Combinatorial Chemotherapeutic Efficacy in non-Hodgkins Lymphoma can be Predicted by a Signaling Model of CD20 Pharmacodynamics

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Abstract

Combination chemotherapy represents the standard-of-care for non-Hodgkin lymphoma. However, the development of new therapeutic regimens is empirical and this approach cannot be used prospectively to identify novel or optimal drug combinations. Quantitative system pharmacodynamic models could promote the discovery and development of combination regimens based upon first principles. In this study, we developed a mathematical model that integrates temporal patterns of drug exposure, receptor occupancy, and signal transduction to predict the effects of the CD20 agonist rituximab in combination with rhApo2L/TRAIL (TNF-Related Apoptosis Inducing Ligand) or fenretinide, a cytotoxic retinoid, upon growth kinetics in non-Hodgkin lymphoma xenografts. The model recapitulated major regulatory mechanisms, including target-mediated disposition of rituximab, modulation of pro-apoptotic intracellular signaling induced by CD20 occupancy, and the relative efficacy of death receptor isoforms. The multi-scale model coupled tumor responses to individual anti-cancer agents with their mechanisms of action in vivo, and the changes in Bcl-xL and Fas induced by CD20 occupancy were linked to explain the synergy of these drugs. Tumor growth profiles predicted by the model agreed with cell and xenograft data, capturing the apparent pharmacological synergy of these agents with fidelity. Together, our findings provide a mechanism-based platform for exploring new regimens with CD20 agonists.
Major Findings

An integrated systems pharmacodynamic model developed from single-agent responses and known mechanisms of drug action is able to predict with fidelity the apparent synergistic antitumor effects of rituximab administered with fenretinide or rhApo2L in a non-Hodgkins lymphoma model. Rituximab binding to tumor CD20 regulates both drug exposure and anti-tumor response. Quantification of Bcl-xL- and Fas modulation is sufficient to explain rituximab synergy without requiring empirical drug interaction parameters. The greater affinity of rhApo2L for death receptor (DR) 5 relative to DR4 can explain relative efficacy of these isoforms, and Fas may serve as a surrogate for rituximab-induced up-regulation of these receptors.
Quick Guide to Equations and Assumptions

The final mathematical model is based on a series of ordinary differential equations that integrate key factors determining antitumor efficacy of rituximab alone and combined with fenretinide or rhApo2L (Figure 1 and S1). Supplementary Materials provides the complete system of equations.

Drug Disposition

Simple pharmacokinetic models describe the time-course of plasma drug concentrations for each agent. These functions drive intermediate cell signaling and the effects of individual or combined drugs on tumor. For rituximab and rhApo2L, the models also account for the loss of drug bound to tumor. Differential equations are specified for total and bound drug in the system. Free concentrations are calculated assuming quasi-equilibrium conditions (1). The free rituximab plasma concentrations ($C_R$) is:

$$C_R = 0.5 \left[ \left( C_{tot,R} - R_{tot,CD20} - K_{D,CD20} \right) + \sqrt{\left( C_{tot,R} - R_{tot,CD20} - K_{D,CD20} \right)^2 - 4 \cdot K_{D,CD20} \cdot C_{tot,R}} \right]$$ (A)

where $C_{tot}$ and $R_{tot}$ represent total rituximab and CD20 concentrations, and $K_D$ is the equilibrium dissociation constant. The solution for rhApo2L is complicated by the presence of two receptors (DR4 and DR5); the resulting cubic polynomial is resolved by obtaining its roots.

The molar concentration of drug receptors (CD20, DR4, and DR5) is calculated as a function of tumor volume ($N$; units of mm$^3$):

$$R_{tot} = \frac{R_{cell} \cdot N \cdot D_N}{N_A \cdot V_c}$$ (B)
where \( R_{cell} \) is cellular receptor density, \( D_N \) is the tumor cell density (9.6x10^5 cells/mm^3 (2)), \( N_A \) is Avogadro’s Number, and \( V_c \) is the volume of plasma in contact with the receptors.

**Signal Transduction**

Fractional CD20 occupancy by rituximab \((f_{b,CD})\) modulates a minimized signal transduction network (Figure S1). The first component is \( RKIP \) induction. The rate of RKIP expression change is:

\[
\frac{dRKIP}{dt} = \int \left\{ \frac{1}{\tau_K} \left[ K_K \cdot f_{b,CD} - x_D - \Delta RKIP \right] \right\} dt \quad (C)
\]

\( \tau_K \) is the mean transit time in the \( RKIP \) compartment, \( K_K \) is a proportionality constant, \( f_{b,CD} = C_R / (K_{D,CD} + C_R) \), and \( \Delta RKIP = RKIP(t) - RKIP(0) \). The dummy state \( x_D \) transforms this second-order process to ordinary differential equations. A simple transit compartment model of signal transduction defines downstream state variables for \( NF\kappa B, Bcl-xL, \) and \( Fas \) (Supplementary Material).

**Tumor growth and efficacy**

The tumor growth function includes both nominal tumor growth and drug effects:

\[
\frac{dN}{dt} = k_{ng} \cdot \left( 1 - \frac{1}{IC_{50,R} + C_R} \right) \cdot N = \sum f_{kill,i} \quad (D)
\]

\( k_{ng} \) is the net first-order rate constant representing cell growth vs. death, \( I_{max} \) is the maximal inhibition of \( k_{ng} \), and \( IC_{50,R} \) is the plasma rituximab concentration mediating half-maximal inhibition of \( k_{ng} \). \( \sum f_{kill,i} \) is the composite cell kill function of the \( i^{th} \) drug (i.e., \( R, \) rituximab, \( H, \) fenretinide, and \( A, \) rhApo2L):
\[
\sum f_{\text{kill,i}} = N \cdot \left\{ k_{\text{kill,R}} \cdot f_{b,CD20} + k_{\text{kill,A}} \cdot \text{Fas}(f_{b,CD20}) \cdot \sum f_{b,DR} + C_H \cdot \left[ 2 - \text{Bcl-xL}(f_{b,CD20}) \right] \right\} \quad (E)
\]

\(k_{\text{kill,i}}\) represents the second-order cell kill rate constant for individual drugs. \(\text{Fas}(f_{b,CD20})\) is the relative fold-change in Fas expression when \(f_{b,CD20}>0\) (otherwise \(f_{b,CD20}=1\)), \(\sum f_{b,DR}\) is the sum of DR4 and DR5 occupancies by rhApo2L, \(C_H\) is the plasma fenretinide concentration, and \(\text{Bcl-xL}(f_{b,CD20})\) is the relative change in Bcl-xL expression when \(f_{b,CD20}>0\) (otherwise \(f_{b,CD20}=1\)).
Introduction

Despite improvements in overall survival over the past 20 years, therapy of indolent and refractory non-Hodgkins lymphoma (NHL) remains an unmet challenge (3). Combination chemotherapy represents standard-of-care, but the repertoire of agents for NHL is expansive (4). Numerous ongoing trials seek to identify drug combination regimens empirically that improve therapeutic outcomes. However, a quantitative, mechanistic approach for predicting the effects of combination therapies, based upon mechanisms of drug action and interaction, could assist development of more efficacious NHL therapies. A component of first-line therapy and rapidly relapsing NHL is rituximab, a CD20 agonist. Its mechanism of action includes induction of apoptosis and inhibition of cellular proliferation (5). Rituximab up-regulates cell surface expression of Fas (Apo1) and Apo2 death receptors (6,7), and reduces Bcl-2 expression, which sensitizes tumor cells to cytotoxic compounds (8). Preclinical NHL models suggest fenretinide, a cytotoxic retinoid that induces apoptosis through caspase activation (9), and rhApo2L/TRAIL (TNF-Related Apoptosis Inducing Ligand), which promotes apoptosis via death receptor activation, synergize with rituximab (10,11). These agents are either approved (rituximab), or in Phase III (fenretinide) or Phase I (rhApo2L) clinical development.

We sought to develop a quantitative, predictive systems pharmacological framework that would integrate commonly-obtained cellular response parameters, such as drug-receptor interactions and cell signaling activity, with outcome measures of efficacy such as tumor burden, thereby establishing a data- and model-based approach to optimize combination chemotherapies. Integrated pharmacokinetic/pharmacodynamic (PK/PD) models can be used to characterize drug concentration-effect systems, generate and test
competing hypotheses, and predict pharmacological outcomes under different experimental conditions (12,13).

Early PD models of chemotherapeutic agents employed system-specific- and drug-specific parameters, and plasma drug exposure usually served as a surrogate for intra-tumor drug concentrations, which ultimately drive pharmacological effects (14). For drugs that elicit rapid, irreversible effects, this approach is accurate and useful. However, many chemotherapeutic agents demonstrate significant temporal dissociation between drug exposure (from measured plasma concentrations) and pharmacological responses. Where specific information about drug mechanisms of action is lacking, several techniques have been employed to account for this apparent hysteresis, including transit compartment models to represent time-dependent signal transduction processes (15,16) or cellular progression through maturation or apoptosis (17,18). Although empirical functions describing drug interactions can extend these semi-mechanistic models (19), the approach nonetheless is driven by specific experimental conditions rather than first-principles or mechanisms of drug action.

Our approach was to develop a dynamic model linking drug exposures with both underlying molecular events occurring at the cellular level, and therapeutically relevant, tissue-level responses such as changes in tumor burden. The macroscopic exposures of rituximab, fenretinide, and rhApo2L, and the efficacy of these agents alone or in combination, have been evaluated in vitro and in murine NHL xenografts (7,8,10,11,20-22). We developed models of known molecular mechanisms of action to link these disparate data quantitatively. The result is a system-level pharmacodynamic model capable of predicting not only efficacy across studies, but also the apparent synergy observed
preclinically with combinations of these agents. This mathematical framework provides improved understanding of the indirect relationships that relate anti-cancer drug exposure and treatment response. The final model can be used to explore new drug combinations, optimize promising regimens, and enhance insight generation from preclinical experiments with CD20 agonists.

Materials and Methods

A mathematical model based on a series of ordinary differential equations was developed to integrate the major factors determining efficacy of rituximab concomitant with fenretinide or rhApo2L. Figure 1 identifies components of the model and mechanistic interconnections between drug disposition and efficacy of these three agents. Pharmacokinetic models, derived for the unique behavior of each agent, provide predictions of plasma drug concentrations that drive intermediate cell signaling and the ultimate therapeutic effects upon tumor burden. In the model, rituximab occupancy of CD20 drives inhibition of tumor cell growth and influences various cellular signal transduction networks. Similarly, fenretinide and rhApo2L influence specific signal transduction pathways, and the resulting tumor cell kill function ($F_{kill,tot}$) is a composite of direct and indirect drug effects acting upon the tumor cell population. Supplementary Materials provides a workflow schematic (Figure S1) and all equations defining this system.

Data for drug exposure, rituximab inhibition of tumor growth, and tumor growth dynamics were extracted from the cited sources using Graphclick (Arizona Software). Parameter estimation and model predictions were performed with MATLAB (R2009a,
MathWorks Inc., Natick, MA). The `fminsearch` function was used to identify the general region of parameters, and `lsqnonlin` was used to identify the final reported estimates and solution summary information. A sequential modeling approach was utilized, in which parameters associated with systemic distribution and elimination, signal transduction associated with binding, and inhibition due to drug plasma concentrations were first identified separately, and then fixed for subsequent estimations of single-agent efficacy.

**Systemic disposition**

Eqs. S1-S3 characterize systemic distribution and elimination of rituximab, fenretinide and rhApo2L. Fenretinide was characterized by a single-compartment model with absorption from a peripheral site. Because Ramos xenografts express CD20 and DR4/DR5 receptors, rituximab and rhApo2L studies (23,24) obtained PK data in tumor-free animals. Rituximab was characterized with a single-compartment model, and rhApo2L was characterized with a two-compartment model.

**Mechanisms of action**

Rituximab efficacy was driven by binding to CD20 receptors (20) and rhApo2L by binding to the proapoptotic receptors DR4 and DR5 (25,26). Receptor interactions were driven by the concentration of the agent in the system, the receptor concentration, and the ligand/receptor binding affinities ($K_D$). Assuming that the rates of association and disassociation are rapid relative to other dynamics of the system, a quasi-equilibrium approach (Eq. A) was used to determine receptor occupancy (1).
Plasma-level exposures were linked with cell-level responses such as apoptosis through CD20 receptor occupancy. The fraction of bound receptors was also the input to a minimal signal transduction cascade model, the output of which is up-regulation of Fas expression and down-regulation of free Bcl-xL. Sequential modeling was performed wherein changes in RKIP activation were modeled first, and the parameters obtained were then fixed and used as an input to the portion of the model describing NFκB signaling. A second-order model was developed for RKIP (Eq. C), which can be defined by a coupled system of first-order differential equations:

\[
\frac{dx_D}{dt} = \frac{1}{\tau_K} : (f_{k,CD20} \cdot RKIP - x_D - \Delta RKIP) \quad \text{(Eq. 1)}
\]

\[
\frac{dRKIP}{dt} = x_D \quad \text{(Eq. 2)}
\]

Finally, with upstream parameters fixed, changes in Fas and Bcl-xL were modeled (Eq. S8).

Fas expression was considered a surrogate for DR4 and DR5 abundance. Up-regulation of Fas was used to up-regulate the surface receptor density of DR4 and DR5 and drive downstream signaling resulting from activation of these receptors. Signaling associated with DR4 and DR5 receptors was driven by receptor occupancy, as determined by the equilibrium binding expression for a ligand having two receptors (Supplementary Materials).

**Efficacy and target-mediated disposition**

An exponential model characterizes nominal tumor growth, consistent with the time frame of the data available. Tumor growth rate was directly inhibited by plasma concentrations of rituximab according to a classic Hill relationship (Eq. D). Kill terms for
rituximab, fenretinide, and rhApo2L were derived to account for cell loss associated with each of these compounds (Eq. E). Rituximab efficacy was proportional to the size of the tumor and the fraction of bound CD20 receptors. The plasma concentration of fenretinide was used to drive its efficacy, and for combination treatment, the proportional effect of efficacy attributable to fenretinide was amplified by the estimated suppression of Bcl-xL levels. The fraction of bound DR4 (f_{b,DR4}) and DR5 (f_{b,DR5}) was used to drive the efficacy of rhApo2L, and for combination treatment, the effect was modulated by Fas expression levels. For simulating the case in which additive interaction effects were hypothesized, Bcl-xL and Fas effects were held at their baseline values.

Data for untreated tumors from the fenretinide data set (10) were used to set the initial tumor volume and estimate nominal tumor growth kinetics. These parameters were then fixed for the estimation of efficacy parameters for single-agent rituximab and fenretinide. Analysis of the rhApo2L data set began by fixing the initial tumor volumes to those reported (11) and then simultaneously estimating the nominal tumor growth rate and rhApo2L efficacy. These parameter estimates were combined with the rituximab efficacy term obtained from the fenretinide data set, and then used to predict efficacy of single-agent rituximab in the rhApo2L data set. As cancer cells die, the volume of the tumor decreases, and it was assumed that the loss of cells also removes the drug bound to their receptors. For small molecule drugs, the target-bound fraction may be inconsequential. However, concentrations of protein-based drugs may be comparable to receptor concentrations, as is the case for rituximab. To account for this, the PK models for rituximab and rhApo2L were augmented to track the total amount of drug in the system.
Terms were then added to account for the loss of bound rituximab as the tumor volume decreased.

Results

Systemic distribution and elimination

The time-course of drug exposure is the initiating driver for subsequent signaling and pharmacological effects. Standard one- and two-compartment models captured rituximab, fenretinide, and rhApo2L pharmacokinetics. These general drug absorption and disposition properties were assumed to operate in parallel with ‘target-mediated drug dispositional’ effects (27) that alter the PK of high affinity ligands such as rituximab when their concentration is stoichiometrically comparable to that of their receptors. Systemic pharmacokinetic profiles were modeled for rhApo2L (Figure 2B) and rituximab (Figure 2D) after intravenous injection in mice. Figure S2 shows fenretinide disposition following intra-peritoneal injection. Terminal phase half-lives for rituximab, fenretinide, and rhApo2L were 22.7 days, 5.1 hours, and 2.6 hours, respectively, highlighting the substantial pharmacokinetic differences among the three compounds. The model-fitted curves captured the data well, and the parameters associated with these profiles (Table S1) were fixed in the subsequent analyses.

Target-mediated dispositional effects of CD20, DR4, and DR5 density

CD20 receptor occupancy was assumed to drive the intensity of rituximab response (28) and DR4/DR5 occupancy was considered to drive rhApo2L responses (11,29). High-
affinity therapeutic ligands often are in a concentration range in which interaction with their receptors alters plasma disposition (27). Changes in tumor volume or the density of tumor-associated receptors can influence pharmacokinetics, as observed for rituximab (23). Therefore, receptor burden was calculated dynamically based on tumor volume, the number of cells per unit volume, and the receptor density. On Ramos cells, CD20 receptor density is approx. 330,000/cell (30), approx. 3726/cell for DR4, and 4790/cell for DR5, based upon relative florescence analysis (11). The influence of tumor volume and CD20 receptor abundance on rituximab PK was observable qualitatively (23), and Figure 2D shows rituximab pharmacokinetics after administration to mice bearing different initial burdens of CD20+ lymphomas. As tumor burden increased, plasma concentrations of rituximab decreased, reflecting an apparent increase in total systemic clearance mediated by the CD20 burden attributable to tumor (Figure 2D). This physiological target-mediated dispositional effect was implemented in the model for systemic PK of rituximab and rhApo2L by eliminating receptor-associated drug on dying cells. The final model predictions for rituximab disposition as a function of hypothetical initial Ramos cell tumor burden show a similar trend as experimental systems across cell lines (Figure 2D).

Rituximab effects upon CD20 signal transduction

With CD20 density effects integrated explicitly in the model, the fraction of bound receptors (fb,CD20) could be calculated and used to drive downstream cell signaling (Figure 1). By assuming that equilibration between free and bound rituximab is rapid compared to the dynamics of drug disposition and disease response, fb,CD20 can be calculated directly from the total rituximab concentration (bound+free; Eq. S10) and the total receptor
number (1). The receptor occupancy model was then used to drive the signal transduction- and tumor-growth inhibition models associated with the pro-apoptotic effects of rituximab.

Exposure of CD20-expressing B cells to rituximab increases expression of Fas/Apo1 and TRAIL-R/Apo2 receptors (20), thereby sensitizing them to apoptotic signaling molecules. CD20 activation also reduces free levels of Bcl-class proteins within the cytosol, via a combination of gene- and protein-level interactions (20). Free Bcl-xL confers resistance to cytotoxic compounds, and conversely, reductions in free Bcl-xL sensitize cells.

Several components of the signal transduction pathway linking CD20 activation with Fas/Apo1- and Bcl-xL modulation have been elucidated (6,7,21,22,31,32) and were incorporated in the model (Figure 3A). The time-course of RKIP activation, NFκB signaling, Fas up-regulation, and free Bcl-xL reduction was obtained from the literature (7,31) and used to construct a minimal model of signal transduction (Figure 3A), which was fit to available data (Figure 3B). The key nodes in the minimal signaling model were identified using discrete dynamic modeling of a much larger signal transduction network (not shown). Once the connectivity was defined and fixed (Figure 3A), several transfer functions, including traditional first-order transit compartments (15), were evaluated to capture the time-course of expression levels (Figure 3B). The second-order system defined in Equations 1 and 2 was found superior to simpler transfer functions. In the model, signal transduction responses are represented as deviations from baseline values that maintain steady-state conditions (7,31), and the dynamic response is driven by the fraction of bound CD20 receptors (Figure 3B). Whereas the model tracks measured Fas levels directly, rituximab also up-regulates DR5 expression (20), for which data are not available. However, because of the mechanistic and temporal similarities between Fas/Apo1 and
other death receptors, the structural model for Fas regulation was also used for predicting rituximab-mediated changes in the abundance of DR4 and DR5 (Eqs. S11, S12).

**Rituximab inhibition of tumor growth**

Rituximab can mediate tumor cell killing through both antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity (33). However, in the available data, efficacy was quantified in immunocompromised animal models. Therefore, these processes were considered negligible and omitted from the final model. Other primary mechanisms of efficacy include inhibition of cell proliferation (34) and induction of apoptosis (35) via CD20 receptor occupancy. The concentration-dependence of Ramos cell growth inhibition by rituximab was described previously (34), and was modeled with a standard saturable function (Eq. D and Figure 1). Figure 4 shows the model fit to data. The capacity of the proliferation inhibitory function (maximal effect attributable to this mechanism) was 14.3%, and the concentration of rituximab mediating half-maximal inhibition of proliferation (IC$_{50}$) was $8.31 \times 10^{-8}$ M. Thus although rituximab inhibition of cell proliferation impacts tumor volume progression, an additional effect representing rituximab-induced apoptosis was required to characterize observed *in vivo* responses (*below*).

**Modeling single agent efficacy**

The strategy for developing a predictive model for rituximab-based combination chemotherapy was to model single-agent effects on the pharmacodynamic markers selected, and then combine models under several hypothesized mechanisms of drug
interaction, including synergy and additivity. A simple exponential growth model was used
to describe unperturbed tumor growth, and a net first-order rate constant represents the
balance of cell proliferation vs. cell death. The effect of treatment is to shift this balance by
retarding the growth function and increasing the cell elimination function.

Two studies of rituximab combinations on Ramos xenografts provided single-agent
data enabling a priori development of a quantitative model for pharmacological
interactions. One investigated rituximab with fenretinide (10) and the other investigated
rituximab with rhApo2L (11). From (10), a tumor doubling time of 142h was estimated in
BALB/c nude mice. Figure 5A shows that the model captured tumor progression data well
for untreated animals and those receiving single-agent rituximab or fenretinide. Data from
(11), which investigated rituximab and rhApo2L in CB17 SCID mice, was then used to test
the model developed for efficacy of single-agent rituximab. A tumor doubling time of 63h
was estimated. By substituting the doubling time appropriate for the specific tumor into
the growth model, the rituximab efficacy term obtained in nude mice (10) provided an
excellent prediction of single-agent rituximab in SCID mice (Figure 5B). Data (11) also
provided results for single-agent rhApo2L. Simultaneous fitting of tumor progression data
for control and rhApo2-treated animals showed that the developed model captured the
data well (Figure 5B). By this approach, appropriate models were developed for all three
drugs as single agents.

Model prediction of combination chemotherapy

The rituximab/fenretinide dataset (10) is relatively rich, permitting estimation of
single-agent effects (Figure 5A) and testing whether simulations based on single-agent
effects could capture tumor responses to the agents combined. The full model was used to simulate two tumor response scenarios for fenretinide and rituximab administered according to the regimen of (10): one hypothesized that drug effects were additive, whereas the other hypothesized synergy arising from rituximab-mediated reduction in Bcl-xL levels (Figure 3), thereby amplifying efficacy. The original study suggested the combination demonstrated supra-additive effects (10). Here, both models predicted the observed data relatively well (Figure 5A), and the regimen of the reported study does not support a conclusion regarding nature of drug interaction.

The data for single-agent rituximab from (11) are sparse, but its efficacy was nonetheless well predicted by the model (Figure 5B), as was efficacy of single-agent rhApo2L. The data for combined rituximab/rhApo2L were richer and enabled testing whether efficacy predictions based upon combination of single-agent responses would capture the efficacy observed for the combinations. As described for simulations with rituximab/fenretinide, simulations of rituximab/rhApo2L included hypotheses of additive vs. synergistic interaction, with synergy postulated to arise through rituximab modulation of Fas expression levels via the temporal relationship of Figure 3. Figure 5B shows that tumor responses to combined rituximab/rhApo2L are best predicted by hypothesizing synergistic interaction; the additivity model seriously underestimated tumor response. Furthermore, the improved fidelity of the model to capture observed data for the combination when rituximab effects on Fas expression were included suggests that Fas modulation may represent a quantitative pharmacodynamic marker for the synergy of interaction.
Finally, the rituximab/rhApo2L interaction model was interrogated to determine whether the relative contributions of DR4 and DR5 to overall efficacy could be discerned. Simulation predicts that DR5 provides the greater contribution to efficacy in the combination regimen (Figure 6), consistent with *in vitro* knockout studies in lung carcinoma cell lines (Kelley et al., 2005). However, the model shows clearly that rhApo2L interaction with both DR4 and DR5 is required to capture the full magnitude of experimentally observed efficacy (11).

**Discussion**

A multi-scale systems pharmacological model was created that informs preclinical development of rituximab-based combination chemotherapies for non-Hodgkin lymphoma. Fenretinide and rhApo2L were selected for analysis based upon prior studies of these agents in combination with rituximab. Fenretinide is a cytotoxic, whereas rhApo2L modulates cellular sensitivity to chemotherapeutic agents. The final model links plasma pharmacokinetics of rituximab, fenretinide, and rhApo2L with pharmacodynamic effect biomarkers (Fas, Bcl-xL RKIP, and NFκB) and overall antitumor efficacy. A target-mediated dispositional PK model was employed to accommodate evidence that rituximab interaction with cell-surface receptors affects PK: Figure 2D demonstrates that rituximab plasma concentrations were lower in animals having higher CD20 body burden due to tumor (24). The model predicts no effect of tumor burden upon disposition of rhApo2L, which was also observed experimentally (not shown).

The final model links drug exposure to efficacy by utilizing informative mechanism-based biomarkers. Components of the cellular models represented the contributions of
both indirect drug interactions through previously-elucidated signal transduction pathways and direct drug effects on tumor progression. The overall structure of the model (Figure 1) may appear complex, but derivation of individual components was driven by well-understood physiological pharmacological processes. The model captures the transient effects of signal transduction as driven by receptor occupancy (Figure 3), and enables testing hypotheses as to whether drug effects upon these pathways promote synergistic or additive therapeutic effects.

Incorporation of quantitative mechanisms of drug action in the system PD model preserves physiological fidelity, and the impact of their inclusion upon the capability of the final model to explain experimentally-observed phenomena underscores their potential pharmacological role. For example, the model suggests that with rhApo2L treatment, DR5 contributes to inhibition of tumor progression to a greater extent than DR4 (Figure 6). Interestingly, there is little separation of DR4- and DR5-only curves at early times, but they diverge around the second treatment course. Comprehensive global sensitivity analysis clearly is warranted to test signals relative to parameter perturbations underlying this prediction. However, this model component represents an explicit, quantitative accounting of drug-target interactions, and is consistent with in vitro and in vivo experimental results. The observed recapitulation of a complex pharmacological system results not from post hoc fitting of experimental data, but rather from a priori, quantitative integration of fundamental cellular and pharmacological response mechanisms, and testing of the model with data not employed in model development.

The model for single-agent rituximab efficacy extrapolated well across multiple experimental studies, and required only determination of the tumor growth kinetics; the
cell-kill term derived from one model system predicted efficacy well with another, underscoring the robustness of the underlying modeling approach. The predictive capability of the model was also confirmed (Figure 5); remarkably, model predictions based on single-agent efficacy captured experimental data for combined rituximab/fenretinide and rituximab/rhApo2L efficacy well. In addition, simulation permitted hypothesis testing regarding the nature of the pharmacological interactions with combination therapy. It suggested that additivity vs. synergy could not be discriminated from existing experimental data for rituximab/fenretinide (Figure 5A), whereas both model predictions and experimental data for rituximab/rhApo2L suggested clearly that rituximab modulation of Fas expression (Figure 3) mediated a synergistic interaction (Figure 5B).

The final model also permitted exploration of the impact signal transduction events have upon scheduling of the combination agents. Evidence for time-dependent processes could identify opportunities to improve efficacy by altering treatment schedule. For example, reconstruction of rhApo2L pharmacokinetics from dosing schemes used experimentally (Figure 2) shows that rhApo2L is eliminated far more rapidly than rituximab. As a result, rhApo2L concentrations fall rapidly below the $K_D$ values of the DR4 and DR5 receptors, thus limiting rhApo2L efficacy in mice to the first hour following administration. Furthermore, the maximum rate of Fas up-regulation by rituximab occurs over the first six hours after drug administration (Figure 3).

As demonstrated here, quantitative, physiologically relevant mathematical models based on underlying mechanisms of drug action can be useful for integrating efficacy and toxicity information across preclinical experimental platforms and identifying important
factors regulating magnitude and time-course of drug response. However, this study and approach has limitations. First, the cell signaling model that predicts antitumor efficacy of combinations with rituximab is specific for CD20+ tumor cells. Second, further research is required to identify sources of inter-tumor variability in signal transduction and their influence on drug responses. Third, our model of CD20-induced signal transduction is deliberately minimal; although Bcl-xL and Fas expression provided insight into rituximab synergy with fenretinide and rhApo2L, prediction of pharmacodynamic interactions with a broader range of anticancer agents will require assessment of additional signaling components. Finally, scaling between preclinical models and humans hinges upon identifying and deconvolving system components vs. pharmacological components (36); the two do not scale in the same manner. The modular nature of the approach presented here is supportive of translational research, but formidable challenges remain in gleaning quantitative insights into human PK and PD from preclinical data. Nevertheless, because the system modeling strategy presented is dynamic in nature, exhaustive evaluation of combination regimens is feasible by simulation and could suggest optimized treatment schedules worthy of experimental evaluation. Approaches to multi-scale integration of systems pharmacology models are in their infancy, but with appropriate extension, could suggest new therapeutic agents or identify targets for combination drug development.

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References


Figure Legends

**Figure 1:** Model describing concomitant therapy of mice bearing Ramos B-lymphoma xenografts with rituximab, rhApo2L, and fenretinide. Supplementary Fig. S1 provides structural model. Pharmacokinetic (PK) functions describe rituximab ($C_R$), fenretinide ($C_H$) and rhApo2L ($C_A$) plasma concentrations. Rituximab contributes to direct tumor growth inhibition ($F_{vg}$), and overall effect (large arrow) on tumor cell killing ($F_{kill, total}$) is composite of individual drug actions and combined modulation of cell signaling events. Dashed lines represent signaling information. Fraction of bound CD20 receptors ($f_{b,CD20}$) drives cell signaling (gray rectangle) and apoptotic effects of rituximab. Expression changes in key signaling proteins (Bcl-xL and Fas) modulate efficacy of fenretinide and rhApo2L directly.

**Figure 2:** Systemic exposure effects of rhApo2L and rituximab in mice. (A) Receptor occupancy ($f_b$) of DR4 (dotted) and DR5 (dashed) by rhApo2L (Eq. S6). (B) Symbols: rhApo2L concentrations observed in (24), and predicted by the model (solid line, Eq. S3) for 10 mg/kg dose and tumor volume of 400 mm$^3$. Lines show $K_D$ values for DR4 (dot-dash) and DR5 (dashed). (C) Rituximab PK model based on target-mediated drug disposition. Receptor expression ($R_{CD20}$) is proportional to tumor volume, and drug removal can occur from central- or target-bound compartments. (D) Effect of CD20$^+$ lymphoma burden on rituximab PK. Symbols: data taken from (23). Initial tumor burden was: no tumor (open squares); $<0.15 \times 10^6$ arbitrary units (AU) (filled circles); $0.15-3 \times 10^6$ AU (filled diamonds); $>3 \times 10^6$ AU (open circles). Solid line: model predictions of PK for 20 mg/kg rituximab, assuming nominal tumor growth rate of $4.87 \times 10^{-3}$ hr$^{-1}$; dashed lines:
systemic PK model predictions for increasing \((400, 1000 \text{ mm}^3)\) tumor burden, simulated with Eqs. S2, S7; profiles are overlaid with observed data and show a similar trend.

**Figure 3:** (A) Schematic of minimal rituximab-modulated signaling cascade. CD20 binding yields up-regulation of RKIP expression and NF\(\kappa\)B reduction, which leads to decreased Bcl-xL levels and increased Fas expression. (B) Rituximab effects on key pharmacodynamic effect markers. Symbols: data from literature \((7,31)\) describing rituximab effects on NF\(\kappa\)B, cell-membrane Fas expression, RKIP phosphorylation, and free Bcl-xL levels in Ramos cells treated with 20 \(\mu\)g/mL rituximab continuously, or for 6h (Fas study). Solid lines: fitted model predictions by Eq. S8; dashed lines represent receptor saturation levels. Tables S2, S3 provide initial conditions, final estimated parameters.

**Figure 4:** Ramos cell growth inhibition as function of rituximab concentration. Symbols: data from \((34)\); line: fitted curve using sub-model for growth inhibition (Hill function; Eq. D). Table S4 provides estimated model parameters.

**Figure 5:** (A,B) Data and model fitting of rituximab alone and in combination on Ramos xenografts. (A) 7.4 mg/kg rituximab and 9.26 mg/kg fenretinide, taken from \((10)\). (B) 4 mg/kg rituximab and 60 mg/kg rhApo2L, from \((11)\). Symbols represent data; lines represent fitted model predictions. Arrows indicate dosing days: rituximab (green) was given at lower frequency than the combination agent (blue). Diamonds/red: untreated controls; circles/green: rituximab alone; squares/blue: combination agent alone; black/inverted triangles: combined agents. Lines show model predictions for additive
(dashes) vs. synergistic (solid) drug interactions. Table S4 provides final estimated parameters for tumor growth profiles.

**Figure 6:** Relative contribution of death receptor isoforms to overall efficacy of rituximab/rhApo2L combination. Dosing schedules (arrows), observed data (symbols), and synergistic model fit to experimental data from (11) (solid line) are as in Figure 5B. Dashed lines show simulated tumor response with rhApo2L occupancy of only DR4 (DR5 inactive; magenta/dash-dot) or only DR5 (DR4 inactive; tan/dashes).
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