial where symptoms of CIPN were moderately decreased.

We hypothesize that dexanabinol, a synthetic cannabinoid derivative, could potentially be repurposed to prevent CIPN. Dexanabinol was first synthesized by Mechoulam and colleagues in 1998 and was initially evaluated in clinical trials for traumatic brain injury. However, dexanabinol failed to show efficacy in humans and was not approved. Dexanabinol has promiscuous pharmacological mechanisms of action, including N-methyl-D-aspartate (NMDA) antagonism and protection of NMDA-induced...
neurotoxic death in mice. In 1995, Eshhar and colleagues showed that dexanabinol has antioxidative properties by acting as a scavenger of peroxy radicals. In 1997, Shohami and colleagues showed that dexanabinol also inhibits the production of TNFα.

**Experimental Models of Chemotherapy-induced Peripheral Neuropathy**

*In vitro* (i.e. cell and tissue cultures) and *in vivo* animal models (i.e. mice and rats) are commonly used to study CIPN. SH-SY5Y and PC12 cells, immortalized neuron-like cell lines, are the two most common types of cells for studying neurotoxic chemicals. There has been an increased interest in the use of human induced pluripotent stem cell (iPSC)-derived neurons to study CIPN and other neurodegenerative diseases. However, results obtained from iPSC-derived neurons may differ owing to differences in the differentiation protocol and the various factors used to maintain cell survival. In contrast to cell-based methods, tissue cultures consisting of dorsal root ganglia (DRG) isolated from embryonic and adult rodents have been used for studying CIPN. DRG explants can be cultured directly or used to isolate sensory neurons for subsequent analyses. The differences between these *in vitro* experimental models and the in vivo physiological system should be considered when translating findings. For example, components of the immune system are often absent in experimental models of CIPN. *In vitro* endpoints typically include changes in cell viability and neurite outgrowth. Proinflammatory cytokines, oxidative stress, and ATF3 could serve as potential biomarkers of nerve injury by chemotherapy.

Rodents are widely used as experimental models to study the neurotoxic effects of drugs. Multiple rat and mouse models have been developed for bortezomib-induced peripheral. Even for the same chemotherapeutics, studies often vary in the dose, frequency/route of administration, outcome measures, and characteristics of the animals (i.e., age, sex, and genetic background). These parameters may play an important role when translating results from rodents to humans. Outcome measures consist of behavioral, electrophysiological, and histological endpoints. Behavioral animal
tests are commonly conducted to assess the dysfunction of peripheral nerves, such as mechanical allodynia measured using von Frey filaments and endurance via a rotarod performance test. Electrophysiological tests that measure action potential amplitude and nerve conduction velocities can be performed to evaluate nerve function. Additionally, histological analyses can be performed to identify nerve damage, mitochondrial swelling, demyelination, changes in vasculature, and intraepidermal nerve fiber density.

Organs-on-chips, or microphysiological systems, have gained tremendous popularity for their use in drug toxicity testing. A microphysiological system of peripheral nerves, peripheral nerve-on-a-chip, has been developed. The incorporation of dorsal root ganglia explants into a 3D dual hydrogel construct creates a microphysiological experimental model that better represents a peripheral nerve and enables the measurement of electrophysiological/histological endpoints in an in vitro system.

**Proteasome Inhibitors for Multiple Myeloma**

Proteasome inhibitors have become an integral part of multiple myeloma therapy, and the development of more effective and safer proteasome inhibitors remains an active area of research. The ubiquitin-proteasome pathway is responsible for degrading proteins that control several cellular processes such as cell-cycle, signal transduction, regulation of transcription, apoptosis, and the immune response. Inhibition of the proteasome has been a useful target for the treatment of hematological cancers, such as multiple myeloma and mantle cell lymphoma. The 26S proteasome is a 2.5 megadalton multicatalytic protease complex that degrades misfolded, abnormal, damaged, or improperly translated proteins. The 26S proteasome consists of the 20S proteasome, a barrel-shaped proteolytic core, that is capped at both ends by 19S regulatory complexes. The 20S catalytic core of the proteasome contains 28 subunits that include the chymotrypsin-like activity of the proteasome subunit β5 (PSMB5), trypsin-like activity of the proteasome subunit β2 (PSMB2), and caspase-like activity of the proteasome subunit
β1 (PSMB1). The mechanism of proteasome inhibition by drugs can be reversible or irreversible and modulate the activity of these three different types of proteasomal subunits to various extents. Inhibition of the proteasome causes the accumulation of proteins, which disrupts homeostatic processes, generates oxidative/endoplasmic reticulum stress, and activates apoptosis.

Proteasome inhibitors can be divided into three classes, boronates, epoxykeytones, and salinosporamides. Bortezomib, a dipeptide boronic acid analogue, was the first-in-class proteasome inhibitor approved in 2003 for the treatment of multiple myeloma. Carfilzomib a second generation proteasome inhibitor, approved in 2012, was the first approved proteasome inhibitor belonging to the epoxykeytone class. Carfilzomib irreversibly binds to the proteasome and selectively inhibits the chymotrypsin-like activity, whereas bortezomib reversibly inhibits the chymotrypsin-like and caspase-like activity of the proteasome. The utility of carfilzomib is that it has been shown to work when myeloma cells have become resistant to bortezomib.

The incidence of peripheral neuropathy is different between bortezomib and carfilzomib. In the phase III APEX trial, 37% of patients undergoing bortezomib therapy developed peripheral neuropathy and 9% had to discontinue treatment due to its severity. In a phase II clinical trial for single agent carfilzomib therapy, only 12% of patients developed peripheral neuropathy, and no patients had to discontinue treatment. The mechanisms underlying the neurotoxicity of proteasome inhibitors remains unclear and likely involves a combination of pharmacokinetic and pharmacodynamic differences. The clinical neurotoxic disparity between bortezomib and carfilzomib had been suggested to result from direct modulation of non-proteasomal targets, such as the inhibition of serine proteases cathepsin A, cathepsin G, chymase, dipeptidyl peptidase II, and HtrA2/Omi. In contrast, Csizmadia and colleagues (2016) showed that neither bortezomib nor carfilzomib inhibited HtrA2/Omi activity. A proteasome-dependent mechanism, in which proteasome inhibitors target different constitutive and immunoproteasome subunits that result in differential substrate specificity, could also be responsible
for neurotoxic disparities across proteasome inhibitors. Decreasing the dosing frequency of bortezomib from twice to once weekly and changing the route of administration from intravenous to subcutaneous injection reduces the incidence and severity of peripheral neuropathy. The systemic exposure of bortezomib and degree of proteasome inhibition in blood was shown to be the same between subcutaneous and intravenous administration. The efficacy of subcutaneous administration was not inferior to intravenous dosing, and the overall incidence of grade ≥3 adverse events were lower with subcutaneous administration. Therefore, maximal plasma concentrations (Cmax) are more reflective of adverse events than total systemic exposure (AUC). However, no relationship was observed between pharmacokinetic parameters and the incidence of severe peripheral neuropathy for the small number of patients for which pharmacokinetic information was available, suggesting that proteasome inhibitor induced neurotoxicity observed in patients cannot be solely explained by differences in pharmacokinetics. Patient-specific risk factors highlight that pharmacodynamic differences may serve to explain why some patients develop CIPN while others do not.

**Mathematical Modeling of Chemotherapy-induced Peripheral Neuropathy**

Models of drug toxicity have been utilized to predict the adverse effects of drugs on human health. A major cause of compound attrition in drug development is non-clinical toxicology, and approximately a quarter of drugs fail during clinical trials due to clinical safety concerns. The ability to predict drug toxicity via computational modeling has gained considerable interest for its potential applications in the drug development process. Computational modeling could complement toxicity testing in order to reduce experimental costs, drug development time, and the use of animals. There are many efforts worldwide to develop and improve models for the prediction of cardiotoxicity and hepatotoxicity, as these are two common types of drug toxicity and frequently result in post-market withdrawal. However, there are few if any predictive models for under-recognized forms of drug-induced toxicities,
such as peripheral neurotoxicity. We briefly discuss mathematical modeling approaches that can be used to predict and potentially resolve chemotherapy-induced peripheral neuropathy.

Quantitative-Structure Toxicity Relationship Modeling

The history of using similarities in chemical structure to quantify toxicity dates back to 1863, with Cros identifying that mammalian toxicity to primary aliphatic alcohols increased as its water solubility decreased. Meyer and Overton separately showed that the anesthetic potency of narcotics correlated with their olive oil/water partition coefficient, reflective of increased membrane permeability owing to greater lipophilicity. Hammett, in 1937, formulated the first quantitative relationship between molecular structure and activity to describe electronic effects of organic reactions, and Hansch and Fujita were credited with the foundation of modern day quantitative-structure activity/toxicity relationships (QSAR/QSTR).

The ability to predict the biological activity and pharmacological/toxicological properties of a compound from its molecular descriptors and physicochemical properties is the underlying goal of QSAR modeling. QSAR models can be utilized in drug development to optimize the molecular structure of a compound in order to achieve a desired activity/property. For example, removing the unwanted off-target inhibition of hERG and BSEP to reduce the risk of cardiotoxicity and hepatotoxicity. Given that cardiac arrhythmias can be induced by many drugs that block hERG channels, QSTR models have played an important role in early drug discovery to design drugs that to not inhibit the hERG channel. QSAR models have been developed to predict drug-induced liver injury (DILI), and BSEP is a transporter of importance since its inhibition has been associated with the development of hepatotoxicity. However, there has been no known efforts to develop and utilize a QSTR model to reduce drug-induced neurotoxicity. QSTR models of drug-induced peripheral neuropathy could be adopted in early discovery to screen out drugs that are highly neurotoxic and identify safer drug candidates to move forward into clinical development.
**Toxicokinetic and Toxicodynamic Modeling**

Pharmacokinetic and pharmacodynamic (PK/PD) models aim to link drug concentrations at the site-of-action to a pharmacological response. PK/PD models are parsimonious, data-driven, and compartmental based on ordinary differential equations. The application of PK/PD principles in relation to chemical toxicity is referred to as toxicokinetics and toxicodynamics (TK/TD). In drug development, TK/TD models aim to provide a quantitative relationship between drug concentration and toxicity in order to prevent unwanted adverse side effects and optimize dosing. TK/TD models could be implemented in clinical development to reduce the incidence and severity of CIPN.

**Network-based Systems Toxicology Modeling**

The study of biology in the context of a system can be traced back to generalized systems theory. Systems biology aims to identify and evaluate how interactions across individual components give rise to the complex emergent behavior observed in biological systems. Systems pharmacology integrates principles from pharmacology and systems biology in order to study the effects of drugs on biological systems. Network models, derived from the principles of graph theory, have been useful for describing the complex relationships between biological components in a systematic/mathematical manner. Nodes of the network models typically represent DNA, RNA, proteins, or other cellular components. Edges describe the regulatory interactions amongst nodes of the network. Topological analyses that measure connectivity, centrality, and clustering, provide information about important components and features of the system. To understand the evolution of the expression of network components throughout time requires the conversion of an interaction network into a dynamical network. Dynamical networks can be constructed using differential equations, Bayesian inferences, fuzzy logic, and Boolean algebra that range from continuous to discrete and deterministic to stochastic.
Network simulations, attractor analyses, and other network analysis techniques can be applied to study how the system responds to drug perturbations.

Boolean networks, originally described in 1969 by Stuart Kauffman, have been used to model gene regulatory networks. Systems biologists and pharmacologists have developed and utilized Boolean network models for therapeutic target identification to understand pathways of cellular differentiation, and to understand how the mutational landscape of cancer cells alter their response to therapy. Network simulations can be performed to predict the effect of a node perturbation on the dynamics of other species in the network, which emulates a pharmacological intervention. The cellular pharmacodynamic effects of drugs can be evaluated by assessing how perturbations alter attractors of the system. Dynamical steady-states, or attractors, in Boolean network models of cellular gene regulation have been shown to correspond with cellular phenotypes, such as differentiation, proliferation, and apoptosis. Calculating the probability of reaching each attractor provides information about long-term behavior. The shift in dynamical trajectory towards attractors is a powerful approach to assess the effects of pharmacological interventions at the cellular level. The development of a network model of pathways of toxicity in peripheral neurons could be applied to understand the cellular toxicodynamics of chemotherapeutics and to identify therapeutic targets for the prevention of CIPN.

Quantitative Systems Toxicology Modeling

The development of a quantitative systems toxicology (QST) model requires the integration of drug exposure, molecular mechanisms of toxicity, a cell/organ model of the physiological system, and clinically relevant biomarkers of toxicity. A QST model of CIPN would include drug exposure in the dorsal root ganglion, network models for the pathways of toxicity in cells of the neuroimmune system, a mechanistic model of the peripheral nerve, and the prediction of electrophysiological endpoints.
Summary

The mechanisms of CIPN are multifactorial and poorly understood, which warrants a systems level approach to understand the complex interplay amongst components of the system. We think that chemotherapy-induced peripheral neuropathy could be resolved through the combined use of computational and experimental models. The following chapters in this thesis have laid the foundation towards the development of a QST model of CIPN. The integration of the QST model of CIPN with machine learning models could aid in optimally designing preventative agents and safer chemotherapeutics. Optimally designed drugs could be experimentally evaluated using rodents and a microphysiological model of the peripheral nerve.
References


Chapter 2:

Machine Learning Models for the Prediction of

Chemotherapy-Induced Peripheral Neuropathy

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Abstract

Chemotherapy-induced peripheral neuropathy (CIPN) is a common adverse side effect of cancer chemotherapy that can be life debilitating and cause extreme pain. CIPN is an unmet medical need of increasing importance, especially as these neurotoxic effects can be irreversible and the number of cancer survivors is growing for most tumor types. The multifactorial and poorly understood mechanisms of toxicity have impeded the identification of novel treatment strategies. In silico models of drug neurotoxicity could be implemented in early drug discovery in order to screen for high risk compounds and select safer drug candidates to move forward into development. In this study, a quantitative-structure toxicity relationship model is developed using machine learning algorithms to predict the incidence of drug-induced peripheral neuropathy. A manually curated library of 95 approved drugs were used to develop the model. The number of aromatic nitrogens was the most important molecular descriptor, which provides insight into structural modifications to reduce the incidence of peripheral neuropathy. The chemical transformation of aromatic nitrogens to carbons reduced the predicted peripheral neuropathy incidence of bortezomib from 32.3% to 21.1%. The model was also used to predict the incidence of peripheral neuropathy for 60 antineoplastic drug candidates that are currently being investigated in the clinic.
Introduction

The discovery and development of more effective anti-cancer agents has led to a significant decline in cancer death rates over the past three decades, translating to over two million fewer deaths.\(^1\) There are approximately 16 million cancer survivors, which is projected to be 26.1 million by 2040, and at least 50% suffer from treatment related side effects.\(^2\) Hence, the number of cancer survivors managing the long-term adverse side effects of cancer therapy is increasing. Chemotherapy-induced peripheral neuropathy (CIPN), a common adverse side effect of cancer treatment, represents damage to peripheral nerves caused by neurotoxic chemotherapeutics. Symptoms of CIPN range from mild (e.g., numbness, tingling, and muscle weakness) to extreme (e.g., burning pain and paralysis), and these effects can be irreversible.\(^3\) The incidence of peripheral neuropathy (PN) varies amongst chemotherapeutic agents and has been reported to occur in approximately 30-40% of patients undergoing chemotherapy.\(^5\) Several knowledge gaps remain regarding the molecular mechanisms by which chemotherapeutics cause neurotoxicity, hindering mechanism-based approaches to identify treatment strategies for CIPN.\(^6\)\(^-\)\(^8\) To date there are no clinically approved drugs for the prophylaxis or treatment of CIPN. A statistical-based approach that relates the structural properties of neurotoxic compounds to a neuropathic endpoint could provide insights into toxicological mechanisms and provide utility during drug candidate selection to reduce the likelihood of nervous system-related adverse events.

There has been an increased interest for the incorporation of machine learning and artificial intelligence approaches in drug discovery and development.\(^9\)\(^-\)\(^11\) The application of computational modeling to complement toxicity testing could reduce experimental costs, drug development time, and the use of animals.\(^12\) There are several types of in silico modeling methods that have
been utilized for toxicity prediction, which range from statistical approaches (based on chemical structure information) to mechanistic approaches involving toxicokinetics, toxicodynamics, and systems toxicology.\textsuperscript{13, 14} Quantitative structure-activity relationship (QSAR) models are a class of models that use information derived from molecular structures to predict activity (QSAR), property (QSPR), and toxicity (QSTR) of compounds. QSAR models have been utilized for a plethora of applications in drug development, such as the prediction of physicochemical (solubility and lipophilicity), pharmacokinetic (absorption, distribution, metabolism, and elimination), and toxicological properties.\textsuperscript{15-17}

The major cause of compound attrition in drug development is non-clinical toxicology and approximately a quarter of drugs fail during clinical trials due to clinical safety concerns.\textsuperscript{18} The ability to predict drug toxicity via computational modeling is becoming feasible in the drug development process. There are many efforts worldwide to develop and improve models for the prediction of cardiotoxicity and hepatotoxicity, as these are two common types of drug toxicity and frequently result in post-market withdrawal.\textsuperscript{19} However, there are few if any predictive models for under-recognized forms of drug-induced toxicities, such as peripheral neurotoxicity. Although QSAR models have been developed for the neurotoxicity of solvents and organophosphates, a QSAR model of drug-induced peripheral neuropathy has yet to be developed.\textsuperscript{20-23}

In this study, we developed a QSTR model of drug-induced PN. Molecular descriptors of 95 drugs reported to cause PN were used to predict their respective incidences. Two quantitative models were developed that use an artificial neural network (ANN) algorithm to predict the incidence of PN. We also developed two classification models using support vector machines (SVM), which
were used to categorize molecules into three groups (high, medium, or low) depending on their propensity to induce PN. Significant molecular descriptors that may provide insight into the molecular mechanisms of drug-induced PN were identified, from which specific structural modifications may be proposed to reduce the neurotoxicity of proteasome inhibitors. Additionally, the model has been applied to predict the incidence of PN of 60 small molecule drugs currently in clinical trials for cancer chemotherapy.

Methods

Data Collection

QSTR models were developed with FDA/EMA approved drugs reported to induce PN. A list of drugs that were previously identified to be associated with PN was obtained by searching three databases (Drugs@FDA, SIDER2, and DailyMed), and this search was extended to include drugs that were approved after the date of the initial analysis (June 2010). The incidence of PN was determined from the most recent package inserts and medical reviews, which were obtained through the Drugs@FDA database. A literature search was performed if there was insufficient information in the package insert and medical review. The overall incidence of PN across all grades was selected. Data were pooled from multiple studies for clinically relevant dose levels. The incidence of PN for intravenous administration was prioritized for cases in which a drug is approved for multiple routes of administration.

Often in package inserts and medical reviews, the incidence of PN is reported as very rare (<0.01%), rare (0.01% to 0.1%), uncommon (0.1% to 1%), common (1% to 10%), or very common (>10%). Compounds with rare and very rare incidences of PN, such as fluoroquinolones and
A literature search was performed for compounds classified into uncommon, common, and very common groups in order to obtain values that were more accurate. If information was not available in the scientific literature, compounds classified as uncommon and common were assumed to have a PN incidence of 0.5% and 5%. No assumptions were necessary for compounds with an incidence of PN greater than 10%; the exact percentage or number of patients that developed PN was always reported. Platinum containing compounds were excluded from the analysis due to the unreliability of the software to predict molecular descriptors of these compounds. Following the specified exclusion criteria, the curated dataset contained 95 drugs, and their respective incidences of PN are listed in Supplementary Table S1.

Software

Molecular descriptors were generated via ADMET Predictor™ version 8.5. Molecular descriptor selection, training/test pool determination, and ANN/SVM model development were performed using ADMET Modeler™ version 8.5. Figures were generated using Adobe Illustrator and GraphPad Prism 7. Structural editing and visualization were performed using MedChem Designer™ version 3.0 and Marvin.

Molecular Descriptors

Molecular descriptors were generated for each of the 95 drugs. MDL MOL files for each drug were obtained from public databases (PubChem, DrugBank, and ChEMBL). MDL MOL files were imported into ADMET Predictor™, and 227 molecular descriptors were generated, which included descriptors from the following categories: electrotopological states, topological indices, charge based, functional groups, hydrogen bonding, simple constitutional, and ionization.
Descriptors from the following categories were excluded: 3D descriptors, Moriguchi descriptors, Meylan flags, pattern recognition flags, indicators, and textual descriptors. Descriptor selection criteria for all machine learning models consisted of a two-step unsupervised and supervised process. The unsupervised process removed descriptors that were highly correlated (>0.95), exhibited insignificant variability (minimum input coefficient of variation = 1%), and were present in at least four compounds (minimum representation = 4). The unsupervised descriptor selection process removed 77 descriptors. A supervised descriptor selection process was performed using a Genetic Algorithm search (max steps = 30,000; max training = 1,000; search = row or cell). The number of descriptors ranged between 1-19 and was searched with a step size of one. The maximum number of descriptors selected (19) was determined based upon an arbitrary rule to not exceed one descriptor for every five compounds in the dataset.

**Training and Test Sets**

The dataset containing 95 drugs was split into training, verification, and test pools using a Kohonen self-organizing map. The Kohonen map was generated by initially scaling all descriptor values between [0, 1] for ANN and [-1, 1] for SVM. Subsequently, an iterative two-stage process was performed that ultimately groups compounds with similar descriptors closer together in a matrix. The matrix is then sampled by selecting one compound from each cell with multiple compounds to be in the training group. The same process is then repeated for the verification pool, followed by the training pool. The remaining compounds are randomly assigned. A test set size of 15% was selected, and an automatic Kohonen size was generated for each individual model. The remaining 85% of drugs were divided into the training and verification pools at a ratio of 2:1.
**Machine Learning Models**

Artificial neural network models were developed for the quantitative prediction of PN incidence, and two ANN models were developed. The first model was trained on PN incidence values that were not transformed, denoted further as the *non-transformed ANN*. The second model was trained on log-transformed PN incidence values, which will be referred to as the *log-transformed ANN*. Each model comprises of 10 individual ANNs that were generated via random seed numbers. Default values were selected for the number of networks per ANN ensemble (33) and network multiplier (5). For network training, the number of Monte Carlo tries for starting points ranged from 1-5, and the maximum number of network weights was set to not exceed 75% of the training dataset size. Several individual models were developed with different random seed numbers, and the top 10 models were selected based upon their overall performance. For each model run, a range of neurons (1-10) and molecular descriptor inputs (1-19) was searched using a step size of one, and the best performing model was reported based upon model selection criteria. The number of hidden layers was set to one owing to software constraints.

Support vector machine models were developed to classify drugs into three groups based upon their propensity to induced PN. The support vector machine algorithm uses molecular descriptor information to solve a binary classification problem, in which 1 (positive) and 0 (negative) indicate that a drug is above or below a defined PN incidence threshold. Two SVM models were developed, referred to as 2% SVM or 10% SVM models. Drugs are classified into two groups depending upon their predicted incidence of PN being ≥ 2% (10%) or < 2% (10%) by the 2% SVM (10% SVM) model. Each model contained 11 individual models to ensure that there
is no tie. A drug is classified as positive if more than half of the 11 individual SVM models were positive. We arbitrarily defined three groups for drugs that have a high (greater than 10%), medium (between 10% and 2%), or low (less than 2%) incidence of PN. A drug has a high (low) incidence of neuropathy if the result of the 10% (2%) SVM model is positive (negative). A drug is classified as medium if more than half of the 2% SVM models and less than half of the 10% SVM models are positive. The number of networks (33) and net pool (40) per SVM ensemble were set to the default values. The number of molecular descriptor inputs ranged from 1-19 and was searched using a step size of one. Several individual models were developed using different random seed numbers and the best performing 11 models were retained.

PN Incidence Prediction for Drugs in Clinical Trials

The machine learning models were applied to predict the incidence of PN for several cancer chemotherapeutics that are in clinical trials. The drug development pipelines for several pharmaceutical companies were searched in order to identify anti-cancer drugs that are being developed. Sixty drugs were classified into groups of low, medium, and high PN incidence using the final SVM models. Additionally, quantitative predictions of PN incidence using the ANN models were performed for each drug. The non-transformed ANN model predicted low PN incidence compounds with a significant amount of error relative to the log-transformed model. However, the log-transformed ANN model poorly predicted high PN incidence compounds. Therefore, in order to improve the overall model predictability, a hybrid approach was used that consisted of recording predictions from the non-transformed ANN model for drugs with a PN incidence greater than 14% and the log-transformed ANN model when predictions were less than 14%. The 14% cutoff value was determined by searching for the cutoff value that produces
the minimal amount of error between the observed and predicted PN incidence for the original 95 compounds. Individual model predictions that were outside of the applicability domain were identified by ADMET Predictor™ and subsequently removed.

Results

Dataset Characteristics

The dataset used for model training and testing contained 95 drugs that have been approved by the FDA or EMA. The indications of these drugs are shown in Figure 1a and are primarily used for the treatment of cancer. The incidence of PN across all drugs followed a power law distribution, in which the majority of drugs show a low incidence of PN, and only a few drugs exhibit a high PN incidence (Figure 1b). The average and range of PN incidence was 8.52% and 0.5%-71%.

Quantitative Model Performance

Artificial neural network models were developed to quantitatively predict the clinical incidence of PN based solely upon molecular descriptor information. For the 10 individual non-transformed ANN models, the number of neurons and molecular descriptors ranged (average) from 2-3 (2.4) and 8-19 (14.6). For the 10 individual log-transformed ANN models, the number of neurons and molecular descriptors ranged (average) from 1-8 (3.2) and 3-16 (10.2). The number of neurons and molecular descriptors used in each individual model are reported in Supplementary Table S2a. Model output statistics (Table 1) and predicted versus observed plots (Figure 2) are shown for both non-transformed and log-transformed ANN models. The square of Pearson’s correlation coefficient ($R^2$) and the coefficient of determination ($Q^2$) across all
pools of the two models range from 0.55-0.82 and 0.61-0.79, which indicates acceptable model performance. As PN incidence increases, the trend lines for the predicted versus observed plots begin to diverge below the line of identity, indicating that the model is slightly under predicting.

*Classification Model Performance*

Support vector machine models were developed to classify drugs into groups based upon their predicted incidence of PN. The number of molecular descriptor inputs in the 11 individual 2% SVM models ranged from 3-8 with an average of 5.9. The number of molecular descriptor inputs in the 11 individual 10% SVM models ranged from 2-14 with an average of 7.2. The number and types of molecular descriptor inputs used in each of the individual models are reported in Supplementary Table S2b-e. 2% SVM (Figure 3a) and 10% SVM (Figure 3b) model predictions of PN incidence compared to reported values are depicted as 2x2 matrices, in which the percentage of true/false positives/negatives are displayed in each of the quadrants. Model output statistics and performance criteria for both models are reported in Table 2. The number of predicted true positives relative to the number of actual true positives (sensitivity) ranged from 0.82-0.90 between both models and training/test pools. The number of predicted true negatives relative to the number of actual true negatives (specificity) ranged from 0.87-0.97 between both models and training/test pools. The model output statistics indicate a strong predictive performance by each of the SVM models.

*Molecular Descriptors*

The top 20 most frequent molecular descriptors used as input across all models are shown in Figure 4. The number of aromatic nitrogens was the most frequent and sensitive molecular
descriptor, occurring in 19 of the 42 individual models. The number of aromatic nitrogens was
the most frequent descriptor for the log-transformed ANN and SVM 2% models, but it occurred
less frequently in the non-transformed ANN and SVM 10% models (Supplementary Figure 1).
The number of aliphatic hydroxyl groups was another structural moiety on the list of frequent
molecular descriptors. Four molecular descriptors (NPA_Q2, NPA_Q3, NPA_Q4, and NPA_Q6)
of the top 20 most frequent descriptors relate to partial atomic charges that were determined
using natural population analysis (NPA).27

Aromatic Nitrogens: Potential to Design Out Neurotoxicity

No correlation was found between the number of aromatic nitrogens and the incidence of PN
(Figure 5a). However, there was a class specific positive correlation between the number of
aromatic nitrogens in proteasome inhibitors and their reported incidence of PN (Figure 5b). The
reported incidence of PN for several of these compounds are from studies with relatively small
sample sizes, and this could confound establishing such relationships. To explore the impact of
aromatic nitrogens on bortezomib, its structure was altered by removing the two aromatic
nitrogens. The final neural network model-predicted incidence of bortezomib-induced PN
decreased from 32.3% to 21.1% when the aromatic nitrogens were absent (Figure 6). The 10%
SVM model predicted that the bortezomib derivative induced PN would be greater than 10%;
however, the 2% SVM model predicts an incidence of less than 2% (data not shown).

Peripheral Neuropathy Incidence Prediction for Drugs in Clinical Trials

The QSTR models were applied to predict the incidence of PN for 60 cancer chemotherapeutic
agents that are under clinical investigation. The SVM models were used to classify drugs into
three categories of high (≥10%), medium (<10% and ≥2%), and low (<2%) incidence. The ANN models were used to predict the percentage of patients that would develop PN from each drug. The classification and quantitative model predictions are reported in Table 3.

The classification models predicted that 19, 32, and 9 compounds may exhibit a high, medium, and low incidence of peripheral neuropathy. Four compounds (delanzomib, fenebrutinib, mavelertinib, and tirabrutinib) were flagged as potentially showing a high incidence of peripheral neuropathy in both the classification and quantitative models. Only three compounds (BMS-986205, AMG176, and GSK2879552) were predicted to have a low incidence of neuropathy in both models.

Discussion

In this study, QSTR models were developed to predict drug-induced PN from molecular descriptors. Two quantitative models were developed using an ANN algorithm, and two classification models were developed using a SVM algorithm to predict and categorize drugs into high, medium, or low incidences of PN. Model performance criteria were acceptable for all models (Tables 1 and 2). The number of aromatic nitrogens in the molecular structure was identified as the most frequent and sensitive parameter across all models. Interestingly, the replacement of an aromatic carbon with a nitrogen is one of the most frequent chemical transformations made by medicinal chemists in lead optimization programs. This substitution affects several molecular properties, such as the redistribution of electron density, alteration of inter-intramolecular forces, and torsional energy. This chemical transformation is often applied to optimize the physicochemical and ADME properties of compounds by increasing the
hydrogen bond acceptors and polar surface area, which alters cellular permeability and improves solubility.\textsuperscript{30-33} The substitution of an aromatic carbon to a nitrogen also can play a substantial role in the pharmacokinetic/pharmacodynamic (PK/PD) properties of a compound. There are clear examples where this single substitution of an aromatic carbon for a nitrogen has improved target binding affinity, biochemical/cellular potency, functional activity, metabolic stability, and plasma retention time.\textsuperscript{29, 34-39}

The QSTR models developed in the present analysis suggest the possibility of a worsening neurological safety profile from the incorporation of aromatic nitrogens. Therefore, considerations could be made in the early stages of drug discovery and lead compound optimization to maximize therapeutic efficacy and minimize neurotoxicity. The possible neurotoxic mechanisms for drugs that contain aromatic nitrogens are incomplete. We hypothesize that metabolism via aldehyde oxidase may play a partial role in development of PN for certain drugs. Aldehyde oxidase is a cytosolic enzyme of the molybdenum hydroxylase family that is responsible for the oxidative hydroxylation of carbon-hydrogen bonds adjacent to aromatic nitrogens.\textsuperscript{40} Aldehyde oxidase-mediated drug metabolism is becoming recognized as an important metabolic pathway since several compounds have been terminated in clinical trials due to toxicity and unfavorable pharmacokinetic properties.\textsuperscript{40} One possible mechanism of neurotoxicity is the production of oxidative radicals. The metabolism of compounds by aldehyde oxidase has been shown to generate reactive oxygen and nitrogen species (\(O_2^{-}\), \(H_2O_2\), and \(NO\)).\textsuperscript{41, 42} Oxidative stress has a well-known role in the pathophysiology of PN.\textsuperscript{43} Another possible mechanism of neurotoxicity could be related to the precipitation of a poorly soluble metabolite.\textsuperscript{44} An additional hypothesis for the neurotoxic mechanism of compounds containing
aromatic nitrogens is through the generation of reactive metabolites, which could cause oxidative stress and hapten-mediated immune activation.$^{45,46}$

One advantage of the final QSTR models is the ability to predict the PN incidence of a compound prior to synthesis and experimental investigation, and these models might show utility during drug candidate selection in the early discovery phase. As a case study, the incidence of PN was predicted for 60 antineoplastic agents in clinical development, which identified compounds that may potentially pose high neurotoxic risks. Additional monitoring of patients in these trials might be warranted. Fenebrutinib and tirabrutinib, two Bruton’s tyrosine kinase inhibitors, were predicted to exhibit a high incidence of PN. It would be of interest to determine if these two compounds are substrates of aldehyde oxidase, especially since the BTK inhibitor GDC-0834 is a known substrate.$^{47}$ To highlight how the model might be implemented, the structure of bortezomib was modified and the incidence of PN was predicted for a structural derivative devoid of aromatic nitrogens. The incidence of PN for the derivative was predicted to be less than bortezomib. A decrease from 32.3% to 21.1% of patients who develop PN might be considered a significant clinical impact, assuming that the pharmacological effect of the drug was not diminished. Delanzomib, a bortezomib derivative, was predicted to have an incidence of peripheral neuropathy of 19.1%, which is similar to the reported value of 21% from the phase I/II study.$^{48}$ Interestingly, delanzomib has one aromatic nitrogen but a lower predicted incidence than the bortezomib derivative with zero aromatic nitrogens. One explanation for this observation could be that delanzomib is not a substrate for aldehyde oxidase since the carbon directly next to the aromatic nitrogen is occupied by a benzene substituent.
One of the main limitation of our model is the lack of pharmacokinetic information. We did not include information regarding the dosing regimen, which is important for understanding the quantitative relationship between drug exposure and neurotoxic effects. The incorporation of pharmacokinetic information and biomarkers of peripheral neurotoxicity might help to bridge the gap between neurotoxic agents and the development of PN. However, this would be challenging due to the multifactorial nature of the mechanisms of toxicity and the lack of adequate biomarkers. The production of oxidative radicals and the expression of proinflammatory cytokines might represent potential biomarkers.\textsuperscript{43, 49}

Another limitation is the lack of confidence about the incidence of PN reported from clinical trials, which has many confounding factors. There are many diverse patient populations with different physical characteristics, genetic background, disease states, and lifestyle/environmental exposures. These conditions could result in predisposed nerve damage that is exacerbated upon drug therapy. Diabetes and alcohol use are two well established etiologies of peripheral nerve damage.\textsuperscript{50} Viral infections alone can induce a painful sensory neuropathy, which is mediated by the activation of immune system components and the release of proinflammatory cytokines in the dorsal root ganglia.\textsuperscript{51} Thus, the reported incidence of neuropathy for antiretroviral therapies may be slightly inflated due to the nature of the disease. In order to test whether the identification of aromatic nitrogens is an artefact of viral-associated PN, several models were generated that excluded antiretroviral drugs in the dataset, and similar results were obtained (data not shown). The NCI-CTCAE scale is commonly used to assess the presence and severity of chemotherapy-induced PN. However, there is known inter-observer disagreement with this method.\textsuperscript{52, 53} The use of patient-reported outcome measures,
such as EORTC QLQ-CIPN20, are becoming more routinely used for the assessment of CIPN. Hence, the reported incidence of PN may deviate from the true incidence due to different and partially subjective assessment methods.

Several classes of compounds were excluded from the final models. Although platinum-based antineoplastic drugs (e.g., cisplatin and oxaliplatin) are known to be highly neurotoxic, we did not include these drugs due to limitations of the software. AMDET Predictor™ generates several molecular descriptors using semi-empirical methods that are not parameterized for heavy metals, such as platinum. Fluoroquinolone antibiotics are known, although seldom, to induce a severe irreversible form of PN.\textsuperscript{26, 54} Statins have also been shown to be associated with PN.\textsuperscript{25, 55} However, fluoroquinolone antibiotics and statins were excluded from this study since the incidence of PN is very low and seems to be idiosyncratic. The final QSTR models were not designed to detect drug-induced peripheral neurotoxicities of this nature.

In summary, QSTR models were constructed that link physicochemical descriptors of compounds with the incidence of PN. Hypotheses regarding the high incidence of PN for select proteasome inhibitors were generated, and the final models were used to anticipate PN for new investigational anti-cancer compounds. CIPN remains an unmet medical need, and the application of \textit{in silico} tools for predicting drug neurotoxicity, such as the final QSTR models in this study, may be useful for developing safer therapeutics and ultimately reducing the clinical burden of CIPN.
Acknowledgements

We would like to thank Simulations Plus, Inc. for providing us with an academic license for ADMET Predictor™, ADMET Modeler™, and MedChem Designer™. Additionally, we would like to acknowledge Michael Lawless, Senior Principle Scientist at Simulations Plus, for his insightful suggestions throughout this project.
## Manuscript Tables:

### Table 1. Model output statistics of artificial neural networks trained on non-/log-transformed data for the quantitative prediction of peripheral neuropathy incidence.

<table>
<thead>
<tr>
<th>Model</th>
<th>Non-Transformed ANN</th>
<th>Log-Transformed ANN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Training</td>
<td>Verification</td>
</tr>
<tr>
<td>Pool</td>
<td>RMSE</td>
<td>MAE</td>
</tr>
<tr>
<td></td>
<td>5.84 +/- 0.74</td>
<td>4.05 +/- 0.54</td>
</tr>
<tr>
<td></td>
<td>5.79 +/- 0.76</td>
<td>4.91 +/- 0.60</td>
</tr>
<tr>
<td></td>
<td>7.78 +/- 1.74</td>
<td>5.46 +/- 0.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.38 +/- 0.02</td>
<td>0.30 +/- 0.02</td>
</tr>
<tr>
<td></td>
<td>0.38 +/- 0.02</td>
<td>0.34 +/- 0.02</td>
</tr>
<tr>
<td></td>
<td>0.39 +/- 0.04</td>
<td>0.31 +/- 0.02</td>
</tr>
</tbody>
</table>

Statistics are reported as the average +/- standard deviation of 10 individual models. Root mean squared error (RMSE); mean average error (MAE); spearman rank correlation coefficient (SRCC); the square of Pearson’s correlation coefficient (R²); coefficient of determination (Q²); slope and intercept for the linear regression of the observed versus predicted PN incidence.

### Table 2. Model output statistics of support vector machine models for the classification of peripheral neuropathy incidence at a cutoff value of 2% (2% SVM) and 10% (10% SVM).

<table>
<thead>
<tr>
<th>Model</th>
<th>2% SVM</th>
<th>10% SVM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Training</td>
<td>Test</td>
</tr>
<tr>
<td>Pool</td>
<td>Sensitivity</td>
<td>0.90 +/- 0.03</td>
</tr>
<tr>
<td></td>
<td>Specificity</td>
<td>0.87 +/- 0.04</td>
</tr>
<tr>
<td></td>
<td>Youden</td>
<td>0.78 +/- 0.04</td>
</tr>
<tr>
<td></td>
<td>MCC</td>
<td>0.78 +/- 0.04</td>
</tr>
<tr>
<td></td>
<td>False Rate</td>
<td>0.11 +/- 0.02</td>
</tr>
</tbody>
</table>

Statistics reported as the average (+/- standard deviation) of 11 individual models. True positive (TP); true negative (TN); sensitivity = TP/(TP+FN); specificity = TN/(TN+FP); Youden’s index J (Youden); Matthew’s correlation coefficient (MCC); fraction of incorrect predictions (false rate).
Table 3: Model predictions of peripheral neuropathy incidence for compounds that are under investigation in clinical trials (Phases I-III) for cancer chemotherapy.

<table>
<thead>
<tr>
<th>Compounds Under Clinical Investigation</th>
<th>Prediction of PN Incidence: Classification Model</th>
<th>Prediction of PN Incidence (%): Quantitative Model</th>
<th>Compounds Under Clinical Investigation</th>
<th>Prediction of PN Incidence: Classification Model</th>
<th>Prediction of PN Incidence (%): Quantitative Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delanzomib</td>
<td>High</td>
<td>19.1</td>
<td>Capmatinib</td>
<td>Medium</td>
<td>6.3*</td>
</tr>
<tr>
<td>Fenebrutinib</td>
<td>High*</td>
<td>16.6</td>
<td>Ipatasertib</td>
<td>Medium</td>
<td>5.9</td>
</tr>
<tr>
<td>Mavelertinib</td>
<td>High</td>
<td>15.1</td>
<td>Citarinostat</td>
<td>Medium</td>
<td>5.6</td>
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<tr>
<td>Tirabrutinib</td>
<td>High</td>
<td>11.0</td>
<td>Veliparib</td>
<td>Medium</td>
<td>5.4</td>
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<tr>
<td>Oprozomib</td>
<td>High</td>
<td>8.8</td>
<td>Entospletinib</td>
<td>Medium</td>
<td>5.4</td>
</tr>
<tr>
<td>Asciminib</td>
<td>High</td>
<td>8.1</td>
<td>Entinostat</td>
<td>Medium</td>
<td>5.3</td>
</tr>
<tr>
<td>AZD2811</td>
<td>High</td>
<td>7.8</td>
<td>Molibresib</td>
<td>Medium</td>
<td>5.0</td>
</tr>
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<td>High</td>
<td>7.5</td>
<td>Selumetinib</td>
<td>Medium</td>
<td>4.8</td>
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<td>7.3</td>
<td>PIM447</td>
<td>Medium</td>
<td>4.7</td>
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<td>6.5</td>
<td>Talazoparib</td>
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<td>4.3</td>
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<td>Vorasidenib</td>
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<td>4.2*</td>
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<td>6.4</td>
<td>Osilodrostat</td>
<td>Medium</td>
<td>4.0</td>
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<tr>
<td>Adavosertib</td>
<td>High</td>
<td>6.4</td>
<td>VX-984</td>
<td>Medium*</td>
<td>3.8*</td>
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<tr>
<td>Alpelisib</td>
<td>High</td>
<td>6.1</td>
<td>AZD6738</td>
<td>Medium</td>
<td>3.4</td>
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<td>BAY1895344</td>
<td>High</td>
<td>5.9</td>
<td>GSK2636771</td>
<td>Medium</td>
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<td>Darolutamid</td>
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<td>4.9</td>
<td>Berzosertib</td>
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<td>LY3381916</td>
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<td>4.9</td>
<td>AZD9496</td>
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<tr>
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<td>4.2</td>
<td>AZD4635</td>
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<td>BMS-813160</td>
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<td>Capivasertib</td>
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<td>Erdaftinib</td>
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<td>10.3</td>
<td>Iberdomide</td>
<td>Low</td>
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<td>AZD5153</td>
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<td>3.7</td>
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<td>Savolitinib</td>
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* More than three individual model predictions were excluded because they were outside of the applicability domain of the model.
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Chapter 2 Figures:

Abstract Figure: Molecular descriptors of drugs known to induce peripheral neuropathy (red, yellow, or green) were used as inputs to train an artificial neural network to predict the incidence of peripheral neuropathy. The model was applied to quantitatively predict the incidence of peripheral neuropathy of investigational drugs (gray) and classify them accordingly.
Figure 1: Dataset characteristics for the 95 drugs used in model development and testing. (top) Drug indications and (bottom) frequency distribution of peripheral neuropathy incidence.
Figure 2: Comparison of model predictions and observed values for the incidence of peripheral neuropathy. ANNs developed with (a) non-transformed data and (b) log-transformed data. The predicted incidence of PN for each drug is depicted as the mean +/- standard deviation (error bars) of 10 individual models. The line of identity and trend line (obtained via linear regression) are shown as solid and dashed black lines. Drugs in the training, test, or a combination of both pools are shown in blue, red, and purple. To improve visualization, the training/test pool error bars are oriented down and the error bars for the combination of both pools are oriented up.
**Figure 3:** Classification model predictions for the incidence of peripheral neuropathy. SVMs developed with a peripheral neuropathy cutoff value of (a) 2% and (b) 10%. The percentages (number) of true/false positives/negatives represent the total of 11 individual models. Drugs in the training and test sets are shown in blue and red.
Figure 4: Frequency and relative sensitivity of the top 20 most frequent molecular descriptors.

Frequency is calculated as the number of times the descriptor was used across all four types of models (non-transformed ANN, log-transformed ANN, 2% SVM, and 10% SVM), and each type consists of 10 or 11 individual models (maximum frequency = 42).

Molecular Descriptor Abbreviations:

1. ArNitrog =N: Number of aromatic nitrogens
2. Pi_MaxQ: Maximum Hückel pi atomic charge
3. NPA_Q4: 4th component of the autocorrelation vector of estimated NPA partial atomic charges
4. EEM_NFnp: Minimum sigma Fukui index on nonpolar atoms
5. T_MiRyy: Topological equivalent of “MiRyy_3D”, but without mass weighting
6. NPA_Q2: 2nd component of the autocorrelation vector of estimated NPA partial atomic charges
7. HBDch: Sum of Estimated NPA Partial Atomic Charges on HB Donor Hydrogens
8. Pi_MaxFmi: Maximum pi Fukui(−) index
9. T_RDmtr: Relative topological diameter: maximal topological distance divided by the number of atoms
10. T_Dipole: Topological equivalent of “Dipole_3D”
11. Pi_Q3: 3rd component of the autocorrelation vector of Hückel pi atomic charges
12. SssCH2: Atom-type E-state index for -CH2- groups
13. ABSQon: Sum of Absolute Values of PEOE Partial Atomic Charges for O and N atoms
14. EEM_MaxF: Maximum sigma Fukui index
15. SsssCH: Atom-type E-state index for >CH- groups
16. NPA_Q6: 6th component of the autocorrelation vector of estimated NPA partial atomic charges
17. NPA_Q3: 3rd component of the autocorrelation vector of estimated NPA partial atomic charges
18. AlHdrl=OH: Number of aliphatic hydroxyl groups
19. SsaasC: Atom-type E-state index for -Caa groups
20. Pi_AQn: Sum of absolute values of Hückel pi atomic charges for N atoms
**Figure 5:** Correlation of peripheral neuropathy incidence and the number of aromatic nitrogens for (a) all 95 drugs in the dataset and (b) proteasome inhibitors that are FDA approved or under clinical development.
Figure 6: Non-transformed ANN model predictions of peripheral neuropathy incidence of bortezomib (left) and an aromatic nitrogen absent derivative (right). Red circles indicate the positions of structural modifications made to remove the aromatic nitrogens from bortezomib.
Supplementary Figure 1. Molecular descriptor frequency and sensitivity of individual models (a) ANN non-transformed, (b) ANN log-transformed, (c) SVM 2% cutoff, and (d) SVM 10% cutoff of the individual models.
References


55. Tierney, E. F.; Thurman, D. J.; Beckles, G. L.; Cadwell, B. L., Association of statin use with peripheral neuropathy in the U.S. population 40 years of age or older. *J Diabetes* 2013, 5, 207-15.
Chapter 3:

In Silico Comparative Systems Toxicology Analysis of

Proteasome Inhibitors
Abstract

After the discovery of bortezomib, proteasome inhibition has emerged as an important mechanism for targeted cancer therapy. The emergence of resistance and dose limiting adverse drug effects have inspired the search for second-generation proteasome inhibitors, such as carfilzomib. The incidence and severity of peripheral neuropathy induced by these two proteasome inhibitors is different. Bortezomib treatment induces peripheral neuropathy in approximately 37% of patients, whereas only 12% of patients taking carfilzomib develop peripheral neuropathy. In this study, an in silico comparative systems pharmacology analysis was performed in order to provide insight into the molecular mechanisms governing the neurotoxic differences between bortezomib and carfilzomib. Interaction networks were developed for each proteasome inhibitor, and a bioinformatics analysis was conducted to annotate genes of the interaction networks functionally and identify significantly enriched cellular signaling pathways. Network simulations were performed to predict the neurotoxic effects of proteasome inhibition and neuroprotective effects of hypothesized therapeutic interventions. Model predictions revealed that the inhibition of IP3R prevented neuronal apoptosis by proteasome inhibition. Cytotoxicity studies were performed to assess the neuroprotective ability of IP3R inhibitors, curcumin and xestospongin C, in preventing bortezomib induced neuronal death. IP3R inhibition did not significantly alter the cytotoxicity of bortezomib on SH-SY5Y cells. The bioinformatic analysis revealed that the unfolded protein response in the endoplasmic reticulum was identified as a significant component of bortezomib neurotoxicity, and the mechanisms of toxicity may be related to the pathogenesis of Guillian-Barré syndrome caused by the Epstein-Barr virus.
Introduction

Chemotherapy-induced peripheral neuropathy (CIPN), or nerve damage caused by cancer chemotherapy, is an unmet medical need of growing concern. Approximately 30-40% of all cancer patients treated with a chemotherapeutic agent will develop peripheral neuropathy (Wolf et al., 2008). Symptoms often manifest as a numbness and tingling sensation in the extremities of the body, which throughout the course of treatment may evolve into an extreme burning pain sensation. Peripheral neuropathy is often reversible upon cessation of treatment; however, some patients suffer from lifelong irreversible effects (Quasthoff and Hartung, 2002; Miaskowski et al., 2017). The prevalence and severity of CIPN are dependent upon several factors, such as the chemotherapy agent, dosing regimen, genetic polymorphisms, and preexisting nerve damage (Kerckhove et al., 2017). The molecular mechanisms of toxicity responsible for the development of CIPN are complex and remain poorly understood.

Taxanes, vinca alkaloids, platinum-containing agents, and proteasome inhibitors are four drug classes that are widely known to cause peripheral neuropathy (Kerckhove et al., 2017). Proteasome inhibitors are relatively new compared to the other three drug classes. The 26S proteasome is a multisubunit protease complex responsible for degradation of ubiquitinated proteins, which is a crucial component for maintaining cellular homeostasis, cell cycle regulation, DNA repair, apoptosis, and the unfolded protein response (Adams, 2003). The 20S catalytic core of the proteasome includes the chymotrypsin-like activity of the proteasome subunit β5 (PSMB5), trypsin-like activity of the proteasome subunit β2 (PSMB2), and caspase-like activity of the proteasome subunit β1 (PSMB1). Bortezomib, a boronic acid dipeptide, was the first in class proteasome inhibitor and had been granted accelerated approval by the FDA in 2003. Bortezomib
therapy resulted in unprecedented survival rates for multiple myeloma patients; however, the most clinically significant adverse effect was peripheral sensory neuropathy (Richardson et al., 2003). In the phase III APEX trial, 37% of patients undergoing bortezomib therapy developed peripheral neuropathy and 9% had to discontinue treatment owing to its severity (Richardson et al., 2009). Second generation proteasome inhibitors, such as the peptide epoxyketone carfilzomib approved in 2012, have been developed to overcome cancerous cells that are resistant to bortezomib therapy (Dick and Fleming, 2010; Ruschak et al., 2011). The overexpression of PSMB5 has been shown to be a mechanism of bortezomib resistance (Oerlemans et al., 2008). Bortezomib is a reversible inhibitor of the chymotrypsin-like and caspase-like activity of the proteasome, whereas carfilzomib is an irreversible inhibitor selective to the chymotrypsin-like activity (Demo et al., 2007). The cytotoxicity of proteasome inhibitors does not correlate solely with the inhibition of the chymotrypsin-like activity; Maximal cytotoxic effects can only be achieved when caspase-like and chymotrypsin-like activities are co-inhibited (Britton et al., 2009). The incidence and severity of peripheral neuropathy for carfilzomib therapy is significantly lower than bortezomib. In a phase II clinical trial for single agent carfilzomib therapy, only 12% of patients developed peripheral neuropathy, and no patients had to discontinue treatment (Siegel et al., 2012). The clinical neurotoxic disparity between bortezomib and carfilzomib has been suggested to result from direct modulation of non-proteasomal targets, such as the inhibition of serine proteases cathepsin A, cathepsin G, chymase, dipeptidyl peptidase II, and HtrA2/Omi (Arastu-Kapur et al., 2011). However, a proteasome-dependent mechanism in which proteasome inhibitors target different constitutive and immunoproteasome subunits that
result in differential substrate specificity could also be responsible for neurotoxic disparities across proteasome inhibitors (Huber et al., 2012).

In this study, a network-based in silico comparative systems pharmacology analysis was performed to provide insights into potential molecular mechanisms by which proteasome inhibitors induce peripheral neuropathy. Interaction networks were constructed for bortezomib and carfilzomib based upon their respective proteasomal subunit targets and known protein-protein interactions with those subunits. Functional annotation and pathway mapping was performed for genes in each of the interaction networks. Hypothetical therapeutic interventions were identified, and one target was investigated via systems pharmacology model predictions and experimental cytotoxicity studies.

**Methods**

*Development of Pharmacological Interaction Networks*

Pharmacological interaction networks were developed for the direct and indirect targets of bortezomib and carfilzomib (Figure 1). Direct targets of bortezomib and carfilzomib were obtained from DrugBank (Law et al., 2013). A bipartite interaction network consisting of proteasome inhibitor and direct targets was constructed using Cytoscape v3.1.1 (Shannon et al., 2003). Proteins that have been reported to interact with the direct drug targets, denoted further as indirect targets, were imported through BioGrid to form a tripartite network consisting of proteasome inhibitors and direct/indirect targets (Stark et al., 2006). BioGrid was selected as the public database to use for this analysis since it contained the largest number of internally-curated interactions (Stark et al., 2006).
Bioinformatic Analysis

Functional annotation and pathway mapping was performed (Figure 2) using the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8 (Dennis et al., 2003). Gene lists were generated from the proteasome inhibitor interaction networks using Cytoscape v3.1.1 (Shannon et al., 2003) and were uploaded to DAVID. Entrez Gene ID was selected as the identifier for the gene list. Homo sapiens was selected as the species of interest. Separate functional annotation charts were generated for bortezomib and carfilzomib for each of the three gene ontology domains: biological processes (BP), cellular components (CC), and molecular function (MF). The top 10 most significant terms were reported, and a chart was generated using the category UCSC_TFBS within protein interactions to determine which transcription factors are most likely responsible for controlling the transcription of genes in the interaction networks. Lastly, genes from each interaction network were mapped to pathways in Kyoto encyclopedia of genes and genomes (KEGG) in order to identify pathways that are significantly enriched (Kanehisa and Goto, 2000). Pathways related to cancer were excluded in the analysis since this is likely a reflection of the drugs indication and not related to its neurotoxicity. For each network, genes that were mapped to pathways related to neurodegenerative diseases were compared in order to identify targets that may be responsible for drug-induced peripheral neurodegeneration.

Systems Pharmacology Network Predictions

A previously developed systems pharmacology model of intracellular signaling and gene regulatory processes in neurons was utilized to predict the neuroprotective effects of select
interventions. Boolean logic functions were transformed into a series of ordinary differential equations using Odefy (Krumsiek et al., 2010). Normalized HillCube functions were used to simulate the dynamics of network species for treatment naïve, proteasome inhibition, and hypothetical therapeutic interventions. Intervention targets were obtained from pathway mapping of interaction network genes to KEGG pathways of neurodegenerative diseases (Kanehisa and Goto, 2000). Model simulations were performed for select therapeutic interventions and the results of IP3R inhibition are presented.

Experimental Investigation

A cytotoxicity assay was performed to investigate the differences in potency between bortezomib and carfilzomib. SH-SY5Y cells were plated on a 96-well plate at an initial seeding density of 100,000 cells/well and were allowed to equilibrate for a day. Concentrations of bortezomib and carfilzomib ranging between 0.001 and 1000 nM were added to each well in triplicate. WST-1 was added after 45 hours of drug exposure and allowed to incubate for 3 additional hours. Absorbance was measured at 440 nm using a SpectraMax i3 plate reader. The same experimental design was used to investigate the effect of IP3R inhibitors, curcumin and xestospongin C (XeC), on bortezomib cytotoxicity.

Results

Proteasome Inhibitor Interaction Networks

Nodes in the proteasome inhibitor interaction networks represent genes that encode the direct and indirect targets of bortezomib/carfilzomib. Direct targets are genes encoding proteasomal
subunits, which were obtained from DrugBank (Wishart et al., 2006; Law et al., 2013). Sixteen
and six direct targets of bortezomib (PSMA6, PSMA5, PSMA4, PSMA3, PSMA2, PSMA1, PSMB2,
PSMB5, PSMB1, PSMD1, PSMD2, PSMB7, PSMB6, PSMB4, PSMB3, PSMA7) and carfilzomib
(PSMB1, PSMB8, PSMB10, PSMB2, PSMB5, PSMB9) were obtained. Indirect targets were
obtained by importing nodes from BioGrid that have been reported to interact with each of the
proteasomal subunits (Stark et al., 2006). All edges in the network are undirected and
unweighted. The bortezomib and carfilzomib interaction networks contain 707 and 224 nodes
(Figures 3 and 4).

*Bioinformatics Analysis: Functional Annotation and Pathway Mapping*

*Gene Ontology*

A gene ontology enrichment analysis was performed for each of the gene lists from the
interaction networks. The top 10 most significant biological processes, cellular components, and
molecular functions for genes in the bortezomib and carfilzomib interaction networks are
displayed in Tables 1 and 2. Nine of the top ten most significant biological processes were shared
across both drugs. Four cellular components relating to the proteasome appeared in both
interaction networks (proteasome complex, proteasome core complex, proteasome accessory
complex, and proteasome regulatory particle). Protein and proteasome binding were two of the
top 10 significant molecular functions across both networks.

The top three most significant biological process exclusive to genes in the bortezomib interaction
network are the unfolded protein response, protein folding, and protein ubiquitination (Table 3).
The myelin sheath was the most significant cellular component exclusive to bortezomib. 32 genes
in the bortezomib interaction network were related to the myelin sheath, which consisted of heat shock proteins (HSPA2, HSPA5, HSPA8, HSPA9, HSPD1), tubulin (TUBA1A, TUBA1B, TUBB4B), and energy production (ATP5B, MDH2, PKM, and SDHA). Microtubules were the 5th most significant cellular component exclusive to bortezomib, which are known to be important for the structure and function of neurons. Binding to unfolded proteins, enzymes, and chaperones were the top three molecular functions exclusive to bortezomib.

**Transcription Factor Binding Sites**

Gene lists from nodes in each of the interaction networks were used to infer transcription factors that are regulated by these genes. The top 10 most significant transcription factor binding sites for genes of each interaction network are reported in Table 4. Three out of the top 10 most significant transcription factors, E2F, ELK1, and NRF2, were shared between both of the interaction networks. MYB, PAX2, and NFKB were the three most significant transcription factors exclusive to genes from the bortezomib interaction network.

**KEGG Pathway Mapping**

Genes in the interaction networks were mapped to pathways in KEGG. The top 10 most significant non-cancer pathways for bortezomib, carfilzomib, and exclusively bortezomib are shown in Table 5. The proteasome was the most significant KEGG pathway across both interaction networks. The Epstein-Barr virus infection, protein processing in the endoplasmic reticulum, and ubiquitin-mediated proteolysis pathways appeared in the top 10 most significant pathways for both bortezomib and carfilzomib. Although these pathways appeared for both proteasome inhibitors, the number of genes from each interaction network mapped to the pathway is different. For example, 45 and 13 genes from the bortezomib and carfilzomib
interaction networks appeared in the protein processing in the endoplasmic reticulum pathway, suggesting that bortezomib likely perturbs this pathway to a greater extent relative to carfilzomib. Protein processing genes in the endoplasmic reticulum pathway that are were present in the bortezomib network, but absent in the carfilzomib network, are shown in Supplementary Table 1. Additionally, 20 genes in the ubiquitin-mediated proteolysis pathway that are exclusive to bortezomib are shown in Supplementary Table 2.

The cell cycle was the most significant non-cancer pathway that was exclusive to genes in the bortezomib interaction network. The Wnt and HIF-1 signaling pathways were also exclusive to bortezomib. Lastly, a few pathways related to pathogenic microorganisms (hepatitis B, Escherichia coli, and measles) only appeared from genes in the bortezomib interaction network. Genes from each interaction network that were mapped to pathways of neurodegenerative diseases were also examined and summarized in a Venn diagram (Figure 6). Four genes (i.e., PARK2, HSD17B10, PSEN1, and POLR2A) mapped to neurodegenerative pathways were shared in both interaction networks. There were 22 genes exclusive to the bortezomib interaction network, and 8 of these genes (ITPR1, ATP5B, TNFSF1A, PPP3R1, PPP3CA, SDHA, TP53, and COX2) appeared in multiple neurodegenerative diseases. The 11 genes exclusive to the carfilzomib interaction network all were DNA-directed RNA polymerase II subunits.

**Systems Pharmacology Model Predictions**

Network model predictions show that proteasome inhibition results in an increase in IP3R activity and subsequent increase in intracellular calcium that leads to neuronal apoptosis (Figure 6: Top). A network simulation of proteasome inhibition in combination with an IP3R inhibitor prevented
the increase in intracellular calcium and neuronal apoptosis (Figure 6: Bottom). Network simulations were performed to investigate the combination of proteasome inhibition with a TNFα receptor inhibitor; however, neuronal apoptosis was not prevented (predictions not shown).

**Cytotoxicity Studies**

The cytotoxicity of bortezomib and carfilzomib was investigated in SH-SY5Y cells. Bortezomib exhibited a more potent effect on cell death than carfilzomib. An inhibitory Hill function was fit to the cytotoxicity data. The estimated IC\textsubscript{50} at 48 hours of exposure to bortezomib and carfilzomib was 5.8 and 48.4 nM.

The cytotoxicity of bortezomib on SH-SY5Y cells in the presence of IP3R inhibitors, curcumin, and XeC, was investigated. The IC\textsubscript{50} of bortezomib (3.06 nM) was not significantly altered when combined with 1 μM of XeC (3.49 nM). Greater concentrations of XeC (25 μM) were also investigated, but resulted in a greater cytotoxic effect. Several concentrations of curcumin were investigated to determine if it decreased the potency of bortezomib-mediated cytotoxicity. Overall, the potency of bortezomib was not significantly decreased by curcumin. Greater concentrations of curcumin (10, 30, and 100 μM) resulted in a greater cytotoxicity.

**Discussion**

A comparative systems pharmacology analysis was performed in order to provide insights into some of the molecular differences that might govern the neurotoxic differences between bortezomib and carfilzomib. A bioinformatic analysis of genes in the respective proteasome
inhibitor interaction networks suggested pharmacological differences and enabled the generation of hypothetical interventions to reduce neurotoxicity, such as the inhibition of IP3R. The inhibition of IP3R was initially assessed via predictions using a previously developed network model of intracellular signaling in peripheral neurons, which showed the prevention of bortezomib-induced neuronal apoptosis. However, these \textit{in silico} predictions did not translate \textit{in vitro}, which may be reflective of an inappropriate experimental model to assess neurotoxicity.

The gene ontology enrichment analysis revealed similarities and differences in biological processes, cellular components, and molecular functions between bortezomib and carfilzomib. Several gene ontology terms that relate to the structure and function of the proteasome were conserved between both networks, which provides reassurance regarding the methodology of this \textit{in silico} comparative approach. Interestingly, the top three most significant biological processes (unfolded protein, protein folding, and protein ubiquitination) and molecular functions (unfolded proteins, enzymes, and chaperones) exclusive to the bortezomib interaction network suggest that the unfolded protein response and ubiquitin-proteasome pathway may attribute to the neurotoxic differences. When genes were mapped from each interaction network to pathways in KEGG, both the protein processing in endoplasmic reticulum and ubiquitin-proteasome pathways contained more genes from the bortezomib network and p-values were significantly different. Proteasome inhibitors have been shown to induce ER stress and interfere with the unfolded protein response (Lee et al., 2003), which are associated with the pathophysiology of neurodegenerative diseases, such as peripheral neuropathy, Alzheimer’s, and Parkinson’s (Scheper and Hoozemans, 2015; Hetz and Saxena, 2017). Therefore, targeting components of the unfolded protein response to prevent peripheral neuropathy induced by
proteasome inhibition could be an attractive target to investigate. Targets could include protein processing genes in the endoplasmic reticulum pathway and ubiquitin-mediated proteolysis pathway present in the bortezomib network but absent in the carfilzomib network (Supplementary Tables 1 and 2). Seven of these genes encode heat shock proteins, specifically from the HSP70 and HSP40 families. Mutations in heat shock proteins 22 and 27 have been shown to cause forms of hereditary motor neuropathy, such as Charcot-Marie-Tooth disease (Evgrafov et al., 2004; Tang et al., 2005; Kijima et al., 2005; Solla et al., 2010). The combination of bortezomib with tanespimycin, an inhibitor of HSP90 and inducer of HSP70, results in a lower incidence of peripheral neuropathy compared to bortezomib monotherapy in both rats and patients (Cavaletti and Jakubowiak, 2010; Richardson et al., 2011; Zhong et al., 2008). Heat shock proteins are dysregulated in other protein misfolding neurodegenerative disorders, such as Alzheimer’s, Parkinson’s, and Huntington’s diseases (Aridon et al., 2011; Hamos et al., 1991; Hoshino et al., 2011; Luo et al., 2010; Perez et al., 1991; Wyttenbach and Arrigo, 2009; Wyttenbach et al., 2000). Two other genes exclusive to bortezomib in the endoplasmic reticulum protein processing pathway were BRCA1 and MDM2. Interestingly, BRCA1 and MDM2 were identified as single nucleotide polymorphisms associated with the development of bortezomib-induced peripheral neuropathy (Corthals et al., 2011).

Two cellular components important in the structure and function of peripheral neurons were exclusive to bortezomib. The myelin sheath and microtubules were the first and fifth most significant cellular components. Bortezomib increases microtubule polymerization and alters axonal transport in peripheral neurons (Poruchynsky et al., 2008; Staff et al., 2013). An increase in tubulin polymerization appears to be a class effect for proteasome inhibitors. To our
knowledge, tubulin polymerization by carfilzomib has not been investigated. However, a naturally occurring structural analog of carfilzomib, epoxomicin, has been shown to significantly increase the percentage of polymerized tubulin in SH-SY5Y and KCNR cells (Poruchynsky et al., 2008). Taxanes and vinca alkaloids are two classes of microtubule inhibitors that are well known inducers of peripheral neuropathy, which highlights the disruption of tubulin polymerization as a prominent mechanism of neurotoxicity (Carozzi et al., 2015). Bortezomib has been shown to decrease the number of myelin fibers and myelin thickness in rodents (Bruna et al., 2010; Cavaletti et al., 2007). However, these affects are likely secondary to the initial peripheral neurodegeneration (Bruna et al., 2010).

The second most significant pathway for both proteasome inhibitors was the Epstein-Barr virus infection pathways, which is particularly interesting since this viral infection is known to cause peripheral neuropathy (Bennett et al., 1996; Fujii et al., 1982; Gavin et al., 1997; Grose et al., 1975). Three other infectious diseases, hepatitis B, Escherichia coli, and measles, were amongst the top 10 significant KEGG pathways exclusive to bortezomib. This suggests that bortezomib-induced neuropathy may be similar in nature to post-infectious immune-mediated neuropathies like Guillain-Barré syndrome (Hughes and Cornblath, 2005; Yuki and Hartung, 2012).

Pathogenic similarities to protein misfolding disorders were evaluated to identify potential therapeutic targets for bortezomib-induced peripheral neuropathy. Genes from proteasome inhibitor interaction networks were mapped to KEGG pathways of Alzheimer’s, Huntington’s, Parkinson’s, and prion diseases. Two genes that were exclusive to bortezomib and appeared across multiple pathways were ITPR1 and TNFSF1A. Inositol 1,4,5-trisphosphate receptor type 1 (ITPR1 or IP3R) is a ligand-gated calcium channel on the ER membrane, which causes an increase
in intracellular calcium upon activation by inositol trisphosphate (Berridge, 1993). The dysregulation of intracellular calcium homeostasis is known to contribute to development of peripheral neuropathy caused by paclitaxel and vincristine (Jaggi and Singh, 2012; Siau and Bennett, 2006). Infusions with calcium/magnesium prevented oxaliplatin-related neurotoxicity in patients (Gamelin et al., 2004). The dysregulation of intracellular calcium has also been shown to be important to bortezomib cytotoxicity (Landowski et al., 2005). Tumor necrosis factor alpha (TNFSF1A or TNFα) is a proinflammatory cytokine produced by macrophages/monocytes and is responsible for various cellular functions including the regulation of cell death (Idriss and Naismith, 2000). The administration of TNFα has been shown to produce neuropathic pain in rats (Ignatowski et al., 1999; Junger and Sorkin, 2000). Neuroinflammatory processes such as cytokine/chemokine production, leukocyte infiltration, and glial cell activation are becoming recognized as major mechanisms underlying chemotherapy-induced peripheral neuropathies (Lees et al., 2017; Makker et al., 2017; Wang et al., 2012).

The pharmacodynamic effects of an IP3R and TNFα inhibitor were predicted for the treatment of proteasome inhibitor induced peripheral neuropathy via network simulations of a systems pharmacology model of intracellular signaling. IP3R inhibition prevented bortezomib-induced neuronal apoptosis; however, TNFα inhibition had no effect. The advantage of this model-based approach is that potential therapeutic targets could be suggested for future experimental studies. However, the model predictions are qualitative in nature and do not provide any sense for the magnitude of inhibition required to produce an efficacious pharmacological effect.

In this study, SH-SY5Y human neuroblastoma cells were used, which is a commonly used cell line to assess drug neurotoxicity and neurodegenerative diseases (Nicolini et al., 1998; Xie et al.,
Bortezomib was more potent than carfilzomib at producing a cytotoxic effect on these cells. Two potential inhibitors of a potential intervention target (IP3R), curcumin and XeC, did not show any sign of neuroprotection. There are a few possible explanations for the discrepancy between model predictions and experimental observations. First, there could be missing components in the network model that govern the cytotoxic effects of bortezomib. The final model does not consider any off-target effects of bortezomib, only its ability to inhibit proteasomal activity. Second, the experimental model may not be an adequate representation of the biological system, because SH-SY5Y cells are cancerous and not representative of healthy primary neurons. Lastly, the information used to generate and analyze the pharmacological interaction networks was obtained via publically available databases/resources (DrugBank, BioGRID, DAVID, and KEGG), which is frequently updated with new knowledge. Therefore, the constructed networks and analyses are dynamic and results of the study could change with time as new information is added into these databases.

Conclusion

Molecular mechanisms that may govern the development of peripheral neuropathy by proteasome inhibitors were evaluated. An advantage for the comparative systems pharmacology approach is that the construction of interaction networks and bioinformatic analysis were performed using publically available resources. Results suggest that (1) the unfolded protein response in the endoplasmic reticulum may be a major component of bortezomib neurotoxicity, and (2) the mechanisms of proteasome inhibitor mediated neurotoxicity may be related to the pathogenesis of Guillain-Barré syndrome caused by the Epstein-Barr virus. Potential therapeutic targets for the treatment of bortezomib-induced peripheral neuropathy were identified through
pathway mapping to other protein misfolding neurodegenerative disorders. Although the inhibition of IP3R did not exhibit neuroprotection, future investigations using a more physiological experimental model or other identified therapeutic targets may be of interest.
Table 1: Top 10 gene ontology biological processes, cellular components, and molecular functions for genes in the bortezomib interaction network

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Table 2: Top 10 gene ontology biological processes, cellular components, and molecular functions for genes in the carfilzomib interaction network

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Table 3: Top 10 gene ontology biological processes, cellular components, and molecular functions that are present in the bortezomib interaction network but absent in the carfilzomib interaction network

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Table 4: Top 10 transcription factor binding sites for genes in interaction networks

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Table 5: Top 10 non-cancer pathways for genes in interaction networks mapped to KEGG pathways

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**Supplementary Table 1:** Genes exclusively in the bortezomib interaction network mapped to the **Protein Processing in Endoplasmic Reticulum** pathway in KEGG

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Supplementary Table 2: Genes exclusively in the bortezomib interaction network mapped to the Ubiquitin Mediated Proteolysis in KEGG

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Figure 1: Interaction network construction methodology. A bipartite interaction network was constructed in Cytoscape, which consists of drugs of interest and their direct targets. The interaction network was extended by including targets of the direct drug targets, which were obtained via BioGRID.
Figure 2: Bioinformatics analysis of genes in the interaction networks. Genes in the bortezomib and carfilzomib interaction networks were uploaded into DAVID in order to perform a functional annotation analysis and to identify pathways that were significantly enriched.
Figure 3: Bortezomib interaction network. Bortezomib is represented as the red middle node. Direct targets (n=16), proteasomal subunits, are depicted in orange. Indirect targets (n=690) are shown in blue.
Figure 4: Carfilzomib interaction network. Carfilzomib is represented as the red middle node. Direct targets (n=6), proteasomal subunits, are depicted in orange. Indirect targets (n=217) are shown in blue.
Figure 5: Venn diagram for different and similar genes between bortezomib (blue) and carfilzomib (yellow) interaction networks. Genes depicted in the Venn diagram represent genes that were mapped to KEGG pathways of neurodegenerative diseases (Alzheimer’s, Huntington's, Parkinson's, Lou Gehrig's, and prion diseases). Genes bolded in red text appeared in multiple neurodegenerative disease pathway.
Figure 6: Systems pharmacology network predictions for the neurotoxic effects of proteasome inhibition by bortezomib/carfilzomib alone (top) and in combination with an IP3R antagonist (bottom).
**Figure 7:** Results from the cytotoxicity of bortezomib (top) and carfilzomib (bottom) on SH-SY5Y cells. WST-1 was added after 45 hours of drug exposure and allowed to incubate for 3 additional hours. After 48 hours of exposure, absorbance was measured at 440 nm using a SpectraMax i3 plate reader.
Figure 8: Results from the cytotoxicity of bortezomib on SH-SY5Y cells in the presence of xestospongic C (top) and curcumin (bottom). WST-1 was added after 45 hours of drug exposure and allowed to incubate for 3 additional hours. After 48 hours of exposure, absorbance was measured at 440 nm using a SpectraMax i3 plate reader.
**Supplementary Text File 1**: List of genes (n=705) in the bortezomib interaction network. Numbers represent Entrez gene identifiers.

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Chapter 4:

Boolean Network Modeling in Systems Pharmacology

Peter Bloomingdale, Van Anh Nguyen, Jin Niu, and Donald E. Mager

This Chapter is published in the Journal of Pharmacokinetics and Pharmacodynamics 45:159-180 (2018)
Abstract

Quantitative systems pharmacology (QSP) is an emerging discipline that aims to discover how drugs modulate the dynamics of biological components in molecular and cellular networks and the impact of those perturbations on human pathophysiology. The integration of systems-based experimental and computational approaches is required to facilitate the advancement of this field. QSP models typically consist of a series of ordinary differential equations (ODE). However, this mathematical framework requires extensive knowledge of parameters pertaining to biological processes, which is often unavailable. An alternative framework that does not require knowledge of system-specific parameters, such as Boolean network modeling, could serve as an initial foundation prior to the development of an ODE-based model. Boolean network models have been shown to efficiently describe, in a qualitative manner, the complex behavior of signal transduction and gene/protein regulatory processes. In addition to providing a starting point prior to quantitative modeling, Boolean network models can also be utilized to discover novel therapeutic targets and combinatorial treatment strategies. Identifying drug targets using a network-based approach could supplement current drug discovery methodologies, and help to fill the innovation gap across the pharmaceutical industry. In this review, we discuss the process of developing Boolean network models and the various analyses that can be performed to identify novel drug targets and combinatorial approaches. An example for each of these analyses is provided using a previously developed Boolean network of signaling pathways in multiple myeloma. Selected examples of Boolean network models of human (patho-)physiological systems are also reviewed in brief.

Keywords

Boolean Network; Quantitative Systems Pharmacology; Systems Biology; Drug Discovery; Modeling and Simulation
Introduction

At the turn of the 21st century, there has been a paradigm shift, from a reductionist viewpoint of biology, where the characteristics of individual components are studied in isolation, back towards one that is holistic and aims to understand how the complex interactions amongst components give rise to emergent properties of biological systems [1]. In order to understand such complexities, the integration of experimental and computational methods is sine qua non in systems science [2]. Advances in experimental methods, such as genomics, proteomics, and metabolomics, have enabled the comprehensive assessment of drug-induced alterations in signaling pathways. Systems pharmacology is an emerging discipline that integrates principles from systems biology and pharmacology to study the effects of drugs on biological systems. To gain a quantitative understanding of how a biological system responds to drug exposure, pharmacokinetic/pharmacodynamic (PK/PD) and systems biology models can be combined to form quantitative systems pharmacology (QSP) models [3,4]. Although QSP is still in its infancy, this field has great potential to impact drug discovery, development, and utilization. QSP models require vast amounts of data in order to characterize the complex dynamics of system components, which are multi-scale (i.e., temporospatial differences from molecular to physiological scales) and often unavailable or highly variable. Therefore, a mathematical framework that does not require knowledge about kinetic parameters, such as Boolean network modeling, could provide an initial foundation for the development of QSP models.

The major challenge in biological network modeling is to map how system components interact amongst each other to give rise to physiological functions, disease processes, and variable responses to therapy. There are several types of biological networks, such as gene regulatory, protein-protein interaction, metabolic, and signal transduction networks, which differ based upon how nodes (vertices) and edges are defined. Nodes often represent genes, proteins, or other biological components, and edges between nodes reflect the regulatory relationship (stimulatory/inhibitory). The principles of graph theory
can be used to model the interactions of intracellular components as a network [5], and topological analyses, such as centrality measures and clustering techniques, can identify important components and functional modules [6]. Network-based analyses serve to evaluate disease and drug effects on the entire system, rather than individual components, which can lead to the identification of novel drug targets, improvement of therapeutic efficacy, and minimization of toxicity [7]. Conceptual biological networks can be converted to dynamical models through the incorporation of a mathematical formalism that allows the concentration or activity of components to change over time. Time is defined as discrete, represented as finite time steps/intervals, or continuous, and the state of components can be qualitative or quantitative. Although quantitative and continuous mechanistic models are preferred [8], most interaction networks have not been studied in such detail, and qualitative logic-based models may provide key insights into systems in the absence of rich quantitative measurements [9]. Boolean networks, originally described by Kauffman in 1969, are the simplest type of discrete dynamic models [10]. Nodes occupy a state of either 1 or 0, which indicates whether the concentration/activity is above or below an activation threshold. The state of each node is governed by the previous states of its regulating nodes through a set of logical functions. Boolean networks have been applied to model signal transduction, gene regulation, and cellular differentiation for several types of physiological and pathophysiological systems, such as the immune system and related diseases [11-23], breast cancer [24-29], gastrointestinal cancers [30-32], hepatic cancer [33,34], lung cancer [35], and several others [36-40].

In oncology, Boolean network modeling can provide a framework for studying system trajectories under pathophysiological conditions and in response to drug treatment. A network state space contains all possible combinations of states for which nodes of the network can reside, and an attractor is a stable set of states that other states evolve towards, manifesting as cellular phenotypes and fates [41,42]. Waddington, inspired by dynamical systems theory, proposed the concept of an “epigenetic landscape” and described a metaphor of a ball traversing a landscape of cellular differentiation processes [43].
Considering network state space, the ball would correspond to an initial set of states, and valleys would correspond to basins of attraction that lead to attractors. Figure 1 is an adaptation of Waddington’s epigenetic landscape showing a normal cell trajectory (blue) and an abnormal trajectory (red), in which a cell becomes cancerous through the accumulation of mutations. In this diagram, a pharmacological intervention (purple) could shift the abnormal trajectory towards one that is favorable, whether it falls back to the normal trajectory or into an apoptotic attractor. Quantifying the frequency at which initial states reach an attractor identifies the relative importance of each attractor and its associated biological phenotype, underscoring the utility of performing attractor analyses on Boolean networks.

In this review, we highlight the applications of Boolean network modeling in systems pharmacology as well as provide examples of various analyses using a previously published Boolean network of signaling pathways in multiple myeloma [23]. The process of Boolean network development is described, which includes construction of an interaction network, conversion of an interaction network into a Boolean framework, determination of initial conditions, network validation, and reduction. Types of Boolean network analyses useful in drug discovery and development are covered, such as dynamic simulations, attractor analysis, and minimal intervention analysis. Finally, a brief overview is provided of previously developed Boolean networks of human intracellular physiology/pathophysiology and their various applications.

Network Development

In this section we will cover the intricacies involved in the development of Boolean network models, which consists of constructing an interaction network, adding Boolean logic, determining initial conditions, network validation, and network reduction.

Constructing and Analyzing an Interaction Network
Network construction begins with compiling a list of nodes relevant to the biological outcome of interest. The type of network is determined according to how vertices (nodes) and edges are defined. Nodes typically represent different biological components, such as DNA, RNA, proteins, and metabolites. The regulatory interactions between these components, either stimulatory or inhibitory, are modeled through the incorporation of edges. The network may contain several different types of regulatory relationships, such as protein-protein interactions observed in signal transduction and DNA-protein interactions in transcriptional and translational processes. Network components are often derived from the literature and supplemented with experimental data. If a pre-existing model, also referred to as a prior knowledge network, is available, then the network could be expanded for a specific purpose, such as to include the mechanism of action of a drug or a pathway of interest. There are two main approaches for identifying nodes and pathways to include in the development of interaction networks. First is a bottom-up knowledge-driven approach that consists of an extensive literature evaluation and the use of biological pathway databases (e.g., KEGG and Reactome) and text mining software (e.g., Chilibot) [44-46]. Second is a top-down data-driven approach, which identifies differentially expressed components (i.e., genes, proteins, and/or metabolites), from omics-based analyses and bioinformatics resources (e.g., DAVID and IPA), to perform functional annotation and biological pathway mapping [47]. A knowledge-driven approach is time intensive, whereas a data-driven approach is more resource intensive. Nodes and pathways that are important to the biological system may be missed with each of these approaches. Developing a network based primarily on information in the literature may overlook an important feature of the system or require selecting between conflicting results. For the data-driven approach, an important system component may not appear to be differentially expressed depending upon the nature of the experimental design, statistical methods, the time of measurement, and the magnitude of biological variability. Ideally, a hybrid knowledge/data-driven approach should be used to identify important components for the construction of an interaction network. Components of the physiological system of
interest could be compiled using a knowledge-based approach and integrated with differentially expressed measurements from a data-driven method. Lastly, the addition of endpoint nodes, representing a biological phenomenon of interest, should be incorporated. For example, networks of cancer typically contain nodes that represent cell proliferation and apoptosis; other endpoint nodes of interest could be DNA damage, epithelial-mesenchymal transition, and metastasis.

Once network components have been complied and edges between them have been defined as either having an activating or inhibiting relationship, a topological analysis could be performed to obtain information about the system. Although this review is focused on dynamical analyses, a brief overview of the graph-theoretical analyses that can reveal nodes of high importance in biological networks is provided, which include measures of closeness, betweenness, degree, and bridging centrality. Closeness centrality of a given node is calculated as the inverse sum of minimal distances between that node and all other nodes [48]. In essence, a central node is one that is, on average, close to other nodes. Betweenness centrality, on the other hand, is the sum of proportions of times that a node is present in the shortest paths between any pair of nodes [49]. Therefore, betweenness indicates the flux of information that is being relayed through a specific point in an active network and reveals the degree to which a node can influence the flow of information in a system [50]. In other words, betweenness centrality allows assessment of functional importance of a node in a wide context of the entire network. If a vertex with high betweenness centrality falls inoperative due to a mutation, the communication between near and distant sites of a network may become less likely or cease completely. Degree centrality is based on the notion that central nodes are ones with the most connections. Node degree refers to the number of edges that a node shares with other nodes, which can be divided into the number of edges that lead to the node (in-degree) and edges that originate from the node (out-degree). These measures are not necessarily correlated. Joy et al. examined a yeast protein interaction network and discovered a wide occurrence of proteins with high betweenness but low connectivity [49]. These proteins may act as essential bridges
between various modules within a network, and their presence could be explained by adaptive evolutionary mechanisms or inherent system constraints. Assessments of bridging centrality can pinpoint elements of the system that might cause network failure if disrupted, and this property may be particularly useful for identifying targets in drug discovery [6]. Aside from centrality measures, clustering analysis can determine the tendency of vertices to create tightly packed regions with dense links, and this tendency is referred to as a clustering coefficient. Global clustering coefficients give an overall indication of the clustering within a network based upon three connected nodes [51]. In contrast, a local clustering coefficient reflects the local density of single nodes [52]. Compared to random networks of equivalent size and degree distributions, nearly all known real networks exhibit high clustering, which gives rise to functional modules [5]. Although topological analyses allow for the identification of important network nodes, an interaction network needs to be converted into a dynamical form in order study how components respond to perturbations and evolve over time.

Converting an Interaction Network to a Boolean network

After an interaction network has been constructed, the next step is to use Boolean logic to describe regulatory interactions amongst species of the network. A Boolean network $G(V, F)$ contains a set of nodes, $V = \{x_1, ..., x_N\}$ and a list of Boolean functions, $F = (f_1, ..., f_n)$. A Boolean function $f_i(x_{i_1}, ..., x_{i_k})$ is assigned to each node $(x_i)$, where $k$ is the in-degree of a node. The spatiotemporal dynamics of Boolean networks are discrete, and the state of each node is binary, $x_i \in \{0,1\}$, $i = (1, ..., N)$. Nodes of the network are updated based upon the states of its regulators at the previous time-step:

$$x_i(t + 1) = f_i(x_{i_1}(t), x_{i_2}(t), ..., x_{i_k}(t))$$  \hspace{1cm} (1)

The state of a node can be either zero (OFF) or one (ON) by solving the Boolean function assigned to that node. Updating nodes for multiple time steps, in a synchronous manner (all nodes are updated at the same time), generates an arbitrary time-course for the dynamics of each node. Other updating methods include asynchronous, probabilistic, and continuous approaches, which are described in detail later.
Boolean functions represent the relationships between a node and its regulators through the incorporation of Boolean operators, such as AND (\(\cap\)), OR (\(\cup\)), and NOT (\(\sim\)). The NOT operator is used to describe an inhibitory edge. For example, if B inhibits A, then the Boolean function assigned to node A would be \(A = \sim(B)\). The AND and OR operators are used when a node is regulated by at least two nodes. For example, if A is activated only when both B and C are present, then the Boolean function assigned to node A would be \(A = (B \cap C)\). If either B or C activates A, then the expression is \(A = (B \cup C)\). The combination of Boolean logic operators to describe complex interactions between biological components provides a simplistic yet robust framework to model the qualitative dynamics of biological systems.

The Boolean operator that best represents the complex interactions between a node and its multiple regulators can be determined through experimental studies. Gene knockout or pharmacological inhibition studies are useful for determining which Boolean operator to assign. For cases in which there are multiple regulators and insufficient experimental data to justify an AND relationship, one approach is to assume an OR relationship for stimulatory edges and AND NOT relationships for inhibitory edges. Flobak et al. describes the application of this approach in the development of a Boolean network for cell fate decisions in gastric cancer [30]. A signaling component is active if any of its regulatory activators are active, while at the same time none of its regulatory inhibitors are active. For example, if node A is affected by activators (B, C, and D) and inhibitors (E, F, and G), then expression of A would be:

\[
A = (B \cup C \cup D) \cap \sim(E \cup F \cup G)
\]  

Multiple Boolean networks could also be generated in order to select the best network that describes experimental findings. CellNOptR (CellNetOptimizerR) is an R package developed for logic-based model selection that utilizes a prior knowledge/interaction network, to identify logic functions to describe an experimental data set [53]. For each node with multiple regulators, CellNOptR generates all possible logic gates and evaluates each against high throughput experimental data in order to determine the most appropriate Boolean network. Saez-Rodriguez et al. (2011) applied this approach to generate
Boolean networks of immediate-early signal transduction pathways in order to compare signaling networks between primary hepatocytes and four hepatocellular carcinoma (HCC) cell lines [33]. Boolean networks that best characterized normal and transformed hepatocytes were determined by minimizing an objective function ($\theta$) based on the sum of the mean squared error between experimental data and model predictions and a penalty for increasing model size:

$$\theta = \frac{1}{n_E} \sum_{k=1}^{s} \sum_{l=1}^{m} \sum_{t=1}^{n}(B_{k,l,t}^M - B_{k,l,t}^E)^2 + \alpha \frac{1}{v^S_e} \sum_{e=1}^{r} v_e P_e$$

with $B_{k,l,t}^M \in \{0,1\}$ and $B_{k,l,t}^E \in \{0,1\}$ as model predictions and discretized observed data. The function is summed across endpoints ($m$), time points ($n$), and experimental conditions ($s$) and is weighted by the total number of data points ($n_E$). The penalty for model size is calculated by weighting each solution $P_e$ by the number of regulating nodes ($v_e$) for each hyperedge ($r$), where hyperedges are defined as edges that allow multiple inputs and have one output. This accounts for the fact that AND gates have multiple inputs and should carry a greater penalty. Hence, an AND gate with two inputs will carry the same penalty as two regulating nodes with an OR operator. The size penalty is also normalized to the total number of tail nodes in the network ($v^S_e = \sum_{r=1}^{r} v_e$) and is multiplied by a tunable parameter ($\alpha$), which was fixed to 0.0001. This optimization algorithm resulted in several models that differed slightly in structure but had nearly the same value of the objective function. Models that best fit the data and differed by 1% in goodness of fit were grouped together and retained as a family of models representing either primary hepatocytes or HCC cells. On comparison, several pathway differences in signaling networks were identified amongst primary hepatocytes and HCC cells. The existence of a new interaction between Jak-Stat and NFkB signaling was inferred, which was found to arise from the polypharmacology of an IkB kinase inhibitor rather than a new protein-protein interaction. This application illustrates an automated approach to converting an interaction network to a Boolean model. To address identifiability concerns the incorporation of several high throughput data sets with multiple perturbations as well as novel model-selection criteria could aid in the identification of a unique model.
Extensions of classic Boolean networks have been implemented in order to address certain limitations. Shmulevich et al. developed probabilistic Boolean networks (PBNs) for gene networks to overcome the deterministic rigidity of classic Boolean models [54]. PBNs are defined as a collection of Boolean networks in which a constituent network governs the states of nodes for a random period of time until another randomly chosen constituent network takes over. In PBNs, \( V = \{x_1, \ldots, x_n\} \), are assigned a set of possible Boolean predictor functions, \( F_i = \{f_{i1}, f_{i2}, \ldots, f_{il(i)}\} \), where \( f_{ij}(1 \leq j \leq l(i)) \) is a possible function determining the value of node \( x_i \) and \( l(i) \) is the number of possible Boolean predictor functions assigned to \( x_i \). Thus, if \( l(i) = 1 \) for all \( i = 1, \ldots, n \), then the PBN reduces to a standard Boolean network. For each time step the probability of choosing \( f_{ij} \) as the predictor function is \( c_{ij} \), where \( 0 \leq c_{ij} \leq 1 \) and \( \sum_{j=1}^{l(i)} c_{ij} = 1 \). A realization of the PBN at a given instant of time is determined by randomly selecting a vector of Boolean predictor functions, where the \( i \)th element of the selected vector contains the predictor function for node \( x_i \). A PBN consisting of \( N \) possible realizations, constituent networks, contains \( N \) vectors of Boolean predictor functions, \( f_1, f_2, \ldots, f_N \) of the form \( f_k = (f_{k1}^{(1)}, f_{k2}^{(2)}, \ldots, f_{kn}^{(n)}) \), for \( k = 1, 2, \ldots, N, 1 \leq k_i \leq l(i) \), and where \( f_{ki}^{(i)} \in F_i (i = 1, \ldots, n) \). The vector function \( f_k: \{0,1\}^n \rightarrow \{0,1\}^n \) acts as a transition function that represents a possible realization of the PBN. Each of the possible \( N \) realizations can be viewed as a standard Boolean network responsible for transitioning the network through its state space prior to randomly switching to another network to drive the updates. In other words, at every state \( x(t) \in \{0,1\}^n \), one of the \( N \) Boolean networks is chosen and used to transition to the next state \( x(t + 1) \in \{0,1\}^n \). Assuming the selection of Boolean functions for each gene are independent of each other, the number of possible realizations is \( N = \prod_{i=1}^{n} l(i) \) and the probability of selecting the \( k \)th Boolean network is defined as \( Pr_k = \prod_{i=1}^{n} c_{ki}^{(i)} \). At each update, a decision is made to switch the constituent network based upon a binary random variable \( (\lambda) \). If \( \lambda = 0 \), then the current network is retained, whereas with \( \lambda = 1 \), a constituent network is randomly selected from
amongst all constituent networks according to its probability distribution \( \{c_k\}_{k=1}^N, \sum_{k=1}^N c_k = 1 \). The switching probability \( q = \Pr(\lambda = 1) \) is a system parameter. When \( q = 1 \), a switch of constituent networks is made, and the PBN is said to be \textit{instantaneously random}. Whereas if \( q < 1 \), the current constituent network is used for transitions until a switch is called for \( (\lambda = 1) \), a case termed as \textit{context sensitive}. The dynamics of PBNs can be framed within the theory of Markov chains, with or without perturbations. Probabilistic Boolean models can be developed and analyzed using the Matlab-based toolbox BN/PBN (https://code.google.com/archive/p/pbn-matlab-toolbox/) or the R-package BoolNet [55]. A Matlab-based toolbox (optPBN) has been developed for the generation and optimization of probabilistic Boolean networks [56]. Overall, this framework introduces stochasticity to overcome the deterministic rigidity of original Boolean networks and enables a different analysis of the systems attractors since state transitions are random. Determining the steady-state probability distribution for nodes in the network provides quantitative insights into the long-term regulation of gene expression [57]. Methods have been developed to solve and approximate the steady-state probability distribution of PBNs [58,59]. The application of PBNs for describing and analyzing complex biological systems has recently been reviewed [60]. PBN modeling has been used as a mathematical framework to describe cancer-specific intracellular signaling of deregulated platelet-derived growth factor in gastrointestinal stromal tumors and the differential regulation of L-plastin phosphorylation by genes of the ERK/MAPK pathway across four breast cancer cell lines [31,61].

To address the spatial limitations of Boolean network modeling, multi-state models can be developed. In a three-state model, nodes may take on three different values, such as \( x \in \{0,1,2\} \) or \( x \in \{-1,0,1\} \). These states represent underactive (downregulated), normal activity, and overactive (upregulated). For example, Flobak et al. constructed a logical network of gastric cancer and allowed two output nodes (i.e., Prosurvival and Antisurvival) to take on four values (0, 1, 2, or 3), and their immediate
upstream nodes could take on three values (0, 1, or 2) [30]. The software packages GINsim and CellNetAnalyzer both support the construction and analysis of multi-state logical models [62,63].

For temporal limitations of Boolean networks, the discrete time scale can be converted into a continuous one. Odefy is a matlab-compatible toolbox that has been developed to convert Boolean networks into a system of ODEs [64]. For example, Chudasama et al. converted a Boolean network of signaling pathways in multiple myeloma to a system of ODEs to simulate the dynamics of species for select inhibitors as well as to ensure the dynamics are consistent between the full and reduced networks [23]. Another method known as the standardized qualitative dynamical systems method (SQUAD) can convert a Boolean network into a continuous dynamical system [65]. This method was used to convert a B cell regulatory network into a continuous dynamical model, which enabled the identification of additional fixed point attractors that could be viewed as intermediate unstable transition states in the differentiation process [14]. The benefit of this approach is that it enables the construction and analysis of species dynamics, in a continuous manner, despite the lack of kinetic information. The conversion of a Boolean network into a series of ODEs could provide an initial framework for the development of quantitative systems pharmacology models [23].

**Determining Initial Conditions**

Once an interaction network has been converted to a Boolean network, the following step is to determine the initial condition of the network, which will be further denoted as the control attractor. The control attractor represents the initial states of nodes in the network when the biological system is at rest, which can be difficult to determine since there is not a formalized method to identify this initial resting state. Quantitative information for each node regarding the basal level of expression and a threshold concentration or activation required to activate/inhibit a downstream component would be ideal. If the concentration or activity of a species at resting conditions, prior to perturbations, is below a threshold of activation, then the initial state of the node would be 0. In contrast, if concentrations are above an
activation threshold, then the initial state would be 1 [66]. Thus, the definition of the initial states should not be confused with the absence or presence of a component. Since quantitative information for all of the processes within a complex system is often unknown, a combination of known \textit{a priori} information about the baseline activity of biological components along with sampling the network state space can be used to determine the control attractor. Determining the initial conditions of network components has been reviewed before [66]. Here we have provided a stepwise process that can be used as a guideline for obtaining initial conditions.

I. Obtain information about the resting concentration/activity and threshold of activation for network components.

II. Convert available \textit{a priori} quantitative data of concentrations/activity of network components into binary qualitative states.

III. Use converted binary values to perform a logical steady state analysis in order to obtain a set of nodes that have fixed states.

IV. Utilize the set of nodes with fixed states to perform an attractor analysis in order to identify attractors in which the system resides.

V. Analyze the identified attractors in the network and select the attractor that best represents a known biological resting condition.

Initial states for input nodes, such as ligands and drugs, are often known. Initial states of the input nodes along with additional \textit{a priori} information about a biological relevant resting state, can be used to determine the fixed states of downstream nodes via its logical steady state. If there are nodes that remain undefined, an exhaustive exploration of all remaining initial states can be performed to identify attractors.

To select an attractor that represents a control attractor, amongst all possible attractors of the system, the attractor with the highest frequency of being reached or the attractor that best corresponds to a relevant biological phenotype can be selected. If discrepancies exist between initial states of the nodes in
the control attractor and known information about a biological resting state, this would indicate a model misspecification in the networks structure or Boolean logic functions.

**Network Validation**

Once the Boolean network has been developed, the network should be validated by comparing model predictions with experimentally obtained data. Network validation is a crucial step in the development process, in order to gain confidence in model predictions. The experimental data sets used in network validation should include several molecular perturbations and cover a broad range of signaling pathways included in the model, to avoid fine-tuning towards a specific scenario and enhance model predictions under new conditions. Several experimental methods can be used to validate components of the network. Small molecule drugs, biologics, and gene editing can alter the expression of specific biological components, and downstream elements can be measured using various immunoassays, such as Western-blot and enzyme linked immunosorbent assays (ELISA). For validation, the qualitative change in expression of proteins or genes downstream of the perturbed target can be compared to network predictions. A time-course for the change in expression is desirable in order to confirm that the model recapitulates intermediate states as well as long-term behavior. Given the size and complexity of biological networks, obtaining experimental data for the behavior of every node under multiple perturbations is not practical with classical methods. However, advanced analytical approaches, such as large-scale transcriptomic and proteomic analyses, can provide insights into how the entire biological system responds to individual or a combination of perturbations. For example, the Gene Expression Omnibus (GEO) is a public data repository that contains microarray, next-generation sequencing, and other high-throughput functional genomic data that could be used for gene network validation [67].

Prior to comparing network simulations with an experimental data set for validation, the initial states of the network should align with a known biological resting state. For anticancer chemotherapy, cancer system nodes pertaining to cell growth, proliferation, and survival should be ON, and nodes
representing senescence or apoptosis should be OFF. Opposite outcomes in the control attractor would, indicate that the network structure is incomplete or there is an error in the Boolean logic functions. Once the control attractor is in agreement with the known resting condition, network simulations can be performed and compared against the trends of differentially expressed genes, proteins, or metabolites for a perturbation of interest. Here we discuss network validation using a synchronous updating scheme due to ease of interpretation. Simulations should be performed until an attractor is reached. The states of nodes in the control attractor, compared to a new attractor, indicate that a node is increasing (0/1 → 1) or decreasing (0/1 → 0) in expression. Since the states of nodes are binary, a node expressing a state of one (or zero) in both the control and new attractors can be viewed as either increasing (or decreasing) or remaining the same. An oscillatory state, as in the case of cyclic attractors, could be viewed as having a state between zero and one. A metric for network validity can be obtained by calculating the number of node states that correspond with differentially expressed components relative to the total number of comparisons. For semi-quantitative validation, an asynchronous updating scheme with random sampling or the conversion to ODEs can project a trajectory for the fractional activation of nodes, which can be compared with experimental measurements.

Network Reduction

Due to the complex nature of gene regulatory and signal transduction networks, network size can lead to problems that are computationally infeasible to solve. Therefore, in order to reduce complexity, while maintaining important properties of the system, a network reduction algorithm can be implemented to reduce the size of the network. The state space of a Boolean network is a finite and discrete number equal to 2^N, where N is the number of nodes in the network. Since the network state space increases exponentially as a function of the number of nodes, network size can limit the ability to perform certain types of analyses. The time that an algorithm takes to identify attractors of the system increases exponentially with the number of nodes: O(2^N) [68,69]. Therefore, the identification of attractors in large
networks by searching entire state spaces can become unfeasible. To address this challenge, Boolean network reduction algorithms have been proposed to reduce the number of nodes and the network state space. Network reduction algorithms can be applied to obtain a final reduced network or as an immediate step prior to further analysis. In either case, the overall goal is to reduce the network to a reasonable size while conserving inherent complex dynamics, topological features, and attractors.

Nodes that exhibit the same state throughout all attractors, initially termed stable variables/elements, are commonly referred to as frozen nodes. Bastolla and Parisi showed that in order for a node to be relevant and have an effect on the dynamics of other nodes, it must be unstable (not frozen) and regulate another unstable node [70]. Therefore, methods have been developed to identify and remove frozen nodes [71,72]. Sampling only a subset of possible initial conditions could potentially miss attractors with a small basin size and possibly result in incorrectly identified frozen nodes [72]. To overcome this limitation, novel algorithms have been developed to find all attractors in Boolean networks [73]. Frozen nodes might also emerge through the introduction of constraints on the system. For example, fixing the values of source nodes and performing a logical steady-state analysis will identify nodes whose state does not change [71,74,75]. However, this simplifying assumption must be biologically justifiable. Similarly, Zañudo and Albert developed a network reduction approach that identifies motifs stabilized in a fixed state and nodes that become frozen due to their regulation by these stable motifs [76]. Additional topological approaches are proposed and non-functional nodes and edges can be removed using Boolean algebra. A common method is to remove mediator nodes, which are defined as nodes that have both an in-degree and out-degree equal to one [77,75]. These nodes are seen as non-functional as they serve only as an intermediate between the information flow of two other nodes. Leaf nodes, also known as terminal nodes, have an out degree of zero and can be removed if they are not important for network analyses [72]. Another method iteratively removes nodes while maintaining self-regulatory nodes, which likely results in an oversimplification of complex network dynamics [78].
A simplistic stepwise reduction method is to:

I. Remove irrelevant source (in-degree = 0) and leaf nodes (out-degree = 0)

II. Remove mediator nodes (in-degree and out-degree = 1)

III. Remove quasi-mediator nodes (in-degree or out-degree = 1)

IV. Remove frozen nodes (fixed to 0 or 1 in all attractors), if frozen nodes are upstream of network perturbations of interest and there are no feedback loops.

Nodes with either an in-degree or out-degree of one, or quasi-mediator nodes, can also be removed. However, the conservation of attractors upon the removal of quasi-mediator nodes has not been mathematically proven. In addition, the order in which quasi-mediator nodes are removed may have an impact on the final reduced network structure. Lastly, frozen nodes can be identified and removed, either through identifying all attractors in the network or by fixing source nodes followed by a logical steady-state analysis. However, since network perturbations can transform attractors and potentially “unfreeze” nodes, frozen nodes should be removed with caution. One suggestion is to only remove frozen nodes that are not in the path of network perturbations of interest.

**Boolean Network Analyses**

In this section, we cover a variety of network simulation and analysis techniques that can be used to understand how drugs affect the qualitative dynamics of species in the network. We describe and compare network simulations using synchronous, asynchronous, and HillCube updating methods. We also describe techniques that are useful for identifying and evaluating drug targets and combination therapies, specifically minimal intervention and attractor analyses.

**Network Simulations**

There are a wide range of perturbations that can be tested, both internal and external, such as small molecule drugs, biologics, and gene modifications. Boolean network simulations are commonly
performed to determine the qualitative effects of therapeutic perturbations. For cancer, the dynamics of intracellular components are simulated to reveal their state in newly reached attractors, which are related to biological phenotypes [42], such as apoptosis, quiescence, differentiation, and proliferation [79]. A comparison of attractors in which the system resides, before and after a perturbation, may provide insight into the probability of therapeutic interventions. The shift from a proliferative attractor towards an apoptotic attractor would indicate a favorable response. In certain cancer networks, other attractors may exist that represent additional complex phenotypes such as the epithelial-mesenchymal transition, which has been described in a Boolean network model of TGFβ signaling in hepatocellular carcinoma [34]. In order to simulate the effect of a drug perturbation, the mechanism of action of the drug must be within the scope of the network, and a node for the drug is introduced and stimulatory/inhibitory edges are drawn from this node to all of the drug targets. Drugs often have multiple direct and indirect targets, and thus it is critical to incorporate all possible mechanisms in order to add confidence in model predictions.

An incomplete understanding of mechanisms of drug action can create a major challenge to implementing Boolean networks for systems pharmacology applications. Networks for drugs that are promiscuous or interact with yet unknown targets are difficult to define. When faced with the problem of trying to model a drug with unknown targets, an option is to identify drug targets through the integration of direct biochemical, genetic interaction, and computational inference methods [80]. The marriage between gene expression signatures of drugs and computational inference methods to identify drug-target interactions has shown success. For example, the Connectivity Map has been used to generate hypotheses about the mechanisms of action of uncharacterized small molecule drugs by comparing their gene expression profile against the gene expression profiles of reference compounds with known targets [81]. This comparative analysis approach was used to identify the mechanism of action of gedunin, an HSP90 inhibitor. Network-based approaches have been used to identify drug-target interactions by using gene expression profiles to infer the mechanisms of drug action based upon the perturbation of gene regulatory and protein-
protein interaction networks. Detecting Mechanism of Action by Network Dysregulation (DeMAND) and Protein Target Inference by Network Analysis (ProTINA) are two network-based methods that infer drug-target interactions from gene expression profiles [82,83]. These two methods were compared, and ProTINA exhibited superiority over DeMAND for predicting known targets of drugs across three datasets: NCI-DREAM drug synergy challenge [84], genotoxicity study [85], and chromosome drug targeting study [86].

In addition to external perturbations, internal perturbations of species that already exist in the network are useful for emulating the effects of mutations and over/under expressed components. For a gain of function mutation (knock-in) or gene/protein overexpression, the state of a particular species would be fixed to one. Regardless of the states of its regulating nodes, the state of the mutated node will always be one. For a loss of function mutation (knockout) or gene/protein repression, the state would be fixed to zero. Alternatively, the transient modification of a node can be incorporated to model a pharmacological intervention and a permanent modification of a node to model a gene knock in/out. For a transient effect, the state of a node could be modulated for a defined number of time-steps. Simulations of multiple perturbations can be performed, which enables the assessment of combinatorial drug regimens, drug effects in the presence of specific gene mutations, and variability in therapeutic response due to individual genetic differences.

Synchronous updating is the original and simplest method used to simulate the dynamics of Boolean networks [10]. In Equation 1, the states of nodes are updated in a time-discretized fashion, in which the future state of a node (t+1) is a function of the current states (t) of its regulators. The states of all nodes are updated at the same time, based upon their previous states. A limitation of the synchronous updating scheme is that it makes the assumption that all biological processes in the network occur at the same rate. This assumption is unrealistic as most biological networks include a temporal distribution of cellular processes, such as receptor binding, signal transduction, and gene transcription. Synchronous
models are deterministic, and the trajectory in a network state space towards an attractor will always be
the same given the same initial conditions.

In contrast, one node is updated at any one time interval in asynchronous updating to account for
the fact that biological processes occur at different rates [87,88]. Asynchronous updating schemes include
stochastic algorithms, such as random order asynchronous and general asynchronous, as well as
deterministic asynchronous algorithms. For random order asynchronous models, all nodes are updated
sequentially at each time step based upon a randomly generated order [88,89]. The state of node \( x_i \) will
be updated according to the most recently updated states of its regulator nodes:

\[
x_i(t + 1) = f_i(x_i(t_{i1}), x_i(t_{i2}), ..., x_i(t_{iN})) \in \{0,1\}, \quad i = 1, ..., N
\]

with \( \tau_{ij} \in \{t, t + 1\} \) and \( j = 1, 2, ..., N \). If node \( x_j \) is updated before node \( x_i \), then \( \tau_{ij} = t + 1 \), otherwise,
\( \tau_{ij} = t \). In random order asynchronous models each node is updated only once during a full round of
updating. In general asynchronous models, a single node is randomly selected to be updated at each time
step and the same node can be updated multiple times in a row [88]. In addition, time is normalized to
the number of nodes in the network. Each time step is \( 1/N \)th of the time step in a random order
asynchronous model. Deterministic asynchronous updating schemes may include fixed individual time
scales and delays. Chaves et al. incorporated fixed individual time scales into a deterministic asynchronous
model where each node is assigned an intrinsic time unit and is updated at intervals of this time unit [90].

Deterministic asynchronous models are particularly useful when information about the kinetics of
biological processes is known. When prior kinetic information is not available, time scales/delays can be
randomly sampled from a time interval that is within biological limitations. This allows nodes associated
with fast processes, such as receptor binding and protein phosphorylation, to be updated more frequently
in comparison to slower processes, such as gene transcription.

Asynchronous simulations have been performed more frequently than synchronous simulations,
as the former often offers a better representation of real biological dynamics. Synchronous simulations
are able to transition to only one other point in a networks state space at each time step. Hence, the
dynamics are deterministically rigid. Asynchronous updating allows for the transition to more than one
point in network state space, and simulation results may be different given the same initial conditions.
Therefore, multiple asynchronous simulations must be performed and the average state of a node, after
a fixed time step or upon reaching an attractor, can be used to represent the node dynamics. In other
words, performing X simulations using an asynchronous updating scheme for Y time-steps and calculating
the fraction at which the state of a node is ON relative to the total number of simulations indicates how
the activation of a species evolves over time. The dynamics of a node fractional activation can be
compared with the shape of experimental time-courses. This is particularly useful if the goal is to compare
how different perturbations affect outcomes of interest.

Boolean networks can be converted into a system of ODEs to transform discrete time steps into
continuous time [64]. Odefy contains three transformation methods, BoolCube, HillCube, and normalized
HillCube, which are all based on multivariate polynomial interpolation. The advantage of these techniques
is that they bridge a gap between the qualitative dynamics of Boolean network models and quantitative
modeling. Discrete dynamics (Eq. 1) are converted into a continuous form:

$$\frac{d\bar{x}_i}{dt} = \frac{1}{\tau_i} (\bar{B}_i(\bar{x}_{i1}, \bar{x}_{i2}, ..., \bar{x}_{iN_i}) - \bar{x}_i)$$ (5)

Which allows nodes to take on any value between 0 and 1, \(x_i \in \{0,1\} \rightarrow \bar{x}_i \in [0,1]\). The function
\(\bar{B}_i\) describes the production of species \(\bar{x}_i\), and \(\tau_i\) is a parameter that represents the species lifetime. For
HillCubes, each species is modeled as a Hill function \((\bar{x}_i) = \frac{\bar{x}_i^n}{\bar{x}_i^n + k^n}\), where \(k\) corresponds to the activation
threshold, and the Hill coefficient \(n\) is a measure of cooperativity. In normalized HillCubes, the Hill function
is normalized to the unit interval. These logic-based mathematical formalisms have been applied in several
models, such as the \(\beta\)-adrenergic signaling pathway and cell growth regulation [91,92].

To provide an example of these different simulation methods, we performed simulations using a
previously developed Boolean network of intracellular signaling in multiple myeloma (Figure 2) [23]. We
simulated the inhibition of the proteasome by bortezomib, a proteasome inhibitor used for the treatment of multiple myeloma, to obtain a profile for the dynamics of a node that represents apoptosis. We performed and compared five different simulation methods: synchronous (BS), general asynchronous (BA), random-order asynchronous (BAR), normalized HillCube with default parameters (NHC), and normalized HillCube with randomly sampled parameter values (NHCR) (Figure 3a). For synchronous updating, one simulation was performed, whereas 1000 simulations were performed for the remaining methods, and the average activation of each node was calculated for every discrete time step. We observed that random order asynchronous (green) and normalized HillCube with randomly sampled parameters (blue) display a delayed and slower increase in apoptosis, whereas synchronous (black) and normalized HillCube (dotted black) simulations exhibit an abrupt increase (Figure 3a). The comparable abrupt increase in apoptosis, between synchronous and normal HillCube methods, can be attributed to the large time scale used in network simulations. Simulation results for all individual nodes using each method are displayed as heat maps in supplementary materials (Supplementary Figure S1). We also compared network simulations with previously published data of the dynamics of select intracellular proteins in U266 multiple myeloma cells exposed to bortezomib (Figure 3b) [93]. Overall, network simulations capture the general trends of the observed data. However, there are a few discrepancies between network predictions and experimental observations (Cas8, p53, and BAD), which indicates that the network could be missing some important mechanisms related to bortezomib's action or cellular regulation. To make note, the Boolean network used in this case study, was the first attempt to characterize the complex intracellular signaling dynamics in U266 multiple myeloma cells, and has recently been refined and extended to account for cellular heterogeneity [94].

Attractor Analysis

As mentioned earlier, an attractor is a stable set of states that other states evolve towards. Attractors represent stable states of the system and, with respect to cellular signaling networks, can be
associated with a biological phenotype or cell fate. Hence, an attractor analysis, which aims to identify all attractors of the network and their relative importance, provides pivotal information about the behavior of the biological system. The basin of attraction, or basin size, of an attractor is the number of initial states that converge towards the attractor. The state space of a network contains a landscape of attractors, where each attractor has its own respective basin of attraction. The network state space can be depicted as a separate network, or state transition graph, where nodes represent sets of states for nodes in the Boolean network and edges indicate the transition of these sets of states towards an attractor. Nodes in Boolean models can take on a value of either one or zero, and a network with N nodes will contain $2^N$ sets of states in the network state space. The total number of possible initial conditions is finite ($2^N$) and will eventually be repeated, indicating the existence of an attractor. Fixed points and limit cycles are two types of attractors in Boolean networks, which can be reached by a synchronous updating scheme. A fixed-point attractor is a finite point in the networks state space that does not change with time, meaning that the set of final states repeats indefinitely. A fixed-point attractor is depicted as a node with a self-loop in the state transition network. A limit cycle attractor is a set of points in the network state space through which transitions repeat. In a state transition network, $n$-limit cycle attractors are depicted as simple loops with $n$ transitions. Fixed point attractors typically correspond with static phenotypes/processes, such as differentiated cell types or activation of apoptosis, whereas limit cycles correspond to oscillatory behaviors, such as calcium signaling, cell cycle, and circadian rhythms. In synchronous Boolean networks, a set of states is able to transition to only one other set of states due to the nature of this updating scheme. In asynchronous Boolean networks, a set of states is able to transition to more than one other set of states. Therefore, different attractors may arise due to differences in the updating scheme between synchronous and asynchronous Boolean networks. In addition to fixed points and limit cycles, asynchronous Boolean networks may also exhibit complex attractors. Complex attractors are sets of states that oscillate irregularly amongst each other.
Analyzing the state space of a network in order to identify attractors is referred to as an *attractor analysis*. In essence, an attractor analysis consists of multiple dynamic simulations with different initial conditions in order to observe the frequency of reaching each attractor and to determine its basin size. To perform an attractor analysis a network's state space must first be sampled by selecting initial sets of states. An exhaustive search, where all possible initial conditions \(2^{N}\) are sampled, is often desired. However, due to computational limitations, simulating all possible initial conditions is unfeasible for large networks. Approaches have been developed to bypass this limitation that are capable of identifying all attractors in a network via model checking, but they are unable to provide any knowledge about the basin size of each attractor [73]. If the basin size of attractors cannot be identified owing to network size limitations, the fraction at which node \(x\) is activate across all attractors \(S_x\) can be calculated:

\[
S_x = \frac{\sum_{i=1}^{n_{ATotal}} x_i}{n_{ATotal}} \quad x_i \in \{0,1\} \quad i = 1, \ldots, n_{ATotal}
\]

with \(S_x\) as the relative fractional activation of node \(x\) across all attractors and \(n_{ATotal}\) is the total number of attractors in the network. In the case where an attractor is cyclical, the state of a node throughout each transition can be averaged. We performed an exhaustive state space search for the multiple myeloma Boolean network to identify all attractors in the network, in the absence of external perturbations and in the presence of bortezomib. This particular algorithm is limited to synchronous updating [73]. In the absence of external perturbations, a total of 210 attractors were identified, which consisted of 68 fixed points and 142 limit cycles. In the presence of bortezomib, the 210 attractors of the network reduced to eight fixed-point attractors. In addition to identifying attractors, we calculated the fractional activation of each node in the absence and presence of bortezomib (Figure 4). The fractional activation of the apoptosis node, across all attractors, shifted from 0.114 to 1 in the presence of bortezomib, indicating that proteasome inhibition results in the activation of apoptosis throughout all eight attractors. In addition, the fractional activation of growth across all attractors decreased from 0.914 to 0.5 in the presence of
bortezomib. We also determined the fractional activation of each node across attractors when a random number of initial conditions were selected ($n = 10^5$) for both synchronous (Figure 5) and general asynchronous (Figure 6) updating schemes. Similar to the synchronous updating algorithm, there was an increase in the fractional activation of apoptosis across attractors when bortezomib was present. In the normal condition (absence of proteasome inhibition), the asynchronous updating scheme results in a greater activation of apoptosis than the synchronous updating scheme (0.352 vs. 0.202). This is likely due to the merging of attractors by the asynchronous updating scheme and the introduction of attractors as an artifact of the synchronous updating scheme [95]. In support of this hypothesis, the asynchronous updating scheme only identified the same 68 fixed-points that where identified by the synchronous updating scheme. Indicating that all 142 limit cycle attractors have merged into fixed-point attractors or formed complex attractors. In the presence of bortezomib, both updating schemes resulted in the same 8 apoptotic fixed-point attractors. The exhaustive state space search (Figure 4) exhibits similar results to the random state space search (Figure 5), despite the vast difference in the sample sizes between these two analyses ($2^{65}$ (approximately $10^{18}$) compared to $10^5$). The synchronous random state space search, in the absence of external perturbations, identified 119 of the 210 attractors in the Boolean network. The fractional activation of apoptosis is smaller in the exhaustive vs. random state space search (Figure 4a vs. 5a; 0.1143 vs. 0.2017), which indicates that the missed attractors likely have a small basin size and apoptosis in the OFF state. As the number of starting initial states increase, the number of identified attractors will approach 210 and the fractional activation of apoptosis will approach 0.1143. Overall, incorporating the effects of bortezomib on the network has shifted the attractors towards those that are representative of apoptosis. Biologically this can be interpreted as proteasome inhibition drives multiple myeloma cells towards an apoptotic fate.

A basin of attraction reflects the importance and robustness of a particular cellular state associated with an attractor. Attractors that have large basin sizes tend to be more robust to perturbations
and represent the predominate phenotype of the system. There are two possible ways to gain information about the basins of attraction in large networks: (1) apply a network reduction technique that decreases network size to make simulations computationally feasible, or (2) sample a random finite set of initial conditions to use for simulations. When the network is sufficiently small, an exhaustive state space search can be performed to simulate all $2^N$ initial conditions, identify every attractor, and determine the basin size for each attractor. If the network is too large for an exhaustive search, one can sample a finite number of random initial conditions to obtain the basins of attraction for each attractor. When the basins of attraction are known, the relative frequency of attractors and relative activation frequency of nodes can be determined. The relative frequency ($v_A$) at which an attractor ($A$) is reached can be calculated:

$$v_{A_i} = \frac{BS_i}{\sum_{i=1}^{n_A} BS_i}, \quad i = 1, \ldots, n_A \quad \sum_{i=1}^{n_A} v_{A_i} = 1$$

(7)

with $v_{A_i}$ and $BS_i$ as the relative frequency and apparent basin size of attractor $A_i$. If initial states are randomly selected, $BS_i$ is an apparent basin size, not the exact basin size of attractor $A_i$, since the entire state space is not sampled and the same initial state can be sampled more than once. Note that $n_A$ represents the total number of attractors identified via the state space search, which can be less than the total number of attractors in the network, $n_A \leq n_{A_{total}}$. For an exhaustive state space search, where all possible initial conditions are sampled $\sum_{i=1}^{n_{A_{total}}} BS_i = 2^N$, $BS_i$ will equal the basin size of attractor $A_i$, and the relative frequency of an attractor will equal its probability: $Pr(A_i) = v_{A_i}$. When the limit of the number of randomly selected initial states approaches infinity, the relative frequency of an attractor will be equal to its probability: $Pr(A_i) = \lim_{\sum_{i=1}^{n_A} BS_i \rightarrow \infty} v_{A_i}$.

The relative frequency of an attractor may shed light on the importance of a certain phenotype to the biological system, since attractors can be associated with biological phenotypes [96]. When multiple attractors exist, they can be binned into groups that relate to a specific phenotype. To give a theoretical example, consider a Boolean network of cell signaling pathways for a type of cancer. This network contains...
1 attractor when no drug is present and 10 attractors when an anticancer agent is present. For the control condition, let us denote the only attractor when no drug is present as proliferation. Hence, 100% of the cancer cells are in a proliferative state in the absence of drug. When drug is introduced, the system divides into 10 attractors. Of the 10 attractors, five, three, and two attractors are associated with apoptosis, proliferation, and cell cycle arrest, respectively. The relative frequencies of the attractors, in each group, can be summed and directly related with the overall frequency of that particular cellular fate. For simplicity, let us also assume that the basin size of each attractor is the same, which results in a relative frequency of 0.10 for each attractor. Therefore, the overall frequency for apoptosis is 0.50, proliferation is 0.30, and cell cycle arrest is 0.20. In a biological context, 50% of the cancer cells would undergo apoptosis, 30% would continue to proliferate, and 20% would enter a state of cell cycle arrest. Quantifying the relative frequency of attractors in biological networks has been applied to identify potential therapeutic targets in T-cell large granular lymphocyte leukemia and breast cancer, evaluate outcomes of molecularly targeted cancer therapies, and describe B-cell differentiation [17,29,97,14].

Frequently, cell fates or other phenotypes of interest, are modeled as nodes in the network. The relative activation frequency of individual nodes can be calculated as:

\[
v_x = \sum_{i=1}^{n_A} (v_{A_i} \times x_i) \quad x_i \in \{0,1\} \quad i = 1, ..., n_A
\]  

(8)

with \(v_x\) as the relative activation frequency of node \(x\). Lu et al. calculated the activation frequencies of nodes in a Boolean network of colitis-associated colon cancer for a non-inflammatory and a pro-tumor inflammatory microenvironment [32]. To simulate the non-inflammatory condition, states of the nodes in the pro-inflammatory microenvironment were set to OFF, and the dynamics were simulated using general asynchronous updating for 5000 initial sets of states. The fractional activation of the proliferation node was plotted over time and eventually reached zero, indicating that the premalignant cells were unable to proliferate in a non-inflammatory microenvironment. The network was then simulated under a pro-tumor inflammatory condition, which resulted in an increase in proliferation and apoptosis reached zero. A node
perturbation analysis was then performed to observe how various interventions modified proliferation and apoptosis, which led to the identification of potential therapeutic targets and provided insight into combinatorial cancer therapies. A node perturbation analysis is a sensitivity analysis in which a perturbation is introduced (by fixing certain nodes to 0 or 1), and the change in relative activation frequency of other nodes is calculated. The sensitivity at which a node changes due to a perturbation can be quantified as a ratio of a node's activation frequency in the presence and absence of a perturbation, known as a perturbation index (PI):

$$PI = \frac{v_x(\text{perturbation})}{v_x(\text{normal})}$$  \hspace{1cm} (9)

Ruiz-Cerda et al. constructed a Boolean network of systemic lupus erythematosus and performed a perturbation analysis coupled with a clustering algorithm to identify potential drug targets, select optimal combination therapies, and identify subpopulations that may have a differential response to drug treatment [21]. SPIDDOR (Systems Pharmacology for efficient Drug Discovery On R) is an R package that can be used to perform such simulations, attractor analyses, and perturbation analyses with clustering of Boolean network models [98].

When the state space of a network is too large to characterize all attractors and basins of attraction, a random finite number of initial conditions can be generated and used for simulations. Since the number of randomly generated initial conditions can be infinitesimal in comparison to the entire state space of large networks, attractors with small basins of attraction are often missed. This raises the concern about whether this sampling approach is an adequate representation of the whole system. However, this approach seems adequate based upon the assumption that attractors with smaller basins are less important than attractors with large basins. In support of this conjecture, the probability of reaching an attractor with a small basin size is very low and will have a negligible effect on the activation frequencies of nodes. A concern with these sampling methodologies, both exhaustive and random, is that some initial conditions may be irrelevant and cannot exist because they are outside of natural biological constraints.
In our multiple myeloma case, $10^5$ initial conditions were sampled and used to determine the relative activation frequency of each node in the presence/absence of bortezomib (Figure 7). Although we observed differences in the relative activation frequencies of certain species in the network, there were only slight differences in the relative activation frequencies of growth and apoptosis between these two conditions. This could be attributed to a variety of factors. First, the network was not constrained by fixing components that are known to be constitutively active or mutated in U266 multiple myeloma cells. There may be certain constitutively active species, such as growth factors, that prevent the system from evolving towards an apoptotic attractor. For example, apoptosis turns off when certain nodes in the PI3k-Akt signaling pathway are fixed (such as PIP3). This pathway is altered across several types of cancers and is associated with survival and growth [99]. Second, there are positive feedback loops in the network that can cause species to become and remain active as a result of randomly sampling initial conditions. Biological systems are known to be robust to transient perturbations, and pathways may dampen and diminish overexpression instead of remaining active. This phenomenon could be due to unrealistic initial conditions, missing regulatory components, or an artifact of the updating scheme [95]. Lastly, the model contains only a small portion of the entire biological regulatory network. Since the network has been constructed to contain pathways that lead to apoptosis in multiple myeloma cells, it is not surprising that sampling random initial conditions results in a high activation frequency of apoptosis.

**Minimal Intervention Analysis**

Minimal intervention analyses can be conducted to identify targets, either single or multiple, for therapeutic intervention. Klamt and Gilles introduced the concept of minimal cut sets for biochemical networks [100]. A minimal cut set was originally defined as a minimal set of reactions whose removal blocks the operation of a chosen objective and has been extended to include structural interventions (removal of nodes) [101]. Minimal intervention analysis identifies the least number of interventions, referred to as *minimal interventions sets*, required to satisfy a user-defined goal. For cancer, a user-
defined goal could be the activation of apoptosis. In this case, the node for apoptosis would be defined as 1 (ON), and the minimal intervention algorithm searches for all possible combinations of node perturbations that result in the activation of apoptosis. A node intervention can represent a permanent activation (1) or deactivation (0), and results are based upon whether the logical steady state satisfies the intervention goal. Thus, interventions will only be identified when no other regulators are influencing the state of the node. In other words, the analysis identifies combinations of node perturbations that results in the node of interest becoming frozen to the defined value. Single or multi-node perturbations can be identified. However, for large networks, identifying combinations greater than three or four nodes starts to become computationally unfeasible. In addition, multiple intervention goals can be defined, and perturbations that satisfy a subset or all of these goals can be identified. CellNetAnalyzer is a Matlab-based toolbox that has been commonly used to perform minimal intervention analyses of cellular networks [62].

Two minimal intervention analyses were conducted on the Boolean network of intracellular signaling in multiple myeloma. In the first analysis, up to two node combinations were identified that result in apoptosis turning ON and growth turning OFF. The analysis identified 52 combinations of two node perturbations that result in apoptosis and growth equaling 1 and 0 (Figure 8a; Supplementary Table S1). Thus, 52 potential drug target combinations may be further investigated to determine their efficacy for the treatment of multiple myeloma. The proteasome was the most frequently inactivated target, which was not surprising since proteasome inhibition is a crucial component of the current standard-of-care treatment for multiple myeloma. For the second analysis, we identified up to three node combinations that result in apoptosis turning ON and growth turning OFF. Here, in addition to the 52 previously identified two node combinations, we found 427 three-node perturbations that result in apoptosis and growth equaling 1 and 0 (Figure 8b; Supplementary Table S2). Caspase-8 (Cas8) was the most frequently activated node, present in approximately 20% (96/479) of all perturbations. This result
was intriguing since activation of the caspase-8-mediated apoptosis pathway is the primary mechanism of action for the cytotoxic effects of lenalidomide, which is another chemotherapeutic agent used in combination with proteasome inhibitors for the treatment of multiple myeloma [102,103]. Overall, this approach has led to the identification of several two-drug combinations and a vast number of three-drug combinations, which may be useful for the treatment of multiple myeloma. Although minimal intervention analysis is a useful tool to narrow down and identify potential drug combinations, information regarding prioritization and differences in efficacy amongst these combinatorial treatments remains unknown. Therefore, performing minimal intervention analyses to identify combination therapies can be seen as a high throughput method, which would subsequently warrant further investigations through \textit{in silico} quantitative analyses, such as the development of a QSP models, and experimental qualifications [9].

In addition to minimal intervention analysis, other network-control methodologies have been employed to identify points of intervention. A \textit{stable motif} is a set of nodes that achieves a single cellular state irrespective of input by the rest of the network. Such a motif, can be used to identify \textit{stable motif control sets}, which are sets of nodes whose states are able to drive the dynamics of the network towards a specific attractor [104]. In essence, the states of nodes in a control set are able to provide insights into the behavior of the system, and perturbing specific nodes of a control set can guide the behavior toward one that is favorable. This stable motif-control methodology has been applied to identify control sets in an epithelial-mesenchymal transition (EMT) network model that drives the system towards an epithelial steady state [105]. Steinway and colleagues identified seven individual targets (all related to E-cadherin transcription), and three targets in combination with SMAD complex inhibition, that were able to suppress EMT. Select targets were then verified to suppress EMT using an \textit{in vitro} cell migration assay. As a comparison, minimal intervention analysis identifies targets that lead to a specific predefined outcome of
select nodes (a steady-state); whereas, network motifs enable the identification of targets that drive the system towards a desired attractor and considers network dynamics as opposed to steady states.

**An Overview of Boolean Network Applications**

Table S3 lists a sample of previously developed Boolean networks that pertain to human physiology and disease. The majority of these networks focused on signaling pathways in cancer, and these networks are grouped based upon the type of cancer, including: the immune system, breast, gastrointestinal, hepatic, and lung cancers. Nodes in these networks are primarily gene products, with the exception of endpoint nodes. Edges represent several types of regulatory interactions within signaling pathways, such as protein-protein, protein-DNA, and metabolic interactions. These networks have been constructed primarily through the utilization of pathway databases and extensive evaluation of the literature, and are listed as knowledge-derived networks (Table S3). Networks that have been constructed based on large datasets, such as genomic, proteomic, and metabolomic studies, are referred to as data-derived networks. In addition, a few studies have extended pre-existing networks to investigate specific interests. The main goals for developing Boolean networks have been to identify potential therapeutic strategies [106,17,18,107,25,26,28-32,34], characterize cellular differentiation [14,11,13,36], understand differential responses to cancer therapies due to mutational differences [27], understand the impact of patient heterogeneity on the response to drug treatments [21,35], and as an initial framework prior to the development of quantitative models [23]. The networks provided in this table could be potentially extended or repurposed for investigating additional features of interest, as opposed to starting from the ground up. A useful resource is the Cell Collective, which is a web-based platform that contains several published biological networks for users to build upon and perform simulations [108]. Previously developed Boolean networks can also be accessed from BioModels, GINsim’s model repository, and PyBoolNet’s model repository [109,63,110]. Additionally, there is a Consortium for Logical Models and
Tools (CoLoMoTo) that provides useful information for logic-based modeling, such as commonly used methods and software tools [111]. Lastly, a tutorial on logic modeling in quantitative systems pharmacology has recently been published [112], which serves as another great resource to help guide the development of network-based systems pharmacology models.

Conclusions

Encoding complex biological processes into mathematical models enables the prediction of responses to various perturbations, which has the potential to uncover emergent properties, discover new therapeutics, and individualize therapy to maximize therapeutic benefits while minimizing adverse drug reactions. These goals can be pursued through a combination of quantitative experiments and mathematical analyses of biological networks. Despite the simplifications and limitations of Boolean network models, they have proven to be effective for qualitatively describing the dynamics of biological systems. Boolean network models of intracellular signal transduction could act as a bridge to connect drug exposure with physiological responses to form a hybrid multiscale model of drug action. Kirouac et al. developed a multiscale model of the ErbB signaling pathway with the objective to design optimal drug combination regimens for ErbB2-positive breast cancer patients [26]. The model contains biological processes occurring at four different time scales: Receptor-ligand binding (minutes), signal transduction (minutes to hours), transcription (hours), and phenotypic effects (days to weeks). Quantitative logic, an extension of Boolean logic, was incorporated in the model to describe intracellular signal transduction events and link the time course of drug exposure to tumor growth dynamics. The rational for selecting a quantitative logic-based modeling formalism was the rapid time scale of signal transduction events relative to clinical phenotypes and the nature of available biochemical data. A combination regimen that targets both ErbB3 and ErbB2 was identified as a potential treatment strategy to improve therapeutic efficacy. The breadth of utility offered by a mathematical model that can accurately predict complex
biological responses to pharmacological perturbations, especially for drug development, has fueled the rise of interest in quantitative systems pharmacology [113]. Quantitative systems pharmacology/toxicology models could improve patient efficacy while minimizing toxicity, reduce late-stage drug attrition, and aid in the movement towards precision medicine [114].

In this review, we have covered one of the most prominent applications of Boolean network modeling in drug discovery, the identification of drug targets and combination therapies. The innovation gap across the pharmaceutical industry, in which drug development costs are increasing exponentially while the number of new molecular entities remains relatively constant, is well appreciated [115]. A novel systems-level approach to drug discovery is needed, which focuses on a comprehensive understanding of drug effects rather than the modulation of a single target. Network-based systems pharmacology approaches, such as Boolean network modeling, may help to fill the innovation gap through the identification of novel drug targets and combination therapies. A quantitative systems-level understanding of cellular physiological responses to pharmacons is key, and novel mathematical modeling approaches and strategies are needed that enable better representation and analysis of biological systems.

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References


Figure and Table Legends

Figure 1. Waddington’s epigenetic landscape from a systems pharmacology perspective. Lines represent the trajectories of the ball towards valleys. Valleys are represented as attractors of normal proliferation, aberrant proliferation, or apoptosis. The unperturbed network (right) is representative of a trajectory towards a healthy normal attractor (blue), whereas a network that has gained mutations in specific nodes (left) is representative of a trajectory towards a neoplastic attractor (red). Yellow bolts indicate nodes that have been mutated. A pharmacological intervention (purple) may shift the trajectory towards one that is favorable, whether it is back towards normal proliferation or apoptosis. Adapted from [43].

Figure 2. Figure adapted from Chudasama et al. (2016) [23]. Boolean network model of cellular signal transduction in U266 multiple myeloma cells. Legend shown to the right of the network.

Figure 3. (a) Comparison of five Boolean network simulation methods. Simulations were performed for the effect of bortezomib (proteasome = OFF) on the Boolean network of signaling pathways in multiple myeloma [23]. The fractional activation of apoptosis is determined by dividing the frequency that apoptosis is activated by the total number of simulations (n=1000) across each time step: Synchronous (BS; black line), general asynchronous (BA; red line), random-order asynchronous (BAR; green line), normalized HillCube with default parameters (NHC; dotted line), and normalized HillCube with randomly sampled parameter values (NHCR; blue line). Default parameters values used for NHC simulations were: $\tau = 1$, $k = 0.5$, and $n = 3$. Parameter values used for NHCR simulations were randomly generated from a Log-normal distribution with a mean (and standard deviation) of: $\tau = 1 (1)$, $k = 0.5 (1)$, and $n = 3 (1)$. (b) Boolean network normalized HillCube simulations compared with intracellular protein dynamics of U266 cells cultured with 3 nM bortezomib for 0, 12, 24, 36, and 48 hours. The data was obtained using a multiplex immunoassay (Luminex® MAGPIX®), which has previously been described in detail [93].
**Figure 4.** Attractor analysis with an exhaustive search and synchronous updating for the multiple myeloma Boolean network [23] for the (a) absence and (b) presence of bortezomib. The y-axis represents the fractional activation of a node across all network attractors, and the x-axis represents all nodes in the network. The fractional activation of growth and apoptosis are represented as blue and red bars.

**Figure 5.** Attractor analysis with a set of random initial states ($10^5$) and synchronous updating for the multiple myeloma Boolean network [23] for the (a) absence and (b) presence of bortezomib. The y-axis represents the fractional activation of a node across all network attractors, and the x-axis represents all nodes in the network. The fractional activation of growth and apoptosis are represented as blue and red bars.

**Figure 6.** Attractor analysis with a set of random initial states ($10^5$) and general asynchronous updating for the multiple myeloma Boolean network [23] for the (a) absence and (b) presence of bortezomib. The y-axis represents the fractional activation of a node across all network attractors, and the x-axis represents all nodes in the Boolean network. The fractional activation of growth and apoptosis are represented as blue and red bars.

**Figure 7.** Attractor analysis with a set of random initial states ($10^5$) and synchronous updating for the multiple myeloma Boolean network [23] for the (a) absence and (b) presence of bortezomib. The y-axis represents the relative activation frequency of each node, and the x-axis represents all nodes in the network. The relative activation frequency of growth and apoptosis are represented as blue and red bars.

**Figure 8.** A minimal intervention analysis of the Boolean network of signaling pathways in multiple myeloma [23]. Network perturbations that result in apoptosis turning ON and growth turning OFF were identified for up to (a) two-node and (b) three-node interventions. The number of times a node was
perturbed relative to the total number of intervention sets (52 and 427 intervention sets for two-node
and three-node perturbations) is shown on the y-axis, and the x-axis depicts the intervention targets.
Red bars indicate the activation of a node (ON), and blue bars indicate the inactivation of a node (OFF).

**Supplementary Figure S1.** Results from for all individual nodes using five simulations methods are
displayed as heat maps. The five simulation methods are synchronous (A), general asynchronous (B),
random-order asynchronous (C), normalized HillCube with default parameters (D), and normalized
HillCube with randomly sampled parameter values (E). Time is expressed as arbitrary time units.
Network simulations were performed in MATLAB using the **odefy** toolbox.

**Supplementary Table S1.** Results from a two-target minimal intervention analysis on the Boolean
network of intracellular signaling in multiple myeloma. 52 combinations were identified for two-targets
that result in apoptosis and growth equaling 1 and 0. Blue and red highlighting indicate an inhibitory and
stimulatory perturbation, respectively.

**Supplementary Table S2.** Results from a three-target minimal intervention analysis on the Boolean
network of intracellular signaling in multiple myeloma. 427 combinations were identified for three-
targets that result in apoptosis and growth equaling 1 and 0. Blue and red highlighting indicate an
inhibitory and stimulatory perturbation, respectively. Due to table size constrains, the table is not shown
but can be accessed online (https://link.springer.com/article/10.1007/s10928-017-9567-4).

**Supplementary Table S3.** Summary of selected Boolean network models for various human
physiological and pathophysiological systems. A brief description of network construction,
reduction/refinement, validation, analyses, and findings are presented. Networks are highlighted based
upon the physiological system that they represent: immune system (blue), breast (pink), gastrointestinal
(brown), liver (green), lung (red), and uncategorized (gray).
Figures and Tables

Figure 1

Aberrant Proliferation  Apoptosis  Normal Proliferation
Figure 2
Figure 3

(a) Fractional Activation of Apoptosis

(b) Network Predictions vs. Experimental Observations

Network Predictions:
- JNK
- Cas8
- Cas9
- Cas3
- p21
- p53
- BAD
- BCL2
- pNFKB
- AKT

Experimental Observations:
- JNK
- Cas8
- Cas9
- Cas3
- p21
- p53
- BAD
- BCL2
- pNFKB
- AKT

Legend:
- Predict: 1
- Observe: 2
- Predict: 0.8
- Observe: 1
- Predict: 0.6
- Observe: 1
- Predict: 0.4
- Observe: 0.8
- Predict: 0.2
- Observe: 0.6
- Predict: 0
- Observe: 0.2
- Predict: -1
- Observe: 0
- Predict: -2
- Observe: 0

Time Steps (Arbitrary Time Units)

Time Steps

Time (hours)
Figure 4
Figure 5

(a) Fractional Activation of Nodes across all Attractors (S)

(b) Nodes in the Boolean Network
Figure 6

(a) Fractional Activation of Nodes across all Attractors (S)

(b) Nodes in the Boolean Network

Growth

Apoptosis

0.7353

0.352

1

0.5

0.4

0.3

0.2

0.1

0

Figure 7

(a) Relative Activation Frequency of each Node (v)

(b) Nodes in the Boolean Network

Growth Apoptosis

0.9920

0.4653

0.4692
Figure 8

(a) Relative Frequency of Intervention

(b) Relative Frequency of Intervention Targets

- activate
- inactivate
## Supplementary Table S1

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**Frequency**
-8 | -7 | -7 | -6 | -6 | -6 | -7 | -6 | 8 | 8 | 8 | 8 | 4 | 8

**Relative Frequency**
0.154 | 0.135 | 0.135 | 0.115 | 0.115 | 0.115 | 0.135 | 0.135 | 0.115 | 0.154 | 0.154 | 0.154 | 0.077 | 0.154
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<th>Physiological/P athological System</th>
<th>Network (A) Construction, (B) Reduction/Refinement, and (C) Validation</th>
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<td>B cell differentiation</td>
<td>(A) Knowledge-derived. The network is composed of 22 nodes and 39 edges. (B) Conversion to ODEs using SQUAD with modifications proposed by Sanchez-Corrales et al. (2010)</td>
<td>(1) Attractor analysis: Exhaustive search and synchronous updating. (2) Perturbation analysis: Gain/loss of function for each node in the discrete and continuous networks</td>
<td>Four fixed-point attractors identified that correspond to types of B cells. Three additional attractors identified through analysis of continuous dynamics, which may represent intermediate differentiated states. Identified Pax5, Bcl6, Bach2, Irf4, and Blimp1 as key regulators of B-cell differentiation.</td>
<td>Mendez A and Mendoza L. PLoS Comput Biol. 2016;12(1):e1004696.</td>
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<td>Multiple myeloma</td>
<td>(A) Knowledge-derived. The network is composed of 67 nodes and 131 edges. (B) Network reduction based on Veliz-Cuba et al. (2011). Conversion to ODEs using ODEFY. (C) Western blotting</td>
<td>(1) Network simulations: Normalized HillCube. Predicted the effects of proteasome inhibition through a transient perturbation.</td>
<td>Network simulations were in agreement with experimental data. Revealed that bortezomib-induced apoptosis is independent of NFkB suppression, in U266 cell line, which contradicts the hypothesized pharmacodynamics.</td>
<td>Chudasama V et al. J Pharmacol Exp Ther. 2015;354(3):448-58.</td>
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<td>T lymphocyte differentiation</td>
<td>(A) Extension of existing networks (Mendoza et al. 2006/2010). The network is composed of 50 nodes and 97 edges. (B) Conversion to ODEs using SQUAD with modifications proposed by Mendoza and Pardo (2010).</td>
<td>(1) Attractor analysis: Random initial states and continuous update. Used the R package deSolve (REF). (2) Perturbation analysis: Gain/loss of function for each node in the discrete and continuous networks</td>
<td>Nine fixed-point attractors, and their respective basins of attraction, were identified that correspond to types of T lymphocytes. Identified a potential threshold for TCR signaling, above which CD4+/CD8+ double positive cells become CD4+ cells, and below which they become CD8+ cells.</td>
<td>Martinez-Sosa P and Mendoza L. Biosystems. 2013;113(2):96-103.</td>
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<td>CD4+ T cell differentiation and plasticity</td>
<td>(A) Knowledge-derived. The full network is composed of 85 nodes. (B) Model reduction using the method in Villarreal et al. (2012) and verified with GINsim. Approximated discrete step-like functions with continuous interaction functions. The reduced network contains 18 nodes. (C) Validated with reported knock-out and over-expression profiles</td>
<td>(1) Attractor analysis: Synchronous updating. (2) Robustness analysis: Altered logic functions to verify function and structural properties of the model. (3) Perturbation analysis: Perturbed each node of all the attractors and measured the number of times that a new attractor was reached.</td>
<td>Identified eleven attractors in a transcriptional-signaling regulatory network that correspond to CD4+ T cell types. System is more sensitive to perturbations in transcription factors than cytokines. T-bet, TGF-β, and SOCS proteins are key network components for CD4+ T cell plasticity.</td>
<td>Martinez-Sanchez M et al. PLoS Comput Biol. 2015;11(6):e1004324.</td>
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<td><strong>T cell receptor signaling</strong></td>
<td><strong>(A)</strong> Extension of an existing network (REF A methodology for the structural and functional analysis of signaling and regulatory networks). The network contains 94 nodes and 123 edges. <strong>(C)</strong> Western blotting.</td>
<td><strong>(1) Logical steady state analysis.</strong> <strong>(2) Minimal intervention analysis:</strong> Assess mutations that would result in uncontrolled proliferation. <strong>(3) Robustness and sensitivity analysis</strong></td>
<td>The activation of the costimulatory molecule CD28 activates JNK in addition to PI3K, but does not activate ERK. The intervention sets identified that induce a sustained T cell proliferation are known oncogenes (ZAP70, PI3K, GAB2, and PLCG1)</td>
<td>Saez-Rodriguez J et al. PLoS Comput Biol. 2007;3(8):e163.</td>
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<td><strong>CD4+ T cells</strong></td>
<td><strong>(A)</strong> Constructed using Cell Collective and extended from existing networks. Naldi et al. (2010). Chaouiya et al. (2013). The network contains 188 nodes and 351 edges. <strong>(C)</strong> Tested the ability of the model to predict 20 different complex input-output phenomena.</td>
<td><strong>(1) Network simulations:</strong> Simulations performed on the Cell Collective platform. Identify protein products most affected by CAV1&lt;sup&gt;−/−&lt;/sup&gt;, CAV1&lt;sup&gt;+/−&lt;/sup&gt;, and CAV1&lt;sup&gt;+/+&lt;/sup&gt; in immunocompetent versus immunocompromised conditions.</td>
<td>Identified ten proteins that are potentially influenced by CAV1 expression. Protein expression of the ten molecules were validated with adult T-Cell leukemia/lymphoma microarray data and immunohistochemical analysis of mouse tissue</td>
<td>Conroy B et al. Front Immunol. 2014;5:599.</td>
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<td><strong>(A)</strong> Knowledge-derived. The network contains 137 nodes (16 HIV-1 and 121 human) and 336 edges. <strong>(C)</strong> Reproduced 21 of 25 global interactions obtained from literature.</td>
<td><strong>(1) Logical steady state analysis:</strong> Performed logical steady state analyses in order to simulate the effects of drugs and mutations on signal transduction pathways.</td>
<td>Identified 26 host cell factors that modulate viral protein dynamics and 9 host cell factors essential to viral replication.</td>
<td>Oyeyemi O et al. Bioinformatics. 2015;31(7):75-83.</td>
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<td>Topic</td>
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<td>Fanconi anemia/breast cancer (FA/BRCA) pathway</td>
<td>(A) Knowledge-derived. The network is composed of 28 nodes and 122 edges. (B) Functional protein complexes are represented as single nodes.</td>
<td>(1) <strong>Network simulations</strong>: Simulated three different types of damage (2) <strong>Attractor analyses</strong>: (a) Exhaustive search and synchronous updating. (b) Random search (10,000 initial states) and asynchronous updating. Determined attractors for all known single gain/loss of function mutations in FA/BRCA.</td>
<td>The wild type FA/BRCA network has only one attractor. For 56 single gain/loss of function mutations, 18 mutations resulted in the wild type attractor, 11 mutations resulted in a new attractor, and 27 contain both the wild type and new attractors. Model suggests alternative DNA repair pathways become active when the FA/BRCA pathway is defective.</td>
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<td>Systemic lupus erythematosus (SLE)</td>
<td>(A) Knowledge-derived. The network consists of 52 nodes and 296 edges. (B) Incorporation of a delayed activation for nodes that require a longer time to become active.</td>
<td>(1) <strong>Network simulations and attractor analysis</strong>: Random order asynchronous updating. Performed simulations for transient (ON for one time step) and continuous (fixed to ON) exposure to DNA damage, under wild type and FA mutant conditions. (2) <strong>Perturbation analysis and clustering</strong>: Identify perturbations that result in alterations exhibited by SLE patients and evaluate therapeutic targets.</td>
<td>Perturbation analysis coupled with clustering highlights the ability to identify potential single and combinatorial drug therapies, and distinguish between responding and nonresponding subpopulations. Model predictions showed that anti-TNFα treatment was ineffective for controlling most SLE-like alterations, in agreement with clinical studies.</td>
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<td>Allergic asthma</td>
<td>(A) Knowledge-derived. (B) Extended the general Boolean framework by including a decay time, where a node turns off after a period of activation.</td>
<td>(1) <strong>Network simulations</strong>: random order asynchronous updating. Calculated the activation frequencies for all nodes across each time step.</td>
<td>Eosinophils and T cells work in cooperative manner to produce IL-13, which regulates T cell recruitment to the lung and development of allergic asthma.</td>
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<td>Mammary epithelial cells: ErbB receptor signal transduction</td>
<td>(A) Knowledge-derived. Constructed in the Cell Collective platform. The network contains 245 nodes and over 1000 edges.</td>
<td>(1) <strong>Network simulations</strong>: Simulations were performed, using the Cell Collective platform, to test whether Src activity affects EGFR endocytosis.</td>
<td>Elevated Src activity will promote the localization of EGFR in internal compartments of the endocytic pathways, regardless of EGF activity.</td>
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<td>Breast cancer</td>
<td>(A) Knowledge-derived. The network contains 31 nodes. (B) Network was divided into three subnetworks and assigned different weights. (C) MTT assay and siRNA transfection</td>
<td>(1) <strong>Stochastic node vulnerability analysis</strong>: Performed deterministic and probabilistic simulations. Select nodes were knockout and the vulnerability is calculated. Vulnerability is the probability that the networks output differs from normal.</td>
<td>Network predictions show that NFkB and eIF4E are not vulnerable, indicating that they would be poor targets for the treatment of breast cancer.</td>
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<td>HER2-positive breast cancer</td>
<td>(A) Knowledge/data-derived, using DDPN (REF). (B) Networks specific to three breast cancer cell lines, for long and short term signaling, were developed.</td>
<td>(1) Attractor and node perturbation analysis: Attractors were identified for all possible treatment combinations for erlotinib, pertuzumab, and trastuzumab. The analysis was performed for all networks, to predict a differential response across cell lines.</td>
<td>Cell-line specific activation patterns of MAPK and PI3K pathways, underlie individual drug response in different breast cancer cell lines. p70S6K, ErbB-3, and PDK1 are essential in the regulation of AKT activity between SKBR3 and HCC1945, which contributes to drug resistance.</td>
<td>von der Heyde S et al. BMC Syst Biol. 2014;8:75.</td>
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<td>Trastuzumab-resistant breast cancer</td>
<td>(A) Knowledge-derived. Training (GIInsim) against experimental data (reverse phase protein assay) (B) Transitive reduction, assisted by jackknife procedure</td>
<td>(1) Network simulations and node perturbation analysis: univariate and multivariate</td>
<td>Phosphorylation of pRB at the transition point can serve as a readout of EGF stimulation. Combinatorial targeting of ERBB family receptors or key signaling components of MAPK (MEK1) and AKT1 pathways is not efficacious in de novo trastuzumab resistance cells.</td>
<td>Sahin O et al. BMC Syst Biol. 2009;3:1.</td>
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<td>p53 pathway (in the context of breast cancer)</td>
<td>(A) Knowledge-derived. (B) State transition probability to extend deterministic model into probabilistic. (C) Single-cell imaging</td>
<td>(1) Attractor and node perturbation analysis: Knockout nodes and observed the change in relative activation frequency of apoptosis. (2) Robustness analysis.</td>
<td>Pulsing or sustained p53 activity is represented as a cyclic attractor or point attractor, corresponding to cell cycle arrest or cell death. The relative activation frequency of apoptosis increased when both Nutlin-3 and a Wip-1 inhibitor were present.</td>
<td>Choi M et al. Sci Signal. 2012;5(251):r83.</td>
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<td>Gastric cancer</td>
<td>(A) Knowledge-derived. (B) Model reduction (Naldi et al. 2011) Incorporation of known AGS cell line mutations. Multi-value states for endpoints. (C) Real-time cell assay</td>
<td>(1) Attractor and node perturbation analysis: Exhaustive search and asynchronous updating.</td>
<td>FOXO may be an important mediator of observed MEK-PI3K, TAK1-PI3K, and TAK1-AKT synergies, but does not play a big role in enhanced efficacy of dual MEK-AKT inhibition.</td>
<td>Flobak A et al. PLoS Comput Biol. 2015;11(8):e1004426.</td>
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<td>Colitis-associated colon cancer (CAC)</td>
<td>(A) Knowledge-derived. (B) Normal and pro-tumor inflammatory microenvironment. Network reduction by removing 33 frozen nodes. (C) Cytotoxicity assays and western blotting</td>
<td>(1) Attractor analysis: General asynchronous updating for 5000 random initial states. Determined the fractional activation of apoptosis in different microenvironments. (2) Node perturbation analysis.</td>
<td>The single target therapy may be less effective in killing tumor cells that had previously developed in CAC cases. Mitochondria outer membrane permeabilization could only be fully activated when ceramide is activated and AKT is inhibited.</td>
<td>Lu J et al. Sci Rep. 2015;5:14739.</td>
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<td>Hepatocellular carcinoma</td>
<td>Hepatocellular carcinoma (HCC): epithelial-to-mesenchymal transition</td>
<td>Small cell lung cancer (SCLC)</td>
<td>Myofibroblast differentiation</td>
<td>Neurons: stress, injury, and apoptosis</td>
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<td><strong>Analysis</strong></td>
<td>(A) Knowledge-derived. (B) Used CellNetOptimizer to select the most optimal network. (C) Cross-validation</td>
<td>(A) Knowledge-derived. The network contains 70 nodes and 135 edges. (B) Reduction based on Saadatpourt et al. (2011) using NET-SYNTHESIS. (C) Patient samples</td>
<td>(A) Knowledge-derived. The network contains 91 nodes and 134 edges. (C) Semi-quantitative: microscopy and image analysis</td>
<td>(A) Knowledge-derived. The network contains 21 nodes and 37 edges. (C) Transfection and inhibitors studies, followed by TUNEL assay and qRT-PCR</td>
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<td><strong>Network Analysis</strong></td>
<td>(1) Drug target and comparative network analysis: Four hepatocellular carcinoma networks were developed and compared.</td>
<td>(1) Network simulations: Asynchronous updating. Determined the fractional activation of nodes over time. (2) Attractor analysis: Exhaustive state space search to characterize the epithelial-to-mesenchymal transition.</td>
<td>(1) Network simulations: Normalized Hill simulations. (2) Sensitivity analysis: Performed by simulating full knockdown of each node and predicting the change in activity of every node in the network. (3) Topological analyses.</td>
<td>(1) Attractor and node perturbation analysis: exhaustive search with synchronous updating. Determined the relative activation frequency of four HSPs in the presence/absence of growth factors and FasL.</td>
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<td><strong>Other Analysis</strong></td>
<td>Jak2 (a known kinase for Stat3) was found to be a target of TPCA-1 (an IKK inhibitor), and this finding may explain differences in potency between TPCA-1 and other IKK inhibitors developed for airway inflammation.</td>
<td>There are eight regulatory feedback motifs that stabilize the mesenchymal state in an independent manner and are associated with a single EMT steady state.</td>
<td>Attractors correlated with cell lines compared to random attractors. Patient samples that did not correlate to any model attractors represented a hybrid phenotype, where single cells exhibit both neuroendocrine/epithelial and mesenchymal-like characteristics. Cells transition towards this hybrid state upon exposure to drug treatment.</td>
<td>Regulation of αSMA by mechanical stimulus is dependent on TGFβ receptor. TGFβ receptor may have a role in sensitizing myofibroblast to differentiation. Antioxidants may be promising in treating fibrosis close to a myocardial infarction, but not in the remote zone.</td>
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<td>Death receptor pathway</td>
<td>(A) Knowledge-derived. (B) Reduction based upon Naldi et al. (2009). Derivation of compact conceptual model. (C) Published reports</td>
<td>(1) <strong>Attractor analysis:</strong> Asynchronous updating and an exhaustive state space search. Reduced the network to investigate which cell fates are reached from individual initial conditions. (2) <strong>Node perturbation analysis.</strong></td>
<td>Identified 27 stable states. RIP1 activity is transient in necrosis but is sustained in survival. The maximum rate of necrosis can be achieved with an optimal duration of death receptor activation by TNF pulse.</td>
<td>Calzone L. PLoS Comput Biol. 2010;6(3):e1000702.</td>
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Chapter 5:

Network-based Systems Pharmacology Model of Intracellular Signaling and Gene Regulation in Peripheral Neurons
Abstract

Cancer chemotherapy is often highly neurotoxic and produces nerve damage known as chemotherapy-induced peripheral neuropathy. The mechanisms of toxicity by which chemotherapeutics induce peripheral neuropathy is multifactorial and remains poorly understood. In this study, a network-based systems pharmacology approach was applied to characterize neuronal signaling pathways that may be disrupted upon neurotoxic insults by cancer chemotherapy. A systems pharmacology network model of intracellular signaling and gene regulation in peripheral neurons was constructed from literature information and pathway databases. Significant cellular signaling pathways specific to drugs that induce peripheral neuropathy were obtained from a prior pharmacological interaction and used as a foundation for the construction of the network. The final network model contains 131 nodes and 252 regulatory interactions. A series of network analyses were conducted to identify novel combinatorial treatment strategies for CIPN. Minimal intervention analysis identified 109 two-target and 115 three-target combinatorial treatment strategies to prevent neuronal apoptosis induced by proteasome inhibition. Attractor analysis suggested that the combinatorial inhibition of TNFα, NMDA receptors, and reactive oxygen species would completely prevent neuronal apoptosis in the presence of proteasome inhibition.
Introduction

Chemotherapy-induced peripheral neuropathy (CIPN) is an adverse side effect of cancer chemotherapy that occurs in approximately 30-40% of patients.\textsuperscript{1} CIPN typically manifests as a tingling/numbness sensation in the extremities of the body, often referred to as a glove-stocking distribution, which can develop into an extreme burning pain.\textsuperscript{2} Muscle weakness, fatigue, and irreversible nerve damage can be life debilitating for many patients.\textsuperscript{3} In severe cases, peripheral neuropathy can become life threatening when nerves controlling autonomic processes (e.g. breathing and heart rate) do not function properly. Improvement in cancer therapy has resulted in a decline in cancer death rates and an increase in the number of cancer survivors managing long-term adverse side effects from treatment.\textsuperscript{3,4} Hence, new strategies are needed to manage CIPN through pharmacological and/or non-pharmacological interventions.

An expert panel of the American Society of Clinical Oncology reviewed the scientific literature and compared outcomes of clinical trials in order to provide guidance on the effectiveness of preventative therapies for CIPN.\textsuperscript{5} The panel concluded that none of the 42 clinical trials conducted between 1946 to April 2013 identified an effective agent that could be recommended for the prevention of CIPN in cancer patients treated with neurotoxic chemotherapy.\textsuperscript{5} Although, there are no clinically approved drugs for the treatment or prophylaxis of CIPN,\textsuperscript{5,6} analgesics such as opioids, cannabis, and gabapentinoids have been used for the treat neuropathic pain caused by CIPN.\textsuperscript{5,8} However, these symptomatic therapies exhibit limited/no efficacy, do not prevent the underlying nerve damage, and exhibit an addictive potential owing to their psychoactive effects. Patients with severe CIPN must either reduce the dose of chemotherapy or completely discontinue treatment, which may affect the outcome of their therapy. The National Cancer Institute sponsored 15 clinical trials to investigate the prevention and symptomatic treatment of chemotherapy-induced peripheral neuropathy.\textsuperscript{6} Duloxetine was the only drug to exhibit efficacy in some patients for treating neuropathic pain caused by CIPN.\textsuperscript{6,9} The exact
molecular mechanisms responsible for the beneficial effects remain to be determined but might involve peripheral delta opioid. Several nutraceuticals and alternative medicine techniques have been evaluated for their potential utility in CIPN, such as vitamin E, goshajinkigan, acetyl-L-carnitine, alphalipoic acid, acupuncture, and electrotherapy. None of the nutraceuticals were effective and in certain cases may have worsened peripheral neuropathy (e.g., acetyl-L-carnitine for taxane-induced PN). Scrambler therapy, a form of electrotherapy, has shown promise in a phase II clinical trial where symptoms of CIPN were moderately decreased.

The molecular mechanisms of toxicity by chemotherapeutics are multifactorial and complex. There are a wide range of cellular components and processes that are disrupted by cancer chemotherapy. Certain mechanisms of neurotoxicity may be exclusive to a specific class of chemotherapeutics (e.g., microtubule or proteasome inhibitors); however, many molecular pathways of toxicity are shared across neurotoxic chemotherapeutics. Jaggi and Singh reviewed several of the possible mechanisms involved in the pathogenesis of CIPN and neuropathic pain. These mechanisms include: mitochondrial changes, alterations in ion channels and ionic current, inflammation, oxidative stress, activation of the intrinsic apoptosis pathway, MAPK pathway alterations, modulation of NMDA receptors, and several others.

The mitotoxicity hypothesis, which includes mitochondrial dysfunction resulting in aberrant Ca\(^{2+}\) signaling, oxidative stress, and apoptosis, has emerged as one of the fundamental causes of CIPN, especially for distal sensory neuropathies. Mitochondria in the sensory axons of dorsal root ganglion (DRG) neurons become swollen and vacuolated when exposed to paclitaxel, oxaliplatin, and bortezomib. Opening of the mitochondrial permeability transition pore (mPTP) increases the permeability of the inner mitochondrial membrane to molecules less than 1500 Da, which results in a loss of membrane potential, mitochondrial swelling, decreased energy production, and rupture of the outer mitochondrial membrane. Rupturing of the outer mitochondrial membrane initiates apoptosis through the release of pro-apoptotic proteins. High levels of Ca\(^{2+}\) and reactive oxygen species (ROS)
can induce opening of the mPTP.\textsuperscript{30-32} Cellular concentrations of Ca\textsuperscript{2+} and ROS are highly regulated by various processes and each other. The endoplasmic reticulum (ER) increases intracellular Ca\textsuperscript{2+} and ROS via IP3 receptors and activation of the unfolded protein response.\textsuperscript{33-35} NMDA receptors increase intracellular Ca\textsuperscript{2+} upon activation and generate ROS through NADPH oxidases. Action potentials cause large influxes of Ca\textsuperscript{2+} into the cell, which demands high energy consumption to restore intracellular Ca\textsuperscript{2+} concentrations by ATP-dependent transporters. Hence, dysfunctional mitochondria can lead to energy deficits that result in aberrant intracellular Ca\textsuperscript{2+} concentrations and oxidative stress.\textsuperscript{36}

Neuroinflammation is another major mechanism underlying the development and progression of CIPN.\textsuperscript{37} Upon peripheral nerve injury, resident macrophages, Schwann cells, satellite glial cells, and recruited immune cells secrete prostaglandins, chemokines, and cytokines.\textsuperscript{38} Neuroinflammatory mediators of the nerve microenvironment drive the complex interplay between cells of the neuroimmune system to promote adaptive (survival and growth) and maladaptive (neuropathic pain) responses.\textsuperscript{38,39} Several of the highly neurotoxic chemotherapeutics (paclitaxel, oxaliplatin, vincristine, and bortezomib) increase pro-inflammatory cytokines (TNF\textalpha and IL-1\beta) and downregulate anti-inflammatory cytokines (IL-10) in the dorsal root ganglion and spinal cord.\textsuperscript{40-46} Pro-inflammatory cytokines (TNF\textalpha, IL-1\beta, IL-6) and chemokines (CCL2) have been proposed as potential biomarkers and therapeutic targets for predicting and preventing CIPN-related pain.\textsuperscript{47} The administration of an anti-TNF\textalpha antibody attenuated CIPN symptoms and exhibited partial neuroprotection in rodent models of bortezomib-induced peripheral neuropathy.\textsuperscript{46,48} CCL2 was significantly increased in the DRG of rats receiving bortezomib, and mechanical allodynia was partially reversed by an anti-CCL2 antibody.\textsuperscript{49} IL-1 receptor antagonism and upregulation of IL-10 both reversed paclitaxel-induced mechanical allodynia in rats.\textsuperscript{43}

The multifactorial nature by which chemotherapeutics induce peripheral neurotoxicity warrants a systems pharmacology approach to understand the complex physiological processes involved in the development of CIPN and to identify novel treatment strategies. Systems biology aims to understand
how interactions across individual components give rise to the complex emergent behavior observed in biological systems. Systems pharmacology integrates principles from systems biology and pharmacology to study the effects of drugs on biological systems. Cellular signal transduction and the regulation of gene expression are controlled by a highly interconnected network of cellular components. Mathematical models are imperative to formalize understanding of complex cellular and biological processes. A variety of mathematical frameworks have been used to characterize dynamical phenomena that occur in biological systems, which include ordinary/partial/stochastic differential equations, fuzzy logic, agent-based, constraint-based, Bayesian methods, and Boolean networks. Boolean networks, originally described in 1969 by Stuart Kauffman, are the least complex mathematical framework, in space and time, that has been used to model gene regulatory networks. Such models have been developed and utilized to identify potential therapeutic targets that lead to a desirable outcome, to identify interventions that shift cellular differentiation towards a cell subtype of interest, and to understand how the mutational landscape of cancerous cells influence the response to therapy. Boolean network simulations can be performed, using a synchronous or asynchronous updating scheme, to predict the effect of a node perturbation on the dynamics of other species in the network, which could emulate a pharmacological intervention. The cellular pharmacodynamic effects of pharmacological interventions can also be evaluated by determining how perturbations alter attractors of the system. Dynamical steady-states, or attractors, in Boolean network models of cellular gene regulation have been shown to correspond with cellular phenotypes, such as differentiation, proliferation, and apoptosis. The identification of attractors and determining the probability of reaching each attractor provides useful information about long-term behavior of the biological system. Understanding how network perturbations alter the dynamical trajectory towards an attractor(s) of interest is a powerful approach to evaluate the effects of pharmacological interventions at the cellular level.
In this study, a Boolean network-based systems pharmacology model of signal transduction and gene regulatory processes in peripheral neurons was constructed to predict the cellular toxicodynamics of neurotoxic agents and to identify potential intervention targets. Since the modulation of a single target will likely be inadequate for preventing CIPN, analyses that consider the entire topology and dynamics of the network were performed to identify combinatorial treatment strategies.

**Methods**

*Boolean Network Model Development*

A bottom-up approach adapted from Albert and colleagues (2012) was utilized for the development of the Boolean network-based systems pharmacology model (Figure 1). The methodology consisted of compiling nodes, determining edges, adding Boolean logic, and determining initial conditions, which were informed from scientific literature, pathway databases, and a prior pharmacological interaction network. An external microarray dataset was used for model qualification by comparing network predictions to experimental observations.

Network construction was initiated using genes specific to drugs that are known to induce peripheral neuropathy, which were identified by a previously developed pharmacological interaction network. The gene list was uploaded to DAVID and mapped to pathways in KEGG to identify significant cellular signaling pathways associated with these genes. Cellular signaling pathways were cross-referenced with literature in order to select pathways associated with peripheral neuropathy and to parse out pathways associated with their therapeutic indications (e.g., chronic myeloid leukemia and pathways in cancer). From this analysis, three cellular signaling pathways were selected (neurotrophin, mitogen-activated protein kinase (MAPK), and apoptosis) as a base for the foundation of the network model. There were considerable overlapping cellular signaling pathways and components within these three pathways,
which were also included in the model. These pathways include the TNFα signaling pathway, calcium signaling pathway, PI3K-Akt signaling pathway, NFκB signaling pathway, p53 signaling pathway, RAS signal pathway, and protein processing in the endoplasmic reticulum.

Literature was searched using, but not limited to, the following keywords: “chemotherapy-induced peripheral neuropathy”, “drug-induced peripheral neuropathy”, “peripheral neuropathy”, “peripheral neurotoxicity”, and “sensory neuropathy”. During network construction, these keywords were searched with each network node to identify additional relevant edges/nodes (e.g. “peripheral neuropathy” + “ROS”; “peripheral neuropathy” + “JNK”). Many of the possible mechanisms by which chemotherapeutics induce nerve damage and neuropathic pain were reviewed by Jaggi and Singh (2012). KEGG was the most extensively used database, but others such as Reactome and WikiPathways were also used in model construction.66-68 Although peripheral neurons are post-mitotic, pathways related to the cell cycle and proliferation were included since aberrant cell cycle reentry can cause neuronal death.69,70

A Boolean network \( G(V, F) \) contains a set of nodes, \( V = \{x_1, ..., x_N\} \) and a list of Boolean functions, \( F = (f_1, ..., f_n) \). Boolean functions \( f_i(x_{i_1}, ..., x_{i_k}) \) were written for each node to describe the relationship between the activation state of a node \( x_i \) and its regulators. The spatiotemporal dynamics of Boolean networks are discrete, and the state of each node is binary, \( x_i \in \{0,1\} \). A state of zero indicates that a node is OFF (inactive) and a state of one indicates that a node is ON (active). Nodes of the network are updated based upon the states of its regulators at the previous time-step:

\[
x_i(t + 1) = f_i(x_{i_1}(t), x_{i_2}(t), ..., x_{i_k}(t))
\]

For nodes with multiple inputs, an OR (|) operator was used unless experimental data suggested the need for an AND (&&) operator. NOT (~) operators were used for inhibitory edges.
An initial condition of the network was obtained through the identification of the control attractor. The states of input nodes were defined and an attractor analysis was performed to identify network attractors. BDNF, NGF, and Proteasome were assumed to be ON. IL-1β, TFGβ, TNFα, IL-6, FasL, and NMDA were assumed to be OFF. An exhaustive SAT-based search was performed using BoolNet. Two attractors were identified. One attractor represented a neuron undergoing oxidative stress, dysregulated calcium, and activation of apoptosis. The other attractor did not exhibit any of these phenotypes. Therefore, the attractor with apoptosis inactive was selected as the control attractor and used as the initial condition for network simulations.

**Boolean Network Model Qualification**

The network was qualified by comparing model predictions with an external transcriptomic dataset that was not used during network construction. A microarray dataset was obtained from Gene Expression Omnibus (GEO), GSE10470, in which gene expression was measured in neurons isolated from ATF4 knockout mice with/without exposure to homocysteate, an NMDA receptor agonist. An analysis in R was performed using Bioconductor packages (Biobase v2.30.0, GEOquery v2.40.0, limma v3.26.8) to identify differentially expressed genes for each of the experimental conditions. Data was log transformed using a base of two. Linear least squares regression was performed for each gene, and differentially expressed genes were identified using an empirical Bayes method with Benjamini and Hochberg FDR adjusted p-values. Network predictions were performed using normalized HillCube functions in Odefy. ATF4 and NMDA were fixed to zero and one to represent the gene knockout and presence of homocysteate. Simulations were performed until a steady-state was reached. Network predictions for the change in node expression were reported as increasing when the initial state changes from 0 or 1 to a final state of 1, and decreasing when the initial state changes from 0 or 1 to a final state of 0. Network predictions were compared to the Log₂ fold-change in gene expression for differentially expressed genes. In cases
where multiple probes exist for the same gene, the probe with the smallest adjusted p-value was selected for comparison. Averaging gene expression across multiple probes provided identical results.

Centrality Analysis

A betweenness centrality analysis was performed in yEd graph editor to identify nodes that are highly connected to network integrity and the flow of information.

Network Simulations

Network simulations were performed to predict the intraneuronal pharmacodynamics of bortezomib. Bortezomib was assumed to only inhibit the proteasome, and non-proteasomal targets were not considered. Boolean logic functions were converted to normalized HillCube differential equations using Odefy and default parameter values were retained. Network simulations were performed for 25 time steps expressed as arbitrary time units, which was a sufficient amount of time to reach steady-state. The MATLAB model code used for network simulations is provided in Appendix I.

Minimal Intervention Analysis

A minimal intervention analysis was performed to identify potential drug combinations for the treatment of proteasome inhibitor-induced peripheral neuropathy. The minimal intervention analysis was performed using CellNetAnalyzer, a MATLAB-based toolbox. The intervention goal was to identify two and three node interventions that retain apoptosis in an OFF (zero) state in the presence of proteasome inhibition (proteasome fixed to zero). Nerve growth factor and brain derived neurotrophin factors were also assumed to be present (fixed to one). The intervention sets that satisfy these conditions were determined. All reactions used for performing minimal intervention analysis in CellNetAnalyzer are reported in Appendix I.

Attractor Analysis
An attractor analysis was performed to identify the steady-states of the system and assess potential therapeutic interventions for decreasing neuronal apoptosis. An exhaustive SAT-based search was performed to identify all attractors in the network. An attractor analysis using synchronous updating and $10^6$ initial start states was performed for eight network perturbations of interest. The proteasome was fixed to zero for all eight analyses. Analyses were performed to assess the effects of a TNFα inhibitor, NMDA receptor antagonist, and ROS inhibitor, alone and in combination. Relative activation frequency was determined for all network components and apoptosis, which can be calculated by the sum of node activation upon reaching an attractor divided by the total number of simulations performed. All analyses were performed using BoolNet, an R package, and the R code that was used to perform attractor analysis using BoolNet is provided in Appendix I.

**Perturbation Analysis**

Perturbation analyses were performed using the R-package SPIDDOR to assess the sensitivity of individual node knockout/knockin perturbations on the activation states of all network nodes. Perturbation analyses were performed for the knockout/knockin of network nodes for treatment naïve and proteasome inhibition conditions. A perturbation index (PI) was calculated to evaluate the effect of an individual node perturbation on the activation state of each node in the network. PI is the ratio of the probability of a node ($x$) being ON in an attractor in the presence of a perturbation versus the probability of a node ($x$) being ON in an attractor under normal conditions.

$$PI_x = \frac{Prob(x)_{Perturbation}}{Prob(x)_{Normal}}$$

An asynchronous updating scheme was used for the perturbation analysis simulations. All analyses consisted of 2000 time-steps and 25 repetitions. Although not shown, perturbations were hierarchical clustered to arrange perturbations according to those that exhibited similar effects. The R code for the perturbation analysis performed in SPIDDOR is provided in Appendix I.
Results

Network Structure

The Boolean network model of neuronal signaling contains 131 nodes and 252 edges (Figure 2). Model equations, initial conditions, and references are provided in Table 1. The network contains 9 input nodes, which are BDNF, NGF, IL-1β, TFGβ, TNFα, IL-6, FasL, NMDA, and Proteasome. The input nodes primarily represent ligands that bind to 9 cell membrane receptors NGFR, TrkA, TrkB, IL-1R, TFGβR, TNFαR, IL-6R, FasR, and NMDAR. There is also one intracellular receptor, inositol 1,4,5-triphosphate (IP3) receptor, that is present on the endoplasmic reticulum membrane. Components of the neurotrophin signaling pathway are clustered in the top left corner of the network model. The activation of nerve growth factor receptor (NGFR; p75NTR) and tropomyosin receptor kinases (TrkA/TrkB) by NGF and BDNF propagates signals through the network to activate downstream pathways that are imperative for cell survival. The downstream effects are primarily facilitated through the PI3K-Akt, PLC/PKC, and RAS/MAPK pathways. TrkA and TrkB both activate phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), which catalyzes the phosphorylation of phosphatidylinositol (4,5)-bisphosphate to phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 subsequently activates protein kinase B (Akt), which directly/indirectly regulates a plethora of pro-apoptotic (Bad, Bax, HtrA2/Omi, Bim, caspases) and pro-survival (NFκB, Bcl-2, Bcl-xL, and XIAP) proteins. PLC/PKC pathway activation initiates via the binding of neurotrophic factors to TrkB, activating phospholipase C-gamma 1 (PLCG1). PLCG1 catalyzes the breakdown of phospholipids into diacylglycerol (DAG) and IP3. DAG activates classic and novel protein kinase C isozymes (cPKC and nPKC), which subsequently activates MAPKs. The presence of calcium is required for the activation of cPKC, whereas nPKC is calcium independent. The MAPK signaling pathway is located in the middle left of the network and nodes representing kinases are pink. Components of the MAPK pathway were organized in a hierarchical manner based upon the three-tier signaling cascade consisting of MAPK kinase kinase (MAP3K), MAPK kinase (MAP2K), and MAPK.
MAP3Ks (MEKK1, MEKK3, ASK1, TAK1, MLK3, c-Raf) phosphorylate/activate MAP2Ks (MEK1/2, MKK3, MKK4, MEK5, MKK6, MKK7).\(^{84,85}\) MAP2Ks then phosphorylate/activate MAPKs (p38, JNK, ERK1/2, ERK5). MKK4/MKK7 are both required for the activation of JNK and MKK3/MKK6 are both required for the activation of p38.\(^{84,85}\) ERK1/2/5 activate RSK2, which activates ATF4 (CREB2).\(^{84,86}\)

The network contains three proinflammatory cytokines and receptors (IL-1β/IL-1R, IL-6/IL-6R, and TNFα/TNFαR). Proinflammatory cytokines are input nodes to the network, which physiologically represents a neuronal microenvironment created by surrounding neuronal-immune system components such as Schwann cells, damaged neurons, macrophages, and other leukocytes. The binding of TNFα to the TNFα receptor (TNFαR; TNFR1) results in activation of TNFR1-associated death domain protein (TRADD) and subsequent recruitment of three adapter proteins, FAS-associated death domain protein (FADD), TNF receptor-associated factor 2 (TRAF2), and receptor-interacting protein kinase 1 (RIPK1).\(^{87}\) TRAF2 activates components of the MAPK pathway (TAK1, ASK1, and MEKK1).\(^{87,88}\) RIPK1 activates the NFκB pathway by directly activating NFκB-inducing kinase (NIK; MAP3K14).\(^{89}\) NIK activates IκB kinase (IKK), which leads to the deactivation of IκB and subsequent activation of NFκB.\(^{90,91}\) FADD activates the extrinsic apoptosis pathway.

Apoptosis consists of two pathways, extrinsic and intrinsic. The extrinsic pathway becomes activated from external signals (TNFα and FasL) that bind to cell surface receptors (TNFαR and FasR) containing an intracellular death domain.\(^{92}\) Death domains, TRADD and FADD, transduce extracellular death signals into a cellular response through the recruitment and activation of two apical cysteine-aspartic proteases, caspases 8 (Casp8) and 10 (Casp10).\(^{92,93}\) Casp8/Casp10 cleave procaspases 3 and 7 into their active forms, caspase 3 (Casp3) and 7 (Casp7).\(^{94}\) The intrinsic apoptosis pathway involves cytotoxic insults that permeabilize the outer mitochondrial membrane and release intermembrane proteins into the cytosol. Although multiple mechanisms are involved in mitochondrial membrane permeabilization during cell death, only activation of the mitochondrial permeability transition pore (mPTP; MPT pore)
was included in the model.\textsuperscript{28} The mPTP becomes active when either Ca\textsuperscript{2+} or ROS are active.\textsuperscript{32} After membrane permeabilization, pro-apoptotic proteins, cytochrome C (Cyc), and the serine protease HtrA2/Omi, are released into the cytosol.\textsuperscript{29} mPTP and BAX are both required for the activation, or release into cytosol, of Cyc and HtrA2/Omi.\textsuperscript{95} HtrA2/Omi cleaves and inhibits the pro-survival protein X-linked inhibitor of apoptosis protein (XIAP).\textsuperscript{96} Cyc activates caspase 9 (Casp9), which activates Casp3 and Casp7.\textsuperscript{77} Effector caspases (Casp3 and Casp7) are responsible for the proteolytic degradation and induction of apoptosis.\textsuperscript{77} The cleavage of poly (ADP-ribose) polymerase 1 (PARP1) by Casp3/Casp7 is used as a surrogate marker of apoptosis.\textsuperscript{97}

The binding of an NMDA agonist, such as glutamate or glycine, to the NMDA receptor (NMDAR) and AMPA receptor-mediated membrane depolarization facilitates the activation of NMDA receptors.\textsuperscript{98} Upon NMDAR activation, Mg\textsuperscript{2+} dissociates from the channel pore and enables Ca\textsuperscript{2+} to traverse through the open pore, which increases intracellular Ca\textsuperscript{2+} concentration.\textsuperscript{99} This cellular process is represented, simplistically, by the incorporation of a stimulatory edge between NMDAR and Ca\textsuperscript{2+}. Intracellular Ca\textsuperscript{2+} plays an important role in a wide array of signal transduction processes. Ca\textsuperscript{2+} activates cPkc, calmodulin (Calm), calpain, and Ras.\textsuperscript{83,100} Ca\textsuperscript{2+} bound Calm activates Ca\textsuperscript{2+}/calmodulin-dependent protein kinases (Camk), which regulate gene transcription via ATF4.\textsuperscript{101} Calpain, a Ca\textsuperscript{2+} dependent cysteine protease, cleaves procaspase 12 into caspase 12 (Casp12), which leads to the downstream activation of intrinsic apoptotic caspases (Casp3, Casp7, and Casp9).\textsuperscript{102,103} NMDAR activation is also a source of ROS production. NMDAR activates NADPH oxidase 2 (Nox2) and neuronal nitric oxide synthase (nNos) to produce ROS and nitric oxide (NO).\textsuperscript{104,105} The production of Nox2-derived ROS requires the activation of nNos-derived NO.\textsuperscript{104,105} NMDAR also activates cyclin dependent kinase-5 (Cdk5)-mediated inhibition of anaphase complex/cyclosome-Cdh1 complex (APCCdh1; APC/C-Cdh1), which results in S-phase reentry and induction of apoptosis in post-mitotic neurons.\textsuperscript{106,107}
Inhibition of the proteasome leads to the accumulation of intracellular proteins and production of oxidative stress. Therefore, an inhibitory edge from the proteasome to ROS was incorporated. Protein accumulation and ROS induces endoplasmic reticulum stress (ER stress), which triggers the activation of components in the unfolded protein response such as inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase R-like endoplasmic reticulum kinase (PERK). In an ER stressed state, in which large amounts of misfolded proteins are present in the ER lumen, binding immunoglobulin protein (Bip; GRP78; HSPA5) dissociates from ATF6 and binds to hydrophobic regions on the misfolded proteins. IRE1, PERK, ATF6, and Bip are activated by ER stress. Upon removal of misfolded proteins in the ER lumen, Bip represses the activation of IRE1, PERK, and ATF6. IRE1 activates TRAF2 and X-box binding protein 1 (XBP1). XBP1, ATF4, ATF6, and p38 all directly activate CCAAT-enhancer-binding protein homologous protein (CHOP). CHOP is a transcription factor that upregulates pro-apoptotic genes (PUMA and Bim) and downregulates anti-apoptotic genes (Bcl-2). PERK activates activating transcription factor 3 (ATF3), which is a marker of tissue/nerve damage. PERK also activates eukaryotic translation initiator factor 2α (eIF2α), which inhibits protein translation.

Lastly, axonal transport was incorporated as another endpoint in the network. Kinesin, a motor protein, is composed of two heavy chains and two light chains. Kinesin light chain (KLC) binds various cargo and kinesin heavy chain (KHC) is responsible for the motor activity. KHC and KLC are regulated by JNK and GSK3B. KLC, KHC, and ATP are all required for axonal transport.

**Network Qualification**

The network was qualified using a gene expression profile from a microarray dataset of neurons isolated from ATF4 knockout mice with/without exposure to homocysteate, an NMDA receptor agonist.
Network-predicted changes in node activation states were compared to the Log$_2$ fold change in gene expression of differentially expressed genes. There were a total of 2,113 differentially expressed genes between ATF4 knockout murine neurons exposed to an NMDA agonist and treatment naïve wildtype neurons. Nineteen genes that exhibited differential expression overlapped with nodes in the network model. Model predictions agree with experimental observations for 16 out of 19 (84%) nodes (Supplementary Figure 1).

Centrality Analysis

Betweenness centrality on the network identified nodes that are important to network structure and information flow (Supplementary Figure 2). The node with the greatest betweenness centrality was c-Jun N-terminal kinase (JNK). Other species in the MAPK pathway also exhibited high betweenness centrality, such as TAK1, MKK4, MKK7, and p38. Components in the stress and unfolded protein response pathway were also highly central (ROS, ER stress, CHOP, and ATF4). A few nodes in the apoptosis pathway exhibited high betweenness centrality, such as HtrA2/Omi, XIAP, and Bax.

Network Simulations

Network simulations were performed to predict intraneuronal pharmacodynamics of proteasome inhibition (Figure 3). Network predictions for treatment naïve and proteasome inhibition are displayed on the left and right columns of Figure 3. Normalized expression of each node is displayed over time. Boolean functions were converted to normalized HillCube differential equations, and the expression of each node is a continuous variable between 0 and 1. Proteasome inhibition causes an accumulation of intracellular proteins and subsequently the production of reactive oxygen species (ROS), which induces the activation of ER Stress and activates components of the unfolded protein response (ATF6, PERK, IRE1). Several transcription factors increased in expression, such as ATF2, ATF3, ATF4, ATF6, XBP1, CHOP, and p53. An increase in intracellular calcium and ROS induce the activation of the mPTP, which
leads to apoptosis via intrinsic mechanisms. Pro-survival proteins (Bcl-2, Bcl-xL, XIAP) decrease and pro-apoptotic proteins (Bad, Bax, Bim, Casp3, Casp7, Casp9, Casp12, Cyc, HtrA2/Omi, and Puma) increase. Lastly, bortezomib resulted in the activation of neuronal apoptosis.

*Minimal Intervention Analysis*

Minimal intervention analysis identified 226 potential treatment strategies that prevent neuronal apoptosis induced via proteasome inhibition. The treatment strategies consist of 2 one-target, 109 two-target, and 115 three-target interventions. Single and two-target interventions are summarized in Table 2. Three-target interventions are summarized in Table 3. A complete list of all two/three-target intervention sets and the frequency of intervention of individual nodes are shown in Supplementary Tables 1 and 2. The frequency of node intervention relative to the total number of intervention sets for up to two-targets (111 intervention sets) and up to three-targets (226 intervention sets) are shown in Figures 4a and 4b. The two one-target interventions were PARP1/cPARP1. The cleavage of PARP1 into cPAPR1 was used as a surrogate marker of apoptosis in the model. Therefore, the inhibition of the cleavage of PARP1 would be the appropriate interpretation of this treatment strategy.

Components of the TNFα signaling pathway, TNFα, TNFαR, TRADD, and FADD, were the most perturbed nodes across all intervention sets (Figures 4a and 4b). The only two-target combination treatment strategy that did not contain an inhibitor of the TNFα signaling axis was the dual inhibition of Casp3 and Casp7. A TNFα inhibitor in combination with a pharmacological agent that modulates one of the 27 targets listed under Target 2 in Table 2 is predicted to prevent proteasome inhibitor induced neuronal apoptosis. The second targets include activation of anti-apoptotic proteins (Bcl-2, Bcl-xL, XIAP) as well as the inhibition of pro-apoptotic proteins (Bad, Bax, Casp12, Casp9, Cyc, HtrA2/Omi). The inhibition of the transcription factors CHOP and NFκB were also second targets. The stimulation of the IL-6/JAK/STAT
signaling axis was predicted to be neuroprotective. A TNFα inhibitor in combination with an inhibitor of ROS was also one of the two-target intervention sets.

Components of the TNFα signaling pathway, TNFα, TNFαR, TRADD, and FADD, were inhibited in 88 of 115 three-target intervention sets. Three-target intervention sets were summarized into five distinct groups (Target 2). The first group consists of the inhibition of nodes in the TNFα pathway (TNFα, TNFαR, TRADD, FADD), NMDA pathway (NMDA, NMDAR, NO, nNos), and MAPK pathway (MEKK1, MKK4, MKK7, TAK1). Intervention sets in the second group consist of a TNFα pathway inhibitor in combination with a Myc or Casp7 inhibitor, and a Bim, calcium, or calpain inhibitor. The third group contains TNFα pathway inhibitors (TNFα, TNFαR, TRADD), IL-1 pathway inhibitors (IL-1, IL-1R), and the inhibition of IRE1. The fourth group contains a FADD inhibitor, PERK or eIF2α inhibitor, and a PI3K-Akt pathway inhibitor (Akt, PI3K, PIP3). Lastly, the fifth group contains 27 of the 115 three-target intervention sets. This group did not require the inhibition of one of the nodes in the TNFα pathway, but required the dual inhibition of caspase 8/10 (Casp8 and Casp10) in combination with one of 27 different perturbations in order to prevent neuronal apoptosis in the presence of proteasome inhibition. The 27 perturbations are identical to the ones listed under Target 2 in Table 2.

**Attractor Analysis**

Using an exhaustive SAT-based search, 276 attractors were identified in the network. These attractors consisted of 266 fixed states, four 4-limit cycles, two 5-limit cycles, and four 10-limit cycles. A random search containing $10^6$ initial start states identified 256 of the 276 attractors. All of the identified attractors were fixed states, which indicates that the limit cycles are rare attractors with a small basin size.

Attractor analyses were performed to predict the therapeutic effect of a TNFα inhibitor, NMDA receptor antagonist, and ROS inhibitor, alone and in combination with each other for decreasing the frequency of
neuronal apoptosis. Results from the analysis are represented in a heatmap, where rows are nodes in the network and columns are the various perturbations (Figure 5a). The heatmap legend spans a continuous range from 0 (blue) to 1 (red), which indicates the relative activation frequency of nodes for each perturbation. In the bortezomib alone group (first column), nodes in the TNFα and NMDA pathways are 0.5. This is expected since bortezomib does not directly alter these pathways and randomly sampling 0 and 1, $10^6$ times, would average to be 0.5. ER stress and ROS increases to 1, which indicates that these nodes are active at steady-state across all $10^6$ simulations. The second-fourth columns (red) represent bortezomib in the presence of NMDA receptor antagonist, ROS inhibitor, or TNFα inhibitor monotherapies. The relative activation frequencies of nodes in the NMDA, ROS, and TNFα pathways decrease to zero in the presence of their respective inhibitors. Each of these single agents have differential effects on various pathways. For example, NMDA and ROS inhibition modulate calcium signaling, whereas TNFα inhibition does not. A trend of decreasing pro-apoptotic (Bax, Casp7, Casp3, HtrA2/Omi, Cyc, Casp9) and increasing pro-survival (Bcl-2, XIAP, Bcl-xL) species was shown. This trend becomes more pronounced for two-drug combination therapies (blue) and is clearly evident for the three-drug combination therapy (purple). The relative activation frequency of apoptosis ($\nu_{\text{apoptosis}}$) was determined for the eight perturbations (Figure 5b). $\nu_{\text{apoptosis}}$ was 0.688 in the bortezomib alone group, and decreased to 0.625, 0.513, and 0.375 in the presence of an NMDA inhibitor, ROS inhibitor, or TNFα inhibitor. $\nu_{\text{apoptosis}}$ decreased further for the two-target combination, and was equal to zero when all three inhibitors were present.

**Perturbation Analysis**

Results from perturbation analyses for the knockout and knockin of network nodes under treatment naïve conditions are shown in Supplementary Figures 3 and 4. Perturbation indexes (PI) are shown in blue, black, and orange, which represent a decrease, no/small change, and increase in node activation as a result of the perturbation. The network appears to be rather insensitive to knockout perturbations.
considering the majority of the matrix cells are black (Supplementary Figure 3). However, the network is highly sensitive to a knockout of the proteasome and Bip. The knockout of the proteasome and Bip result in increases in ROS and activation of the unfolded protein response. However, the knockout of Bip, unlike the proteasome, does not result in the activation of apoptosis. The network appears to be sensitive to knockin perturbations since several nodes became upregulated as shown in orange (Supplementary Figure 4). Thirty knockin perturbations resulted in similar effects and the activation of apoptosis. These perturbed nodes can be grouped into the following signaling pathways: TNFα/FAS death receptor pathway (TNFα, TNFαR, TRADD, TRAF2, FasR, FasL, DAXX), NMDA pathway (NMDAR, NMDA, NO, nNos), oxidative/ER stress related pathways (XBP1, ROS, ER stress, eIF2α, IRE1, CHOP, ATF6, ATF4, PERK), calcium regulation (Camk, Calm, Calcium), MAPK pathway (MAPKAPK2, JNK, ASK1, p38, MLK3), and the IL-1 pathway (IL-1β, IL-1R).

Network sensitivity changes were monitored in the presence of proteasome inhibition, which may provide insight into potential therapeutic interventions to prevent neurotoxicity associated with proteasome inhibition (Figure 5). The knockout of ROS and ER stress often results in the opposite effects of proteasome inhibition. Interestingly, the inhibition of the BDNF-TrkB axis decreased apoptosis and resulted in similar effects as the ROS and ER stress knockouts. Thirty nodes exhibited similar neuroprotective effects and decreased apoptosis in the presence of proteasome inhibition. These neuroprotective knockout perturbations are grouped into the following pathways: MAPK pathway (MKK4, JNK, MKK7, MEKK1, TAK1, MKK3, p38, MKK6, ASK1), ER stress pathway (CHOP, Bip, IRE1), apoptosis pathway (Casp12, Cyc, Casp9, mPTP, HtrA2/Omi, Bax, PUMA, Bad, ATM, p53), calcium regulation (Calpain, IP3, IP3R, Calcium), and other (TRAF2, PLCG1, RAS). Results from the knockin perturbations in the presence of proteasome inhibition are shown in Supplementary Figure 6. Seven nodes exhibited neuroprotective effects and decreased apoptosis in the presence of proteasome.
inhibition. These nodes consist of pro-survival proteins (Bcl-2, Bcl-xL, XIAP) and the IL-6 signaling pathway (IL-6, IL-6R, JAK, STAT).

Discussion

Peripheral neuropathy caused by cancer chemotherapy remains an unmet medical need. In this study, a network-based systems pharmacology model of intracellular signaling and gene regulation in peripheral neurons was constructed and analyzed, which enabled the identification of novel combinatorial treatment strategies that potentially could prevent CIPN. The network analyses that were conducted focused on the inhibition of the proteasome, which emulates the direct on-target mechanism of toxicity of proteasome inhibitors (e.g., bortezomib, carfilzomib, and ixazomib). However, the network model can be expanded to incorporate additional mechanisms of toxicity in order to predict the neurotoxic effects of other chemotherapeutics, such as taxanes, vinca alkaloids, and platinum agents. For example, nodes that represent α/β-tubulin, and components involved in the regulation/dysfunction of microtubule dynamics, would need to be incorporated to extend the model to capture the neurotoxic effects of antimitotic agents (e.g., paclitaxel, vincristine, and monomethyl auristatin E). Nodes representing the formation of DNA adducts, activation of DNA damage response, and DNA repair mechanisms could be incorporated to account for toxicity of platinum agents (e.g., cisplatin and oxaliplatin). Considering the paucity of molecular signaling data from human primary peripheral sensory neurons, the network model was constructed using multiple sources of data ranging across species and cell types. Efforts were made during model construction to prioritize data obtained from immortalized neurons of human origin (e.g. SH-SY5Y and PC12 cells) and primary cultures of rodent-derived neurons. Several of the cellular transduction pathways modeled are well conserved across species and present in all mammalian cells.¹²⁰ Network predictions indicated that proteasome inhibition resulted in the production of oxidative/ER stress and activation of the intrinsic apoptosis pathway. However, in the model, proteasome inhibition
does not activate the TNFα signaling pathway, which assumes that proteasome inhibitor-induced neuronal apoptosis can occur in the absence of proinflammatory cytokines. This is reasonable since peripheral neuropathy still manifests in immunodeficient mice exposed to bortezomib.\textsuperscript{121,122} Cytokines serve as model inputs as they are not regulated by any nodes of the network. Macrophages, satellite glial, and other immune components that are responsible for the production of proinflammatory cytokines, and regulation of the peripheral nerve microenvironment could be incorporated into the model to more accurately represent the \textit{in vivo} system.

Key findings of the \textit{in silico} model are:

1. Single target therapy is likely inadequate for the prevention of CIPN.
2. The TNFα signaling pathway must remain inactive in order to prevent neuronal apoptosis and the development of CIPN.
3. The combinatorial inhibition of TNFα, NMDAR, and ROS may prevent the activation of proteasome inhibitor-induced neuronal apoptosis across all network attractors.
4. The network model is sensitive to proteasome inhibition, oxidative/ER stress, proinflammatory cytokines (TNFα, IL-6, and IL-1β), NMDAR activation, and JNK/p38 MAPK pathway activation.

The inhibition of the cleavage of PARP1 was identified as the only single target treatment strategy via the minimal intervention algorithm; however, this should be interpreted with caution since the cleavage of PARP1 was used as a marker of apoptosis in the network model. Results from minimal intervention analysis identified 109 two-target and 115 three-target interventions that prevent neuronal apoptosis in the presence of proteasome inhibition. Although it would be impractical experimentally to evaluate all 224 possible combinations, the number of combinations is significantly smaller compared to two \(8,515; \frac{131!}{2! \times 129!}\) and three \(366,145; \frac{131!}{3! \times 128!}\) target combinations selected from the network at
random. Considering the number of possible two-/three-target combinations in the druggable genome (~10^7-10^8), 224 is small in comparison.\textsuperscript{123,124}

Minimal intervention analysis results suggest that the TNFα signaling pathway could be an important pathway for neuronal apoptosis and the development of CIPN. All two-target combinations inhibited at least one node in the TNFα signaling pathway (TNFα, TNFαR, TRADD, FADD) and the three-target combinations that did not inhibit one of these nodes required the dual inhibition of caspase 8/10. Hence, it is likely essential for TNFα-mediated extrinsic apoptosis pathway to remain inactive in order to prevent neuronal apoptosis in the presence of proteasome inhibition. Proinflammatory cytokines and immune cells are important components in the pathogenesis of CIPNs. However, the temporal dynamics and relative contribution of the immune system to the development/severity of CIPN and the temporal dynamics remains to be elucidated. The upregulation of TNFα in DRG neurons following peripheral nerve injury and its role in neuropathic pain is well known.\textsuperscript{125,126} The molecular etiologies of neuropathic pain and cytokine expression in the DRG following neurotoxic chemotherapies have been studied less. Bortezomib has been shown to increase the expression of proinflammatory cytokines in the DRG, and the co-administration of an anti-TNFα antibody exhibited partial neuroprotection in rodents.\textsuperscript{46,48} The partial restoration in murine sensory nerve action potential is in agreement with the results from the model, which indicates that anti-TNFα therapy should be combined with the modulation of another target in order to achieve a full response. The gut microbiota in mice was shown to be important for development of oxaliplatin-induced mechanical hyperalgesia, which is likely in part due to decreased inflammatory cytokines produced by macrophages in the DRG.\textsuperscript{127} The administration of the anti-inflammatory cytokine IL-10 attenuated paclitaxel induced allodynia in mice, and CD8+ T cells are responsible for the resolution of CIPN.\textsuperscript{128}

Several testable hypotheses were generated for potential combination treatment strategies with anti-TNFα therapy, which are listed under Target 2 in Table 2. Among these are proapoptotic/antiapoptotic
proteins, MAPKs, oxidative/ER stress related targets, and others. Antioxidants that decrease ROS, such as vitamin E, alpha lipoic acid, and acetyl-L-carnitine, have been tried clinically with mixed success. Based upon model simulations, one hypothesis is that antioxidant therapy may successfully prevent, or delay the onset, of CIPN in patients with a low immunological burden on the DRG. In other words, patients that exhibit a strong local immune response in the DRG after chemotherapy would not respond well to antioxidant therapy.

An attractor analysis was conducted to determine the relative activation frequency of apoptosis for the combination of anti-TNFα and antioxidant therapy in the presence of proteasome inhibition. The activation of apoptosis was not completely inhibited, which was different than results from the minimal intervention analysis. This discrepancy likely is based on the nature of minimal intervention analyses, which is a model checking algorithm based on satisfying the defined goal at a logical steady-state. This is in contrast to an attractor analysis that considers the dynamical trajectory across a network state space. For triple combinations, the NMDA signaling pathway was a frequently inhibited pathway, and NMDA receptors have been considered as a potential target for the treatment of bortezomib-induced neuropathic pain.\textsuperscript{129,130} The combinatorial inhibition of TNFα, ROS, and NMDAR resulted in an activation frequency of apoptosis equal to zero, which suggests that this treatment strategy may be useful for the prevention of neuronal apoptosis and peripheral neuropathy caused by proteasome inhibition.

The construction of a quantitative systems pharmacology model, based on ordinary differential equations, is limited by the availability of data required to parameterize reaction rates with meaningful precision. Although Boolean network models do not require prior knowledge of parameter values for kinetic processes, this mathematical framework vastly simplifies biological processes. Time is expressed as arbitrary units or time steps that cannot be related to physical time. The expression of network nodes is constrained to zero and one, which restrains predictions to be qualitative in nature. However, despite the temporal and spatial limitations, the Boolean network could be used to generate testable
hypotheses that could lead to an effective treatment strategy for the prevention of CIPN. The combinatorial inhibition of TNFα, NMDAR, and ROS may prevent the development of proteasome inhibitor induced-peripheral neuropathy, which could be evaluated in experimental models of CIPN. The model presented in this study could be used as the foundation for the future development of a quantitative model as data become available. The model could be extended also to include components of the peripheral nervous system, immune system, and other physiologically relevant systems. Lastly, the network model potentially could be modified to study diabetic neuropathy and central neurodegenerative diseases.
Figures and Tables

Figure 1: Workflow for Boolean network development methodology. Constructed started with compiling nodes and determining edges, followed by addition of Boolean logic and selection of initial conditions. These steps were informed using information in the scientific literature, referencing various pathway databases, and using a prior pharmacological interaction network. Following the construction of the Boolean network, network was revised and qualified using external datasets prior to performing analyses.

Figure 2: Network-based systems pharmacology model of intracellular signaling in peripheral neurons. The network contains 131 nodes and 252 edges. Nodes: transcription factors (blue), GTPases (purple), enzymes (dark green), kinases (pink), phosphatases (light green), endpoints (light blue), pro-apoptosis (red), pro-survival (lime green), not categorized (gray), ligands (tan diamond), receptors (tan rotated flags). Edges: stimulatory edge (black arrowhead) and inhibitory edge (white diamond).

Figure 3: Systems pharmacology network model simulations of intraneuronal signaling in the absence (left column) and presence (right column) of proteasome inhibition. The Boolean network model was converted to normalized HillCube differential equations using Odefy and default parameter values were retained (\( \tau = 1; k = 0.5; n = 3 \)). Simulation was performed for 25 time steps expressed as arbitrary time units. Network nodes are shown on the y-axis and the heatmap represents the activation state of each node. The activation states of nodes are continuous between 0 (dark blue) and 1 (dark red) as shown in the legend on the right.

Figure 4: Results from minimal intervention analysis. Network perturbations that result in apoptosis remaining in the OFF state in the presence of proteasome inhibition were identified for up to (a) two-node and (b) three-node interventions. The x-axis depicts the perturbed nodes and the number of times a node was perturbed relative to the total number of intervention sets is shown on the y-axis.
potential treatment strategies were identified: 2 one-target, 109 two-target, and 115 three-target interventions. Red bars indicate the inhibition of a node (OFF) and blue bars indicate the stimulation of a node (ON).

**Figure 5:** Results from attractor analysis. Heatmap representing the relative activation frequency of (a) select network nodes and (b) apoptosis across identified attractors for eight network perturbations. The eight perturbations are proteasome inhibition (bortezomib) in combination with a TNFα inhibitor, NMDA receptor antagonist, and/or ROS inhibitor. Monotherapies, two-drug combinations, and the three-drug combination are highlighted in red, blue, and purple.

**Supplementary Figure 1:** Boolean network predictions compared against an experimental microarray dataset of ATF4 knockout murine neurons exposed to an NMDA agonist (GSE10470). Model predictions for 16 of 19 nodes agree with experimental observations. Y = yes (agree); N= no (disagree).

**Supplementary Figure 2:** Betweenness centrality for each node was determined using yEd graph editor. Nodes with high betweenness centrality are larger in size and red in color, whereas nodes with low betweenness centrality are depicted smaller and blue in color.

**Supplementary Figure 3:** Results from a knockout perturbation analysis under treatment naïve conditions. The `KO_matrix.f` function in the R-package SPIDDOR was used to evaluate the effect of single node knockouts on the network by computing a perturbation index (PI), where PI is the ratio of fractional activation of a node in the presence of a perturbation versus normal conditions. Asynchronous updating for 2000 time steps and 25 repetitions. Knockout perturbations are listed as columns of the matrix. The effect individual knockouts on all network nodes are shown in rows of the matrix. The color of each cell indicates the degree of perturbation. Black has a perturbation index value between 0.8 and 1.25, blue is less than 0.8, and orange is greater than 1.25.
**Supplementary Figure 4:** Results from an overexpression perturbation analysis under treatment naïve conditions. The OE_matrix.f function in the R-package SPIDDOR was used to evaluate the effect of single node overexpression (knockin) on the network by computing a perturbation index (PI), where PI is the ratio of fractional activation of a node in the presence of a perturbation versus normal conditions. Asynchronous updating for 2000 time steps and 25 repetitions. Knockin perturbations are listed as columns of the matrix. The effect individual knockouts on all network nodes are shown in rows of the matrix. The color of each cell indicates the degree of perturbation. Black has a perturbation index value between 0.8 and 1.25, blue is less than 0.8, and orange is greater than 1.25.

**Supplementary Figure 5:** Results from a knockout perturbation analysis under proteasome inhibition. The KO_matrix.f function in the R-package SPIDDOR was used to evaluate the effect of single node knockouts on the network by computing a perturbation index (PI), where PI is the ratio of fractional activation of a node in the presence of a perturbation versus normal conditions. Note that the normal condition in this analysis is proteasome inhibition. Asynchronous updating for 2000 time steps and 25 repetitions. Knockout perturbations are listed as columns of the matrix. The effect individual knockouts on all network nodes are shown in rows of the matrix. The color of each cell indicates the degree of perturbation. Black has a perturbation index value between 0.8 and 1.25, blue is less than 0.8, and orange is greater than 1.25.

**Supplementary Figure 6:** Results from an overexpression perturbation analysis under proteasome inhibition. The OE_matrix.f function in the R-package SPIDDOR was used to evaluate the effect of single node overexpression (knockin) on the network by computing a perturbation index (PI), where PI is the ratio of fractional activation of a node in the presence of a perturbation versus normal conditions. Note that the normal condition in this analysis is proteasome inhibition. Asynchronous updating for 2000 time steps and 25 repetitions. Knockin perturbations are listed as columns of the matrix. The effect individual knockouts on all network nodes are shown in rows of the matrix. The color of each cell indicates the
degree of perturbation. Black has a perturbation index value between 0.8 and 1.25, blue is less than 0.8, and orange is greater than 1.25.

**Table 1**: Logic functions, initial conditions, and references for nodes in the Boolean network-based systems pharmacology model of cellular signal transduction of gene regulatory processes in neurons.

**Table 2**: Results from Minimal Intervention Analysis for Two-Target Interventions that Prevent Neuronal Apoptosis in the Presence of Proteasome Inhibition.

**Table 3**: Results from Minimal Intervention Analysis for Three-Target Interventions that Prevent Neuronal Apoptosis in the Presence of Proteasome Inhibition

**Supplementary Table 1**: List of Minimal Intervention Sets for Two-Target Interventions that Prevent Neuronal Apoptosis in the Presence of Proteasome Inhibition

**Supplementary Table 2**: List of Minimal Intervention Sets for Three-Target Interventions that Prevent Neuronal Apoptosis in the Presence of Proteasome Inhibition
Figures and Tables

Figure 1:

1) Pathway Databases
2) Scientific Literature
3) Prior Network
Figure 3

Proteasome Inhibition

Treatment Naïve

Time (arbitrary units)

Time (arbitrary units)
Figure 5b
Supplementary Figure 1

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Table 2: Results from Minimal Intervention Analysis for Two-Target Interventions that Prevent Neuronal Apoptosis in the Presence of Proteasome Inhibition

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Table 3: Results from Minimal Intervention Analysis for Three-Target Interventions that Prevent Neuronal Apoptosis in the Presence of Proteasome Inhibition

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Chapter 6:

Pharmacodynamics of Dexanabinol in SH-SY5Y Cells and

a Microphysiological Model of Peripheral Nerves

Peter Bloomingdale¹, Kevin Pollard², Michael J. Moore², and Donald E. Mager¹

1. Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, The State University of New York, Buffalo, NY

2. Department of Biomedical Engineering, School of Science and Engineering, Tulane University, New Orleans, LA
Abstract

In the previous chapter, a network-based systems pharmacology model was developed to identify therapeutic targets for chemotherapy-induced peripheral neuropathy (CIPN). Network analyses suggested that a combinatorial treatment strategy consisting of a TNFα inhibitor, reactive oxygen species (ROS) inhibitor, and N-methyl-D-aspartate (NMDA) receptor antagonist would prevent the activation of bortezomib-induced neuronal apoptosis. Dexanabinol is a cannabinoid derivative that has been shown to inhibit all three of these targets. The potential of dexanabinol for preventing bortezomib neurotoxicity was evaluated, and network simulations were performed to confirm that dexanabinol prevents neuronal apoptosis caused by bortezomib. Initial experiments consisted of cytotoxicity studies using SH-SY5Y cells, which exhibited no sign of neuroprotection. However, dexanabinol partially restored bortezomib-induced decreases in proximal action potential amplitude and distal nerve conduction velocity in a microphysiological system of peripheral nerves.
Introduction

Chemotherapy-induced peripheral neuropathy (CIPN) is a common adverse side effect of cancer chemotherapy that can be life debilitating and cause extreme pain. Approximately 30-40% of patients undergoing cancer chemotherapy develop peripheral neuropathy, and these effects can be irreversible. Symptoms range from numbness and tingling to extreme pain. In severe cases, peripheral neuropathy can be life threatening when autonomic functions, such as breathing and heartbeat, become compromised. There are no clinically approved drugs for the treatment or prophylaxis of CIPN. When symptoms of CIPN become severe, the present treatment options are to either reduce the dose of chemotherapy or completely discontinue therapy. The National Cancer Institute sponsored 15 clinical trials to investigate the prevention and symptomatic treatment of chemotherapy-induced peripheral neuropathy. Duloxetine was the only drug to exhibit efficacy in some patients for treating neuropathic pain caused by CIPN. However, no drugs have been found that prevent the development of CIPN.

The use of Cannabis sativa has a long history for its therapeutic use in the treatment of pain. Cannabinoids, a class of compounds that act on cannabinoid receptors, have been evaluated clinically for a wide range of therapeutic uses including appetite stimulation, anxiety, glaucoma, nausea/vomiting, sleep, and pain. The antinociceptive effects of cannabinoids have been assessed in animal models of nerve injury and various disease-related origins of neuropathic pain. There has been success by various cannabinoids (WIN55,212-2, MDA7, (R)-AM1241, AM1714) for reversing paclitaxel and vincristine induced mechanical allodynia. However, tolerance due to receptor desensitization, physical withdrawal, and unwanted psychoactive effects of centrally-acting cannabinoid agonists that activate cannabinoid receptors in the brain has limited their therapeutic potential.

Experimental models of CIPN include in vitro cell/tissue cultures as well as in vivo animal models. Primary dorsal root ganglion (DRG) isolated from embryonic and adult rodents have been used to assess
the neurotoxicity of chemotherapeutics. Additionally, primary neurons can be co-cultured with Schwann cells to promote myelination. An alternative to primary rodent cultures is the use of human SH-SY5Y neuroblastoma cells, which have been used to study chemotherapy-induced peripheral neuropathy and neurodegenerative diseases. The use of human induced pluripotent stem cells (iPSCs) derived neurons has gained attention for their potential applications in drug neurotoxicity testing. Rodent models of chemotherapy-induced peripheral neuropathies are very common. However, even for the same chemotherapeutics, rodent studies often vary in the dose, frequency/route of administration, outcome measures, and characteristics of the animals (e.g., age, sex, and genetic background). Outcome measures may consist of behavioral, electrophysiological, and histological endpoints. Behavioral tests are commonly used to assess symptoms associated with peripheral neuropathy, such as mechanical allodynia using von Frey filaments. Electrophysiological tests that measure action potential amplitude and nerve conduction velocities can be performed to evaluate nerve function. Histological endpoints include measuring intraepidermal nerve fiber density and cross sections of nerves to identify degeneration of myelin and mitochondrial swelling.

Organs-on-chips, or microphysiological systems, have been proposed for in vitro toxicity testing. A microphysiological system of peripheral nerves, nerve-on-a-chip, has been developed by Dr. Moore’s lab. The incorporation of dorsal root ganglia explants into a 3D dual hydrogel construct creates a microphysiological experimental model that better represents a peripheral nerve and enables the measurement of electrophysiological endpoints in an in vitro system.

A prior network-based systems pharmacology model (Chapter 5) identified that a combinatorial treatment strategy consisting of a TNFα inhibitor, ROS inhibitor, and NMDA receptor antagonist may have therapeutic potential for preventing bortezomib-induced peripheral neuropathy. The synthetic cannabinoid derivative, dexanabinol, has been shown to inhibit all three of these targets. Dexanabinol does not exhibit any psychoactive effects and it not prone to cannabinoid receptor
desensitization since it does not bind to cannabinoid receptors.\textsuperscript{29,30} Dexanabinol was previously investigated for traumatic brain injury, but the phase III clinical trial failed to show efficacy.\textsuperscript{31} Dexanabinol exhibited a favorable pharmacokinetic profile and was safe in humans.\textsuperscript{31-33}

The goal of this study is to investigate the therapeutic potential of dexanabinol for preventing bortezomib neurotoxicity. A network simulation confirmed that dexanabinol prevents neuronal apoptosis caused by bortezomib, and the cytotoxicity on SH-SY5Y cells was assessed. The neuroprotective effects of dexanabinol was also investigated using the peripheral nerve-on-a-chip model.

**Methods**

*Network Simulation*

A Boolean network model of intracellular signaling in peripheral neurons was developed previously (Chapter 5). The Boolean network model was converted to normalized HillCube differential equations and default parameter values were retained (\( \tau = 1; k = 0.5; n = 3 \)). The number of time steps was set to 25 and expressed as arbitrary time units. The pharmacodynamic effect of bortezomib was incorporated into the model by adding an inhibitory edge from a newly created bortezomib node to the proteasome. The pharmacodynamic effects of dexanabinol were incorporated into the model by adding an inhibitory edge from a newly created dexanabinol node to ROS, TNFR, and NMDAR. The Boolean operators used for the drug effects were ‘AND NOT’. Model simulations were performed in MATLAB using the Odefy toolbox.

*Cytotoxicity Study in SH-SY5Y Cells*
Cytotoxicity studies were performed using SH-SY5Y neuroblastoma cells for single agent treatment as well as the combination. SH-SY5Y cells were grown at 37°C and 5% CO$_2$ in DMEM:F12 media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (10,000 U/mL; 10,000 μg/mL) until a sufficient number of cells were obtained. SH-SY5Y cells were seeded at a density of 100,000 cells/well in 96 well plates and were allowed to equilibrate for a day. Various concentrations of bortezomib (0.001, 0.01, 0.1, 1, 10, 100, 1000 nM) and dexanabinol (1, 10, 25 μM) were added to their respective wells. The concentration ranges were selected in order to achieve a wide range of concentrations above the IC$_{50}$ of bortezomib and spanning the potential therapeutic range of dexanabinol. Cell viability was measured using a colorimetric reagent (WST-1). After 48 hours of drug exposure, 10% v/v of WST-1 was added to each well. Plates were briefly shaken and placed into the incubator for 2 hours. Absorbance was measured using a SpectraMax 190 microplate reader at a wavelength of 450 nm (690 nm reference wavelength). The inhibitory Hill function was fit to the cell viability versus bortezomib concentration (C$_{Bort}$) profiles:

\[
\text{Cell Viability(\%) = } E_0 \times \left(1 - I_{\text{max}} \times \frac{C_{Bort}^V}{IC_{50}^V + C_{Bort}^V}\right)
\]  

(1)

Modeling was performed using the weighted least squares function, with general weighting, in ADAPT 5.

**Dexanabinol Neuroprotection using Nerve-on-a-Chip**

All animal handling and tissue harvesting procedures were performed under observation of guidelines set by NIH and the Institutional Animal Care and Use Committee (IACUC) at Tulane University. Constructs were micropatterned using an apparatus for dynamic mask projection photolithography. 35,36

6-well collagen-coated PTFE cell culture inserts were soaked overnight in adhesion media consisting of Neurobasal medium supplemented with penicillin/streptomycin, nerve growth factor (NGF), 10% fetal bovine serum, and L-glutamine. DRG were harvested from Day 15 embryonic rats and incorporated into
a 3D dual (polyethylene glycol and gelatin methacrylate) hydrogel construct. Tissue explants were grown for 1 day in neural growth media (Neurobasal medium supplemented with NGF, penicillin/streptomycin, L-glutamine, and B27). Nerves were grown for 7 days in premyelination media to permit Schwann cell proliferation. Nerves were then grown in myelination media for 14 days to promote myelination. The constructs were split into four groups: (1) treatment naïve, (2) dexanabinol, (3) bortezomib, and (4) dexanabinol + bortezomib. Nerves were exposed to various concentrations of bortezomib and dexanabinol for 48 hours. Distal (2.25 mm) and proximal (1.5 mm) measurements of nerve conduction velocity and action potential amplitudes were recorded for each treatment group.

Statistical Analyses

All statistical analyses were performed using GraphPad Prism version 7.04 for Windows (La Jolla, California). The change in nerve-on-a-chip NCV and action potential amplitude for various concentrations of bortezomib was assessed using one-way ANOVA with Dunnet’s correction for multiple comparisons. Comparisons were made for each group with respect to the vehicle control. The change in nerve-on-a-chip NCV and action potential amplitude for the combination of dexanabinol (10 and 25 μM) and bortezomib (100 nM) was assessed using one-way ANOVA with Tukey’s correction for multiple comparisons. Comparisons were made across all groups.

Results

Network Model Prediction of Dexanabinol Neuroprotection

The intraneuronal pharmacodynamics of bortezomib in the absence and presence of dexanabinol were simulated using a network model of cellular signaling in peripheral neurons (Figure 1). Bortezomib caused a decrease in activity of the proteasome, an increase in ROS, increases in caspases, and apoptosis (Figure 1, top). Model predictions suggest that the combination of bortezomib and dexanabinol prevents the activation of ROS, intracellular calcium, caspases, and neuronal apoptosis (Figure 1, bottom).
In Vitro Cytotoxicity Studies in SH-SY5Y Cells

SH-SY5Y cell viability as a function of bortezomib concentrations in the absence of dexamabinol is shown in Figure 2 (red), and the estimated IC$_{50}$ was 4.40 nM. Figure 2 also includes SH-SY5Y cell viability across various bortezomib concentrations in the presence of 1 μM (green) or 10 μM (blue) of dexamabinol. The estimated IC$_{50}$ of bortezomib in the presence of 1 μM and 10 μM dexamabinol was 7.00 and 4.62 nM. An increase in the IC$_{50}$ of bortezomib in the presence of dexamabinol was expected, which would have indicated a neuroprotective effect. Although the IC$_{50}$ of bortezomib did slightly increase in the presence of dexamabinol, the increase was not statistically significant.

Investigation of Dexamabinol for CIPN using Nerve-on-a-Chip

Nerve-on-a-chip neurite outgrowth staining is shown in Figure 3. Red and green color indicates neurites and myelin, and yellow indicates their co-localization. NCV and action potential amplitudes were stimulated distal (2.25 mm) and proximal (1.5 mm) to the recording site at the neuronal soma (Figure 3, bottom).

Nerve-on-a-chip NCV and action potential amplitude was measured for various concentrations of bortezomib to obtain dose-response profiles (Figures 4a-d). Proximal and distal recordings of action potential amplitudes exhibit a decreasing trend for increasing concentrations of bortezomib (Figures 4a and b). However, there were no statistically significant differences in amplitudes between the control and any of treatment groups. The overall trend for the change in proximal and distal recordings of NCV was also decreasing with increasing concentrations of bortezomib. Proximal NCV at 1000 nM of bortezomib decreased; however, this was not statistically significant (p-value = 0.054, Figure 4c). There was a significant decrease in distal NCV for the 100 and 1000 nM concentrations of bortezomib (Figure 4d).
The effects of dexanabinol on action bortezomib-induced changes in potential amplitude (Figure 5) and NCV (figure 6) were tested. Electrophysiological endpoints were normalized to their control values. The effects of 10 and 25 μM dexanabinol on NCV and action potential amplitude were not significantly different than control, which indicates that dexanabinol is not neurotoxic at the selected concentrations. Nerves exposed to 100 nM of bortezomib for 48 hours exhibited a significant decrease in the proximal amplitude and distal NCV. The treatment group of 100 nM of bortezomib in combination with 10 μM of dexanabinol resulted in NCVs and amplitudes that were no longer significantly different than control. However, there was no significant difference observed in distal NCV between bortezomib alone and in combination with 10 μM of dexanabinol (p-value = 0.070). The combination of bortezomib with a higher concentration of dexanabinol (25 μM) decreased amplitudes further and did not affect NCV.

Discussion

In this chapter, network-based in silico predictions, in vitro cytotoxicity studies, and electrophysiological examinations using a microphysiological peripheral rat nerve were studied to assess the pharmacological efficacy of dexanabinol for alleviating bortezomib-induced neurotoxicity. The overall results suggest that dexanabinol exhibits signs of neuroprotection against the neurotoxic effects of bortezomib.

Network simulations suggested that dexanabinol prevents bortezomib-induced increases in ROS, calcium, and the intrinsic apoptosis pathway (Figure 1). However, due to limitations of the modeling framework, the extent by which dexanabinol modulates the cellular pharmacodynamics of bortezomib is incompletely known. For example, dexanabinol likely acts by decreasing, but not completely preventing, the production of oxidative species by bortezomib. Dose-response relationships between bortezomib/dexanabinol exposure and cellular pharmacodynamics (ROS production, TNFα activity, and NMDAR activation) is important to characterize the potential for dexanabinol to reduce bortezomib
neurotoxicity. However, elucidation of the extent by which each of these individual pathways are modulated by dexamabinol and contribute to the neurotoxicity of bortezomib is beyond the objective of this study.

Dexamabinol was previously shown to prevent the inhibition of LPS-induced TNFα production in macrophages and inhibit NMDA-induced neurotoxicity in rat-derived neurons.\textsuperscript{29,37} The EC\textsubscript{50} for inhibiting TNFα production and NMDA-induced neurotoxicity was 9.05 and 3.57 μM (Appendix II). This suggests that dexamabinol is more potent for decreasing NDMA-induced neurotoxicity than inhibiting the production of TNFα, but the EC\textsubscript{50} values are still within 2-3 fold. The ability of dexamabinol to scavenge oxidative species is lower (EC\textsubscript{50} = 29.7 μM) relative to its other pharmacodynamic effects (Appendix II). Dexamabinol therapy could be considered with an additional antioxidant (e.g., vitamin E, N-acetylcysteine, and acetyl-L-carnitine) to compensate for the relatively lower antioxidant activity. However, all of the EC\textsubscript{50} values are well below observed plasma concentrations in humans.\textsuperscript{32}

Results from the \textit{in vitro} study using SH-SY5Y cells suggested that dexamabinol is not neuroprotective for altering bortezomib-induced cell death. Additional greater concentrations of dexamabinol were tested, and cytotoxic effects were observed when concentrations were above 25 μM. Although the IC\textsubscript{50} of bortezomib in the presence of dexamabinol did not change, maximal cell viability was greater when SH-SH5Y cells were exposed to 10 μM of dexamabinol compared to bortezomib alone (123% vs. 105%). Since cell viability was measured using a colorimetric reagent and normalized to control, percentages can exceed 100%. The 95% confidence interval around the E\textsubscript{0} for SH-SY5Y cells exposed to bortezomib and 10 μM of dexamabinol was 107% to 139%, which does not include the estimated E\textsubscript{0} for bortezomib alone (105%). This suggests that low concentrations of dexamabinol may be altering the growth of these cells.

One limitation of using SH-SY5Y cells for neurotoxicity testing is that these cells are immortalized and are characteristically different from the physiological system of interest (i.e., human peripheral neurons).
The anticancer properties of dexanabinol could be masking any neuroprotective effects. Dexanabinol is currently being investigated in clinical trials for hepatocellular carcinoma and pancreatic cancer. A preclinical study investigating the combination of anti-CTLA4 antibody with dexanabinol in an immune-competent mouse model of colon cancer exhibited a significant improvement in partial/complete tumor regression compared to anti-CTLA4 monotherapy.38

The neuroprotective effects of dexanabinol were also tested using the peripheral nerve-on-a-chip model. 3D microphysiological systems have been gaining popularity since they better represent in vivo physiology compared to traditional 2D in vitro models.26,39,40 One advantage of this experimental model is the ability to obtain electrophysiological measurements of NCV and action potential amplitude. Nerve conduction studies are important for the objective assessment of peripheral nerve damage and diagnosis/grading of peripheral neuropathy.41,42 A dose-response relationship for bortezomib exposure and electrophysiological endpoints (NCV and action potential amplitude) was obtained (Figure 4). Higher concentrations of bortezomib resulted in a significant decrease in NCV and amplitudes. The magnitude of change in NCV and action potential amplitude for distal measurements were often lower than proximal measurements. This observation may reflect the common dying back phenomena associated with CIPN, where longest and most vulnerable neurons are affected first.2,43,44 Bortezomib neurotoxicity was evaluated at 100 nM; however, clinically relevant steady-state plasma concentrations of bortezomib are in the range of 5-25 nM. Long term exposure (several weeks/months) at lower concentrations of bortezomib would be ideal, but it is currently limited by experimental constraints regarding the ex vivo lifespan of the tissue. Both high (25 μM) and low (10 μM) concentrations of dexanabinol alone did not exhibit signs of toxicity. The lower concentration of dexanabinol reversed bortezomib-induced decreases in proximal amplitude and distal NCV. The higher concentration of dexanabinol was not neuroprotective and appeared to exacerbate bortezomib-induced decreases in action potential amplitudes. This may be due to a narrow therapeutic window for NMDA receptor activity, where low concentrations of
Dexanabinol are neuroprotective and high concentrations are neurotoxic. NMDA receptors are important for the growth and survival of neurons, and long-term antagonism causes neurodegeneration.⁴⁵-⁴⁸ A transient inhibition of NMDA receptors during the most prominent time of nerve injury, administration of bortezomib (Cmax), followed by a washout period to enable glutamate mediated neurogenesis could be a rational dosing strategy. In contrast to traumatic brain injury, this dosing strategy might be possible for chemotherapy-induced peripheral neuropathy.⁴⁵

Conclusion

Dexanabinol exhibits signs of neuroprotection against the neurotoxic effects of bortezomib. Considering the unmet medical need of preventing or treating CIPN, this evidence may warrant further testing of dexanabinol for preventing bortezomib-induced peripheral neuropathy in preclinical models of CIPN.
**Figures and Tables**

**Figure 1:** Network model simulations of the intraneuronal pharmacodynamics for bortezomib in the (a) absence and (b) presence of dexanabinol. Boolean network model was converted to normalized HillCube differential equations using Odefy, and default parameter values were retained \((\tau = 1; k = 0.5; n = 3)\). Time is expressed as arbitrary time units (AU). Proteasome (green), TNFα receptor (purple), reactive oxygen species (blue), caspase 3 (orange), cytochrome C (light blue), calcium (pink), NFκB (gray), and apoptosis (red).

**Figure 2:** Cytotoxicity of SH-SY5Y cells exposed to various concentrations of bortezomib in the absence (red) or presence of 1 μM (green) and 10 μM (blue) dexanabinol for 48 hours.

**Figure 3:** (Top) Nerve-on-a-chip neurite outgrowth staining. Red/green stains mark neurites/myelin, and yellow indicates their co-localization. (Bottom) Nerve-on-a-chip action potentials. Recordings from proximal (left) and distal (right) simulations are shown. Distal and proximal nerve conduction velocity and action potential amplitude was recorded 2.25 mm and 1.5 mm from the stimulation site. Vehicle and bortezomib treated constructs are shown in red and green.

**Figure 4:** Nerve-on-a-chip proximal/distal (a/c) action potential amplitude and proximal/distal (b/d) nerve conduction velocity measured across various concentrations of bortezomib \((n = 4-6)\). GraphPad prism v7.04 was used to perform a one-way ANOVA with Dunnet’s correction for multiple comparisons. Comparisons were made for each group with respect to the vehicle control. P-values are reported as: * \((<0.05)\), ** \((<0.01)\), and *** \((<0.001)\).

**Figure 5:** The effect of dexanabinol on bortezomib-induced decreases in proximal (top) and distal (bottom) action potential amplitude using nerve-on-a-chip \((n = 4-20)\). Action potential amplitudes were normalized to treatment naïve (black). The effect of dexanabinol \((10 \mu M \text{ and } 25 \mu M)\), bortezomib \((100 nM)\), and the combination of both drugs are shown in blue, red, and purple. GraphPad prism v7.04 was
used to perform a one-way ANOVA with Tukey’s correction for multiple comparisons. Comparisons were made across all groups with respect to each other, but only the significant comparisons to control are displayed. P-values are reported as: * (<0.05), ** (<0.01), and *** (<0.001).

**Figure 6**: The effect of dexanabinol on bortezomib-induced decreases in proximal (top) and distal (bottom) nerve conduction velocity using nerve-on-a-chip (n = 4-20). Nerve conduction velocities were normalized to treatment naive (black). The effect of dexanabinol (10 μM and 25 μM), bortezomib (100 nM), and the combination of both drugs are shown in blue, red, and purple. GraphPad prism v7.04 was used to perform a one-way ANOVA with Tukey’s correction for multiple comparisons. Comparisons were made across all groups with respect to each other, but only the significant comparisons to control are displayed. P-values are reported as: * (<0.05), ** (<0.01), and *** (<0.001). The p-value for the not significant (ns) comparison was 0.070.

**Table 1**: Parameter estimates from SH-SY5Y cytotoxicity after 48 hours of exposure to (a) bortezomib alone and in the presence of (b) 1 μM and (c) 10 μM of dexanabinol. $E_0$ is maximal cell viability, $I_{max}$ is maximal cell viability inhibition by drug, $IC_{50}$ is the concentration of drug that results in half of the maximal cell viability inhibition, and $\gamma$ is the Hill coefficient.
Figure 1
Figure 2

![Graph showing cell viability against bortezomib concentration with various conditions: Bortezomib Alone, Bortezomib + 10μM Dexanabinol, Bortezomib + 1μM Dexanabinol.](image)
Figure 3

[Diagram showing somatic and neurite outgrowth with measurements of 1.3mm (Height) and 6.1mm (Length). Diagram includes a section labeled "(Record)" and another labeled "(Proximal)" with an arrow pointing towards the proximal stimulation, and a section labeled "(Distal)" with an arrow pointing towards the distal stimulation.]

Proximal Stimulation

- Red line: Treatment Naïve
- Green line: Bortezomib (1000 nM)

Distal Stimulation

- Red line: Treatment Naïve
- Green line: Bortezomib (100 nM)
Figure 4

A. Proximal Response Amplitude

B. Distal Response Amplitude

C. Proximal Nerve Conduction Velocity

D. Distal Nerve Conduction Velocity
Table 1a: Parameter Estimates from Bortezomib Cytotoxicity in SH-SY5Y cells after 48 hours
of exposure.

<table>
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<th>CV (%)</th>
<th>Confidence Interval (95%)</th>
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</tr>
<tr>
<td>γ</td>
<td>-</td>
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<tr>
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Table 1b: Parameter Estimates from SH-SY5Y Cytotoxicity of Bortezomib in the Presence of 1
μM Dexanabinol after 48 hours of exposure.

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<tr>
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Table 1c: Parameter Estimates from SH-SY5Y Cytotoxicity of Bortezomib in the Presence of 10
μM Dexanabinol after 48 hours of exposure.

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<tr>
<td>γ</td>
<td>-</td>
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<td>123</td>
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<td>[ 107 - 139 ]</td>
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</table>


Chapter 7:

Network and Preclinical Pharmacokinetic-Pharmacodynamic Modeling of Dexanabinol and Bortezomib in Multiple Myeloma

Peter Bloomingdale\textsuperscript{1}, Louise Carlson\textsuperscript{2}, Wensheng Liu\textsuperscript{2}, Kelvin P. Lee\textsuperscript{2}, and Donald E. Mager\textsuperscript{1}

1. Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, The State University of New York, Buffalo, NY

2. Department of Immunology, Roswell Park Comprehensive Cancer Center, University at Buffalo, The State University of New York, Buffalo, NY
Abstract

In the previous chapter, the potential utility of using dexanabinol as a therapeutic agent for the prevention of chemotherapy-induced peripheral neuropathy (CIPN) was investigated. Prior to a clinical investigation assessing the efficacy of dexanabinol for reducing the neurotoxicity of select cancer chemotherapeutics, preclinical investigations of possible drug-drug interactions that could diminish anticancer activity are critical. The potential for dexanabinol to interfere with the pharmacodynamics of bortezomib in the treatment of multiple myeloma was evaluated. *In silico, in vitro, and in vivo* studies were performed. A previously developed network model of signaling in multiple myeloma was utilized to predict whether dexanabinol could prevent bortezomib-induced apoptosis in multiple myeloma cells. There was no change in the activation of apoptosis between bortezomib monotherapy and in combination with dexanabinol. Cytotoxicity studies using U266 multiple myeloma cells were performed to determine the combinatorial relationship between dexanabinol and bortezomib. A pharmacodynamic interaction model suggested an antagonistic relationship. However, there were no significant differences in tumor volume between mice treated with bortezomib alone compared to mice treated with the combination of bortezomib and dexanabinol. A pharmacodynamic interaction suggests that dexanabinol modulates a model parameter and may delay the resistance to bortezomib therapy.
Introduction

Chemotherapy-induced peripheral neuropathy (CIPN), or nerve damage caused by cancer chemotherapy, is an unmet medical need of growing concern. 30-40% of all cancer patients treated with a cancer drug, and approximately 37% of patients taking bortezomib, will develop peripheral neuropathy.¹² Symptoms often manifest as a numbness and tingling sensation in the extremities of the body, which throughout the course of treatment may evolve into an extreme burning pain.³ Currently, there are no clinically approved treatment options for the treatment or prevention of CIPN.⁴ There are also no well-established biomarkers or well-characterized predictors of CIPN, which makes it difficult to determine which patients will develop CIPN.⁴

In the previous chapter, dexamabinol was shown to modulate some markers of bortezomib-induced peripheral neuropathy. In order to move this potential therapy candidate forward, preclinical studies are needed to determine if dexamabinol diminishes the anticancer efficacy of bortezomib. Bortezomib has remained an important drug in the treatment of multiple myeloma, which is a cancer of terminally differentiated plasma cells and the second most common hematological malignancy.⁵-⁷ In this chapter in silico, in vitro, and in vivo preclinical studies were conducted to determine if dexamabinol alters the antineoplastic activity of bortezomib. A previously developed network model of signaling in multiple myeloma was utilized to predict if dexamabinol prevents bortezomib-induced apoptosis in multiple myeloma cells. Cytotoxicity studies using U266 multiple myeloma cells were performed to determine the combinatorial relationship between dexamabinol and bortezomib. The pharmacodynamics of bortezomib and dexamabinol on in vivo tumor volume was also evaluated in a multiple myeloma SCID mouse model.

Methods

Multiple Myeloma Signaling Network Model: Simulation and Attractor Analysis
A Boolean network model that incorporates cellular signaling pathways important to the growth, proliferation, and apoptosis of multiple myeloma has been previously developed. The network model was utilized to predict the cellular pharmacodynamic effects of bortezomib in the absence/presence of dexanabinol. The network model diagram was constructed using yEd graph editor and is displayed in Figure 1.

Network simulations were performed in MATLAB using the Odefy toolbox. Boolean logic functions were converted to normalized HillCube differential equations, and default parameter values were retained (\( \tau = 1; k = 0.5; n = 3 \)). The number of time steps were selected to ensure that the model converged to a new steady-state. Network simulations were performed for the four following conditions: (1) treatment naïve, (2) bortezomib, (3) dexanabinol, and (4) bortezomib + dexanabinol. Bortezomib was modeled by inhibiting the proteasome and activation of receptor interacting protein kinases (RIP), which is how the mechanism of action of bortezomib was described in the original network model.

Dexanabinol was modeled by inhibiting TNF\( \alpha \), TNF\( \alpha \) receptor (TNF\( \alpha \)R), and reactive oxygen species (ROS). An attractor analysis was performed on the network to identify steady-states of the system upon various network perturbations. The analysis was performed in R using the package BoolNet. A SAT exhaustive search with synchronous updating, which identifies all attractors of the network, was performed for the four following conditions: (1) treatment naïve, (2) bortezomib, (3) dexanabinol, and (4) bortezomib + dexanabinol. The relative fractional activation of each node, including proliferation and apoptosis, across all attractors was calculated according to the following equation:

\[
S_x = \frac{\sum_{i=1}^{n_{A_{Total}}} x_i}{n_{A_{Total}}} \quad x_i \in \{0,1\} \quad i = 1, \ldots, n_{A_{Total}}
\]

with \( S_x \) as the relative fractional activation of node \( x \) across all attractors, and \( n_{A_{Total}} \) is the total number of attractors in the network for each of the respective perturbations.
In Vitro U266 Cytotoxicity Studies

Cytotoxicity studies were performed using U266 multiple myeloma cells for single agent treatment as well as the combination. U266 cells were grown at 37°C and 5% CO₂ in RPMI-1640 media supplemented with 15% fetal bovine serum and 1% penicillin-streptomycin (10,000 U/mL; 10,000 μg/mL) until a sufficient number of cells were obtained. U266 cells were seeded at a density of 25,000 cells/well in 96 well plates and were allowed to equilibrate for one day. Various concentrations of bortezomib (0.001, 0.01, 0.1, 1, 10, 100, 1000 nM) and dexanabinol (1, 3, 10, 30, 100, 300 μM) were added to their respective wells. The concentration ranges were selected in order to achieve a wide range of concentrations above and below the IC₅₀ of each drug. Cell viability was measured using a colorimetric reagent (WST-1). After 24, 48, or 72 hours of drug exposure, 10% v/v of WST-1 was added to each well. Plates were briefly shaken and placed into the incubator for 2 hours. Absorbance was measured using a SpectraMax 190 microplate reader at a wavelength of 450 nm (690 nm reference wavelength).

Pharmacodynamic Modeling of Bortezomib and Dexanabinol Cytotoxicity

For single agents, the decrease in cell viability (%) as a function of drug concentration was modeled using an inhibitory Hill function:

\[
Cell\ Viability\ (%) = E_0 \times \left( 1 - I_{max} \times \frac{[c]^\gamma}{I_{C50}^\gamma + [c]^\gamma} \right)
\]

with \(E_0\) as the maximal cell viability in the absence of drug, \(I_{max}\) is the fraction of maximal cell viability inhibition by drug, \(I_{C50}\) is the concentration of drug that results in half of \(I_{max}\), and \(\gamma\) is the Hill coefficient.

For the combinatorial effect of two drugs, cell viability was modeled using a modified version of the Ariens equation, which was described by Chakraborty and Jusko.
Cell Viability (%) = \( E_0 \times \left[ 1 - \frac{I_{\text{max}}B \times \left( \frac{[B]}{\Psi_{\text{vit}} \times IC_{50B}} \right)^{\gamma B} + I_{\text{max}}D \times \left( \frac{[D]}{IC_{50D}} \right)^{\gamma D} + (I_{\text{max}}B + I_{\text{max}}D + I_{\text{max}}B \times I_{\text{max}}D) \times \left( \frac{[B]}{\Psi_{\text{vit}} \times IC_{50B}} \right)^{\gamma B} \times \left( \frac{[D]}{IC_{50D}} \right)^{\gamma D} \right]}{(3)}\)

with \([B]\) and \([D]\) representing the concentrations of bortezomib (nM) and dexanabinol (μM). \(I_{\text{max}}\), \(IC_{50}\), and \(\gamma\), have the same definition as in Equation 2, but subscripts B or D represent if the parameter relates to bortezomib or dexanabinol pharmacodynamics. The \(\Psi_{\text{vit}}\) parameter was introduced to account for the \textit{in vitro} synergistic (\(\Psi_{\text{vit}} < 1\)), antagonistic (\(\Psi_{\text{vit}} > 1\)), or additive (\(\Psi_{\text{vit}} = 1\)) relationship of the drug combination on U266 cell viability. For this model, \(\Psi_{\text{vit}}\) was solely incorporated on the \(IC_{50}\) of bortezomib. Parameters were estimated from single agent treatments and fixed while fitting the Chakraborty equation to cell viability data from the drug combination to obtain an estimate of \(\Psi_{\text{vit}}\).

\textbf{In Vivo Pharmacodynamics of Bortezomib and Dexanabinol on Murine Tumor Volume}

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Roswell Park Comprehensive Cancer Center. Twenty SCID female mice at age of 4 weeks were irradiated with 300 Rads using a Mark II Cesium irradiator. Twenty-four hours after irradiation, animals were injected subcutaneously with 5 million MM1S cells in the left flank. Animals were monitored for tumor formation and tumor sizes were measured with a digital caliper twice weekly. Tumor volumes were calculated using the modified ellipsoidal formula: \(\text{tumor volume} = 0.5 \times \text{length} \times \text{width} \times \text{width} \). Treatments were started when the tumor volumes reached approximately 100 mm\(^3\). Treatments were administered twice a week via IP injection at the doses of 1 and 10 mg/kg for bortezomib and dexanabinol. The study was conducted for four weeks, and mice were sacrificed when tumors grew to the limit enforced by IACUC.

\textbf{Pharmacokinetic Modeling of Bortezomib and Dexanabinol in Mice}
A physiologically-based pharmacokinetic model of bortezomib concentrations in mice was previously described. Plasma concentrations of bortezomib for mice that received an IV (intrapenile) dose of 0.25 or 1 mg/kg were modeled using an in-house model shown in Figure 2. The model was used to simulate a multiple dosing regimen of 1 mg/kg bortezomib administered twice weekly. The average body weight of all mice in the bortezomib treatment group was used to determine the dose for each administration.

To our knowledge, the pharmacokinetics of dexanabinol in mice has not been previously reported in the literature. However, there is information available for the pharmacokinetics of dexanabinol in rats and humans. Therefore, the pharmacokinetics of dexanabinol in rats and humans were allometrically scaled to predict mouse pharmacokinetics. A two-compartment pharmacokinetic model was fit to rat and human plasma concentrations of dexanabinol:

\[
\frac{dC}{dt} = \frac{(CL_D \times C_t - CL_D \times C - CL \times C)}{V} \\
\frac{dC_t}{dt} = \frac{(CL_D \times C - CL_D \times C_t)}{V_t}
\]

The well-known power law relationship was used for parameter scaling:

\[
X_{Species} = \alpha \left( BW_{species} \right)^{\beta}
\]

with \( \alpha \) and \( \beta \) calculated for each parameter (X) of the two compartment pharmacokinetic model using the species body weight (BW). The values for \( \alpha \) and \( \beta \), along with the average body weight of mice in the dexanabinol treated group, were used to predict the plasma pharmacokinetics of dexanabinol in mice. Model predicted half-life of dexanabinol for each species was calculated by dividing the natural log of 2 by the slope of the elimination phase.

**Pharmacodynamic Modeling of Bortezomib and Dexanabinol on Murine Tumor Volume**

Tumor volume (TV) was modeling using the following equations:

\[
\frac{dkg}{dt} = -kgr \times kg
\]
\[
\frac{dT_V}{dt} = k_g(t) \times TV - K_{Dex} \times C_{Dex} \times TV - K_{Bort} \times e^{-(\Psi_{viv} \times kres \times t)} \times C_{Bort} \times TV
\]  

Tumor volume in the absence of drug was modeled using an exponential growth function.\(^{25}\) The pharmacodynamic effects of bortezomib and dexanabinol on tumor volume were modeled as second-order elimination processes governed by the rate constants \(K_{bort}\) and \(K_{dex}\). A function that decreases the rate of elimination of tumor volume by bortezomib was included \((kres)\), which greatly improved the fit of the model. The interaction term \((\Psi_{viv})\) was incorporated on the parameter governing bortezomib resistance, \(kres\). \(\Psi_{viv}\) was introduced to account for the \textit{in vivo} synergistic \((\Psi_{viv} < 1)\), antagonistic \((\Psi_{viv} > 1)\), or additive \((\Psi_{viv} = 1)\) relationship of the drug combination on tumor volume pharmacodynamics.

\section*{Results}

\textit{Multiple Myeloma Network Model Predictions}

The Boolean network model of intracellular signaling in multiple myeloma contains 92 nodes and 209 regulatory interactions. Results from network simulations for the four treatment conditions (i.e., control, bortezomib, dexanabinol, and bortezomib + dexanabinol) are displayed as separate columns in a heatmap (Figure 3). Normalized expression and time are both continuous since Boolean equations have been converted to ordinary differential equations. The dynamics of select nodes for bortezomib monotherapy versus the combination of bortezomib and dexanabinol are shown in Figures 3b and 3c. Bortezomib resulted in a decrease in proteasome function, increase in ROS, release of cytochrome c, increase in caspase expression, and subsequent activation of apoptosis. Dexanabinol decreased TNF\(\alpha\) and prevented the increase in bortezomib-induced ROS. However, the increase in expression of cytochrome c, caspase 3, and apoptosis was not prevented by dexanabinol. Interestingly, the time of onset for the increase of these components was slightly delayed.

Results from the attractor analysis are shown in Figure 4. The relative fractional activation of nodes (Eq. 1) are reported for each of the four treatment groups. The fractional activation is relative to the total
number of network attractors and is reported as a value between \([0,1]\), where zero (one) indicates that the activation state of the node of interest is OFF (ON) throughout all possible steady-states in the dynamical system. The fractional activation of proliferation decreased to zero in the presence of bortezomib, and remained zero for the combination (Figure 4b). Additionally, the fractional activation of proliferation was slightly lower for dexanabinol compared to treatment naïve conditions. The fractional activation of apoptosis increased to one in the presence of bortezomib, and remained one for the combination (Figure 4c). Additionally, the fractional activation of apoptosis was slightly lower for dexanabinol compared to treatment naïve conditions.

**In Vitro Cytotoxicity Studies in U266 Multiple Myeloma Cells**

The results of the cytotoxicity study for the combination of bortezomib and dexanabinol in U266 multiple myeloma cells is shown in Figures 5 and 6. A BLISS independence model was used to assess the nature of the pharmacological relationship of the drug combination at 24 (Figure 5a), 48 (Figure 5b), and 72 (Figure 5c) hours. As highlighted in red, there appeared to be a more pronounced antagonist relationship at earlier times (24 and 48 hours) around the IC$_{50}$ of bortezomib for low concentrations of dexanabinol. The cytotoxicity curves for U266 multiple myeloma cell viability as a function of bortezomib or dexanabinol concentrations are shown in Figures 6a and 6b. An inhibitory Hill function (Equation 2) was fit to cell viability data from single drug exposures, and parameter estimates are listed in Tables 1a-c, for 24, 48, and 72 hours of exposure. The IC$_{50}$ for dexanabinol ranged from 18.0 to 20.7 μM, and the IC$_{50}$ for bortezomib ranged from 1.07 to 2.10 nM. The Hill coefficient (\(γ\)) for dexanabinol was fixed to 5 since it was estimated as a high value, and the amount of data points were not sufficient for estimating a precise value. The Hill coefficient (\(γ\)) for bortezomib was estimated around 1. The Hill coefficient for 48 hours of bortezomib exposure was fixed to 1 because it was unidentifiable. The estimated values of Imax for bortezomib and dexanabinol were around 1, and Imax was fixed to 1 in cases where it was estimating over 1.
The effects of both drugs on cell viability was modeled using Equation 3. The estimated values of $\Psi_{vit}$ were 2.71, 2.10, and 1.40 for 24, 48, and 72 hours of exposure. All of the $\Psi_{vit}$ values were greater than one, which suggests an antagonistic relationship between dexanabinol and bortezomib on *in vitro* cell viability. The confidence interval around $\Psi_{vit}$ at 24 hours did not include 1, but confidence intervals around $\Psi_{vit}$ at 48/72 hours included 1. This suggests a statistically significant antagonistic relationship at 24 hours, but not at 48 or 72 hours.

**PK/PD of Bortezomib and Dexanabinol in a Multiple Myeloma SCID Mouse Model**

The pharmacokinetic model in Figure 2 was fit to bortezomib plasma concentrations for two doses (Figure 7a). Parameter values were shared across both doses, and the final estimates are reported in Table 2. All parameters were estimated with reasonable precision. After parameter estimation, the model was used to predict plasma concentrations in mice given bortezomib twice weekly (Figure 7b). The pharmacokinetic profile exhibits a rapid distributive phase followed by a prolonged elimination phase. The predicted Cmax after the last dose was 2.06 $\mu$M, and the trough concentration prior to the last dose was 0.083 $\mu$M.

The pharmacokinetics of dexanabinol in mice were scaled down from data in humans and rats. A two-compartment pharmacokinetic model was fit to plasma concentrations from rats and humans that were administered with various amounts of dexanabinol (Figures 8a/b). Estimated parameters and the respective body weights of species were used to calculate $\alpha$ and $\beta$ of the allometric equations. The $\alpha$ and $\beta$ terms were determined from parameters of the human/rat pharmacokinetic models and were used to calculate parameters of the mouse model. The predicted plasma concentration-time profile of dexanabinol in mice is shown in Figure 8c. All estimated and scaled parameter values are reported in Table 3. The pharmacokinetics of dexanabinol in mice exhibited a very fast distributive phase. The predicted Cmax after the last dose was 28.1 $\mu$M, and the model predicted half-life of dexanabinol in
humans, rats, and mice was 4.4, 4.1, and 10.8 hours. Therefore, trough concentrations of dexanabinol were practically zero prior to the administration of each dose.

The pharmacokinetics of bortezomib and dexanabinol in mice were fixed when estimating parameters of the pharmacodynamic model of tumor volume. The pharmacodynamic model fittings and parameter estimates are shown in Figure 9 and Table 4. All parameters were estimated with good precision. Tumor volume in the dexanabinol alone group was slightly lower than the control, however these changes were not significant. Bortezomib alone and in combination with dexanabinol both resulted in a statistically significant decrease in tumor volume. Model performance, especially during the first two weeks of treatment, greatly improved when incorporating an exponential decay of the second order elimination rate constant (Kbort) governing the elimination of tumor volume by bortezomib exposure. The exponential decay parameter (kres) was estimated to be 0.0076 1/hr. The interaction term (Ψviv) was placed on the kres term and estimated to be 0.883 (CV% = 4.7) with a 95% confidence interval of 0.80 to 0.97, which indicates a synergistic interaction by dexanabinol for slowing the decay rate of tumor volume elimination by bortezomib. Mice body weights were not significantly different across treatment groups indicating the mice were in good health (Figure 10).

In addition to the pharmacodynamic interaction of bortezomib and dexanabinol on tumor volume, the Koch model was also evaluated. The Koch model places Ψviv on the second-order elimination rate constant governing the change in tumor volume by another drug. When comparing these two models head-to-head, both models accurately described the data with great precision. However, the final model in Equations 8 and 9 (AIC = 328.8; BIC = 336.6) slightly outperformed the Koch model (AIC = 332.3; BIC = 340.1). Interestingly, in the Koch model, Ψviv was estimated to be 1.13 (CV% = 6.1) with a 95% confidence interval of 0.98 to 1.27. Hence, an additive interaction was observed when applying the Koch model versus the synergistic interaction observed with the model.
**Discussion**

Preclinical studies consisting of *in silico*, *in vitro*, and *in vivo* analyses were used to test pharmacodynamic relationships between dexanabinol and bortezomib in the treatment of multiple myeloma. These analyses suggest that dexanabinol does not compromise the anticancer properties of bortezomib. The time- and concentration-dependent antagonistic relationship between bortezomib and dexanabinol *in vitro* was not observed in the *in vivo* experiment.

Overall network predictions suggest that dexanabinol will not prevent the cytotoxicity of bortezomib in multiple myeloma cells. Interestingly, the onset of activation of components in the intrinsic apoptosis pathway (i.e. cytochrome C and caspase 3/9) was slightly delayed. However, due to the qualitative nature of this mathematical framework, the time frame of this theoretical delay is not known. Experimentally, in the multiple myeloma mouse model, we observed that tumor volume in the combination group were slightly larger than bortezomib alone for the first week of treatment. Another interesting observation is that tumors in the bortezomib alone group necrotized earlier, approximately one week, then tumors in the combination group.

Results from the attractor analysis indicated that dexanabinol did not alter the decrease in proliferation or increase in apoptosis by bortezomib. The fractional activation of proliferation and apoptosis was slightly lower for dexanabinol compared to treatment naïve, which may reflect the complex dual roles of reactive oxygen species and tumor necrosis factor signaling pathway in survival and death. For the attractor analysis, a SAT exhaustive search was performed over a random search because the activation frequency of apoptosis was approximately 1, even in the absence of drug perturbations. Therefore, to assess drug effect without placing constraints on the system, a SAT exhaustive search was conducted and fractional activations of proliferation and apoptosis were compared across all network attractors.
Based upon in vitro results, the pharmacodynamic relationship between bortezomib and dexanabinol on multiple myeloma cell viability appears to be antagonistic. However, this antagonistic relationship exists only at low concentrations of dexanabinol, near the IC_{50} of bortezomib, and seems to lessen over the time of exposure. In addition, the antagonistic interaction by dexanabinol did not alter the efficacy of bortezomib treatment in the multiple myeloma mouse model. The reason for this discrepancy is likely due to the fact that plasma concentrations of bortezomib in mice are predicted to be well above its estimated IC_{50}, which is outside the observed range of antagonism.

For the bortezomib pharmacokinetic model, a more simplistic model was used as compared to the PBPK model developed Zhang and Mager (2015), since we are only interested plasma exposure.\textsuperscript{21} Plasma concentrations of dexanabinol in humans appears to be triphasic.\textsuperscript{22} However, a two-compartment model was chosen over three in order to retain the same model structure across species for allometric scaling.

Treatment naïve tumor growth in mice were first modeled using a simple first-order growth term. However, the Gompertz function, which consists of a time-dependent first-order growth term, significantly improved model fitting criteria. Initially, the pharmacokinetics of each drug were incorporated on the growth term using both linear and nonlinear functions. However, model performance was superior when drug concentrations were incorporated on the second-order elimination of tumor volume. Parameters for nonlinear elimination of tumor volume were unidentifiable. The inclusion of a resistance term that decreases the second-order elimination of tumor volume over time significantly improved effects of bortezomib on tumor volume. Without this term, the model predicted pharmacodynamic effects of bortezomib did not capture the observed data, especially during the first two weeks of the study. The abrupt increases in tumor volume in the bortezomib and combination groups at 22 and 29 days likely reflects mice that were sacrificed due to ulcerations from necrotized tumors. All of the data from this study were reported, as opposed to censoring data points.
once mice had been sacrificed from a group. Interestingly, tumors in the bortezomib alone groups
became necrotized earlier than tumors in the combination group. This phenotypic difference is likely
attributed to the molecular mechanisms of action of dexanabinol, which decreases the production of
tumor necrosis factor. In the pharmacodynamic model, an interaction term was incorporated on the
bortezomib resistance term in order to account for possible pharmacodynamic interaction between
dexanabinol and bortezomib. Interestingly, the interaction term was significantly lower than 1, which
indicates a synergistic interaction by dexanabinol for prolonging the onset of bortezomib resistance. The
molecular mechanism of action for this phenomenon could be through suppressing the activation of
NFkB by dexanabinol. Dexanabinol has previously been shown to inhibit the TNFα-dependent activation
of NFkB in neurons by decreasing the degradation rate of IkB, nuclear translocation, and transcriptional
activity. Additionally, aberrant NFkB signaling is a well-known molecular mechanism of bortezomib
resistance. In the Koch model, the interaction term was estimated to be approximately 1, indicating
an additive effect. An explanation for this discrepancy could be that the interaction term in our model
better characterizes the slope of tumor volume regrowth (progression), whereas the interaction term in
the Koch model is more representative of the initial declining slope of tumor volume (stabilization or
regression).

Additional studies that measure the pharmacological effects at the molecular level, such as a
transcriptomic or proteomic analysis, could provide more insight into the mechanisms governing the
complex concentration-time dependent in vitro antagonism and possible in vivo effects on bortezomib
resistance. Investigations using a multiple myeloma mouse model that is more representative of the
human disease, such as a VK*MYC model, may provide a better assessment for the pharmacodynamics
of the in vivo drug combination.

Conclusion
Based upon network predictions and results of experimental investigations, dexanabinol does not appear to alter the anticancer activity of bortezomib. This provides evidence to warrant a clinical investigation of this drug combination to prevent bortezomib-induced peripheral neuropathy without fear of compromising the efficacy of bortezomib for treating patients with multiple myeloma.
Figures and Tables

Figure 1: Boolean network modeling of intracellular signaling in multiple myeloma. The network contains 92 nodes and 209 regulatory interactions, which does not include nodes/edges for bortezomib and carfilzomib. Model equations were obtained from Ramakrishnan and Mager (2018). Model diagram was constructed in yEd using the orthogonal edge layout. The legend contains information regarding the color scheme of nodes and edge types.

Figure 2: Pharmacokinetic model of bortezomib concentrations in mouse plasma. Model was obtained from Zhang and Mager (2015). Two-compartment pharmacokinetic model with reversible binding to red blood cell proteasomes in the central compartment. Rmax and Kd represent the maximal binding to red blood cell proteasome and dissociation rate constant for proteasomal binding. Equations are shown below the model diagram.

Figure 3: Multiple myeloma signaling network simulations. Boolean network model was converted to normalized HillCube differential equations using Odefy, and default parameter values were retained (\( \tau = 1; k = 0.5; n = 3 \)). (a) Model predictions for the activation state of network components over time are displayed as heatmaps for (from left to right): control, dexamabinol monotherapy, bortezomib monotherapy, and the combination of dexamabinol and bortezomib. The key on the right-hand side of the figure indicates the magnitude of activation for network species, which ranges between zero (blue) and one (red). Model predicted activation state dynamics of select network components are displayed for (b) bortezomib monotherapy and (c) the combination of bortezomib and dexamabinol therapy. Proteasome (green), TNF\( \alpha \) receptor (purple), reactive oxygen species (blue), caspase 3 (orange), cytochrome C (light blue), and apoptosis (red).

Figure 4: Attractor analysis of the Boolean network model of multiple myeloma signaling. Attractor analysis was performed using the SAT exhaustive function inBoolNet. (a) The fractional activation of all
92 network components are displayed as heatmaps for (from left to right): control, bortezomib monotherapy, dexanabinol monotherapy, and the combination of dexanabinol and bortezomib. The key on the right-hand side of the figure indicates the magnitude of fractional activation, which ranges between zero (blue) and one (red). The fractional activation of (b) proliferation and (c) apoptosis were calculated for the absence of drug (black), bortezomib monotherapy (red), dexanabinol monotherapy (blue), and combination of bortezomib and dexanabinol (purple).

**Figure 5:** Cytotoxicity study for the combination of bortezomib and dexanabinol in U266 multiple myeloma cells at (a) 24, (b) 48, and (c) 72 hours of drug exposure. A BLISS independence model was used to assess the nature of the pharmacological relationship. Analyses were performed in MATLAB using Combenefit. The combination of drug concentrations that result in an antagonistic, synergist, and additive relationship are highlighted on the 3D cytotoxicity curve in red, blue, and green.

**Figure 6:** Cytotoxicity of U266 multiple myeloma cells exposed to various concentrations of (a) bortezomib or (b) dexanabinol for 24, 48, and 72 hours. The IC\textsubscript{50} of bortezomib and dexanabinol is approximately 2.5 nM and 20 μM across all sampling times.

**Figure 7:** Bortezomib pharmacokinetics in mice. (a) Pharmacokinetic model fits to bortezomib plasma concentrations from mice that were administered with 1 mg/kg (red) and 0.25 mg/kg (blue) of bortezomib via the penile vein. (b) Predicted multiple dosing pharmacokinetic profile of bortezomib plasma concentrations in mice administered 1 mg/kg on days 1, 5, 8, 12, 15, and 19. Observed plasma concentrations and model estimated profiles are displayed as solid markers and dashed lines.

**Figure 8:** Interspecies scaling of dexanabinol pharmacokinetics from rats and humans to mice. (a) Pharmacokinetic model fits to plasma concentrations from rats administered IV with 4.5 mg/kg of dexanabinol. (b) Pharmacokinetic model fits to plasma concentrations from human volunteers administered IV with 48, 100, and 200 mg of dexanabinol. (c) Scaled and predicted multiple dosing
pharmacokinetic profile of dexanabinol plasma concentrations in mice administered 10 mg/kg on Days 1, 5, 8, 12, and 15. Observed plasma concentrations and model estimated profiles are displayed as solid markers and dashed lines.

Figure 9: Pharmacodynamics of bortezomib and dexanabinol on tumor volume in a multiple myeloma SCID mouse model. Tumor was grown to approximately 100 mm³ prior to drug administration. Mice (n=20) were split into four treatment groups: treatment naïve (black), dexanabinol (blue), bortezomib (red), and the combination of dexanabinol and bortezomib (purple). Bortezomib and dexanabinol were administered IP twice weekly at a dose of 1 and 10 mg/kg. Tumor volume measurements and pharmacodynamic model estimated profiles are displayed as solid markers and dashed lines. Asterisks indicate statistical significance from control (corrected p-value < 0.5), which were obtained via multiple t-tests with Holm-Sidak Bonferroni correction (GraphPad Prism v7.04).

Figure 10: Change in body weight over time in MM1.S multiple myeloma-bearing SCID mice for each of the treatment groups (see Figure 9 legend).
**Table 1:** Parameter estimates from bortezomib and dexanabinol cytotoxicity in U266 cells after (a) 24, (b) 48, and (72) hours of exposure. Model parameters, Imax, IC_{50}, and γ, end with the letter B or D to represent that the parameter relates to bortezomib or dexanabinol. E_0 is maximal cell viability, Imax is maximal cell viability inhibition by drug, IC_{50} is the concentration of drug that results in half of the maximal cell viability inhibition, and γ is the Hill coefficient. Ψ is the pharmacodynamic interaction term governing the relationship between dexanabinol and bortezomib.

**Table 2:** Parameter estimates for the pharmacokinetics of bortezomib in mice. Parameter notation: central compartment volume of distribution (V1), clearance (CL), rate constant of drug distribution from the central to peripheral compartment (K12), rate constant of drug distribution from the peripheral to central compartment (K12), maximal binding to red blood cell proteasomes (Rmax), and dissociation rate constant for proteasomal binding (Kd).

**Table 3:** Pharmacokinetic modeling and allometric scaling of dexanabinol. Parameter estimates are reported from maximum likelihood estimation of a two-compartment pharmacokinetic model that describes human and rat plasma concentrations of dexanabinol. Allometric coefficients were calculated using estimated parameter values and body weights of the corresponding species. The scaled parameters for the two-compartment model of dexanabinol pharmacokinetics in mice are reported.

**Table 4:** Parameter estimates from a pharmacodynamic model of bortezomib and dexanabinol effects on tumor volume in a multiple myeloma SCID mouse model. The pharmacodynamic model includes a time-dependent change in growth, which is governed by the first-order growth rate constant (kgr). Parameters of the model include the initial growth rate (k_{g0}), second-order elimination of tumor volume via dexanabinol/bortezomib (K_{dex}/K_{bort}), first-order rate constant for the emergence of bortezomib resistance (kres), and the modulation of bortezomib resistance via dexanabinol (Ψ).
Figure 1
\[
\begin{align*}
\frac{dA_p}{dt} &= -CL \cdot C_{pf} - k_{12} \cdot C_{pf} \cdot V_1 + k_{21} \cdot A_t \\
\frac{dA_i}{dt} &= k_{i2} \cdot C_{pf} \cdot V_1 - k_{21} \cdot A_t \\
C_{pf} &= 0.5 \cdot \left[\left(\frac{A_p}{V_1} - \beta \cdot R_{max} - K_D\right) + \sqrt{\left(\frac{A_p}{V_1} - \beta \cdot R_{max} - K_D\right)^2 + 4 \cdot K_D \cdot \frac{A_p}{V_1}}\right]
\end{align*}
\]
Figure 3a

The figure shows a heatmap representing the temporal expression levels of various genes or proteins across different conditions: Control, Dexanabinol, Bortezomib, and Dex & Bort. The heatmap uses a color scale ranging from 0 to 1 to indicate the level of expression over time, with time points marked horizontally. Genes/proteins listed vertically include AKAP, JNK, MAPK, etc., and their expression dynamics are visualized across the timeline.

The overall trend indicates differences in expression patterns under each condition, with some genes showing increased expression in certain conditions compared to others. The heatmap provides a comprehensive view of how gene expression changes over time under different treatments.
Figure 3b

Bortezomib

Normalized Expression vs. Time

0.0  0.5  1.0

Prot  TNFR  ROS  Cas3  Cytc  Apo

Figure 3c

Bortezomib and Dexanabinol

Normalized Expression vs. Time

0.0  0.5  1.0

Prot  TNFR  ROS  Cas3  Cytc  Apo

270
Figure 4b

Fractional Activation of Proliferation ($S_{Pr0}$)

- Bortezomib
- Dexanabinol

Figure 4c

Fractional Activation of Apoptosis ($S_{Apo}$)

- Bortezomib
- Dexanabinol
Figure 5c
Figure 7a

Figure 7b
Figure 8c

Predicted Dexanabinol Plasma Concentration

Dexanabinol Plasma Concentration in Mice (µM)

Time (Days)
Figure 10

![Graph showing weight changes over time for different treatments: Control, Dexanabinol, Bortezomib, and Dex + Bort. The graph displays weight in grams on the y-axis and time in days on the x-axis. The control group shows a slight decrease in weight, the Dexanabinol group shows an overall increase, the Bortezomib group shows a decrease followed by an increase, and the Dex + Bort group shows a steady increase.]
Table 1a: Parameter Estimates from Bortezomib and Dexanabinol Cytotoxicity in U266 cells after 24 hours of exposure.

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<td>[0.878 - 1.02]</td>
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<td>γD</td>
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<tr>
<td>ψ</td>
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<td>2.71</td>
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Table 1b: Parameter Estimates from Bortezomib and Dexanabinol Cytotoxicity in U266 cells after 48 hours of exposure.

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<td>IC$_{50}$B</td>
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<tr>
<td>ψ</td>
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Table 1c: Parameter Estimates from Bortezomib and Dexanabinol Cytotoxicity in U266 cells after 72 hours of exposure.

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<tr>
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<td>[ 0.983 - 1.02 ]</td>
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<tr>
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Table 2: Bortezomib Pharmacokinetics in Mice

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<tr>
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Table 3: Pharmacokinetic Modeling and Allometric Scaling of Dexanabinol

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<tr>
<td>BW (kg)</td>
<td>kg</td>
<td>0.400</td>
<td>-</td>
<td>77.4</td>
</tr>
<tr>
<td>Half-life</td>
<td>hr</td>
<td>4.13</td>
<td>-</td>
<td>4.44</td>
</tr>
</tbody>
</table>

Table 4: Pharmacodynamics of Bortezomib and Dexanabinol on Tumor Volume in a Multiple Myeloma SCID Mouse Model

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Units</th>
<th>Value</th>
<th>CV (%)</th>
<th>Confidence Interval (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>kgr</td>
<td>1/hr</td>
<td>1.57E-03</td>
<td>26.5</td>
<td>[0.707E-03, 0.244E-02]</td>
</tr>
<tr>
<td>Kdex</td>
<td>1/(hr*μM)</td>
<td>5.42E-04</td>
<td>Fixed</td>
<td>Fixed</td>
</tr>
<tr>
<td>Kbor</td>
<td>1/(hr*nM)</td>
<td>2.82E-04</td>
<td>24.7</td>
<td>[0.139E-03, 0.427E-03]</td>
</tr>
<tr>
<td>kres</td>
<td>1/hr</td>
<td>7.60E-03</td>
<td>21.3</td>
<td>[0.423E-02, 0.110E-01]</td>
</tr>
<tr>
<td>ψ</td>
<td>-</td>
<td>0.883</td>
<td>4.7</td>
<td>[0.796 , 0.970]</td>
</tr>
<tr>
<td>kg₀</td>
<td>1/hr</td>
<td>9.82E-03</td>
<td>6.8</td>
<td>[0.844E-02, 0.112E-01]</td>
</tr>
<tr>
<td>σ</td>
<td>-</td>
<td>0.143</td>
<td>13.8</td>
<td>[ 0.102 , 0.185]</td>
</tr>
</tbody>
</table>
References


Conclusion

In this dissertation, a combination of mathematical and experimental models was used to study and gain insights toward resolving chemotherapy-induced peripheral neuropathy (CIPN). A machine learning model that links physicochemical descriptors of compounds with the incidence of peripheral neuropathy was successfully conducted. The final model can be applied in early drug discovery to develop therapeutics that are less neurotoxic. A Boolean network-based systems model of signal transduction and gene regulatory processes in peripheral neurons was also developed. Although the Boolean network model is a simplified representation of the complex biological processes occurring in peripheral neurons, the model could be used to generate testable hypotheses that could lead to an effective treatment strategy for the prevention of CIPN. Based on the \textit{in silico} results of modeling endeavors, the combinatorial inhibition of TNF\(\alpha\), NMDAR, and ROS might prevent the development of proteasome inhibitor induced-peripheral neuropathy. However, one limitation is the qualitative framework of the network model. A quantitative understanding of the cellular transduction and gene regulatory processes is needed.

Ultimately, the goal is to use the Boolean network model as the base structure for the development of a quantitative systems toxicology (QST) model as data become available. Additionally, we would like to link physicochemical descriptors of chemotherapeutics and neuroprotective agents with pharmacodynamic parameters (e.g., EC\textsubscript{50}/IC\textsubscript{50} and Emax/Imax) that govern the activity of network nodes. In essence, the network model would act as a mechanistic interface between physicochemical descriptors of drugs and clinical endpoints of peripheral neuropathy. Sensitivity analysis could be performed on a calibrated quantitative systems model in order to understand the relative importance of individual network components on neuronal apoptosis and clinically relevant biomarkers of peripheral neurotoxicity. A QST model of CIPN could be used to identify sensitive drug targets and the extent of
modulation required to obtain optimal efficacy. Future directions to expand the QST model include the incorporation of biophysical processes in peripheral nerves that govern action potential propagation and scaling from a single neuron to a peripheral nerve to predict electrophysiological endpoints. Additionally, the incorporation of other components of the peripheral nervous system and immune system, such as satellite glia cells, Schwann cells, macrophages, and T-cells, would better represent the physiological system. The QST model could be used to inform the optimal design of drugs to prevent CIPN and provide a bridge toward personalized medicine when individual patient information is available.

An interesting finding from QSTR modeling was the identification that the number of aromatic nitrogens was a frequent and sensitive molecular descriptor used in the machine learning models of CIPN. The incorporation of aromatic nitrogens in the molecular structure of drug candidates is frequently performed to improve physicochemical characteristics of drugs. Therefore, using the final QSTR model, one could assess how the incorporation or removal of an aromatic nitrogen alters the safety profile of a drug. Elucidating the possible mechanisms underlying the association between aromatic nitrogens and CIPN could be an area of future investigation. We hypothesize that metabolism via aldehyde oxidase could be partially responsible, since the enzyme has been shown to produce reactive oxygen/nitrogen species and historically has resulted in the failure of drug candidates in clinical trials due to the generation of poorly soluble metabolites. The combination of the QSTR model with in silico models to predict the likelihood of aldehyde oxidase metabolism would be an interesting area of investigation.

The efficacy of dexanabinol for preventing bortezomib neurotoxicity was assessed in a microphysiological model of a peripheral rat nerve. Dexanabinol exhibited signs of neuroprotection and partially prevents bortezomib-mediated changes in nerve conduction velocity and action potential amplitude. However, confirmatory studies in vivo, such as an assessment of dexanabinol for preventing bortezomib-induced peripheral neuropathy in rats, are necessary. Lastly, dexanabinol does not alter the
anticancer efficacy of bortezomib in a multiple myeloma mouse model. Follow up studies with additional dose levels and assessments using other in vivo multiple myeloma models would provide more confirmatory evidence of a pharmacodynamic interaction. There exists substantial scientific promise for methods that bridge gaps between microphysiological and in vivo systems. The incorporation of microfluidics in the nerve-on-a-chip would better emulate the true drug exposure-time profile occurring in vivo. Obtaining a time-course of drug effect using the microphysiological model of a rat peripheral nerve could be used to predict effects seen in the rat models CIPN.

In summary, in silico tools have been developed that can be used to predict the clinical incidence of CIPN, simulate the neuronal toxicodynamics of chemotherapeutics, and identify novel therapeutic strategies for the prevention of CIPN. Based upon network analyses, the potential for repurposing, or revival, of dexanabinol for the treatment of CIPN became an attractive strategy. Promising preclinical results were obtained using a microphysiological model of peripheral nerves, however confirmatory studies to assess neuroprotection in a rat model of peripheral neurotoxicity is needed. These successful in vivo results warrant a clinical investigation for the use of dexanabinol to prevent peripheral neuropathy in multiple myeloma patients undergoing bortezomib therapy.
Appendix I:

Boolean Network Model Code: Intracellular Signaling and Gene Regulation in Peripheral Neurons

Peter Bloomingdale

Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences,

University at Buffalo, The State University of New York, Buffalo, NY
Overview

In this appendix we provide model code for the Boolean network model of intracellular signaling and gene regulation in peripheral neurons. MATLAB was used to perform network simulations using O defy. R was used to perform network attractor analyses using BoolNet. R was used to perform network perturbation analyses using SPIDDOR. Lastly, the reactions used when performing minimal intervention analysis in CellNetAnalyzer are provided.

MATLAB Code: Network Simulations

```matlab
% ExpressionsToOdefyGenerateateanOdefymodelfromBooleanexpressions.
% MODEL=ExpressionsToOdefy(EXPR) generates an Odefy model from the set of
% Boolean equations EXPR. EXPR can be (a) an array of strings or (b)
% a file containing one Boolean equation per line.
% MODEL=ExpressionsToOdefy(EXPR,MODELNAME) directly assigns the name
% MODELNAME to the resulting Odefy model.
% Each expression must have the form
% varname=expression

%-----------------------------------------------------------------------
%%%%% Logical Operators and Input Species
% Logical AND = &&
% Logical OR = ||
% Logical NOT = ~
% Input species = <>
%%%%% Example Call
% model = ExpressionsToOdefy({'a=a||b','b=a&&b'})
%%%%% Accepted Model Types
% boolcube, hillcube, hillcubenorm, boolsync, boolasync, boolrandom
%-----------------------------------------------------------------------

close all;
clear all;

InitOdefy

model = ExpressionsToOdefy({

'APCdh1 = ~ Cdk5',
'Akt = Pip3 || ~ Trib3',
'Apoptosis = cParp1',
'Arf = Myc && Foxo3',
'Ask1 = Daxx || Traf2',
'Atf2 = Jnk || p38',
'Atf3 = Atf2 || Perk || Atf4',
'Atf4 = (Camk && Rsk2) || Mapkapk2 || Eif2s1 || Jnk',
'Atf6 = ERStress || ~ Bip',
'Atm = ROS',
'ATP = ~ mPTP',
'AxonalTransport = (Khc&&ATP) || (Klc&&ATP)',
'Bad = ~ Akt || p53 || Jnk',
'Bax = Bid || (~ Bcl2 && ~ Bclxl && Myc) || (Bim && Puma && ~ Bcl2 && ~ Bclxl)',

);```
'nNos = Nmdar',
'Nox2 = Nmdar && Atf4 && cPkc',
'NO = nNos',
'Bort = <>'
}, 'PNBNFinalHumanV3Model');

%-----------------------------------------------------------------------%

simstruct = CreateSimstruct(model);

%Define time of simulation
simstruct.timeto = 100;

%Define type of model. Types of models: boolsync, boolasync, boolrandom,
%boolcube, hillcube, hillcubenorm
simstruct.type = 'hillcubenorm';

%Define parameter values for hill functions
%simstruct.params = DefaultParameters(simstruct.model,#Tau, #N, #K)

%Define Initial Conditions
%Initial condition: Control condition (No drug)
simstruct.initial = [1 1 0 0 0 0 0 0 0 0 1 1 0 0 1
1 0 1 0 0 1 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0
0 0 0 0 1 0 0 0 0 1 1 0 0 0 0 0 0 0 0 1 0 0
1 1 0 0 1 1 0 0 0 0 1 1 0 1 1 1 1 1 1 0 0
0 0 0 0 1 0 1 1 1 1 0 0 0 1 0 0 0 0 0 0 0 0
1 1 0 1 1 1 0 1 0 0 1 1 0 0 1 0 0 0 0 0 0 0
0 0 0 0 0 0 1 1 0 1 0 1 0 0 0 0 0 0 0 0 0 0];

%Initial condition: Proteasome inhibitor ON
%simstruct.initial = [1 1 0 0 0 0 0 0 0 0 1 1 0 0 1
1 0 1 0 0 1 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0
0 0 0 0 1 0 0 0 0 1 1 0 0 0 0 0 0 0 0 1 0 0
1 1 0 0 1 1 0 0 0 0 1 1 0 1 1 1 1 1 1 0 0
0 0 0 0 1 0 1 1 1 1 0 0 0 1 0 0 0 0 0 0 0 0
1 1 0 1 1 1 0 1 0 0 1 1 0 0 1 0 0 0 0 0 0 0
0 0 0 0 0 1 1 0 1 0 1 0 0 0 0 0 0 0 0 0 1];

%use [T,Y] for a dynamic simulation. Use [Y] for Boolean
[T,Y] = OdefySimulation(simstruct,1,1)
R Code: Attractor Analysis

#Model code is executed using a stepwise process
# 1) Load your network using loadNetwork function
# 2) Decide what type of attractor analysis to run using the getAttractors function
# 3) Write the output to a text file
# 4) Parse the data using the data extraction functions below.

#Load BoolNet
library(BoolNet)

#Load Data Extraction Tools
library("plyr")
library(stringr)

#Load network from text file
setwd("C:/")
PNBNFull = loadNetwork('HumanBN Final 3.0 BoolNet.txt')

##################################################################
####### Attractor Analysis: SAT Exhaustive Synchronous
##################################################################

###***Bortezomib Alone***
AttrSATFullBortON = getAttractors(PNBNFull, "synchronous", "sat.exhaustive",
genesisOFF = c("Proteasome"), genesON = c())
sink("AttrSATFullBortON.txt")
AttrSATFullBortON
sink()

###***Bortezomib + TNFalpha Inhibitor***
AttrSATFullBortON_TNFOFF = getAttractors(PNBNFull, "synchronous", "sat.exhaustive",
genesisOFF = c("Proteasome","Tnfa"), genesON = c())
sink("AttrSATFullBortON_TNFOFF.txt")
AttrSATFullBortON_TNFOFF
sink()

###***Bortezomib + ROS Inhibitor***
AttrSATFullBortON_ROSOFF = getAttractors(PNBNFull, "synchronous", "sat.exhaustive",
genesisOFF = c("Proteasome","ROS"), genesON = c())
sink("AttrSATFullBortON_ROSOFF.txt")
AttrSATFullBortON_ROSOFF
### Bortezomib + NMDA Inhibitor

```r
AttrSATFullBortON_NMDAOFF = getAttractors(PNBNFull, "synchronous", "sat.exhaustive", genesOFF = c("Proteasome", "Nmda"), genesON = c())
sink("AttrSATFullBortON_NMDAOFF.txt")
```

### Bortezomib + ROS Inhibitor + TNFalpha Inhibitor

```r
AttrSATFullBortON_ROSOFF_TNFOFF = getAttractors(PNBNFull, "synchronous", "sat.exhaustive", genesOFF = c("Proteasome", "ROS", "Tnfa"), genesON = c())
sink("AttrSATFullBortON_ROSOFF_TNFOFF.txt")
```

### Bortezomib + ROS Inhibitor + NMDA Inhibitor

```r
AttrSATFullBortON_ROSOFF_NMDAOFF = getAttractors(PNBNFull, "synchronous", "sat.exhaustive", genesOFF = c("Proteasome", "ROS", "Nmda"), genesON = c())
sink("AttrSATFullBortON_ROSOFF_NMDAOFF.txt")
```

### Bortezomib + TNFalpha Inhibitor + NMDA Inhibitor

```r
AttrSATFullBortON_TNFOFF_NMDAOFF = getAttractors(PNBNFull, "synchronous", "sat.exhaustive", genesOFF = c("Proteasome", "Tnfa", "Nmda"), genesON = c())
sink("AttrSATFullBortON_TNFOFF_NMDAOFF.txt")
```

### Bortezomib + ROS Inhibitor + TNFalpha Inhibitor + NMDA Inhibitor

```r
AttrSATFullBortON_ROSOFF_TNFOFF_NMDAOFF = getAttractors(PNBNFull, "synchronous", "sat.exhaustive", genesOFF = c("Proteasome", "ROS", "Tnfa", "Nmda"), genesON = c())
sink("AttrSATFullBortON_ROSOFF_TNFOFF_NMDAOFF.txt")
```

# Attractor Analysis: Random (n=10^6) Synchronous

```r
sink()
```
AttrRandFullBortON = getAttractors(PNBNFull, "synchronous", "random", startStates = 1000000, 
\hspace{1cm} genesOFF = 
\hspace{1cm} c("Proteasome"), genesON = c())
sink("AttrRandFullBortON.txt")
AttrRandFullBortON
sink()

### Bortezomib + TNFalpha Inhibitor***
AttrRandFullBortON_TNFOFF = getAttractors(PNBNFull, "synchronous", "random", 
\hspace{1cm} startStates = 1000000, 
\hspace{1cm} genesOFF = 
\hspace{1cm} c("Proteasome","Tnfa"), genesON = c())
sink("AttrRandFullBortON_TNFOFF.txt")
AttrRandFullBortON_TNFOFF
sink()

### Bortezomib + ROS Inhibitor***
AttrRandFullBortON_ROSOFF = getAttractors(PNBNFull, "synchronous", "random", 
\hspace{1cm} startStates = 1000000, 
\hspace{1cm} genesOFF = 
\hspace{1cm} c("Proteasome","ROS"), genesON = c())
sink("AttrRandFullBortON_ROSOFF.txt")
AttrRandFullBortON_ROSOFF
sink()

### Bortezomib + NMDA Inhibitor***
AttrRandFullBortON_NMDAOFF = getAttractors(PNBNFull, "synchronous", "random", 
\hspace{1cm} startStates = 1000000, 
\hspace{1cm} genesOFF = c("Proteasome","Nmda"), 
\hspace{1cm} genesON = c())
sink("AttrRandFullBortON_NMDAOFF.txt")
AttrRandFullBortON_NMDAOFF
sink()

### Bortezomib + ROS Inhibitor + TNFalpha Inhibitor***
AttrRandFullBortON_ROSOFF_TNFOFF = getAttractors(PNBNFull, "synchronous", 
\hspace{1cm} "random", startStates = 1000000, 
\hspace{1cm} genesOFF = 
\hspace{1cm} c("Proteasome","ROS", "Tnfa"), genesON = c())
sink("AttrRandFullBortON_ROSOFF_TNFOFF.txt")
AttrRandFullBortON_ROSOFF_TNFOFF
sink()

### Bortezomib + ROS Inhibitor + NMDA Inhibitor***
AttrRandFullBortON_ROSOFF_NMDAOFF = getAttractors(PNBNFull, "synchronous", 
"random", startStates = 1000000, 

genesOFF =
c("Proteasome", "ROS", "Nmda"), genesON = c())
sink("AttrRandFullBortON_ROSOFF_NMDAOFF.txt")
AttrRandFullBortON_ROSOFF_NMDAOFF
sink()

###Bortezomib + TNFalpha Inhibitor + NMDA Inhibitor***
AttrRandFullBortON_TNFOFF_NMDAOFF = getAttractors(PNBNFull, "synchronous", 
"random", startStates = 1000000, 

genesOFF =
c("Proteasome", "Tnfa", "Nmda"), genesON = c())
sink("AttrRandFullBortON_TNFOFF_NMDAOFF.txt")
AttrRandFullBortON_TNFOFF_NMDAOFF
sink()

###Bortezomib + ROS Inhibitor + TNFalpha Inhibitor + NMDA Inhibitor***
AttrRandFullBortON_ROSOFF_TNFOFF_NMDAOFF = getAttractors(PNBNFull, "synchronous", 
"random", startStates = 1000000, 

genesOFF = c("Proteasome", "ROS", 
"Tnfa", "Nmda"), genesON = c())
sink("AttrRandFullBortON_ROSOFF_TNFOFF_NMDAOFF.txt")
AttrRandFullBortON_ROSOFF_TNFOFF_NMDAOFF
sink()

#+++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
###### DATA EXTRACTION: Read Attractor Analysis Output File
#+++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++

#DataExtraction
y = readLines('AttrSATFullBortON.txt')
y = readLines('AttrSATFullBortON_ROSOFF.txt')
y = readLines('AttrSATFullBortON_TNFOFF.txt')
y = readLines('AttrSATFullBortON_NMDAOFF.txt')
y = readLines('AttrSATFullBortON_ROSOFF_TNFOFF.txt')
y = readLines('AttrSATFullBortON_ROSOFF_NMDAOFF.txt')
y = readLines('AttrSATFullBortON_TNFOFF_NMDAOFF.txt')
y = readLines('AttrRandFullBortON.txt')
y = readLines('AttrRandFullBortON_ROSOFF.txt')
y = readLines('AttrRandFullBortON_TNFOFF.txt')
y = readLines('AttrRandFullBortON_NMDAOFF.txt')
y = readLines('AttrRandFullBortON_ROSOFF_TNFOFF.txt')
y = readLines('AttrRandFullBortON_ROSOFF_NMDAOFF.txt')
y = readLines('AttrRandFullBortON_TNFOFF_NMDAOFF.txt')
y = readLines('AttrRandFullBortON_ROSOFF_TNFOFF_NMDAOFF.txt')

#################################################################################
######### DATA EXTRACTION: SYNCHRONOUS SAT EXHAUSTIVE
#########  *Note: Read the attractor analysis files one at a time. Then run the
#########   data extraction functions below. Then write to an excel file.
#########  *Functions work with BoolNet version 2.1.1
#################################################################################

# Start Data Extraction
attrsummary<- function(y, text){
  nodes <- str_split(gsub(".*order: ", "", y[grep(".*order:\\", y)[1]]), "\"")[[1]]
  n = length(y[grep("Attractor\", y)])
  substrattr <- gsub(".*basin\ of\ ", "", y[grep("Attractor\ ", y)])
  temp2 <- gsub("\ .*", "", gsub(".*of ", "", substrattr )
  df<- data.frame(Attr = c(1:n),
                  freq = 1 / n)
  a = grep("V", y)
  b = list()
  c = list()
  d = list()
  e = matrix(0, nrow = (length(a)/2), ncol=length(nodes))

  for (i in 1: (length(a)/2)){
    b[[i]] = c( (a[2*i-1]+1) : (a[2*i]-1) )
    c[[i]] = as.data.frame( gsub("\\", "", gsub("\ ", "", y[b[[i]]] )) )
    d[[i]] = str_split_fixed(as.character(c[[i]][,1]),"", n = length(nodes))
    class(d[[i]])<-"numeric"  #colnames(d[[i]]) <- nodes
    e[i, ] = colMeans(d[[i]]) # mean for node value in an attractor (averaged by
                                #attractor size itself)
  }
  colnames(e) <- nodes
  return(e)
}

# End Data Extraction

# Write to an Excel CSV file
AttrRandFullBortON_TNFOFF_NMDAOFF=attrsummary(y,"")
write.csv(AttrRandFullBortON_TNFOFF_NMDAOFF, file = "AttrRandFullBortON_TNFOFF_NMDAOFF.csv")

# Start Data Extraction
attrsummary2 <- function(y, text){
  nodes <- str_split(gsub(".*order: ", "", y[grep(".*order:\n ", y)][1]]), "\"
  ][[1]]  
  # allattr <- gsub("\", ", gsub("\", ", y[grep("[0-9]{65}", y)])
  n = length(y[grep("Attractor\n ", y)])
  substrattr <- gsub(".*basin\n of\n ", "", y[grep("Attractor\n ", y)])
  temp2 <- gregexpr("[0-9]+", substrattr)
  df <- data.frame(Attr = c(1:n),
                   Basin=as.numeric(unlist(regmatches(substrattr, temp2))))
  
  df$BF <- df$Basin / sum(df$Basin)
  
  a = grep("V", y)
  b = list()
  c = list()
  d = list()
  e = matrix(0, nrow = (length(a)/2), ncol=length(nodes )),
  for (i in 1: (length(a)/2)){
    b[[i]] = c( (a[2*i-1]+1) : (a[2*i]-1) )
    c[[i]] = as.data.frame( gsub("\\","", gsub("\", ", y[b[[i]]] )))
    d[[i]] = str_split_fixed(as.character(c[[i]][1]),"", n = length(nodes))
    class(d[[i]])<-"numeric"
    #colnames(d[[i]]) <- nodes
    e[i, ] = colMeans(d[[i]]) # mean for node value in an attractor
  }
  colnames(e) <- nodes
  f <- t( sapply(1:nrow(e), function(x) e[x, ] * df[x, 3] ) ) # mean value * freq
  return(f)
}
# End Data Extraction
# Write to an Excel CSV file

```r
AttrRandFullBortON_ROSOFF_TNFOFF_NMDOFF=attrsummary2(y,"")

write.csv(AttrRandFullBortON_ROSOFF_TNFOFF_NMDOFF, file = "AttrRandFullBortON_ROSOFF_TNFOFF_NMDOFF.csv")
```
R Code: Perturbation Analysis

########## Perturbation Analysis: SPIDDOR ##########

# Help functions
?read.Boolean.functions
?KO_matrix.f

# Install Packages
install.packages("gdata")
install.packages("gtools")
install.packages("caTools")
install.packages("latin1")

# Load SPIDDOR
library(SPIDDOR)
library(ggplot2)

# Set working directory
setwd("D:/Google Drive/PhD Dissertation/Chapter 4 - Boolean Network Modeling of CIPN/Sensitivity Analysis")

# Connect to Rtools (write your path to Rtools):
Connect2Rtools(path="C:\\Rtools")

# Load Peripheral Neuropathy Boolean Network Model
PNBNmodel = read.Boolean.functions(file="HumanBN Final 3.0 SPIDDOR.txt")

# Summary of Model
print(PNBNmodel)

# ***** NOTE: (1) Run an Initial Condition of Interest and (2) Run the Perturbation Analysis of Interest *****

###### Define Initial Conditions
# Healthy State

# Healthy State + Proteasome Inhibition
(Note: Proteasome initial condition must be OFF for the knockout function to work correctly)

Only Input Nodes ON
PNBNmodel$Initial_conditions = c("Bdnf", "Ngfb", "Proteasome", "Il1b", "Tgfb", "Tnfa", "Nmda", "Il6", "Fasl")

List of all nodes
# PERTURBATION ANALYSES

## Knock-Out Treatment Naive

```r
KO.m = KO_matrix.f(PNBNmodel, time.steps=2000, asynchronous=TRUE, repetitions = 25)
Create_heatmap(KO.m)
```

## Knock-Out Proteasome Inhibition

```r
KO.m = KO_matrix.f(PNBNmodel, time.steps=2000, Knockouts="Proteasome", asynchronous=TRUE, repetitions = 25)
Create_heatmap(KO.m)
```

## Knock-In Treatment Naive

```r
OE.m = OE_matrix.f(PNBNmodel, time.steps=2000, asynchronous=TRUE, repetitions = 25)
Create_heatmap(OE.m)
```

## Knock-In Proteasome Inhibition

```r
OE.m = OE_matrix.f(PNBNmodel, time.steps=2000, Knockouts="Proteasome", asynchronous=TRUE, repetitions = 25)
Create_heatmap(OE.m)
```

## Knock-Out Proteasome Inhibition (TNF present)

```r
KO.m = KO_matrix.f(PNBNmodel, time.steps=2000, Knockouts="Proteasome", Over_expr="Tnfa", asynchronous=TRUE, repetitions = 25)
Create_heatmap(KO.m)
```

## Knock-In Proteasome Inhibition (TNF present)

```r
OE.m = OE_matrix.f(PNBNmodel, time.steps=2000, Knockouts="Proteasome", Over_expr="Tnfa", asynchronous=TRUE, repetitions = 25)
Create_heatmap(OE.m)
```
CellNetAnalyzer Interactions

Reaction 1: Pip3 ==> Akt
Reaction 2: !Trib3 ==> Akt
Reaction 3: cParp1 ==> Apoptosis
Reaction 4: Foxo3 + Myc ==> Arf
Reaction 5: Daxx ==> Ask1
Reaction 6: Jnk ==> Atf2
Reaction 7: Atf2 ==> Atf3
Reaction 8: Eif2s1 ==> Atf4
Reaction 9: ER_Stress ==> Atf6
Reaction 10: !Bip ==> Atf6
Reaction 11: ROS ==> Atm
Reaction 12: !mPTP ==> ATP
Reaction 13: ATP + Khc ==> Axonal_Transport
Reaction 14: ATP + Klc ==> Axonal_Transport
Reaction 15: !Akt ==> Bad
Reaction 16: p53 ==> Bad
Reaction 17: Jnk ==> Bad
Reaction 18: Bid ==> Bax
Reaction 19: !Bcl2 + !Bclxl + Myc + Puma ==> Bax
Reaction 20: !Bcl2 + !Bclxl + Bim + Puma ==> Bax
Reaction 21: Stat ==> Bcl2
Reaction 22: !Chop + Nfkb ==> Bcl2
Reaction 23: !Bad ==> Bcl2
Reaction 24: !Bad ==> Bclxl
Reaction 25: Nfkb + !Puma ==> Bclxl
Reaction 26: E2f1 ==> BCMyb
Reaction 27: Casp8 ==> Bid
Reaction 28: Chop ==> Bim
Reaction 29: BCMyb + Foxo3 + Jun ==> Bim
Reaction 30: ER_Stress ==> Bip
Reaction 31: Bip + ER_Stress + IP3R ==> Calcium
Reaction 32: NmdaR ==> Calcium
Reaction 33: Calcium ==> Calm
Reaction 34: Calcium ==> Calpain
Reaction 35: Calm ==> Camk
Reaction 36: Calpain ==> Casp12
Reaction 37: Casp7 ==> Casp12
Reaction 38: Casp9 + !Xiap ==> Casp3
Reaction 39: Casp8 ==> Casp3
Reaction 40: Casp8 ==> Casp7
Reaction 41: Casp9 + !Xiap ==> Casp7
Reaction 42: Fadd ==> Casp8
Reaction 43: Casp12 + Cyc + !Xiap ==> Casp9
Reaction 44: NgfR ==> Cdc42Rac1
Reaction 45: CycE ==> Cdk2
Reaction 46: CycD ==> Cdk4_6
Reaction 47: Calpain + NmdaR ==> Cdk5
Reaction 48: Atf4 ==> Chop
Reaction 49: Atf6 ==> Chop
Reaction 50: Parp1 ==> cParp1
Reaction 51: Bax + mPTP ==> Cyc
Reaction 52: !Gsk3b + !p15 + !p21 + !Pcna ==> CycD
Reaction 53: !NgfR ==> CycD
Reaction 54: Cdk4_6 + CycD + !p21 ==> CycE
Reaction 55: Plcg1 ==> Dag
Reaction 56: FasR ==> Daxx
Reaction 57: !pRb ==> E2f1
Reaction 58: Perk ==> Eif2s1
Reaction 59: !Gadd34 + Perk ==> Eif2s1
Reaction 60: ROS ==> ER_Stress
Reaction 61: Mek1_2 ==> Erk1_2
Reaction 62: Mek5 ==> Erk5
Reaction 63: FasR ==> Fadd
Reaction 64: Tradd ==> Fadd
Reaction 65: FasL ==> FasR
Reaction 66: !Akt ==> Foxo3
Reaction 67: Chop ==> Gadd34
Reaction 68: p53 ==> Gadd45
Reaction 69: Trkb ==> Grb2sos
Reaction 70: !Rsk2 ==> Grb2sos
Reaction 71: !Akt ==> Gsk3b
Reaction 72: !Bdnf ==> Gsk3b
Reaction 73: NmdaR ==> Gsk3b
Reaction 74: !Akt + Bax + mPTP ==> Htra2Omi
Reaction 75: Bax + mPTP ==> Htra2Omi
Reaction 76: !Ikk ==> Ikba
Reaction 77: Nfkb ==> Ikba
Reaction 78: !Cdk5 ==> APCC_Cdh1
Reaction 79: Akt + Nik ==> Ikk
Reaction 80: Il6 ==> IL6R
Reaction 81: Plcg1 ==> IP3
Reaction 82: IP3 ==> IP3R
Reaction 83: ER_Stress ==> Ire1
Reaction 84: !Bip ==> Ire1
Reaction 85: IL6R ==> Jak
Reaction 86: Mkk4 + Mkk7 ==> Jnk
Reaction 87: Jnk ==> Jun
Reaction 88: !Jnk ==> Khc
Reaction 89: !Gsk3b ==> Klc
Reaction 90: p38 ==> Mapkapk2
Reaction 91: !Arf ==> Mdm2
Reaction 92: Mekk1 ==> Mek1_2
Reaction 93: Mekk3 ==> Mek5
Reaction 94: Cdc42Rac1 ==> Mekk1
Reaction 95: Ras ==> Mekk3
Reaction 96: Ask1 ==> Mkk3
Reaction 97: Mlk3 ==> Mkk3
Reaction 98: Ask1 + Mekk1 + Tak1 ==> Mkk4
Reaction 99: Mlk3 ==> Mkk4
Reaction 100: Ask1 ==> Mkk6
Reaction 101: Mlk3 ==> Mkk6
Reaction 102: Ask1 + Mekk1 + Tak1 ==> Mkk7
Reaction 103: Mlk3 ==> Mkk7
Reaction 104: !Akt + !NgfR ==> Mlk3
Reaction 105: Calcium ==> mPTP
Reaction 106: !Gsk3b ==> Myc
Reaction 107: Stat ==> Myc
Reaction 108: !Smad4 ==> Myc
Reaction 109: !Arf ==> Myc
Reaction 110: Atf3 ==> Nerve_Damage
Reaction 111: Eif2s1 ==> Nfkb
Reaction 112: !Gsk3b ==> Nfkb
Reaction 113: Bdnf ==> NgfR
Reaction 114: Ngf ==> NgfR
Reaction 115: Ripk1 ==> Nik
Reaction 116: Mekk1 + Tak1 ==> Nik
Reaction 117: Nmda ==> NmdaR
Reaction 118: !Myc ==> p15
Reaction 119: !Akt ==> p21
Reaction 120: p53 ==> p21
Reaction 121: Stat ==> p21
Reaction 122: APCC_Cdh1 ==> p21
Reaction 123: Mkk3 + Mkk6 + Ras ==> p38
Reaction 124: !Mdm2 ==> p53
Reaction 125: !Atm + Jnk + p38 ==> p53
Reaction 126: Casp3 ==> Parp1
Reaction 127: Gadd45 ==> Pcna
Reaction 128: ER_Stress ==> Perk
Reaction 129: !Bip ==> Perk
Reaction 130: Trka ==> Pi3k
Reaction 131: Jak ==> Pi3k
Reaction 132: Trkb ==> Pi3k
Reaction 133: Ras ==> Pi3k
Reaction 134: Pi3k ==> Pip3
Reaction 135: !Pten ==> Pip3
Reaction 136: Dag ==> nPkc
Reaction 137: Trkb ==> Plcg1
Reaction 138: !Cdk4_6 + !CycD ==> pRb
Reaction 139: !Cdk2 + !CycE ==> pRb
Reaction 140: !pRb ==> Proliferation
Reaction 141: !PI ==> Proteasome
Reaction 142: p53 ==> Pten
Reaction 143: Chop + Nfkβ + p53 ==> Puma
Reaction 144: cPkc ==> Raf
Reaction 145: Ras ==> Raf
Reaction 146: !Erk_2 ==> Ras
Reaction 147: Jak ==> Ras
Reaction 148: Calcium ==> Ras
Reaction 149: Grb2sos ==> Ras
Reaction 150: Tradd ==> Ripk1
Reaction 151: Chop ==> ROS
Reaction 152: !Proteasome ==> ROS
Reaction 153: Erk_2 ==> Rsk2
Reaction 154: TgfbR ==> Smad2_3
Reaction 155: Jnk ==> Smad4
Reaction 156: Jak ==> Stat
Reaction 157: Traf2 ==> Tak1
Reaction 158: Traf6 ==> Tak1
Reaction 159: !Xiap ==> Tak1
Reaction 160: Tgfb ==> TgfbR
Reaction 161: Tnfa ==> TnfR
Reaction 162: TnfR ==> Tradd
Reaction 163: Ire1 ==> Traf2
Reaction 164: Tradd ==> Traf2
Reaction 165: IL1R ==> Traf2
Reaction 166: NgfR ==> Traf6
Reaction 167: TgfbR ==> Traf6
Reaction 168: IL1R ==> Traf6
Reaction 169: Bdnf ==> Trkb
Reaction 170: Foxo3 + !NgfR ==> Trib3
Reaction 171: Atf4 + Chop ==> Trib3
Reaction 172: Ngf ==> Trka
Reaction 173: Ire1 ==> Xbp1
Reaction 174: !Htra2Omi + Nfkβ ==> Xiap
Reaction 175: !Ikba ==> Nfkβ
Reaction 176: IL1 ==> IL1R
Reaction 177: Traf2 ==> Ask1
Reaction 178: Smad2_3 ==> Smad4
Reaction 179: Camk + Rsk2 ==> Atf4
Reaction 180: ROS ==> mPTP
Reaction 181: Bip ==> Bip_2
Reaction 182: Bip_2 ==> Bip
Reaction 183: Xbp1 ==> Chop
Reaction 184: p38 ==> Chop
Reaction 185: Jnk ==> Atf4
Reaction 186: Mapkapk2 ==> Atf4
Reaction 187: Calcium + Dag ==> cPkc
Reaction 188: Atf4 + NmdaR + cPkc ==> Nox2
Reaction 189: NmdaR ==> nNos
Reaction 190: nNos ==> NO
Reaction 191: Nox2 + NO ==> ROS
Reaction 192: NO ==> Jnk
Reaction 193: Traf2 ==> Mekk1
Reaction 194: Raf ==> Mek1_2
Reaction 195: nPkc ==> Mek1_2
Reaction 196: Erk5 ==> Rsk2
Reaction 197: Casp7 ==> Parp1
Reaction 198: Perk ==> Atf3
Reaction 199: Atf4 ==> Atf3
Reaction 200: p38 ==> Atf2
Reaction 201: Casp10 ==> Bid
Reaction 202: Casp10 ==> Casp7
Reaction 203: Casp10 ==> Casp3
Reaction 204: Fadd ==> Casp10
Reaction 205: Foxo3 + Jun ==> FasL
Appendix II:

Pharmacodynamic Properties of Dexanabinol

Peter Bloomingdale

Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences,

University at Buffalo, The State University of New York, Buffalo, NY
Overview

In this appendix we cover the previous work that has been done regarding the promiscuous pharmacological mechanisms of action of dexanabinol. We discuss the history of this drug candidate for its initial promise as a treatment for traumatic brain injury and potential drug revival for chemotherapy-induced peripheral neuropathy. The pharmacodynamic modeling that was performed enabled us to assess the potency of dexanabinol for inhibiting each of its targets, which ultimately was used to help inform the selection of a dosing regimen for an in vivo toxicity study using a rat model of bortezomib-induced peripheral neuropathy. All models were developed and fit to literature-derived data using parameter estimation methods in Adapt 5.

Brief History and Pharmacodynamics of Dexanabinol

Dexanabniol (HU-211) and its enantiomer HU-210 are synthetic cannabinoid derivative that were first synthesized in 1988 by Mechoulam et al. HU-210 binds to cannabinoid receptors and has been reported to be 80-800 times more potent than natural Δ9-THC. Interestingly, dexanabinol does not bind to cannabinoid receptors 1/2 (CB1/CB2). Therefore, dexanabinol is pharmacological different than most cannabimimetics as it does not cause sedation, immobility, reduction in body temperature, or psychoactive effects that are associated with THC-type activity.

Dexanabinol has a promiscuous pharmacological activity profile. Initially, dexanabinol was shown to be an NMDA antagonist since it blocked NMDA-induced neurotoxic death of mice. Afterwards Eshhar et al. (1995) showed that dexanabinol has antioxidative properties by acting as a scavenger of peroxo radicals. Then in 1997 Shohami et al. showed that dexanabinol inhibited the production of TNFα.

Clinically, dexanabinol was a promising candidate from traumatic brain injury (TBI) considering the successes in rodent models of TBI. However, the preclinical rodent models did not translate to human, which resulted in a phase III clinical trial failure in 2006. Currently, dexanabinol is being assessed for its potential utility in a variety of cancer types, which include brain, pancreas, and liver cancers. Also dexanabinol has recently been assessed as a combination therapy with an anti-mCTLA4 antibody.

Equations

\[
\text{TNFa Inhibition} \ (\%) = E_0 \times \frac{[\text{Dex}]^γ}{EC_{50}^γ + [\text{Dex}]^γ}
\]

\[
\text{Cell Viability} \ (\%) = E_0 \times \frac{[\text{Dex}]^γ}{EC_{50}^γ + [\text{Dex}]^γ}
\]

\[
\text{LDH Release} \ (\%) = E_0 \times \left(1 - I_{\text{max}} \times \frac{[\text{Dex}]^γ}{IC_{50}^γ + [\text{Dex}]^γ}\right)
\]

\[
\text{Ca}^{2+}\text{Uptake} = 1 + E_0 \times \left(1 - I_{\text{max}} \times \frac{[\text{Dex}]^γ}{IC_{50}^γ + [\text{Dex}]^γ}\right)
\]

\[
\text{Ca}^{2+}\text{Uptake Rate} = E_0 \times \left(1 + \frac{E_{\text{max}}}{E_0} \times \frac{[\text{NMDA}]^γ}{EC_{50}^γ + [\text{NMDA}]^γ}\right) \times \left(1 - I_{\text{max}} \times \frac{[\text{Dex}]^γ}{IC_{50}^γ + [\text{Dex}]^γ}\right)
\]

\[
\text{Oxygen Consumption} = E_0 \times \left(1 - I_{\text{max}} \times \frac{[\text{Dex}]^γ}{IC_{50}^γ + [\text{Dex}]^γ}\right)
\]
Figures and Tables

Figure 1: Inhibition of LPS-induced TNFα production in Macrophages by Dexanabinol.

![Graph showing inhibition of TNFα production](image1)

Figure 2: Inhibition of NMDA-induced Neurotoxicity in Rat-derived Cortical Neurons by Dexanabinol

![Graph showing cell viability](image2)
Figure 3: Inhibition of LDH Release in Rat-derived Cortical Neurons by Dexanabinol

![Graph showing inhibition of LDH release](image)

Figure 4: Inhibition of $^{45}\text{Ca}^{2+}$ Uptake in Rat-derived Cortical Neurons

![Graph showing inhibition of $^{45}\text{Ca}^{2+}$ uptake](image)
Figure 5 Inhibition of $^{45}\text{Ca}^{2+}$ Uptake Rate in Rat-derived Cortical Neurons

Figure 6 Methyl Linoleate Oxidation Assay to Measure Oxygen Radical Scavenging by Dexanabinol
Table 1: Summary of Publications that Investigate the Pharmacodynamics of Dexanabinol

<table>
<thead>
<tr>
<th>Publication</th>
<th>Experimental Model</th>
<th>Pharmacodynamic Effect</th>
<th>Experimentally Measured Biomarker</th>
<th>Concentration(s) Investigated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallily (1997)</td>
<td>Macrophages (RAW 264.7)</td>
<td>TNFα Inhibition</td>
<td>LPS induced TNFα Production</td>
<td>5-20 μM</td>
</tr>
<tr>
<td>Eshhar (1993)</td>
<td>Rat-derived Cortical Neurons</td>
<td>NMDA antagonism</td>
<td>NMDA-induced neurotoxicity (XTT)</td>
<td>1-10 μM</td>
</tr>
<tr>
<td>Nadler (1993a)</td>
<td>Rat-derived Cortical Neurons</td>
<td>NMDA antagonism</td>
<td>LDH Release</td>
<td>5-50 μM</td>
</tr>
<tr>
<td>Nadler (1993b)</td>
<td>Rat-derived Cortical Neurons</td>
<td>NMDA antagonism</td>
<td>$^{45}$Ca$^{2+}$ Uptake</td>
<td>5-50 μM</td>
</tr>
<tr>
<td>Nadler (1993b)</td>
<td>Rat-derived Cortical Neurons</td>
<td>NMDA antagonism</td>
<td>$^{45}$Ca$^{2+}$ Uptake Rate</td>
<td>5-50 μM</td>
</tr>
<tr>
<td>Schwaninger (2004)</td>
<td>Human Glioblastoma (U373 MG)</td>
<td>NFKB Inhibition</td>
<td>Transcriptional Activity (Luciferase Reporter)</td>
<td>3-33 μM</td>
</tr>
<tr>
<td>Schwaninger (2004)</td>
<td>Murine-derived Cortical Neurons</td>
<td>NFKB Inhibition</td>
<td>Transcriptional Activity (Luciferase Reporter)</td>
<td>1-33 μM</td>
</tr>
</tbody>
</table>

Table 2: Dexanabinol Mediated Inhibition of LPS-induced TNFα production in Macrophages

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Units</th>
<th>Value</th>
<th>CV (%)</th>
<th>Confidence Interval (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{\text{max}}$</td>
<td>%</td>
<td>100</td>
<td>Fixed</td>
<td>Fixed</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>-</td>
<td>1.26</td>
<td>2.1</td>
<td>[0.929 - 1.58]</td>
</tr>
<tr>
<td>$EC_{50}$</td>
<td>μM</td>
<td>9.05</td>
<td>1.0</td>
<td>[7.85 - 10.3]</td>
</tr>
</tbody>
</table>

Table 3: Dexanabinol Mediated Inhibition of NMDA-induced Neurotoxicity in Rat-derived Cortical Neurons

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Units</th>
<th>Value</th>
<th>CV (%)</th>
<th>Confidence Interval (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{\text{max}}$</td>
<td>%</td>
<td>100</td>
<td>Fixed</td>
<td>Fixed</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>-</td>
<td>1.83</td>
<td>11.2</td>
<td>[-0.778 - 4.45]</td>
</tr>
<tr>
<td>$EC_{50}$</td>
<td>μM</td>
<td>3.57</td>
<td>11.5</td>
<td>[-1.63 - 8.77]</td>
</tr>
</tbody>
</table>
### Table 4: Dexanabinol Mediated Inhibition of LDH Release in Rat-derived Cortical Neurons

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Units</th>
<th>Value</th>
<th>CV (%)</th>
<th>Confidence Interval (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_0$</td>
<td>%</td>
<td>100</td>
<td>Fixed</td>
<td>Fixed</td>
</tr>
<tr>
<td>$\gamma$</td>
<td></td>
<td>1</td>
<td>Fixed</td>
<td>Fixed</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>$\mu$M</td>
<td>8.97</td>
<td>6.2</td>
<td>[7.43 - 10.5]</td>
</tr>
<tr>
<td>Imax</td>
<td></td>
<td>1</td>
<td>Fixed</td>
<td>Fixed</td>
</tr>
</tbody>
</table>

### Table 5: Dexanabinol Mediated Inhibition of $^{45}$Ca$^{2+}$ Uptake in Rat-derived Cortical Neurons

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Units</th>
<th>Value</th>
<th>CV (%)</th>
<th>Confidence Interval (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_0$</td>
<td></td>
<td>0.943</td>
<td>3.4</td>
<td>[0.874 - 1.01]</td>
</tr>
<tr>
<td>$\gamma$</td>
<td></td>
<td>2.58</td>
<td>18.6</td>
<td>[1.55 - 3.62]</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>$\mu$M</td>
<td>30.4</td>
<td>8.6</td>
<td>[24.8 - 36.0]</td>
</tr>
<tr>
<td>Imax</td>
<td></td>
<td>1</td>
<td>Fixed</td>
<td>Fixed</td>
</tr>
</tbody>
</table>

### Table 6: Dexanabinol Mediated Inhibition of $^{45}$Ca$^{2+}$ Uptake Rate in Rat-derived Cortical Neurons

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Units</th>
<th>Value</th>
<th>CV (%)</th>
<th>Confidence Interval (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_0$</td>
<td>nmol/mg/min</td>
<td>10.1</td>
<td>8.2</td>
<td>[8.09 - 12.0]</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>-</td>
<td>3</td>
<td>Fixed</td>
<td>Fixed</td>
</tr>
<tr>
<td>Emax</td>
<td>nmol/mg/min</td>
<td>9.03</td>
<td>15.2</td>
<td>[5.77 - 12.3]</td>
</tr>
<tr>
<td>EC$_{50}$</td>
<td>$\mu$M</td>
<td>43.2</td>
<td>20.6</td>
<td>[22.2 - 64.3]</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>$\mu$M</td>
<td>105</td>
<td>30.8</td>
<td>[28.4 - 181]</td>
</tr>
<tr>
<td>Imax</td>
<td>-</td>
<td>1</td>
<td>Fixed</td>
<td>Fixed</td>
</tr>
</tbody>
</table>

### Table 7: Methyl Linoleate Oxidation Assay to Measure Oxygen Radical Scavenging by Dexanabinol

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Units</th>
<th>Value</th>
<th>CV (%)</th>
<th>Confidence Interval (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_0$</td>
<td>-</td>
<td>1</td>
<td>Fixed</td>
<td>Fixed</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>-</td>
<td>0.4301</td>
<td>21.0</td>
<td>[0.179 - 0.681]</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>$\mu$M</td>
<td>29.7</td>
<td>50.5</td>
<td>[-12.0 - 71.4]</td>
</tr>
<tr>
<td>Imax</td>
<td>-</td>
<td>1</td>
<td>Fixed</td>
<td>Fixed</td>
</tr>
</tbody>
</table>
References

Appendix III:

Boolean Network Model Code of Multiple Myeloma Signaling

Peter Bloomingdale

Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences,

University at Buffalo, The State University of New York, Buffalo, NY
Model Application:
The code for the Boolean network model of intracellular signaling in multiple myeloma cells is shown below. Equations for the model were obtained from Ramakrishnan and Mager (2018). The model was applied to simulate the cellular pharmacodynamic effects of bortezomib and/or dexanabinol in multiple myeloma cells. The following code is written in MATLAB. Odefy, a MATLAB based toolbox, was used to perform network simulations. Additionally, an attractor analysis was performed on the network model, which was performed in R (source code provided).

The only edit to the original published model was changing the error in the following relationship:

\[ Cdc25A = \neg (Chk2 \text{ || Chk2}) \Rightarrow Cdc25A = \neg (Chk1 \text{ || Chk2}) \]

MATLAB Model Code: Used for Network Simulations

% ExpressionsToOdefy generates an Odefy model from Boolean expressions.
% MODEL = ExpressionsToOdefy(EXPR) generates an Odefy model from the set of Boolean equations EXPR. EXPR can be (a) a cell array of strings or (b) a file containing one Boolean equation per line.
% MODEL = ExpressionsToOdefy(EXPR, MODELNAME) directly assigns the name MODELNAME to the resulting Odefy model.
% Each expression must have the form
% varname = expression

%%%%%%%%%%%%%%%%%%%%%%%%%%%%
% Logical Operators and Input Species
% Logical AND = &&
% Logical OR = ||
% Logical NOT = ~
% Input species = <>
% Example Call
% model = ExpressionsToOdefy({'a=a||b','b=a&&b'})
% Accepted Model Types
% boolcube, hillcube, hillcubenorm, boolsync, boolasync, boolrandom
%%%%%%%%%%%%%%%%%%%%%%%%%%%%

close all;
clear all;

% Initialize Odefy
InitOdefy

% Define Network Functions
NetworkDex = ExpressionsToOdefy({
    'IL6 = pNFKB',
    'gp130 = ~Cas3',
    'TNFR = ~Dex',
    'TNF = ~Dex',
    'RIP = (TNF && TNFR) || Bort',
    'NIK = ~TRAF3',
    'JAK1 = IL6 && gp130 && ~SHP1',
    'JAK2 = IL6 && gp130 && ~SHP1',
    'IKK = ((RIP && NIK) || AKT) || (ROS && ATM && DNAdam)',
    'pIKB = IKK',
    'pNFKB = (pIKB && Prot) || pIKB || pSTAT3 || ~GSK3B',
    'FLIP = pNFKB',
    'Prot = ~Bort',
    'XIAP = (pNFKB || pSTAT3) && ~(Smac || p53)',
    'BclXL = (pNFKB || pSTAT3) && ~(BAX || BAD || p53)',
    'Bcl2 = (pNFKB || pSTAT3) && ~BAD || p53',
    'CYCD = (MYC || AKT || ERK || P90RSK || cMAF) && ~GSK3B',
    'CYCE = MYC',
    'MDM = (p53 || AKT) && ~(ATM || ATR)',
    'MYC = (pSTAT3 || ERK || MEKK || IRF4) && ~BLIMP1',
    'STAT3 = ~Cas3',
    'pSTAT3 = ((JAK1 && JAK2 && STAT3) || (JAK1 && JAK2 && STAT3 && ~IKK)) && ~JNK',
    'AKT = mTORC2 || (PIP3 && mTORC2)',
    'PI3K = (IL6 && gp130) || IRS',
})
'Pip3 = PI3K & ~PTEN',
'pRb = (CDK4 & CDK6) || (CDK4 & CDK2 & CDK6) || (CDK4 & CDK2 & CDK6 & CYCB)',
'CIAP = TNFR & ~Dex',
'Ras = (IL6 & gp130) || SHP1 || IRS',
'RAF = Ras',
'MEK1 = RAF',
'MEKK = MEK1',
'MAPK = MEK1',
'ERK = MAPK',
'CDK2 = (CYCE & Cdc25A) & ~p21 & p27',
'CDK4 = (CYCD & Cdc25A) & ~p21 & p27',
'CDK6 = CYCD',
'Proliferation = pRb',
'DEPTOR = (cMAF || (cMAF & ~mTORC1 || mTORC2))',
'mTORC2 = ((~DEPTOR & PI3K) || PI3K)',
'SGK1 = mTORC2',
'mTORC1 = ((Rheb) || ((Rheb) & ~DEPTOR || PRAS40))',
'P70S6K = mTORC1',
'IRS = ((IGF1R & IGF1) || (IGF1R & (IL6 & gp130))) || ~P70S6K || JNK',
'Rheb = ~TSC',
'P90RSK = ERK',
'cdc25bc = ~(Chk1 || Chk2)',
'Cdc25A = ~(Chk2 || Chk2)',
'CDK1 = ~p21 & p27 & cdc25bc',
'CYCB = CDK1',
'cMAF = ~GSK3B',
'IGF1 = <>',
'IGF1R = <>',
'IRF4 = IRF4 || MYC || pNFKB',
'Bc16 = pSTAT3 || (pSTAT3 & ~BLIMP1)',
'Viace1 = Proliferation & ~Apo',
'PRAS40 = ~AKT',
'TSC = ~AKT || ERK || P90RSK & (GSK3B || STRESS)',
'GSK3B = ~(P70S6K || AKT || P90RSK)',
'ATR = DNA adam',
'Chk1 = ATR',
'Chk2 = ATM',
'ROS = (~MnSOD2 & (MITO || STRESS)) & ~Dex',
'MnSOD2 = pNFKB & ROS',
'BLIMP1 = IRF4 & ~Bc16',
'FasL = FasR || cJun',
'FasR = FasL || p53',
'BAD = JNK || ~AKT || P90RSK || P70S6K',
'TRAF3 = ~CIAP',
'PTEN = p53',
'SHP1 = <>',
'p27 = p53 & (~MYC || ~CDK4 || ~AKT || ~Bc16)',
'p21 = p53 & (~MYC || ~MDM || ~AKT || ~CDK4 || ~Bc16)',
'RAC = STRESS',
'MEKK2 = RAC',
'MKK4 = MEKK2',
'JNK = MKK4 & ~Prot',
'DNA adam = Cas3 || STRESS || ROS',
'ATM = DNA adam || Cas3',
'DNAPK = ATM || ATR',

320
\[ p53 = (\text{DNAPK} \mid \text{JNK} \mid \text{Chk1} \mid \text{Chk2}) \&\& ((\neg (\text{MDM} \& \text{Prot} \& \text{Bcl6})) \mid \neg (\text{MDM} \& \text{Prot})) \],
\[ cJun = \text{JNK} \],
\[ \text{BAX} = (p53 \mid \text{BID}) \&\& \neg (\text{BclxL} \mid \text{Bcl2}) \],
\[ \text{BID} = (\text{Cas8} \mid \text{STRESS} \mid \text{FasR}) \&\& \neg (\text{Bcl2} \mid \text{BclxL}) \],
\[ \text{Cas8} = (\text{FasL} \& \text{FasR}) \mid \neg \text{FLIP} \],
\[ \text{MITO} = \text{BAX} \mid \text{ROS} \],
\[ \text{Smac} = \text{MITO} \],
\[ \text{Cytc} = \text{MITO} \],
\[ \text{Cas9} = \text{Cytc} \&\& \neg \text{XIAP} \],
\[ \text{Cas3} = (\text{Cas8} \mid \text{Cas9}) \&\& \neg \text{XIAP} \],
\[ \text{CL PARP} = \text{Cas3} \],
\[ \text{STRESS} = (\text{DNA} \& \neg \text{Prot} \& \text{ROS}) \],
\[ \text{Apo} = \text{CL PARP} \],
\[ \text{Bort} = <> \],
\[ \text{Dex} = <> \].
%simstruct.initial = [1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
1];

%use [T,Y] for a dynamic simulation. Use [Y] for Boolean
[T,Y] = OdefySimulation(simstruct,1,1)
R Code: Used for Attractor Analysis

#Model code is executed using a stepwise process
# 1) Load your network using loadNetwork function
# 2) Decide what type of attractor analysis to run using the getAttractors function
# 3) Write the output to a text file
# 4) Parse the data using the data extraction functions below.

#Set working directory and load packages
setwd("C:/Google Drive/PhD Dissertation/Chapter 6 - Pharmacodynamics of Dexanabinol in MM/MM Boolean Network/MM Network Attractor Analysis/")
setwd("C:/Users/peterblo/Google Drive/PhD Dissertation/Chapter 6 - Pharmacodynamics of Dexanabinol in MM/MM Boolean Network/MM Network Attractor Analysis")

library("plyr")
library(stringr)

#Load Networks
MMNetwork = loadNetwork('Final Vidya Network BoolNet.txt')
MMNetwork_BortRIP = loadNetwork('Final Vidya Network BoolNet _ Bort RIP.txt')
MMNetwork_BortRIP_NoBort = loadNetwork('Final Vidya Network BoolNet _ Bort RIP Test.txt')

#Perform Attractor Analyses
#(1) Random synchronous attractor analysis with 1,000,000 start states
#(2) SAT.exhaustive synchronous attractor analysis
# Note that both types of analyses were ran for the condition where (1) bortezomib only inhibits proteasome and (2) bortezomib inhibits both the proteasome and RIP
###Control***
MMNetAttrRandSync = getAttractors(MMNetwork, "synchronous", "random", startStates = 1000000,
                                 genesOFF = c("Bort"), genesON = c())
sink("MMNetAttrRandSync.txt")
MMNetAttrRandSync
sink()

###(NO BORT) Control*** True control, removed bortezomib from equations
MMNetAttrRandSync_NoBort_NoRIP = getAttractors(MMNetwork_BortRIP_NoBort,
                                             "synchronous", "random", startStates = 1000000,
                                             genesOFF = c(), genesON = c())
sink("MMNetAttrRandSync_NoBort_NoRIP.txt")
MMNetAttrRandSync_NoBort_NoRIP
sink()

###(NO BORT) Only Dex (TNFOFF and ROSOFF)
MMNetAttrRandSync_NoBort_NoRIP_ROSOFF_TNFOFF = getAttractors(MMNetwork_BortRIP_NoBort, "synchronous", "random", startStates = 10000000,
         genesOFF = c("ROS", "TNF", "TNFR"), genesON = c())
sink("MMNetAttrRandSync_NoBort_NoRIP_ROSOFF_TNFOFF.txt")
MMNetAttrRandSync_NoBort_NoRIP_ROSOFF_TNFOFF
sink()

###Bortezomib Alone: Synchronous***
MMNetAttrRandSyncBortONTest = getAttractors(MMNetwork, "synchronous", "random",
         startStates = 100,
         genesOFF = c(), genesON = c("Bort"))
sink("MMNetAttrRandSyncBortONTest.txt")
MMNetAttrRandSyncBortONTest
sink()

sink('BasinOfAttraction.txt')
getBasinOfAttraction(MMNetAttrRandSyncBortONTest, 30)
sink()

###Dexanabinol Alone (ROS and TNFalpha Inhibition)***
MMNetAttrRandSyncBortOFF_ROSOFF_TNFOFF = getAttractors(MMNetwork, "synchronous", "random", startStates = 10000000,
         genesOFF = c("ROS", "TNF", "TNFR"), genesON = c())
sink("MMNetAttrRandSyncBortOFF_ROSOFF_TNFOFF.txt")
MMNetAttrRandSyncBortOFF_ROSOFF_TNFOFF
sink()

###Bortezomib + Dexanabinol (ROS and TNFalpha Inhibition)***
MMNetAttrRandSyncBortON_ROSOFF_TNFOFF = getAttractors(MMNetwork, "synchronous", "random", startStates = 10000000,
         genesOFF = c("ROS", "TNF", "TNFR"), genesON = c("Bort"))
sink("MMNetAttrRandSyncBortON_ROSOFF_TNFOFF.txt")
MMNetAttrRandSyncBortON_ROSOFF_TNFOFF
sink()

###(NO RIP) Bortezomib Alone: Synchronous***
MMNetAttrRandSyncBortON_NoRIP = getAttractors(MMNetwork_BortRIP, "synchronous", "random", startStates = 1000000, genesOFF = c(), genesON = c("Bort"))
sink("MMNetAttrRandSyncBortON_NoRIP.txt")
MMNetAttrRandSyncBortON_NoRIP
sink()

#**(NO RIP) Bortezomib + Dexanabinol (ROS and TNFalpha Inhibition)**
MMNetAttrRandSyncBortON_ROSOFF_TNFOFF_NoRIP = getAttractors(MMNetwork_BortRIP, "synchronous", "random", startStates = 1000000, genesOFF = c("ROS", "TNF", "TNFR"), genesON = c("Bort"))
sink("MMNetAttrRandSyncBortON_ROSOFF_TNFOFF_NoRIP.txt")
MMNetAttrRandSyncBortON_ROSOFF_TNFOFF_NoRIP
sink()

#Attractor Analysis SAT Exhaustive

#**(NO BORT) Control** True control, removed bortezomib from equations
MMNetAttrSATSync_NoBort_NoRIP = getAttractors(MMNetwork_BortRIP_NoBort, "synchronous", "sat.exhaustive", genesOFF = c(), genesON = c())
sink("MMNetAttrSATSync_NoBort_NoRIP.txt")
MMNetAttrSATSync_NoBort_NoRIP
sink()

#**(NO RIP) Control**
MMNetAttrSATSync_NoRIP = getAttractors(MMNetwork_BortRIP, "synchronous", "sat.exhaustive", genesOFF = c("Bort"), genesON = c())
sink("MMNetAttrSATSync_NoRIP.txt")
MMNetAttrSATSync_NoRIP
sink()

#**(NO BORT) Only Dex (TNFOFF and ROSOFF)**
MMNetAttrSATSync_NoBort_NoRIP_ROSOFF_TNFOFF = getAttractors(MMNetwork_BortRIP_NoBort, "synchronous", "sat.exhaustive", genesOFF = c("ROS", "TNF", "TNFR"), genesON = c())
sink("MMNetAttrSATSync_NoBort_NoRIP_ROSOFF_TNFOFF.txt")
MMNetAttrSATSync_NoBort_NoRIP_ROSOFF_TNFOFF
sink()
### Bortezomib Alone: Synchronous***

MMNetAttrSATSyncBortON = getAttractors(MMNetwork, "synchronous", "sat.exhaustive",
   genesOFF = c(), genesON = c("Bort"))
sink("MMNetAttrSATSyncBortON.txt")
MMNetAttrSATSyncBortON sink()

### Bortezomib + Dexanabinol (ROS and TNFalpha Inhibition)***

MMNetAttrSATSyncBortON_ROSOFF_TNFOFF = getAttractors(MMNetwork, "synchronous", "sat.exhaustive",
   genesOFF = c("ROS", "TNF", "TNFR"), genesON = c("Bort"))
sink("MMNetAttrSATSyncBortON_ROSOFF_TNFOFF.txt")
MMNetAttrSATSyncBortON_ROSOFF_TNFOFF sink()

### (NO RIP) Bortezomib Alone: Synchronous***

MMNetAttrSATSyncBortON_NoRIP = getAttractors(MMNetwork_BortRIP, "synchronous", "sat.exhaustive",
   genesOFF = c(), genesON = c("Bort"))
sink("MMNetAttrSATSyncBortON_NoRIP.txt")
MMNetAttrSATSyncBortON_NoRIP sink()

### (NO RIP) Bortezomib + Dexanabinol (ROS and TNFalpha Inhibition)***

MMNetAttrSATSyncBortON_ROSOFF_TNFOFF_NoRIP = getAttractors(MMNetwork_BortRIP, "synchronous", "sat.exhaustive",
   genesOFF = c("ROS", "TNF", "TNFR"), genesON = c("Bort"))
sink("MMNetAttrSATSyncBortON_ROSOFF_TNFOFF_NoRIP.txt")
MMNetAttrSATSyncBortON_ROSOFF_TNFOFF_NoRIP sink()

-------------------------------------------------------------
#### DATA EXTRACTION: SYNCHRONOUS RANDOM (LIMIT CYCLES)####
-------------------------------------------------------------

y = readLines("MMNetAttrRandSync.txt")
y = readLines("MMNetAttrRandSyncBortON.txt")
y = readLines("MMNetAttrRandSyncBortON_ROSOFF_TNFOFF.txt")
```r
y = readLines('MMNetAttrRandSyncBortOFF_ROSOFF_TNFOFF.txt')
y = readLines('MMNetAttrRandSyncBortON_NoRIP.txt')
y = readLines('MMNetAttrRandSyncBortON_ROSOFF_TNFOFF_NoRIP.txt')
y = readLines('MMNetAttrRandSync_NoBort_NoRIP.txt')
y = readLines('MMNetAttrRandSync_NoBort_NoRIP_ROSOFF_TNFOFF.txt')

attrsummary2 <- function(y, text){
  nodes <- str_split(gsub(".*order: ", "", y[grep(".*order:
", y)][1]]), "\ 
")[[1]]
  # allattr <- gsub("\|", "", gsub("\| ", "", y[grep("[0-9]{65}", y)]))
  n = length(y[grep("Attractor\ 
", y)])
  substrattr <- gsub(".*basin\ of\ ", "", y[grep("Attractor\ 
", y)])
  temp2 <- gregexpr("[0-9]+", substrattr)
  df<- data.frame(Attr = c(1:n), Basin=as.numeric(unlist(regmatches(substrattr, temp2))))
  df$BF <- df$Basin / sum(df$Basin)
  a = grep("V", y)
  b = list()
  c = list()
  d = list()
  e = matrix(0, nrow = (length(a)/2), ncol=length(nodes))

  for (i in 1: (length(a)/2)){
    b[i] = c( (a[2*i-1]+1) : (a[2*i]-1) )
    c[i] = as.data.frame( gsub("\|", "", gsub("\| ", "", y[b[i]])) )
    d[i] = str_split_fixed(as.character(c[[i]][,1]), ",", n = length(nodes))
    class(d[[i]])<-"numeric"          #colnames(d[[i]]) <- nodes
    e[i, ] = colMeans(d[[i]]) # mean for node value in an attractor
  }
  colnames(e) <- nodes
  f<-t( sapply(1:nrow(e), function(x) e[x, ] * df[x, 3] ) ) # mean value * freq

  return(f)
}

MMNetAttrRandSync_NoBort_NoRIP_ROSOFF_TNFOFF=attrsummary2(y,""

write.csv(MMNetAttrRandSync_NoBort_NoRIP_ROSOFF_TNFOFF, file = "MMNetAttrRandSync_NoBort_NoRIP_ROSOFF_TNFOFF.csv")
```
```r
y = readLines('MMNetAttrSATSyncBortON.txt')
y = readLines('MMNetAttrSATSyncBortON_ROSOFF_TNFOFF.txt')
y = readLines('MMNetAttrSATSyncBortON_NoRIP.txt')
y = readLines('MMNetAttrSATSyncNoBortNoRIP_ROSOFF_TNFOFF.txt')
y = readLines('MMNetAttrSATSyncNoBortNoRIP.txt')
y = readLines('MMNetAttrSATSyncNoBortNoRIP_ROSOFF_TNFOFF.txt')

attrsummary <- function(y, text){
  nodes <- str_split(gsub(".*order: ", "", y[grep(".*order:\s", y)][1]]), "\"")[1]
  n = length(y[grep("Attractor\s", y)])
  substrattr <- gsub(".*basin\sor\s", "", y[grep("Attractor\s", y)])
  temp2 <- gsub("\s", "", gsub("\sor\s", "", substrattr) )
  df <- data.frame(Attr = c(1:n), freq = 1 / n )

  a = grep("V", y)
  b = list()
  c = list()
  d = list()
  e = matrix(0, nrow = (length(a)/2), ncol=length(nodes ))

  for (i in 1: (length(a)/2)){
    b[[i]] = c( (a[2*i-1]+1) : (a[2*i]-1) )
    c[[i]] = as.data.frame( gsub("\", "", gsub("\", "", y[b[[i]]] ) )
    d[[i]] = str_split_fixed(as.character(c[[i]][1]),"", n = length(nodes))
    class(d[[i]])<-"numeric" #colnames(d[[i]]) <- nodes
    e[i, ] = colMeans(d[[i]]) # mean for node value in an attractor (averaged by
    attractor size itself)
  }
  colnames(e) <- nodes
  return(e)
}

MMNetAttrSATSyncNoBortNoRIP_ROSOFF_TNFOFF=attrsummary(y,"")

write.csv(MMNetAttrSATSyncNoBortNoRIP_ROSOFF_TNFOFF, file = "MMNetAttrSATSyncNoBortNoRIP_ROSOFF_TNFOFF.csv")
```

Appendix IV:

Model Code: Pharmacodynamic Modeling of Bortezomib and Dexanabinol U266 Cytotoxicity

Peter Bloomingdale

Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences,

University at Buffalo, The State University of New York, Buffalo, NY
Adapt code for the pharmacodynamic interaction of bortezomib and dexanabinol on U266 cell viability. Model was adapted from Chakraborty and Jusko (2012).\textsuperscript{1}


Subroutine SYMBOL
Implicit None

Include 'globals.inc'
Include 'model.inc'.

NDEqs = 0  ! Enter # of Diff. Eqs.
NSParam = 8  ! Enter # of System Parameters.
NVparam = 2 ! Enter # of Variance Parameters.
NSecPar = 0 ! Enter # of Secondary Parameters.
NSecOut = 0 ! Enter # of Secondary Outputs (not used).
Ieqsol = 3 ! Model type: 1 - DIFEQ, 2 - AMAT, 3 - OUTPUT only.
Descr = ' bort saha comb eq 2 ' 

C Enter Symbol for Each System Parameter (eg. Psym(1)=’Kel’)    C
Psym(1)='I\text{max}_D'
Psym(2)='IC50_D'
Psym(3)='y_D'
Psym(4)='I\text{max}_B'
Psym(5)='IC50_B'
Psym(6)='y_B'
Psym(7)='psi'
Psym(8)='E0'

C Enter Symbol for Each Variance Parameter {eg: PVsym(1)=’Sigma’}  C
PVsym(1)=’Int’
PVsym(2)=’Slope’

C Enter Symbol for Each Secondary Parameter {eg: PSsym(1)=’CLt’}  C

C-------------------------------
Return
End

C*****************************************************************************C
Subroutine Diffeq(T,X,XP)
Implicit None

Include 'globals.inc'
Include 'model.inc'

Real*8 T,X(MaxNDE),XP(MaxNDE)

CC
C--------------------------------------C
C Enter Differential Equations Below {e.g. XP(1) = -P(1)*X(1) } C
C---------------------------C

C--------------------------------------C
C--------------------------------------C
C--------------------------------------C
C
Return
End
C****************************************

Subroutine Output(Y,T,X)
Implicit None

Include 'globals.inc'
Include 'model.inc'

Real*8 Y(MaxNOE),T,X(MaxNDE)

real*8 Imax_D, Imax_B
real*8 IC50_B, IC50_D, Y_D, Y_B, psi
real*8 P1, P2, P3, P4, P5, A(2,21), E0

CC
C--------------------------------------C
C Enter Output Equations Below {e.g. Y(1) = X(1)/P(2) } C
C---------------------------C

C--------------------------------------C

Cc
Bortezomib Concentration (nM)
A(1,1)=0
A(1,2)=0
A(1,3)=0
A(1,4)=0
A(1,5)=0
A(1,6)=0
\[ A(1, 7) = 0.01 \]
\[ A(1, 8) = 0.001 \]
\[ A(1, 9) = 0.001 \]
\[ A(1, 10) = 0.001 \]
\[ A(1, 11) = 0.001 \]
\[ A(1, 12) = 0.001 \]
\[ A(1, 13) = 0.001 \]
\[ A(1, 14) = 0.001 \]
\[ A(1, 15) = 0.01 \]
\[ A(1, 16) = 0.01 \]
\[ A(1, 17) = 0.01 \]
\[ A(1, 18) = 0.01 \]
\[ A(1, 19) = 0.01 \]
\[ A(1, 20) = 0.01 \]
\[ A(1, 21) = 0.01 \]
\[ A(1, 22) = 0.1 \]
\[ A(1, 23) = 0.1 \]
\[ A(1, 24) = 0.1 \]
\[ A(1, 25) = 0.1 \]
\[ A(1, 26) = 0.1 \]
\[ A(1, 27) = 0.1 \]
\[ A(1, 28) = 0.1 \]
\[ A(1, 29) = 1 \]
\[ A(1, 30) = 1 \]
\[ A(1, 31) = 1 \]
\[ A(1, 32) = 1 \]
\[ A(1, 33) = 1 \]
\[ A(1, 34) = 1 \]
\[ A(1, 35) = 1 \]
\[ A(1, 36) = 10 \]
\[ A(1, 37) = 10 \]
\[ A(1, 38) = 10 \]
\[ A(1, 39) = 10 \]
\[ A(1, 40) = 10 \]
\[ A(1, 41) = 10 \]
\[ A(1, 42) = 10 \]
\[ A(1, 43) = 100 \]
\[ A(1, 44) = 100 \]
\[ A(1, 45) = 100 \]
\[ A(1, 46) = 100 \]
\[ A(1, 47) = 100 \]
\[ A(1, 48) = 100 \]
\[ A(1, 49) = 100 \]
\[ A(1, 50) = 1000 \]
\[ A(1, 51) = 1000 \]
\[ A(1, 52) = 1000 \]
\[ A(1, 53) = 1000 \]
\[ A(1, 54) = 1000 \]
\[ A(1, 55) = 1000 \]
\[ A(1, 56) = 1000 \]

\[ A(2, 1) = 0 \]
\[ A(2, 2) = 1 \]
\[ A(2, 3) = 3 \]
\[ A(2, 4) = 10 \]
\[ A(2, 5) = 30 \]
\[ A(2, 6) = 100 \]
\[ A(2, 7) = 300 \]
\[ A(2, 8) = 0 \]
\[ A(2, 9) = 1 \]
\[ A(2, 10) = 3 \]
\[ A(2, 11) = 10 \]
\[ A(2, 12) = 30 \]
\[ A(2, 13) = 100 \]
\[ A(2, 14) = 300 \]
\[ A(2, 15) = 0 \]
\[ A(2, 16) = 1 \]
\[ A(2, 17) = 3 \]
\[ A(2, 18) = 10 \]
\[ A(2, 19) = 30 \]
\[ A(2, 20) = 100 \]
\[ A(2, 21) = 300 \]
\[ A(2, 22) = 0 \]
\[ A(2, 23) = 1 \]
\[ A(2, 24) = 3 \]
\[ A(2, 25) = 10 \]
\[ A(2, 26) = 30 \]
\[ A(2, 27) = 100 \]
\[ A(2, 28) = 300 \]
\[ A(2, 29) = 0 \]
\[ A(2, 30) = 1 \]
\[ A(2, 31) = 3 \]
\[ A(2, 32) = 10 \]
\[ A(2, 33) = 30 \]
\[ A(2, 34) = 100 \]
\[ A(2, 35) = 300 \]
\[ A(2, 36) = 0 \]
\[ A(2, 37) = 1 \]
\[ A(2, 38) = 3 \]
\[
\begin{align*}
A(2,39) &= 10 \\
A(2,40) &= 30 \\
A(2,41) &= 100 \\
A(2,42) &= 300 \\
A(2,43) &= 0 \\
A(2,44) &= 1 \\
A(2,45) &= 3 \\
A(2,46) &= 10 \\
A(2,47) &= 30 \\
A(2,48) &= 100 \\
A(2,49) &= 300 \\
A(2,50) &= 0 \\
A(2,51) &= 1 \\
A(2,52) &= 3 \\
A(2,53) &= 10 \\
A(2,54) &= 30 \\
A(2,55) &= 100 \\
A(2,49) &= 300 \\
\end{align*}
\]

\[
\begin{align*}
\text{Imax}_D &= P(1) \\
\text{IC50}_D &= P(2) \\
y_D &= P(3) \\
\text{Imax}_B &= P(4) \\
\text{IC50}_B &= P(5) \\
y_B &= P(6) \\
\psi &= P(7) \\
E0 &= P(8) \\
\end{align*}
\]

\[
\begin{align*}
P1 &= (A(1,T) \times y_B) \\
P2 &= (\psi \times (\text{IC50}_B \times y_B)) \\
P3 &= (A(2,T) \times y_D) \\
P4 &= (\text{IC50}_D \times y_D) \\
P5 &= (\text{Imax}_B + \text{Imax}_D - \text{Imax}_B \times \text{Imax}_D) \\
\end{align*}
\]

\[
\begin{align*}
Y(1) &= E0*(1 - ((\text{Imax}_B \times P1/P2)+(\text{Imax}_D \times P3/P4)+P5*(P1/P2) \times (P3/P4))) \\
\$ &= ((P1/P2) + (P3/P4) + (P1/P2) \times (P3/P4) + 1)) \\
\end{align*}
\]

\[
\begin{align*}
\text{C} &\text{-----------------------------------------------C} \\
\text{C} &\text{-----------------------------------------------C} \\
\text{C} \\
\text{Return} \\
\text{End} \\
\end{align*}
\]
Subroutine VARMOD(V,T,X,Y)
Implicit None

Include 'globals.inc'
Include 'model.inc'

Real*8 V(MaxNOE),T,X(MaxNDE),Y(MaxNOE)

C
C Enter Variance Model Equations Below
C { e.g. V(1) = (PV(1) + PV(2)*Y(1))**2 }  
C
V(1) = (PV(1) + PV(2)*Y(1))**2

C
C
C
C
Return
End

Subroutine PRIOR(Pmean,Pcov,ICmean,ICcov)
Implicit None

Include 'globals.inc'
Include 'model.inc'

Integer I,J
Real*8 Pmean(MaxNSP+MaxNDE), ICmean(MaxNDE)
Real*8 Pcov(MaxNSP+MaxNDE,MaxNSP+MaxNDE), ICcov(MaxNDE,MaxNDE)

C
C Enter Nonzero Elements of Prior Mean Vector
C { e.g. Pmean(2) = 10.0 }
C
C

Subroutine SPARAM(PS,P,IC)
Implicit None

Include 'globals.inc'

Real*8 PS(MaxNSECP), P(MaxNSP+MaxNDE), IC(MaxNDE)

Enter Equations Defining Secondary Parameters
{ e.g. PS(1) = P(1)*P(2) }

Return
End

Subroutine AMAT(A)
Implicit None

Include 'globals.inc'
Include 'model.inc'
Integer I, J
Real*8 A(MaxNDE, MaxNDE)

DO I=1, Ndeqs
   Do J=1, Ndeqs
      A(I, J) = 0.0D0
   End Do
End Do

CC
C---------------------------------------------------------------C
C   Enter non zero elements of state matrix {e.g. A(1,1) = -P(1)} C
C---------------------------------------------------------------C
C---------------------------------------------------------------C
C---------------------------------------------------------------C
C
C   Return
End

C%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%C
Appendix V:

Model Code: Bortezomib and Dexanabinol PK and Multiple Myeloma Tumor Volume PD in Mice

Peter Bloomingdale

Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, The State University of New York, Buffalo, NY
Adapt code for the pharmacodynamic interaction of bortezomib and dexanabinol on tumor volume in mice.

**Subroutine SYMBOL**

**Implicit None**

**Include 'globals.inc'**

**Include 'model.inc'**

**NDqs = 13**  ! Enter # of Diff. Eqs.
**NSParam = 11**  ! Enter # of System Parameters.
**NVParam = 4**  ! Enter # of Variance Parameters.
**NSecPar = 0**  ! Enter # of Secondary Parameters.
**NSecOut = 0**  ! Enter # of Secondary Outputs (not used).
**Ieqsol = 1**  ! Model type: 1 - DIFFEQ, 2 - AMAT, 3 - OUTPUT only.
**Descri = 'simple direct effect model'**
Enter Symbol for Each System Parameter (eg. Psym(1)='Kel')

Psym(1) = 'CL'
Psym(2) = 'V1'
Psym(3) = 'K12'
Psym(4) = 'K21'
Psym(5) = 'Rmax'
Psym(6) = 'kd'
Psym(7) = 'kgr'
Psym(8) = 'Kbort'
Psym(9) = 'Kdex'
Psym(10) = 'kres'
Psym(11) = 'psi'

Enter Symbol for Each Variance Parameter {eg: PVsym(1)='Sigma'}

PVsym(1) = 'slope1'
PVsym(2) = 'intercept1'
PVsym(3) = 'slope2'
PVsym(4) = 'intercept2'

Enter Symbol for Each Secondary Parameter {eg: PSym(1)='CLt'}

Refine
End
Include 'globals.inc'
Include 'model.inc'

Real*8 T,X(MaxNDE),XP(MaxNDE)
Real*8 CL,V1,K12,K21,Rmax,Kd, V2
Real*8 dAp1,dAt1,Ap1,At1,dAp2,dAt2,Ap2,At2
Real*8 Cp1,Cp2,kgr
Real*8 Vdex,VTdex,CLdex,CLDdex
Real*8 Kbort,Kdex,kres,psi

CC
C----------------------------------------------------------------------
C Enter Differential Equations Below {e.g. XP(1) = -P(1)*X(1) }  C
C----------------------------------------------------------------------

C1   = P(1)
V1   = P(2)
K12  = P(3)
K21  = P(4)
Rmax = P(5)
Kd   = P(6)
kgr  = P(7)
Kbort = P(8)
Kdex = P(9)
kres = P(10)
psi  = P(11)
Ap1  = X(1)
At1  = X(2)
Ap2  = X(3)
At2  = X(4)

C Peripheral volume
V2   = V1 * K12/K21

C Central compartment free drug concentration at 0.25mg/kg
Cp1  = 0.5 * ( (Ap1/V1 - Rmax - Kd) + (Ap1/V1 - Rmax -Kd)**2 +
$ 4 * Kd * Ap1/V1 )**0.5 

C Central compartment free drug concentration at 1mg/kg
Cp2  = 0.5 * ( (Ap2/V1 - Rmax - Kd) + (Ap2/V1 - Rmax -Kd)**2 +
$ 4 * Kd * Ap2/V1 )**0.5 

C DOSE 0.25mg/kg
dAp1 = - CL* Cp1 - K12 * Cp1 * V1 + K21 * At1

dAt1 = K12 * Cp1 * V1 - K21 * At1
C DOSE 1 mg/kg

dAp2 = - CL * Cp2 - K12 * Cp2 * V1 + K21 * At2

dAt2 = K12 * Cp2 * V1 - K21 * At2

C Bortezomib Pharmacokinetics

XP(1) = dAp1
XP(2) = dAt1
XP(3) = dAp2
XP(4) = dAt2

C Dexanabinol Allometrically Scaled Parameters (Units: L & hr)

Vdex = 0.016175
CLdex = 0.012230
VTdex = 0.051392
CLDdex = 0.053178

C Dexanabinol Pharmacokinetics 10 mg/kg: Monotherapy

XP(5) = -CLDdex/Vdex*X(5) - CLdex/Vdex*X(5) + CLDdex/VTdex*X(6)
XP(6) = CLDdex/Vdex*X(5) - CLDdex/VTdex*X(6)

C Dexanabinol Pharmacokinetics 10 mg/kg: Combination

XP(12) = -CLDdex/Vdex*X(12) - CLdex/Vdex*X(12)
$ + CLDdex/VTdex*X(13)
XP(13) = CLDdex/Vdex*X(12) - CLDdex/VTdex*X(13)

C Tumor Volume Dynamics: Control

XP(7) = X(10) * X(7)

C Tumor Volume Dynamics: Bortezomib Monotherapy

XP(8) = X(10) * X(8) - (Kbort*exp(-kres*T)) * Cp1 * X(8)

C Tumor Volume Dynamics: Dexanabinol Monotherapy

XP(9) = X(10) * X(9) - KDex * X(5)/Vdex * X(9)

C Gompertz Growth Function (Time Dependent kg)

XP(10) = -kgr * X(10)

C Tumor Volume Dynamics: Bortezomib and Dexanabinol Combination

XP(11) = X(10) * X(11) - (Kbort*exp(-kres*psi*T)) * Cp1 * X(11)
$ - KDex * X(12)/Vdex * X(11)
Return
End

Subroutine OUTPUT(Y,T,X)
Implicit None

Include 'globals.inc'
Include 'model.inc'

Real*8 Y(MaxNOE),T,X(MaxNDE)
Real*8 CL,V1,k12,k21,Rmax,Kd, V2
Real*8 dAp1,dAt1,Ap1,At1,dAp2,dAt2,Ap2,At2
Real*8 Cp1,Cp2,kgr,Vdex
Real*8 Kbort,Kdex,kres,psi

C Enter Output Equations Below {e.g. Y(1) = X(1)/P(2) } C
C----c-----------------------------------------------------------------

CL = P(1)
V1 = P(2)
K12 = P(3)
K21 = P(4)
Rmax = P(5)
Kd = P(6)
kgr = P(7)
Kbort = P(8)
Kdex = P(9)
kres = P(10)
psi = P(11)
Ap1 = X(1)
At1 = X(2)
Ap2 = X(3)
At2 = X(4)
Vdex = 0.016175

C Central Compartment Bortezomib Concentration 1 mg/kg (Monotherapy)
Cp1 = 0.5 * ( (Ap1/V1 - Rmax - Kd) + (Ap1/V1 - Rmax -Kd)**2 +
* 4 * Kd * Ap1/V1 )**0.5 )

C Central Compartment Bortezomib Concentration 1 mg/kg (Combination)
Cp2 = 0.5 * ( (Ap2/V1 - Rmax - Kd) + (Ap2/V1 - Rmax -Kd)**2 +
* 4 * Kd * Ap2/V1 )**0.5 )
C Bortezomib Pharmacokinetics 1 mg/kg (Monotherapy and Combination)
   Y(1) = Cp1
   Y(2) = Cp2

C Dexanabinol Pharmacokinetics 10 mg/kg (Monotherapy and Combination)
   Y(3) = X(5)/Vdex
   Y(4) = X(12)/Vdex

C Tumor Growth Dynamics: Control
   Y(5) = X(7)

C Tumor Growth Dynamics: Bortezomib Monotherapy
   Y(6) = X(8)

C Tumor Growth Dynamics: Dexanabinol Monotherapy
   Y(7) = X(9)

C Tumor Growth Dynamics: Dexanabinol Monotherapy
   Y(8) = X(11)

C
C------------------------------------------------------------------
C
C   Enter Variance Model Equations Below                           C
C         {e.g. V(1) = (PV(1) + PV(2)*Y(1))**2 }                  C
C------------------------------------------------------------------
C
V(1) = (PV(2)+PV(1)*Y(1))**2.0
V(2) = (PV(2)+PV(1)*Y(2))**2.0
V(3) = (PV(2)+PV(1)*Y(3))**2.0

C Subroutine VARMOD(V,T,X,Y)
Implicit None

Include 'globals.inc'
Include 'model.inc'

Real*8 V(MaxNOE),T,X(MaxNDE),Y(MaxNOE)
real*8 slope1,intercept1,slope2,intercept2

C
C------------------------------------------------------------------
C
C Enter Variance Model Equations Below                             C
C             {e.g. V(1) = (PV(1) + PV(2)*Y(1))**2 }              C
C------------------------------------------------------------------
C
V(1) = (PV(2)+PV(1)*Y(1))**2.0
V(2) = (PV(2)+PV(1)*Y(2))**2.0
V(3) = (PV(2)+PV(1)*Y(3))**2.0
\[ V(4) = (PV(2) + PV(1) \cdot Y(4))^{*2} \]
\[ V(5) = (PV(4) + PV(3) \cdot Y(5))^{*2} \]
\[ V(6) = (PV(4) + PV(3) \cdot Y(6))^{*2} \]
\[ V(7) = (PV(4) + PV(3) \cdot Y(7))^{*2} \]
\[ V(8) = (PV(4) + PV(3) \cdot Y(8))^{*2} \]

---

\[ \text{Subroutine} \ \text{PRIOR}(P_{\text{mean}}, P_{\text{cov}}, IC_{\text{mean}}, IC_{\text{cov}}) \]
\[ \text{Implicit} \ \text{None} \]

\[ \text{Include 'globals.inc'} \]
\[ \text{Include 'model.inc'} \]

\[ \text{Integer} \ I, J \]
\[ \text{Real}^{*8} \ P_{\text{mean}}(\text{MaxNSP} + \text{MaxNDE}), IC_{\text{mean}}(\text{MaxNDE}) \]
\[ \text{Real}^{*8} \ P_{\text{cov}}(\text{MaxNSP} + \text{MaxNDE}, \text{MaxNSP} + \text{MaxNDE}), IC_{\text{cov}}(\text{MaxNDE}, \text{MaxNDE}) \]

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\[ \text{Subroutine} \ \text{SPARAM}(PS, P, IC) \]
\[ \text{Implicit} \ \text{None} \]
Include 'globals.inc'

Real*8 PS(MaxNSECP), P(MaxNSP+MaxNDE), IC(MaxNDE)

C---------------------------------------------------------------C
C   Enter Equations Defining Secondary Parameters               C
C       { e.g. PS(1) = P(1)*P(2) }                            C
C---------------------------------------------------------------C

C---------------------------------------------------------------C
C---------------------------------------------------------------C
C
C     Return
End
C######################################################################C

Subroutine AMAT(A)
Implicit None

Include 'globals.inc'
Include 'model.inc'

Integer I,J
Real*8 A(MaxNDE,MaxNDE)

DO I=1,Ndeqs
   Do J=1,Ndeqs
      A(I,J)=0.0D0
   End Do
End Do

C
C---------------------------------------------------------------C
C   Enter non zero elements of state matrix  {e.g. A(1,1) = -P(1) }  C
C---------------------------------------------------------------C
C---------------------------------------------------------------C
C---------------------------------------------------------------C
C
C     Return
End
C######################################################################C