Logic-based and Cellular Pharmacodynamic Modeling of Bortezomib Responses in U266 Human Myeloma Cells

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Introduction: 747 words (limit 750)
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Abbreviations
NF-κB: nuclear factor kappa B
JAK/STAT: janus kinase/signal transducers and activator of transcription
PI3K/Akt: phosphatidylinositol 3-kinase and protein kinase B
MAPK: mitogen activated protein kinase
TNFα: tumor necrosis factor α

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Abstract

Systems models of biological networks show promise for informing drug target selection/qualification, identifying lead compounds and factors regulating disease progression, rationalizing combinatorial regimens, and explaining sources of inter-subject variability and adverse drug reactions. However, most models of biological systems are qualitative and are not easily coupled with dynamical models of drug exposure-response relationships. In this proof of concept study, logic-based modeling of signal transduction pathways in U266 multiple myeloma (MM) cells is used to guide the development of a simple dynamical model linking bortezomib exposure to cellular outcomes. Bortezomib is a commonly used first-line agent in MM treatment; however, knowledge of the signal transduction pathways regulating bortezomib-mediated cell cytotoxicity is incomplete. A Boolean network model of 66 nodes was constructed that includes major survival and apoptotic pathways and was updated using responses to several chemical probes. Simulated responses to bortezomib were in good agreement with experimental data, and a reduction algorithm was used to identify key signaling proteins. Bortezomib-mediated apoptosis was not associated with suppression of NFκB protein inhibition in this cell line, which contradicts a major hypothesis of bortezomib pharmacodynamics. A pharmacodynamic model was developed that included 3 critical proteins (pNFκB, BclxL, and cleaved PARP). Model-fitted protein dynamics and cell proliferation profiles agreed with experimental data, and the model-predicted IC₅₀ (3.5 nM) is comparable to the experimental value (1.5 nM). The cell-based pharmacodynamic model successfully links bortezomib exposure to MM cellular proliferation via protein dynamics, and this model may show utility in exploring bortezomib-based combination regimens.
Introduction

The fields of systems biology and pharmacokinetic/pharmacodynamic (PK/PD) modeling have evolved largely in parallel, and there is an emerging consensus that an effective integration of these disciplines is needed in order to fully realize the promise of each in bringing new therapeutic molecules and combination regimens to the bedside (Sorger et al., 2011). Traditional PK/PD models of drug action utilize compartmental structures to integrate the time-course of drug exposure, pharmacological properties (capacity, sensitivity, and transduction of drug-target interactions), and (patho)-physiological turnover processes (Mager et al., 2003). Such semi-mechanistic models contain a minimal number of identifiable parameters to describe temporal profiles of macro-scale therapeutic and adverse drug effects. When coupled with nonlinear mixed effects modeling of relatively large clinical trials, a covariate analysis can be used to identify patient specific characteristics (e.g., genetic polymorphisms) that explain the inter-individual variability in model parameters (Pillai et al., 2005) Although a major component of model-based drug development and therapeutics (Milligan et al., 2013), this approach can be limited by specific study designs and is rarely sufficient for recapitulating multiple, complex genotype-phenotype relationships.

Significant insights have been realized from the recognition that both drugs and disease processes give rise to complex and dynamic clinical phenotypes by altering natural interconnected biochemical networks and support the emergence of systems pharmacology models of drug action (Zhao and Iyengar, 2012; Huang et al., 2013; Jusko, 2013). Multi-scale models that combine PK/PD principles and signaling networks can serve as a platform for integrating genomic/proteomic factors that regulate drug effects and clinical outcomes – so called enhanced PD (ePD) models (Iyengar et al., 2012). Two major challenges to the
development of ePD models include the lack of complete mathematical models of signal transduction networks (e.g., concentrations and reaction rate constants) and unknown quantitative relationships between individual genomic/proteomic differences and model parameters. Mechanistic network models are preferred over empirical structures (Birtwistle et al., 2013); however, such models may not be defined or calibrated to specific pharmacological and/or disease systems. Logic-based modeling techniques provide a global perspective of system properties in the absence of kinetic parameters through the integration of qualitative a priori knowledge of network connections (Albert and Wang, 2009). In this proof of concept study, a reduction algorithm is applied to a mathematical network to guide the development of a small signaling model for bortezomib, a potent proteasome inhibitor, which may ultimately serve as an ePD model for its use in multiple myeloma (MM).

Multiple myeloma is a B-cell neoplasm associated with several co-morbidities including hypercalcemia, renal insufficiency, anemia, and bone lesions (Caers et al., 2008; Blade et al., 2010). The prognosis for advanced stages of MM is poor despite multiple treatment options, with median survival of advanced stage patients of less than 10 months (Richardson et al., 2003; Richardson et al., 2005). Bortezomib is commonly prescribed alone or in combination with other anti-myeloma agents (Oancea et al., 2004), and the addition of bortezomib has significantly improved overall survival of MM patients (Caers et al., 2008). However, almost all patients relapse and become refractory to all treatment options (Richardson et al., 2005; San Miguel et al., 2008), and better understanding of MM disease progression and mechanisms of drug action are critical for improving the treatment of MM.

Bortezomib modulates both survival and apoptotic cellular pathways in MM cells (Figure 1), and multiple mechanisms of action are proposed for inducing cell death, including the
inhibition of proteasome and the pNFκB pathway (Hideshima et al., 2001; Hideshima et al.,
2002; Hideshima et al., 2003a; Hideshima et al., 2003b). However, recent reports suggest that
bortezomib stimulates RIP (a signaling protein upstream of the NFκB pathway) leading to
activation of pNFκB protein expression (Hideshima et al., 2009). Despite extensive qualitative
information on bortezomib-induced intracellular signaling, mathematical models linking
bortezomib exposure to intracellular protein dynamics have not been established. Systems-level
modeling may facilitate the rational design of single and combinatorial dosing regimens,
approaches to overcome drug resistance, and prevent sub-optimal dosing (Berger and Iyengar,
2011; Zhao and Iyengar, 2012). Mechanistic models are emerging in which chemotherapy
exposure is connected to ultimate responses through target occupancy and biomarker signal
transduction (Yamazaki et al., 2011; Harrold et al., 2012; Kay et al., 2012; Kirouac et al., 2013).
The purpose of this study is to integrate network systems analysis and PK/PD modeling
principles to study bortezomib effects on signal transduction in MM cells. Although the current
study is conducted in U266 cells, the strategic framework may be extended to other cell-lines and
applied to other therapeutic areas.
Methods

**Tissue Culture Materials.** The human myeloma cell line U266 (TIB-196™) was purchased from ATCC®. IKKi (PS-1145) and JAKi (Jak inhibitor I) were purchased from Santa Cruz Biotechnology, Inc®. IκBi (BAY11-7085) was purchased from Sigma-Aldrich®. The clinically available formulation of bortezomib was used for all experiments (Millennium Pharmaceuticals, Inc). The primary monoclonal antibodies to phospho-p65 (p-p65), p-IκBα, p-Stat3, Bcl-xL, PARP, p65, Stat3, IκBα, and β-actin were purchased from Cell Signaling Technology® (Danvers, MA). α-tubulin primary and rabbit and mouse secondary antibodies were purchased from Santa Cruz Biotechnology, Inc®. WST-1 reagent assay kit was purchased from Roche®, HRP-conjugate was purchased from BioRad®, and ECL-substrate was purchased from Thermo Scientific®. RPMI-1640 and FBS were purchased from ATCC®.

**Cell Culture Experimental Design.** All experiments were conducted in U266 myeloma cells. Cells were cultured in RPMI 1640 medium supplemented with 15% FBS and 1% pen/strep antibiotics. Treatment protocols included 1) IKKi (10 µM), 2) JAKi (10 µM), 3) IκBi (10 µM), 4) IKKi and JAKi (10 µM each), 5) IκBi and JAKi (10 µM each), 6) bortezomib (20 and 2 nM), and 7) DMSO vehicle control. All treatments were continuous for 48h, with the exception of IκBi experiments (10h). A transient exposure to bortezomib (20 nM) was also conducted, in which cells were incubated for either 1 or 2h followed by removal of drug from the culture media and subsequent incubation with vehicle control for up to 48h.

**WST-1 Cell Proliferation Assay.** Approximately 10,000 cells per well were incubated with a range of concentrations (from 0.001-100 µM) of JAKi, IKKi, or a combination of IKKi and JAKi. Incubation experiments with bortezomib alone ranged from 0.001-100 nM. Cell viability was measured at 0, 24, 48, 72, and 96h according to manufacturer instructions.
Absorbance was measured at 450 and 690 nm after 2h incubation with WST-1 reagent with a 
Microplate spectrophotometer (Molecular Devices, Sunnydale, CA).

**Western Blot Analysis.** Relative protein expression levels of pStat3, pNFκB, pIkBα, 
cleared PARP, and BclxL were measured following treatment regimens as specified in 
Supplemental Table S1. Cells (5×10⁶/10mL culture media) were incubated in 10 cm² culture 
dishes. Cells were collected at the end of the treatment duration, washed with ice cold PBS, and 
lysed at 4 °C in RIPA buffer (Cell Signaling Technology®) supplemented with protease and 
phosphatase inhibitor cocktail (BioRad®) and PMSF (BioRad®). Protein lysates were stored at - 
80 °C until used. Equal amounts of proteins were separated on SDS-PAGE gels and transferred 
to nitrocellulose membranes. Immunoblotting was performed according to manufacturer 
instructions, and the relative intensity of bands was assessed by densitometric analysis of 
digitized images using ImageJ software (National Cancer Institute, Bethesda, MD). All 
experiments were conducted in at least duplicate.

**Drug Stability.** Bortezomib degradation in cell culture was assessed at different time-
points (0, 12, 24, 48, and 72h). Bortezomib concentrations were measured after incubation (20 
nM) at 37 °C in culture medium (RPMI1640 supplemented with 15% FBS and 1% Pen/strep) 
using an LC-MS/MS assay previously validated in our lab (unpublished). Bortezomib 
degradation kinetics were fit with a monoexponential (λ) decay model, and the half-life was 
calculated as: ln (2) / λ.

**Boolean Network Assembly and Simulations.** The Boolean network model (Figure S1) 
was drawn with SmartDraw software (http://www.smartdraw.com) and was implemented in 
Odefy (http://www.helmholtz-muenchen.de/cmb/odefy) (Wittmann et al., 2009), a MATLAB® 
(The Mathworks, Natick, MA) compatible toolbox. Odefy converts Boolean relationships into a
continuous framework using a Hill type function. In the hypothetical case in which node B stimulates A, the relationship can be described by \( \frac{dA}{dt} = \frac{1}{\tau} \left( B^n \left( B^n + k^n \right) - A \right) \). Default parameter values for \( k \) (0.5), \( n \) (3), and \( \tau \) (1) were used for all nodes. All model simulations were performed using the ‘HillCubeNorm’ option within Odefy (MATLAB model code is provided in Supplemental materials). Each node in the model was initialized as ON (1) or OFF (0) based on baseline activity in the cell. For example, if the state is constitutively active in the cell, it was initialized as 1. A list of Boolean transfer functions and initial state values are summarized in Table S2 (Supplemental materials). An example of ODEs corresponding to each node, which were generated using Odefy, are listed in Supplemental materials (Eqs. S1 to S5).

Three specific probes, JAKi, IκBi, and IKKi, were used to develop and update the network model. The effect of the JAKi was added such that it inhibits nodes JAK1 and JAK2. The IκBi inhibitor was added by direct inhibition of pIκB node activation. Similarly, the IKKi effect was added such that it inhibits node IKK. Model simulations were performed to predict bortezomib outcomes, and drug effect was incorporated as direct inhibition of the proteasome node and stimulation of the RIP node. For simulations of transient bortezomib exposure, the bortezomib node was activated for only 1 or 2 iterations.

**Network Reduction.** The final full Boolean network model developed for U266 cells (Figure S1) was reduced using a model reduction algorithm (Veliz-Cuba, 2011) to identify critical proteins involved in bortezomib-mediated signal transduction. Figure 2 shows examples of the steps taken to reduce the network. First, nodes with only one input and one output are eliminated and the pathways are reconnected (Figure 2A). For example, RAF was removed and RAS and MEK1 were directly connected (Figure 2B). In another network reduction rule, non-functional edges are identified and removed based on the Boolean relationships (algorithm S
from (Veliz-Cuba, 2011)). For example, Figure 2C shows nodes A, B, and C, with the following Boolean functions: \( f(A) = B \ OR \ (B \ AND \ NOT \ C) \), \( f(B) = (A \ AND \ C) \), and \( f(C) = A \). Hence node C is non-functional in \( f(A) \) because of the ‘OR’ relationship, and the B node is sufficient to elicit the same effect without invoking C. The resulting diagram is shown (block arrow, Figure 2C) with the Boolean relationships: \( f(A) = B \), \( f(B) = (A \ AND \ C) \), and \( f(C) = A \). p21 is an example of non-functional edges (Figure 2D), and Boolean relationships for p21 (\( f(p21) = p53 \ AND \ (NOT \ AKT \ OR \ NOT \ MDM \ OR \ NOT \ MYC \ OR \ NOT \ CKD4) \)) were refined using step 2 to \( f(p21) = p53 \ AND \ NOT \ AKT \) to remove non-functional edges. AKT is the sole connection to BAD; therefore, the p21 connection to AKT is retained (Figure 2D).

Finally, non-functional nodes are identified and removed from the network (algorithm R from Veliz-Cuba, 2011). Before applying this rule, the network is re-wired and the first rule is re-applied. Nodes that do not have any impact on other nodes are considered non-functional and removed. In Figure 2E, node C is regulating node B, but at the same time node A is regulating node C. Therefore, the connection from node C to B can be removed, and consequently node C can be removed. As an example, the only node modulated by MYC is CYCE, as the MYC connection to CYCD can be removed because of the AKT connection to CYCD (Figure 2F). pStat3, MEKK, and ERK stimulate MYC independently, and MEKK was removed following the first network reduction rule (Figure 2A). Similarly, ERK was removed as it does not modulate any other node besides MYC. pStat3 modulates several other nodes (e.g., BclxL and XIAP) and stimulates MYC. MYC can be removed following the one input-one output rule, and therefore CYCE is directly modulated by pStat3 (Figure 2F), resulting in 4 final nodes. This entire process was repeated until no further nodes could be removed from the Boolean network. Boolean
Simulations of the final reduced network were compared to simulations of the full network using Odefy (Wittmann et al., 2009).

**Dynamical Model Development.** Several critical proteins identified using the model reduction algorithm were incorporated into a reduced cellular PD model based on mechanisms of bortezomib action and signal transduction pathways. The reduced dynamic model of bortezomib is depicted in Figure 3. In brief, bortezomib stimulates protein expression of pNFκB and cleaved PARP (following delays) and inhibits expression of BclxL. A transit compartment (M1_B) was added to account for the slight delay in stimulation of pNFκB protein expression (Mager and Jusko, 2001). Bortezomib stimulates the synthesis rate of M1_B (k_{syn,M1B}) via a linear stimulation coefficient (S_{m,M1B}), and the rate of change in expression of M1_B is defined as:

\[
\frac{d(M1_B)}{dt} = k_{syn,M1_B} \cdot (1 + S_{m,M1_B} \cdot C_B) - k_{deg,M1_B} \cdot M1_B; \quad M1_B(0) = 1
\]

with \( C_B \) as the concentration of bortezomib, and \( k_{deg,M1B} \) is a first-order degradation rate constant of M1_B. pNFκB protein expression increases but returns toward the baseline after 24 hours of continuous bortezomib exposure. To capture this trend, a precursor-dependent indirect response model was proposed (Sharma et al., 1998), with stimulation of the first-order transfer rate constant from the precursor pool (pre_{pNFκB}) to the pNFκB compartment (k_{tr,NFκB}). The rate of change of pre_{pNFκB} and pNFκB are described by the following equations:

\[
\frac{d(pre_{pNFκB})}{dt} = k_{syn,NFκB} - k_{tr,NFκB} \cdot pre_{pNFκB} \cdot M1_B; \quad pre_{pNFκB}(0) = \frac{k_{syn,NFκB}}{k_{tr,NFκB}}
\]

\[
\frac{d(pNFκB)}{dt} = k_{tr,NFκB} \cdot pre_{pNFκB} \cdot M1_B - k_{deg,NFκB} \cdot pNFκB; \quad pNFκB(0) = 1
\]

with \( k_{syn,NFκB} \) as a zero-order production rate constant of pre_{pNFκB}, and \( k_{deg,NFκB} \) is a first-order degradation rate constant of pNFκB. Changes in cleaved PARP expression follow a
significant delay, and the protein expression profile was best characterized using a time-dependent transduction model (Mager and Jusko, 2001), with negative feedback from the last compartment to the synthesis rate of first cleaved PARP compartment in order to characterize the downward phase of protein expression. Bortezomib stimulates the synthesis rate \((k_{\text{syn,PARP}})\) of the first cleaved PARP compartment \((\text{cPARP}_1)\) with a linear stimulation coefficient \((S_{m,PARP})\):

\[
\frac{d(\text{cPARP}_1)}{dt} = k_{\text{syn,PARP}} \cdot \left(1 + S_{m,PARP} \cdot C_B\right) \cdot \text{cPARP}_1 \cdot \text{cPARP}_1(0) = 1 \quad [4]
\]

The three subsequent transit compartments are described by the general equation:

\[
\frac{d(\text{cPARP}_n)}{dt} = k_{u,PARP} \cdot (\text{cPARP}_{n-1} - \text{cPARP}_n); \quad \text{cPARP}_n(0) = 1 \quad [5]
\]

with \(n\) representing the compartment number \((n = 2 \text{ to } 4)\). pNF\(\kappa\)B is a pro-survival transcription factor that upregulates anti-apoptotic proteins (e.g., BclxL), whereas PARP is a pro-apoptotic factor that results in apoptosis when activated (cleaved PARP) and stimulates the degradation of BclxL. The rate of change of BclxL is defined as:

\[
\frac{d(\text{BclxL})}{dt} = k_{\text{syn,Bcl}} \cdot \text{pNFkB} - k_{\text{deg,Bcl}} \cdot \text{cPARP}_4 \cdot \text{BclxL}; \quad \text{BclxL}(0) = 1 \quad [6]
\]

with \(k_{\text{syn,Bcl}}\) as a zero-order synthesis rate constant of BclxL, \(k_{\text{deg,Bcl}}\) is a first-order degradation rate constant of BclxL, and \(\lambda\) is a power coefficient for the cleaved PARP effect on BclxL. Cell proliferation is dependent on the balance between apoptotic and anti-apoptotic signals. Therefore, cell proliferation \((N)\) was modulated by BclxL and cleaved PARP expression profiles, with the first-order natural cell death rate constant \((k_d)\) inhibited by BclxL and stimulated by cleaved PARP. \(N\) represents cell proliferation:

\[
\frac{d(N)}{dt} = N \cdot \left(k_e - k_d \cdot \text{cPARP}_4 \cdot (2 - \text{BclxL})\right); \quad N(0) = 1 \quad [7]
\]
with $k_g$ representing a first-order growth rate constant. Secondary equations defining zero-order production rate constants are listed in Supplemental materials (Eqs. S6-S9).

**Data Analysis.** The dynamical model (Figure 3) was first fitted to mean pNFκB and cleaved PARP data, followed by pooling of all relative changes in these protein expression patterns (naïve pooled data approach). Initially, pNFκB and cleaved PARP protein dynamics were fit separately. BclxL data were subsequently included and all the data including BclxL, pNFκB, and cleaved PARP protein dynamics were fitted simultaneously. Next, cell proliferation was introduced to the existing cellular dynamic model and all of the data (three biomarkers and cell proliferation) were fitted simultaneously. The *in vitro* degradation half-life of bortezomib was included for all model runs. Parameters were estimated using MATLAB (fminsearch function, Maximum Likelihood algorithm, and ode23s) and a model development framework (Harrold and Abraham, 2014). All protein dynamics were described using a proportional error variance model $Y_{obs} = Y_{pred} \cdot \sigma$ and cell proliferation was fitted using an additive plus proportional error variance model: $Y_{obs} = Y_{pred} \cdot \sigma + \varepsilon$, with $Y_{obs}$ as the observation at time $t$, $Y_{pred}$ is the model predicted value at time $t$, and $\sigma$ and $\varepsilon$ are estimated variance model parameters.

**Model Qualification.** The final cell-based model and parameter estimates were used to simulate protein dynamics and cell proliferation after continuous exposure to a 10-fold lower bortezomib concentration (2 nM). Simulations were compared to the experimentally measured cell proliferation and protein expression profiles. Only parameters associated with natural cell proliferation ($k_g$ and $k_d$) were estimated. Cell proliferation was measured at 0, 24, 48, and 72h and protein expression measurements were made at 0, 1, 4, 6, 8, 11, 24, 33, and 48h. The final model was also used to predict cell proliferation at 48h for a range of bortezomib concentrations.
(0.001-100 nM). The experimentally estimated IC₅₀ value was compared to the IC₅₀ obtained from the simulated data.
Results

Network Development with IKK and JAK Inhibitors. The final Boolean network model incorporates major survival and apoptotic pathways in U266 cells (Figure S1), and Table S2 summarizes all node descriptions, Boolean inter-nodal relationships, and initial values. The green connections in Figure S1 indicate how the initial network was updated based on cellular responses to the pathway probes. Nodes that are constitutively active under baseline cell conditions were set as being active (1) or otherwise inactive (0) in the network. An initial Boolean network was constructed, and the performance of the initial model was tested with two pathway specific probes, IKKi and JAKi, to inhibit the NFκB and JAK/STAT pathways. In the Boolean network, the effect of each inhibitor was added such that IKKi inhibits node IKK, and JAKi inhibits nodes JAK1 and JAK2. Simulations of the initial Boolean network show that nodes representing pIκBα, pNFκB, pStat3, and BclxL expression decrease gradually upon inhibition of node IKK (Figure S2A, black dash-dotted lines). Inhibition of pStat3 expression appears to be due to IL6 inhibition (major stimulus of JAK/STAT3 pathway) via pNFκB expression. However, in vitro western blot experiments show that expressions of pNFκB, pStat3, and BclxL remained unchanged after 48h exposure to the IKKi, whereas pIκBα expression decreased (Figure S2B, black symbols and dotted lines). In contrast, simulations of the same proteins using the initial model following JAKi exposure (Figure S2A, green dashed lines) were comparable to observed experimental profiles over 48h (Figure S2B, green symbols and dashed lines). In order to account for the observed trend in pNFκB expression, the initial network was modified such that pStat3 also stimulates pNFκB (Figure S1). Although not yet confirmed in U266 or other MM cell lines, studies suggest cross-talk between NFκB and STAT3 signaling in tumors (Squarize et al., 2006; Lee et al., 2009; Saez-Rodriguez et al., 2011). To
further test this hypothesis, model simulations were compared for the combination of IKKi and JAKi treatment with measured temporal expression profiles of pNFκB. Simulations of pNFκB following combination treatment with JAKi and IKKi using the initial Boolean network showed a steady decrease over time (data not shown). However, pNFκB expression measured by western blot analysis only transiently decreased with a return to baseline values (Figure S2B, blue symbols and dash-dot lines). A detailed evaluation of pNFκB is described in the next section. Overall, cellular protein dynamic simulations using the final model (Figure S2C) reasonably agree with the experimental results (Figure S2B).

**NFκB Dynamics.** As the expression of pNFκB was not suppressed by the IKKi, either as a single agent or in combination with the JAKi, cellular responses to another NFκB pathway inhibitor (IκBi) were evaluated alone or in combination with the JAKi. Using the initial Boolean network, inhibition of node pIκBα by IκBi resulted in simulations showing inhibition of pNFκB expression (data not shown). However, continuous IκBi exposure to U266 cells for up to 10h (alone and in combination with JAKi) maintained pIκBα expression below baseline values (Figure S3A), whereas pNFκB expression transiently decreased followed by a steady increase above the baseline (Figure S3A). Based on the lack of pNFκB suppression with either the IKKi or IκBi, alone or in combination with JAKi, additional factors were assumed to govern pNFκB expression in U266 cells outside of IKK, pIκBα, and pStat3 signaling. This phenomenon was emulated by incorporating a dummy node ‘X’ to constantly stimulate pNFκB in the final network (Figure S1). The addition of this factor stabilized the Boolean network and reconciled simulations (Figures S2C, S2D, and S2F) with all experimental data (Figures S2B, S2E, and S2G). These results highlight the need to inhibit NFκB activity directly rather than through upstream pathways. Both final model simulations and experimental data confirm inhibition of
pIκBα expression following continuous 48h exposure to the IKKi (10 µM), as well as the lack of suppression of pNFκB and BclxL expression levels (Figures S2B and S2C, black symbols and dotted lines). Analogously, continuous exposure to the JAKi (10 µM) suppressed expression of pStat3, whereas pNFκB and BclxL expression levels remained unchanged (Figures S2B and S2C, green symbols and dashed lines). In addition, final model simulations of U266 cellular proliferation and apoptosis agreed well with experimental cellular responses (Figure S2D-G). A range of IKKi and JAKi concentrations (0.001 to 100 µM) was used to evaluate cell viability, and neither induction of apoptosis nor decreased cell viability was observed after 72h continuous exposure alone or in combination (Figure S2E and S2G).

**Bortezomib Pharmacodynamics.** Once the final Boolean network model was updated using protein dynamics and cellular responses after exposures to probe inhibitors, the model was used to evaluate bortezomib pharmacodynamics. Bortezomib effect was introduced into the network such that it directly inhibits the ‘Proteasome’ node and stimulates the ‘RIP’ node (Figure S1). Stimulation of RIP activates a downstream cascade leading to activation of pIκBα followed by pNFκB. In the Boolean network, a state initialized as 1 (active) cannot be further stimulated; therefore, pIκBα and pNFκB signal intensities remain unchanged (Figure S4A, blue dash-dot line). However, our immunoblot analysis revealed transient increases in pNFκB and pIκBα expression levels, followed by a gradual return to baseline values (Figure S4B, blue symbols and lines), which is in agreement with published profiles (Hideshima et al., 2009). Comparing steady-state values for pNFκB and pIκBα before and after treatment suggests no change in expression levels, which is in agreement with Boolean network simulations (Figure S4A). Furthermore, inhibition of proteasome results in accumulated cellular stress that activates the JNK pathway as well as the apoptotic pathway. The mitochondrial apoptotic pathway
activates caspase-3 leading to activation of p53 followed by p21, resulting in the inhibition of the Bcl-2 family proteins (e.g., BclxL and Bcl-2) (Figure S4B, blue symbols and lines). The activated JNK pathway inversely regulates pStat3 leading to down regulation of pStat3 expression (Figure S4B, blue symbols and lines). Simulations using the final Boolean network model correctly predicted the inhibition of pStat3 and BclxL in U266 cells after bortezomib exposure (Figure S4A, blue symbols and lines). In addition, final model simulations of U266 cellular outcomes to bortezomib exposure were in good agreement with *in vitro* measurements of cell viability and apoptosis measured via cleaved PARP expression (Figure 4).

In order to further test the fidelity of the Boolean network model to predict bortezomib pharmacodynamics, we compared Boolean network simulations with experimental results of cellular responses upon transient bortezomib exposures. Simulations of transient drug exposure were achieved by maintaining the Bortezomib node as active (1 = ON) for a limited number of iterations, and the dynamics and steady-states of protein and cellular response nodes were monitored. Transient experiments were conducted in which U266 cells were briefly exposed (i.e., for 1 and 2h) to bortezomib. Interestingly, some nodes (e.g., proteasome) returned to baseline values once bortezomib was removed from the system, whereas other nodes (e.g., cleaved PARP or Cl. PARP) achieved a new steady-state even after drug was removed. Furthermore, steady-state outcomes for several nodes varied depending on the duration of simulated bortezomib exposure. For example, a relatively short duration of proteasome suppression produces a transient induction of apoptosis, whereas longer durations of bortezomib exposure resulted in steady-states of apoptosis induction and inhibited cell growth that are similar to simulated outcomes following continuous drug exposure. A shorter duration of bortezomib exposure was associated with a delay in apoptosis induction as compared with
continuous drug exposure (Figure S5A), and similar trends were observed experimentally (Figure S5B). Western blot analysis of BclxL and cleaved PARP expression shows a reduced magnitude in changes from baseline values between transient and continuous bortezomib exposure. The magnitude of cleaved PARP induction was reduced from 22- to 6-fold after 24h of bortezomib treatment. Model simulations of selected proteins were comparable with experimental results (Figure S5A and B). However, stimulation of cleaved PARP is associated with apoptosis induction, down-regulation of total NFκB expression, and subsequent suppression of pNFκB expression. In contrast, incubation of U266 cells with bortezomib (20 nM) for 2h failed to inhibit pNFκB expression (Figure S5C, bottom panel) despite activation of cleaved PARP for over 48h (Figure S5B, Apoptosis, black triangles). A specific threshold of cleaved PARP activation might be required to inhibit NFκB expression, which is not incorporated into the current network model, and further studies are needed to test this hypothesis.

**Network Reduction.** A Boolean network reduction approach (Veliz-Cuba, 2011) identified 8 critical nodes (Figure S6A), 5 of which are survival pathway nodes (AKT, pNFκB, BclxL, proteasome, and pRB) and 3 are apoptotic pathway nodes (Caspase3, JNK, and p21). Boolean simulations of the reduced network are identical to the steady-state values of the full network, which further supports the reduction algorithm (Figure S6B). Although Caspase3 was identified as a critical protein through the Boolean network reduction, cleaved PARP expression was measured as a marker for apoptosis due to Caspase3 assay limitations.

**Bortezomib Cellular Pharmacodynamic Model.** Three critical proteins (i.e., pNFκB, BclxL, and cleaved PARP) were selected from the reduced Boolean network model (Figure S6A) for measurement and inclusion in the cellular pharmacodynamic model. The final cellular model (Figure 3) integrates the time-courses of bortezomib exposure, protein dynamics, and cell...
proliferation. Bortezomib elicits its effects on pNFκB via stimulation of upstream proteins in pNFκB pathway and on cleaved PARP via stress accumulation due to proteasome inhibition. Upon continuous exposure of U266 cells to bortezomib, pNFκB protein expression is stimulated after a slight delay with a return toward the baseline (Figure 5A). The slight delay in the stimulation of pNFκB was well characterized by adding a simple transit compartment (M1B, Eqs. 1 and 2). pNFκB expression was well described by a precursor model, in which the delayed signal (M1B) stimulates the first-order transfer from the precursor to the pNFκB compartment (Eqs. 2 and 3). Parameters associated with M1B and pNFκB (k_{deg,M1B} and S_{m,M1B}) were not estimated with good precision, therefore M1B associated parameters were fixed to the estimates during an initial model run. This had no impact on model performance (i.e., model fits were not compromised and parameter values did not change substantially), but precision of the estimated parameters was significantly improved (Table 1). A relatively long delay was observed before cleaved PARP expression increased (Figure 5B), and a transit-compartment model was selected to describe this delay. Four transit compartments were found to be adequate, and the total transit time was well estimated at 26.8h (Table 1, k_{tr,parp}). BclxL expression starts decreasing after 12h (Figure 5C), and this delay was also well characterized by the proposed model. The initial phase of BclxL was maintained at steady-state by pNFκB, and the eventual decrease in BclxL expression was successfully described using cleaved PARP stimulation of the BclxL degradation rate constant. An exponential growth model well captured the natural cell proliferation under control conditions (Figure 5D, triangles). The final signal transduction model reasonably captured the delay in cell death, and parameters were estimated with good precision (Table 1). Overall, the pharmacodynamic model well characterized the time-courses of protein expression and cell proliferation profiles after exposure to bortezomib at 20 nM.
**Sensitivity to Dose and Model Qualification.** The final pharmacodynamic model (Figure 3) was developed using several cellular biomarkers after continuous exposure to a single, relatively high concentration of bortezomib (20 nM). Simulations of protein expression profiles and cell proliferation following exposure to different bortezomib concentrations (0.1 to 20 nM) were performed to evaluate the role of bortezomib dose in cellular outcomes (Figure S7). As bortezomib concentration is increased, the magnitude of pNFκB expression is also increased and the time to peak response is decreased (Figure S7A). The extent of induced cleaved PARP is also increased (Figure S7B), along with greater suppression of BclxL expression (Figure S7C) as bortezomib concentration increases. For cell proliferation, cell growth is suppressed up to 2 nM (Figure S7D, red line), but greater concentrations result in cell death (Figure S7D). As an external predictive check, protein expression profiles and cell proliferation after low-dose bortezomib exposure (2 nM) were measured and compared with model simulations, which were obtained using the final model (Figure 3) and parameter estimates (Table 1) from the high-dose bortezomib (20 nM) experiments. The low concentration of bortezomib (2 nM) was chosen as it is close to its IC50 value (1.5 nM) from the in vitro exposure-response relationship at 48h. Thus, the selected concentration is relatively low but should still elicit a pharmacological response.

Model predicted pNFκB, cleaved PARP, and BclxL profiles and cell proliferation dynamics are in good agreement with the experimental data (Figure S8).

The final model was also used to predict the IC50 for bortezomib after 48 hours of continuous exposure. Although the model was developed based on temporal profiles after a single concentration of bortezomib, the model predicted IC50 was comparable to that obtained from the in vitro concentration-effect experiments (3.5 vs. 1.5 nM; Figure 6).
Discussion

Network-based approaches are increasingly used in drug discovery and development (Csermely et al., 2013; Harrold et al., 2013). Pharmacological networks are now commonly utilized for target identification (Sahin et al., 2009), evaluating signaling networks between normal and diseased conditions (Saez-Rodriguez et al., 2011), and understanding interactions among pathways (Thakar et al., 2007; Ge and Qian, 2009; Mai and Liu, 2009). For example, a Boolean network was used to identify novel targets to overcome trastuzumab resistance in breast cancer cell lines (Sahin et al., 2009), in which c-MYC was identified as a potential therapeutic target. In another example, Boolean network modeling was used to analyze immune responses due to the presence of virulence factors in lower respiratory tract infection (Thakar et al., 2007). Three distinct phases of Bordetellae infection were identified, which was not possible using traditional experimental methods (i.e., biochemical and molecular biology techniques). A similar approach is used in the present analysis, in which a Boolean network model is applied for evaluating bortezomib signal transduction pathways in U266 cells.

Our initial Boolean network model was developed based on the literature; however, available studies focus on either NFκB (Bharti et al., 2003b) or JAK/STAT3 (Bharti et al., 2003a; Park et al., 2011) pathways independently, and joint effects of these pathways have not been previously evaluated in U266 cells (e.g., apoptosis and cell proliferation). In addition, cellular protein dynamics and outcomes following exposure to specific probe compounds were also unavailable. Therefore, in vitro experiments and computational modeling were combined with pathway specific inhibitors to evaluate the roles of both pathways individually and simultaneously. The Boolean network model confirmed that stress accumulation due to
proteasome inhibition is a major pathway of myeloma cell death despite the activation of pNFκB protein expression, which contradicts a major proposed hypothesis of bortezomib effects.

Whereas logic-based models provide qualitative insight into network connectivity and drug induced signal transduction, a quantitative dynamical model that includes important cellular biomarkers allow for the integration of critical factors responsible for cell death upon chemotherapy exposure (Yamazaki et al., 2011; Kay et al., 2012; Zhang et al., 2013). It is not yet practical to quantify all components in cellular signaling pathways; therefore, it is necessary to identify critical proteins for dynamical model development. A Boolean network reduction algorithm (Veliz-Cuba, 2011) identified critical factors regulating bortezomib cell death that guided development of a reduced PD model. Among the biomarkers (Figure S6), 2 anti-apoptotic proteins and one pro-apoptotic protein (pNFκB, BclxL, and cleaved PARP) were integrated into a reduced pharmacodynamic model (Figure 3). pNFκB was integrated in the model as bortezomib increases its expression, contradictory to proposed mechanisms. BclxL was selected as a major anti-apoptotic protein that inhibits apoptosis, and cleaved PARP is a primary pro-apoptotic marker. The factor p21 was not incorporated in the current model as Boolean simulations suggest that bortezomib effects on apoptosis precede cell growth arrest.

The final cellular PD model (Fig. 3) was successfully qualified using the external data set following a low concentration of bortezomib (2 nM). Despite the fact that the cellular model was developed based on a single bortezomib concentration (20 nM) and linear coefficients, the final model reasonably predicted responses to the lower bortezomib exposure (Figure S8) and the dose-response curve for bortezomib at 48h (Figure 6). Although there is a slight mis-fit for BclxL and cleaved PARP profiles (Figure S8B and S8C), overall simulated profiles reasonably agree with experimental data. Most commonly, exposure-response relationships of protein
expression data are sigmoidal in nature (Bharti et al., 2003a; Bharti et al., 2003b; Park et al., 2008; Park et al., 2011). For example, the inhibition of pNFkB activity by curcumin is 10% at 1 μM and 40% at 10 μM (Bharti et al., 2003b). Similarly, cleaved PARP activation is about 1% at 10 μM of curcumin but 100% at 50 μM of curcumin (Park et al., 2008). It is therefore interesting that although our model was developed using a single drug concentration and simple linear coefficients (e.g., Sm_M1B and Sm_PARP in Eqs. 1 and 4), it reasonably predicts profiles to a lower bortezomib concentration (Figure S8) and a sigmoidal concentration-effect relationship (Figure 6). Furthermore, the predicted exposure-response relationship for BclxL can be overlaid with observed cell proliferation at 48 h (Figure S9). This suggests that BclxL could serve as a potential biomarker to predict efficacy in MM and warrants further study. However, a potential limitation is that the proteins measured in this study reflect relative fold-change and not absolute values of protein concentrations. Grounding on relative changes in protein expression maintains a degree of modularity, but more advanced proteomic methods are needed to quantitatively measure low-abundance signaling proteins. Cell growth and death parameters were also compared to available values from the literature. Natural cell growth and death were modeled using first-order growth rate constants (k_g and k_d). The estimated net growth rate constant (k_g – k_d) in the final model (0.00490 h⁻¹) is comparable to net growth rate constants from U266 xenografts at 0.00487 h⁻¹ (Siveen et al., 2014) and 0.00450 h⁻¹ (Rhee et al., 2012) that were obtained from fitting an exponential model to digitized control curves (data not shown).

Preliminary experiments were conducted to measure pNFkB in the nucleus and cytoplasm of U266 cells after bortezomib exposure. Both cellular fractions showed a similar trend of increased relative pNFkB expression (data not shown), and so total cellular pNFkB was measured by western blot analysis of cell lysates, revealing transient increased expression
For the stimulation of pNFkB protein expression, apoptosis is induced and the model is able to well capture this phenomenon. The NFkB pathway is one of the major cell survival pathways and is deregulated in many types of cancer (Karin, 2009). Bortezomib stimulates the upstream cascade of the NFkB pathway (Hideshima et al., 2009) leading to activation of pNFkB protein expression. Apoptosis is still induced through cellular stress resulting from the inhibition of proteasome (Figure 4D) (Hideshima et al., 2001). The cellular model was able to well describe both mechanisms. Aberrant expression of pNFkB is responsible for the lack of efficacy and resistance in some cases of MM (Hideshima et al., 2002; Bharti et al., 2004). Greater expression of pNFkB results in greater expression of anti-apoptotic proteins (e.g. BclxL) (Karin, 2002), with crosstalk between survival and apoptotic pathways determining cellular fate. Hence, greater drug concentrations are required to activate the apoptotic pathway. Initially, BclxL inhibition of cleaved PARP activation and cleaved PARP stimulation on removal of BclxL was incorporated. However, since BclxL exerts a negative feedback on cell death, as incorporated in the model, the BclxL effect on cleaved PARP was removed to simplify the model and reduce model redundancy.

In summary, systems pharmacology is an emerging field that seeks to couple systems biology and PD modeling, which could promote the discovery, development, and effective use of drugs based upon first principles. Computational tools based on graph theory (Liu et al., 2013), discrete dynamic relationships (Albert and Wang, 2009), and others, can be used to identify critical system components within networks at multiple organizational levels, thus providing guidance for multi-scale model construction and evaluation. We have used an integrated approach to investigate bortezomib signal transduction and exposure-response relationships in MM cells. A cell-based model of bortezomib in U266 human myeloma cells was
developed that incorporates one of the major survival pathway proteins pNFkB, anti-apoptotic protein BclxL, and an apoptotic marker (cleaved PARP) to link bortezomib exposure to cell proliferation. The final model-predicted in vitro IC₅₀ for bortezomib reasonably agrees with the experimental IC₅₀ at 48h. Although the Boolean and dynamical models are relatively simple and specific to U266 cells and bortezomib, the overall strategic approach can be easily extended with slight modification to other myeloma cell lines (e.g., MM.1S and RPMI 8226) or bortezomib-based combination regimens (e.g., HDAC inhibitors). This model may serve as a basis for studying bortezomib combinations with anti-myeloma agents and to optimize xenograft combination studies, which is a focus of current research and will be reported separately. In addition, the model structure can be easily extended to describe responses in other cancer types that share similar pathways.
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Authorship Contributions

Design and execution of experiments: VLC

Mathematical model development and data analysis: VLC and DEM

Network analysis: VLC and MO

Wrote or contributed to the writing: VLC, MO, DRA, and DEM
References


Thymoquinone overcomes chemoresistance and enhances the anticancer effects of bortezomib through abrogation of NF-kappaB regulated gene products in multiple myeloma xenograft mouse model. Oncotarget.


Footnotes

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Legends for Figures

Figure 1 Signal transduction pathways in multiple myeloma disease pathology.

Figure 2 Boolean network reduction steps. A) Removal of nodes with one input and one output (see node Y). B) Specific example of removing nodes with one input and one output, in which nodes RAS and MEK1 are connected after removing RAF. C) General example of identification and removal of non-functional edges in a network, in which the edge from node C to node A is identified as a non-functional edge and removed. D) Specific sub-network example of removing a non-functional edge for p21. E) General example of identification and removal of non-functional nodes, in which node C is removed after applying the rule in Figure 1A. F) Specific sub-network example for removing non-functional nodes (e.g., MYC). Block arrows indicate the final network after applying the specific rule. ‘OR’ relationship is represented by ‘||’, ‘AND’ by ‘&&’, and ‘NOT’ by ‘~’.

Figure 3 Signal transduction model of bortezomib effects in multiple myeloma. Bortezomib compartment represents bortezomib concentration in vitro with a degradation half-life of 144 h. M1B is a signaling compartment for stimulating the conversion of the precursor compartment (pre-NFκB) to pNFκB, which represents relative pNFκB protein expression. cPARPn represents transit compartment n for cleaved PARP. The Bcl-xL compartment represents relative BclxL protein expression, and N is cell proliferation in vitro. Yellow highlighted boxes are compartments for which experimental data are measured. Open and closed rectangles represent stimulation and inhibition processes.
Figure 4 Comparison of model simulated and observed U266 cellular outcomes with continuous bortezomib exposure. A) Cell proliferation over 96 h after bortezomib (20 nM) exposure in U266 cells, B) Time-course of apoptosis as measured by cleaved PARP protein expression under constant exposure to bortezomib (20 nM) in U266 cells, C) Final Boolean model (Figure S1) simulations of cell proliferation, and D) Apoptosis under control conditions (red, solid) and after constant exposure to bortezomib (blue, dash-dot). Symbols are mean observed data and error bars represent standard deviation.

Figure 5 Time-course of cellular protein dynamics and cell proliferation after continuous bortezomib exposure (20 nM). Relative protein expression profiles are shown for A) pNFκB, B) cleaved PARP, and C) BclxL. Protein expression was measured using western blot in whole-cell lysate. D) Cell proliferation measured using WST-1 assay kit. Measurements following bortezomib treatment and vehicle control are represented by circles and triangles. Solid lines are model-fitted profiles.

Figure 6 Comparison of model predicted and observed bortezomib concentration-effect profiles at 48 h. Circles represent mean observed data (error bars are standard deviation) at 48 h. Lines represent either a fitted curve to observed data using a Hill-type function (red) or the model predicted data at 48 h (blue).
Table 1 Final Bortezomib Parameter Estimates in U266 Cells

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