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/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 proapoptotic intracellular signaling induced by CD20 occupancy, and the relative efficacy of death receptor isoforms. The multiscale model coupled tumor responses to individual anticancer 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 pharmacologic synergy of these agents with fidelity. Together, our findings provide a mechanism-based platform for exploring new regimens with CD20 agonists.

Introduction

Despite improvements in overall survival over the past 20 years, therapy of indolent and refractory non-Hodgkin lymphoma (NHL) remains an unmet challenge (1). Combination chemotherapy represents standard-of-care, but the repertoire of agents for NHL is expansive (2). 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 (3). Rituximab upregulates cell surface expression of Fas (Apo1) and Apo2 death receptors (4, 5), and reduces Bcl-2 expression, which sensitizes tumor cells to cytotoxic compounds (6). Preclinical NHL models suggest fenretinide, a cytotoxic retinoid that induces apoptosis through caspase activation (7), and rhApo2L/TRAIL (TNF-related apoptosis inducing ligand), which promotes apoptosis via death receptor activation, synergize with rituximab (8, 9). 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 pharmacologic framework that would integrate commonly obtained cellular response parameters, such as drug–receptor interactions and cell-signaling activity, with outcome

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-Hodgkin lymphoma model. Rituximab binding to tumor CD20 regulates both drug exposure and antitumor 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 upregulation of these receptors.
Quick Guide to Main Model 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 (Fig. 1 and Supplementary Fig. S1). Supplementary Materials provides the complete system of equations.

Drug disposition

Simple PK 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 (10). The free rituximab plasma concentrations (C_R) is:

\[ C_R = 0.5 \left( C_{tot,R} - R_{tot,CD20} - K_{D,CD20} \right) + \frac{\left( C_{tot,R} - R_{tot,CD20} - K_{D,CD20} \right)^2 + 4 \cdot K_{D,CD20} \cdot C_{tot,R}}{2} \]  \hspace{1cm} (A)

in which 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 2 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 (V_t units of mm^3):

\[ R_{tot} = \frac{R_{cell} \cdot N \cdot D_N}{N_A \cdot V_c} \]  \hspace{1cm} (B)

in which R_cell is cellular receptor density, D_N is the tumor cell density (9.6 \times 10^5 cells/mm^3; ref. 11)), 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_{CD20}) modulates a minimized signal transduction network (Supplementary Fig. S1). The first component is RKIP induction. The rate of RKIP expression change is:

\[ \frac{dRKIP}{dt} = \left\{ \frac{1}{\tau_R} \left[ K_K \cdot f_{CD20} - x_D - \Delta RKIP \right] \right\} dt \]  \hspace{1cm} (C)

\( \tau_R \) is the mean transit time in the RKIP compartment, \( K_K \) is a proportionality constant, \( f_{CD20} = C_K \left( K_D,CD20 + C_K \right) \), 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-κB, Bcl-xL, and Fas (Supplementary Material).

Tumor growth and efficacy

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

\[ \frac{dN}{dt} = k_{agr} \cdot \left( 1 - \frac{I_{max} \cdot C_R}{R_{50,R} + C_R} \right) \cdot N - \sum f_{kill,i} \]  \hspace{1cm} (D)

\( k_{agr} \) is the net first-order rate constant representing cell growth versus death, \( I_{max} \) is the maximal inhibition of \( k_{agr} \) and \( R_{50,R} \) is the plasma rituximab concentration mediating half-maximal inhibition of \( k_{agr} \). \( \sum f_{kill,i} \) is the composite cell kill function of the \( i^{th} \) drug (i.e., R, rituximab, H, fenretined, and A, rhApo2L):

\[ \sum f_{kill,i} = N \cdot \left\{ k_{kill,R} \cdot f_{CD20} + k_{kill,A} \cdot Fas(f_{CD20}) + \sum f_{kill,H} + C_H \cdot [2 - BclxL(f_{CD20})] \right\} \]  \hspace{1cm} (E)

\( k_{kill,H} \) represents the second-order cell kill rate constant for individual drugs. \( Fas(f_{CD20}) \) is the relative fold-change in Fas expression when \( f_{CD20} \geq 0 \) (otherwise \( Fas = Fas(0) \)), \( \sum f_{kill,D} \) is the sum of DR4 and DR5 occupancies by rhApo2L, \( C_H \) is the plasma fenretined concentration, and \( BclxL(f_{CD20}) \) is the relative change in Bcl-xL expression when \( f_{CD20} \geq 0 \) (otherwise \( BclxL = BclxL(0) \)).
mechanisms of drug action. Specifically, models (19), the approach nonetheless is driven by functions describing drug interactions can extend these semi-through maturation or apoptosis (17, 18). Although empirical signal transduction processes (15, 16) or cellular progression ing transit compartment models to represent time-dependent been employed to account for this apparent hysteresis, includ- ing therapeutic agents show signi- cant temporal dissociation between drug disposition and efficacy of these agents alone or in combination, have been evaluated in vitro and in murine NHL xenografts (5, 6, 8, 9, 20– 22). We developed models of known molecular mechanisms of action to link these disparate data quantitatively. The result is a system-level PD 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 anticancer 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 fenreti- nide or rhApo2L. Figure 1 identifies components of the model and mechanistic interconnections between drug disposition and efficacy of these 3 agents. PK 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 (Fkill,total) is a composite of direct and indirect drug effects acting upon the tumor cell population. Supplementary Materials provides a workflow schematic (Supplementary Fig. 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 carried out with MATLAB (R2009a; MathWorks Inc.). The fminsearch function was used to identify the general region of parameters, and laponlin was used to identify the final reported estimates and solution summary information. A sequential modeling approach was used, in which parameters associated with systemic disposition 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

Supplementary 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 2-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 dissociation are rapid relative to other dynamics of the system, a quasi-equilibrium approach (Eq. A) was used to determine receptor occupancy (10).

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 upregulation of Fas expression and downregulation of free Bcl-xL. Sequential modeling was carried out 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 NFkB signaling. A second-order model was developed for RKIP (Eq. C), which can be defined by a coupled system of first-order differential equations:

\[
\frac{dx_D}{dt} = \frac{1}{\tau_k} \left( f_{D,CD20} \cdot RKIP - x_D - \Delta RKIP \right) \quad (F)
\]

\[
\frac{dRKIP}{dt} = x_D \quad (G)
\]

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

Fas expression was considered a surrogate for DR4 and DR5 abundance. Upregulation of Fas was used to upregulate 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 2 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_{D,DR4}) and DR5 (f_{D,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 (8) 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 (9) 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 with 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 (bound + free; Supplementary Eqs. S13 and S14; ref. 10). 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 pharmacologic effects. Standard 1- and 2-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 with that of their receptors. Systemic PK profiles were modeled for rhApo2L (Fig. 2B) and rituximab (Fig. 2D) after intravenous injection in mice. Supplementary Fig. S2 shows fenretinide disposition following intraperitoneal 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 PK differences among the 3 compounds. The model-fitted curves captured the data well, and the parameters associated with these profiles (Supplementary 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 (9, 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 approximately 330,000 per cell (30), approximately 3,726 per cell for DR4, and 4,790 per cell for DR5, based upon relative fluorescence analysis (9). The influence of tumor volume and CD20 receptor abundance on rituximab PK was observable qualitatively (23), and Fig. 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 (Fig. 2D). This physiologic 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 showed a similar trend as experimental systems across cell lines (Fig. 2D).

**Rituximab effects upon CD20 signal transduction**

With CD20 density effects integrated explicitly in the model, the fraction of bound receptors \( f_{b,CD20} \) could be calculated and used to drive downstream cell signaling (Fig. 1). By assuming that equilibration between free and bound rituximab is rapid compared with the dynamics of drug disposition and disease response, \( f_{b,CD20} \) can be calculated directly from the total rituximab concentration (bound + free; Supplementary Eq. S10) and the total receptor number (10). The receptor occupancy model was then used to drive the signal transduction and tumor growth inhibition models associated with the proapoptotic 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 (4, 5, 21, 22, 31, 32) and were incorporated in the model (Fig. 3A). The time course of RKIP activation, NF\( \kappa \)B signaling, Fas upregulation, and free Bcl-xL reduction was obtained from the literature (5, 31) and used to construct a

![Figure 2.](image)

Figure 2. Systemic exposure of rhApo2L and rituximab in mice. A, receptor occupancy \( f_{b} \) of DR4 (dash-dot) and DR5 (dashed) by rhApo2L (Supplementary Eq. S6). B, symbols: rhApo2L concentrations observed in Kelley and colleagues (24), and predicted by the model (solid line, Supplementary Eq. S3) for 10 mg/kg dose and tumor volume of 400 mm\(^3\). Lines show \( K_D \) values for DR4 (dash-dot) 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 Dayde and colleagues (23). Initial tumor burden was: no tumor (open squares); <0.15 x 10^6 arbitrary units (AU; filled circles); 0.15 x 10^6 to 3 x 10^6 AU (filled diamonds); more than 3 x 10^6 AU (open circles). Solid line: model predictions of PK for 20 mg/kg rituximab, assuming nominal tumor growth rate of 4.87 x 10^−2/h; dashed lines: systemic PK model predictions for increasing (400, 1,000 mm^3) tumor burden, simulated with Supplementary Eqs. S2, S7; profiles are overlaid with observed data and show a similar trend.
minimal model of signal transduction (Fig. 3A), which was fit to available data (Fig. 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 (Fig. 3A), several transfer functions, including traditional first-order transit compartments (15), were evaluated to capture the time course of expression levels (Fig. 3B). The second-order system defined in Equations F and G was found superior to simpler transfer functions. In the model, signal transduction responses were represented as deviations from baseline values that maintain steady-state conditions (5, 31), and the dynamic response was driven by the fraction of bound CD20 receptors (Fig. 3B). Whereas the model tracks measured Fas levels directly, rituximab also upregulated 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 (Supplementary Eqs. S11 and 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 Fig. 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 $8.31 \times 10^{-8}$ mol/L. 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 PD markers selected and then combine models under several hypothesized mechanisms of drug

![Figure 3](https://example.com/image3.png)

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

![Figure 4](https://example.com/image4.png)

**Figure 4.** Ramos cell growth inhibition as function of rituximab concentration. Symbols: data from Vega (34); line: fitted curve using submodel for growth inhibition (Hill function; Eq. D). Supplementary Table S4 provides estimated model parameters.
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 versus 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 pharmacologic interactions. One investigated rituximab with fenretinide (8) and the other investigated rituximab with rhApo2L (9). From Gopal and colleagues (8), a tumor doubling time of 142 hours 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 Daniel and colleagues (9), which investigated rituximab and rhApo2L in CB17 severe-combined immunodeficient (SCID) mice, was then used to test the model developed for efficacy of single-agent rituximab. A tumor doubling time of 63 hours was estimated. By substituting the doubling time appropriate for the specific tumor into the growth model, the rituximab efficacy term obtained in nude mice (8) provided an excellent prediction of single-agent rituximab in SCID mice (Fig. 5B). Data (9) also provided results for single-agent rhApo2L. Simultaneous fitting of tumor progression data for control and rhApo2L-treated animals showed that the developed model captured the data well (Fig. 5B). By this approach, appropriate models were developed for all 3 drugs as single agents.

**Model prediction of combination chemotherapy**

The rituximab/fenretinide dataset (8) is relatively rich, permitting estimation of single-agent effects (Fig. 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 2 tumor response scenarios for fenretinide and rituximab administered according to the regimen of Gopal and colleagues (8): one hypothesized that drug effects were additive, whereas the other hypothesized synergy arising from rituximab-mediated reduction in Bcl-xL levels (Fig. 3), thereby amplifying efficacy. The original study suggested the combination showed supra-additive effects (8). Here, both models predicted the observed data relatively well (Fig. 5A), and the regimen of the reported study does not support a conclusion with regard to nature of drug interaction.

The data for single-agent rituximab from Daniel and colleagues (9) are sparse, but its efficacy was nonetheless well predicted by the model (Fig. 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 versus synergistic interaction, with synergy postulated to arise through rituximab modulation of Fas expression levels via the temporal relationship of Fig. 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 PD marker for the synergy of interaction.

**Figure 5.** A and 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 Gopal and colleagues (8). B, 4 mg/kg rituximab and 60 mg/kg rhApo2L, from Daniel and colleagues (9). 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) versus synergistic (solid) drug interactions. Supplementary Table S4 provides final estimated parameters for tumor growth profiles.
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 (Fig. 6), consistent with \textit{in vitro} knockout studies in lung carcinoma cell lines (36). However, the model shows clearly that rhApo2L interaction with both DR4 and DR5 is required to capture the full magnitude of experimentally observed efficacy (9).

Discussion

A multiscale systems pharmacologic 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 induces apoptosis through interaction with death receptors. The final model links plasma pharmacokinetics of rituximab, fenretinide, and rhApo2L with PD 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 shows 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 using 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 (Fig. S1) may appear complex, but derivation of individual components was driven by well-understood physiologic and pharmacologic processes. The model captures the transient effects of signal transduction as driven by receptor occupancy (Fig. 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 physiologic fidelity, and the impact of their inclusion upon the capability of the final model to explain experimentally observed phenomena underscores their potential pharmacologic role. For example, the model suggests that with rhApo2L treatment, DR5 contributes to inhibition of tumor progression to a greater extent than DR4 (Fig. 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 \textit{in vitro and in vivo} experimental results. The observed recapitulation of a complex pharmacologic system results not from \textit{post hoc} fitting of experimental data, but rather from \textit{a priori}, quantitative integration of fundamental cellular and pharmacologic 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 (Fig. 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 with regard to the nature of the pharmacologic interactions with combination therapy. It suggested that additivity versus synergy could not be discriminated from existing experimental data for rituximab/fenretinide (Fig. 5A), whereas both model predictions and experimental data for rituximab/rhApo2L suggested clearly that rituximab modulation of Fas expression (Fig. 3) mediated a synergistic interaction (Fig. 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 (Fig. 2) shows that rhApo2L is eliminated far more rapidly than rituximab. As a result, rhApo2L concentrations fall rapidly to explain experimentally observed phenomena underscores their potential pharmacologic role. For example, the model suggests that with rhApo2L treatment, DR5 contributes to inhibition of tumor progression to a greater extent than DR4 (Fig. 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 \textit{in vitro and in vivo} experimental results. The observed recapitulation of a complex pharmacologic system results not from \textit{post hoc} fitting of experimental data, but rather from \textit{a priori}, quantitative integration of fundamental cellular and pharmacologic 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 (Fig. 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 with regard to the nature of the pharmacologic interactions with combination therapy. It suggested that additivity versus synergy could not be discriminated from existing experimental data for rituximab/fenretinide (Fig. 5A), whereas both model predictions and experimental data for rituximab/rhApo2L suggested clearly that rituximab modulation of Fas expression (Fig. 3) mediated a synergistic interaction (Fig. 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 (Fig. 2) shows that rhApo2L is eliminated far more rapidly than rituximab. As a result, rhApo2L concentrations fall rapidly below the $K_i$ 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 upregulation by rituximab occurs over the first 6 hours after drug administration (Fig. 3).
As shown 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 intertumor 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 PD 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 versus pharmacologic components (37); 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 pharmacokinetics and pharmacodynamics 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 multiscale 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: J.M. Harrold and D.E. Mager.
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.M. Harrold, R.M. Straubinger, and D.E. Mager.
Writing, review, and/or revision of the manuscript: J.M. Harrold, R.M. Straubinger, and D.E. Mager.
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.M. Harrold.
Study supervision: D.E. Mager.

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