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RESEARCH PAPER

Interactions of Everolimus and Sorafenib in Whole Blood Lymphocyte Proliferation

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ABSTRACT

Purpose Everolimus is an immunosuppressant that blocks growth factor-mediated proliferation of hematopoietic cells by targeting the mammalian target of rapamycin (mTOR). Sorafenib is a multikinase inhibitor that inhibits cell proliferation by arresting cells in the G0-G1 phase of the cell cycle. These agents are under investigation as combination therapy for various cancers. Because the two drugs individually inhibit lymphocyte proliferation, this study examined the effects of everolimus and sorafenib on lymphocyte proliferation in order to anticipate possible immunosuppression.

Methods Inhibition of lymphocyte proliferation was evaluated *ex vivo* over a range of concentrations of these drugs, alone and in combination. Data analysis, using a population approach to characterize interactions, employed the Ariens noncompetitive interaction model, which was modified to accommodate interactions of the two drugs.

Results Everolimus alone caused partial inhibition of lymphocyte proliferation, with a mean IC_{50} of 4.5 nM for females and 10.5 nM in males. Sorafenib alone caused complete inhibition, with a mean IC_{50} of 11.4 μM and no difference between genders.

Conclusion The population estimate for the interaction term was greater than 1, suggesting that the two drugs exert slight antagonism in terms of inhibition of lymphocyte proliferation.

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INTRODUCTION

Everolimus (Certican®, RAD-001) is an orally active rapamycin derivative that is clinically approved for immunosuppression following organ transplantation (1). Everolimus binds to the FKBP-12 protein to form a complex that binds to the mammalian target of rapamycin (mTOR), and exerts its immunosuppressive effect by blocking interleukin (IL)-2- and IL-15 -mediated proliferation of T and B lymphocytes (2). Everolimus is under investigation as an anti-cancer agent because it inhibits growth-promoting signaling downstream of mTOR. It is approved by the FDA for renal cell carcinoma (3). Sorafenib (Nexavar®) is a multikinase inhibitor that exerts its effects by inhibiting signaling pathways that are involved in angiogenesis and proliferation, including the Raf/mitogen-activated protein kinase (MAPK), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) pathways. Sorafenib inhibits T-cell proliferation via a MAPK-independent mechanism (4), and inhibits phytohemagglutinin- (PHA) induced proliferation of T-cells at therapeutic concentrations (5). Multiple trials evaluating these agents in combination are underway for a variety of cancers, such as melanoma, renal, pancreatic, hepatic, thyroid, and other solid tumors (*according to the clinicaltrials.gov;* accessed 01/25/2012) based on their potential for complimentary mechanisms of action.

The whole blood lymphocyte proliferation (WBLP) assay developed by Piekoszewski *et al* (6) employs a small quantity of blood and has been extensively used for both single drug effects and to characterize drug interactions (8–13). The assay conditions and reagents have been optimized for various species (14). The WBLP method performed *in vitro*

allows use of the natural milieu of blood and thus is reflective of immune suppression *in vivo*. The effects of everolimus and sorafenib on T-cell proliferation were evaluated here using the WBLP assay to lend insight into their potential to cause immunosuppression as an adverse effect.

MATERIALS AND METHODS

Subjects

Healthy female (n=6) and male (n=6) volunteers of ages 25–50 year were enlisted for this study. The volunteers were not taking any medications chronically. Blood was collected by professional phlebotomists on the morning of experiments. The study was approved by the Institutional Review Board of the University at Buffalo, State University of New York.

Reagents

Everolimus and sorafenib p-toluenesulfonate salt were purchased from LC Laboratories (Woburn, MA). PHA was purchased from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 (Invitrogen; Grand Island, NY) was used for serial dilutions of drugs.

Whole Blood Lymphocyte Proliferation

Blood was collected in evacuated heparinized glass tubes and diluted 1:20 (v/v) with RPMI 1640 medium supplemented with 2 mML-glutamine, 20 mM HEPES, 100 U/mL penicillin, and 0.25 mM 2-mercaptoethanol and 165 μL was dispensed into each well of 96 well plates. The concentration of PHA required to induce cell proliferation was optimized beforehand, and was added to cell-containing wells to a final concentration of 3 μg/mL. Drug combinations were tested in triplicate with a total volume of 200 μL in each well. The cultures were incubated at 37°C for 72 h and then pulsed with 1 μCi of [^{3}H] thymidine (New England Nuclear, Boston, MA) per well for 20 h. The cells were then harvested onto microplates, washed with 3% hydrogen peroxide, dried, and counted in liquid scintillation fluid (Microscint-20, Packard) using a Top Count microplate scintillation counter (Perkin Elmer, Waltham, MA).

Drug Interactions

Drug interaction experiments were carried out with various combinations of the drugs evaluated in triplicate, along with 4 control wells (no drug) and four background wells (no drug or mitogen). Single drug effect profiles were characterized using 8 different concentrations. Drug combination experiments included at least 24 different combinations of the two drugs with concentrations that spanned the entire range tested for inhibition of lymphocyte proliferation using single agents.

Data Analysis

The PHA-stimulated incorporation of ^{3}H-thymidine in the presence of drug, expressed as counts per minute (CPM_D) and normalized based on the background radioactivity (CPM_N; no drug and no mitogen) and the radioactivity from drug-free control samples (CPM_M), was used to obtain the % maximum inhibition drug response (%DR):

$$\%DR = 100 \bullet \left[\frac{CPM_D - CPM_N}{CPM_M - CPM_N}\right] \quad (1)$$

The concentration-effect profiles of the individual drugs were modeled using the sigmoidal Hill function:

$$R = R_0 \bullet \left[1 - \frac{I_{\max} \bullet C^{\gamma}}{IC_{50}^{\gamma} + C^{\gamma}}\right] \quad (2)$$

where R_0 is response in the absence of drug, I_{max} is the maximum possible inhibition due to the drug, C is the drug concentration, IC_{50} is the drug concentration causing 50% of the maximum effect, and γ is the Hill coefficient. All model parameters were fitted to the %DR for each of the three individual wells for each concentration.

Data that captured the maximum drug effect for sorafenib was sparse for several individuals, and therefore a population approach was used. The effect of everolimus alone was modeled using equation (2) with variability in IC_{50}, I_{max}, γ, and R_0. An exponential error model was used for the parameters and the residual error model used was proportional. The effect of gender upon the IC_{50} of each drug was also tested with gender being a categorical variable.

The effects of the two drugs combined on lymphocyte proliferation was modeled using a modified non-competitive interaction model (7, 8). The published method was modified to estimate R_0. The value of $\Psi < 1$ signifies synergism, $\Psi = 1$ indicates additivity, and $\Psi > 1$ denotes antagonism. In the equation R_0 is percent of control in the absence of the drug.

$$R = R_0 \bullet \left[1 - \frac{\begin{array}{c}\left(\frac{I_{\max,E} \bullet C_E^{\gamma_E}}{(\psi \bullet IC_{50,E})^{\gamma_E}}\right) + \left(\frac{I_{\max,S} \bullet C_S^{\gamma_S}}{(\psi \bullet IC_{50,S})^{\gamma_S}}\right) \\ +(I_{\max,E} + I_{\max,S} - I_{\max,E} \bullet I_{\max,S}) \\ \times \left(\frac{C_E^{\gamma_E}}{(\psi \bullet IC_{50,E})^{\gamma_E}}\right) \bullet \left(\frac{C_S^{\gamma_S}}{(\psi \bullet IC_{50,S})^{\gamma_S}}\right)\end{array}}{\begin{array}{c}\left(\frac{C_E^{\gamma_E}}{(\psi \bullet IC_{50,E})^{\gamma_E}}\right) + \left(\frac{C_S^{\gamma_S}}{(\psi \bullet IC_{50,S})^{\gamma_S}}\right) \\ +\left(\frac{C_E^{\gamma_E}}{(\psi \bullet IC_{50,E})^{\gamma_E}}\right) \bullet \left(\frac{C_S^{\gamma_S}}{(\psi \bullet IC_{50,S})^{\gamma_S}}\right) + 1\end{array}}\right] \quad (3)$$

where R is the $\%DR$, R_0 is the baseline $\%DR$, subscripts $_E$ and $_S$ reflect everolimus and sorafenib parameters. An exponential error model was used to describe variability in the ψ term and

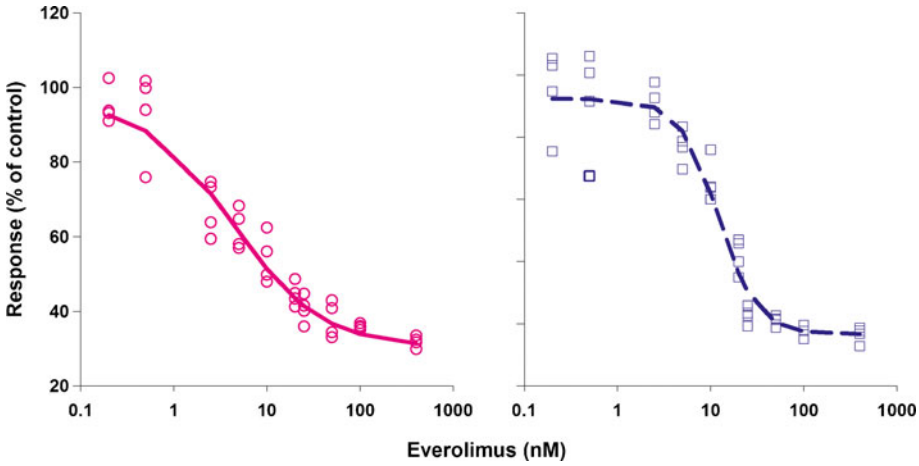

Fig. 1 Inhibition of lymphocyte proliferation by everolimus for a typical female (*left*) and male subject (*right*). Symbols represent experimental data, lines depict fit of the model using Eq. 2.

in the baseline. The residual error model used was proportional.

Population analysis was performed using NONMEM VI (level 1.1, Icon Development Solutions, Ellicott City, MD) (15), and the Intel Fortran Compiler 9.0. NONMEM runs were executed using Wings for NONMEM (Version 6.11, http://wfn.sourceforge.net). The first-order conditional estimation (FOCE) method with the interaction estimation option in NONMEM was used for all analyses.

RESULTS

Single Drug Effects

Data for inhibition of cell proliferation by everolimus are shown for representative male and female subjects in Figure 1, and Fig. 2 shows representative data for sorafenib. The concentration-response relationship for both drugs, represented by the smooth curve through the data, was described using eq. (2). The parameter estimates, along with their precisions, are listed in Table I. The population mean for maximum inhibition (I_{max}) by everolimus was estimated to be 0.7, with an inter-individual variability of 9%. The IC_{50} for everolimus differed for the two genders, with a population mean of 10.5 nM in males and 4.5 nM in females ($p<0.05$). The parameter γ, indicating steepness of the concentration-effect curve, was 1.2 for everolimus. The maximum inhibition (I_{max}) by sorafenib was fixed to 1. The mean IC_{50} for sorafenib was estimated to be 12.4 μM with 15% inter-individual variability, and there was no difference between the genders. The parameter γ was 9.72 for sorafenib.

For everolimus, data from twelve subjects (n=6 male, n=6 female) were used. Diagnostic plots for the analysis of everolimus data are shown in Fig. 3. Figure 3a and b are plots of observed *vs.* population predicted- and individual predicted responses. The predictions are reasonable, given that the values hover near the line of unity. Figure 3c plots the weighted residuals, which are uniformly distributed across the predicted concentrations.

For sorafenib, data from 8 individuals were available (n=4 male, n=4 female). The diagnostic plots for the analysis of sorafenib data are shown in Fig. 4. Figure 4a and b are observed *vs.* population predicted and

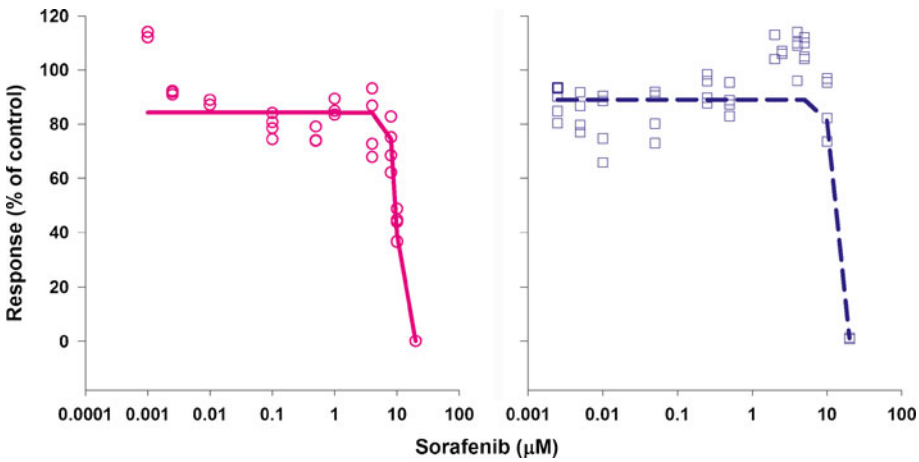

Fig. 2 Inhibition of lymphocyte proliferation by sorafenib for a typical female (*left*) and male subject (*right*). Symbols represent experimental data, lines depict fit of the model using Eq. 2.

Table I Population Parameter Estimates for Individual Drug Effects

		Estimate	%RSE	Inter-individual variability (%)	%RSE
Everolimus	$IC_{50\ (female)}$ (nM)	4.49	15.1	37.0	42.5
	$IC_{50\ (male)}$ (nM)	10.5	19.8		
	I_{max}	0.73	2.9	9.1	39.0
	γ	1.2	12.4	38.9	67.8
	R_0 (%)	94.5	2.5	6.9	77.0
	Residual error	15.8	27.5	–	–
Sorafenib	IC_{50} (μM)	12.4	5.0	15.0	37
	I_{max}	1	(Fixed)	–	–
	γ	9.72	8.7	–	–
	R_0 (%)	90.3	2.4	7.0	78.2
	Residual Error (%)	27.9	21.4	–	–

individual predicted responses. The predictions are reasonable, given that the values hover near the line of unity. Figure 4c plots the weighted residuals, which are uniformly distributed across the predicted concentrations. The population model reasonably described observations for the effect of everolimus and sorafenib as single agents on lymphocyte proliferation.

Combination Drug Effects

The effect of combined everolimus and sorafenib on lymphocyte proliferation was assessed using 14 combinations of the two drugs. Three-dimensional surface plots were used to display the effects of the two drug combination based on individually estimated parameters as analyzed using equation 3. The fitted surface describes well the observed data for the drug combinations, as shown in representative plots (Fig. 5). As listed in Table II, the population mean for the interaction term Ψ was estimated to be 1.15, with 7.4% variability among individuals. A residual variability of 21.8% was estimated with a good precision for the combination. There was no significant difference between genders for the intensity of interaction (Ψ) between the two drugs. Diagnostic plots for the population analysis of data for the drugs in combination are shown in Fig. 6. The plots of observed *vs.* population- (Fig. 6a) and individual- (Fig. 6b) predicted responses show a uniform trend around the line of unity, except for several high-response observations. Figure 6c plots the weighted residuals, which also show a uniform spread over the range of predicted concentrations, except for a slight bias at the low responses. Overall the population model reasonably describes the observations from the studies testing combinations of everolimus and sorafenib on lymphocyte proliferation. All parameters associated with single- (Table I) and combination- (Table II) drug effects were estimated with high precision as indicated by low % relative standard errors (RSE). The population estimate of the interaction term Ψ, which was greater than 1, signifies a slight antagonistic interaction between the two drugs on lymphocyte proliferation. These results indicate that the combination of everolimus and sorafenib should inhibit lymphocyte proliferation to an extent no more than would the individual drugs alone.

DISCUSSION

Everolimus binds to the FKBP12 protein complex and inhibits mTOR activation of cellular growth and proliferation signaling pathways, resulting in cell-cyle arrest in the late G1-S stage (16). It is FDA-approved as an immunosuppressant and lymphocyte suppression is observed in humans after single doses of 0.75–

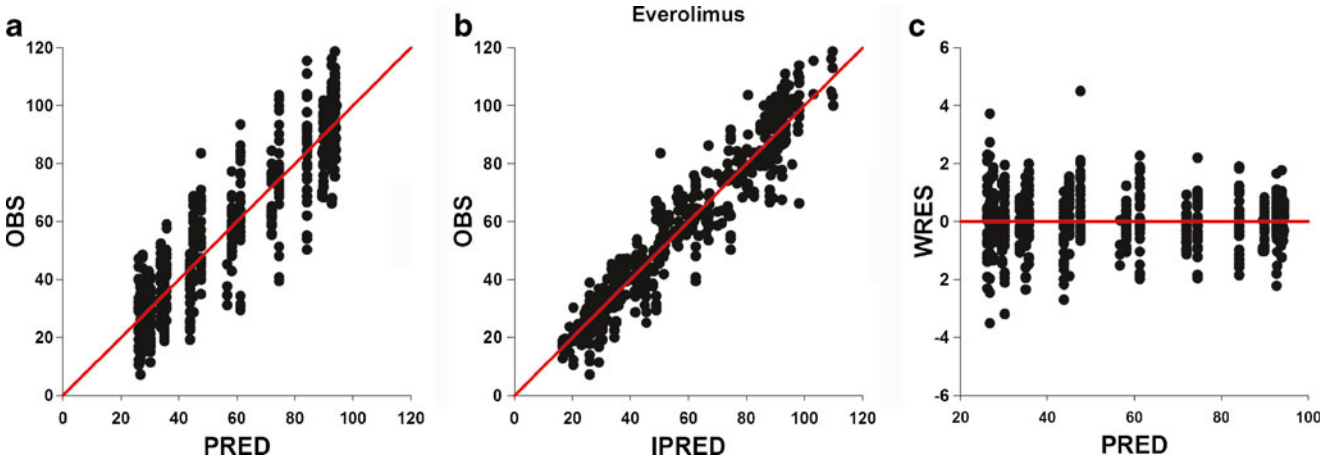

Fig. 3 Goodness-of-fit plots for the modeling of inhibition of lymphocyte proliferation by everolimus. (**a**) Population predicted vs. observed responses; (**b**) individual predicted vs. observed responses; (**c**) weighted residuals vs. predicted responses.

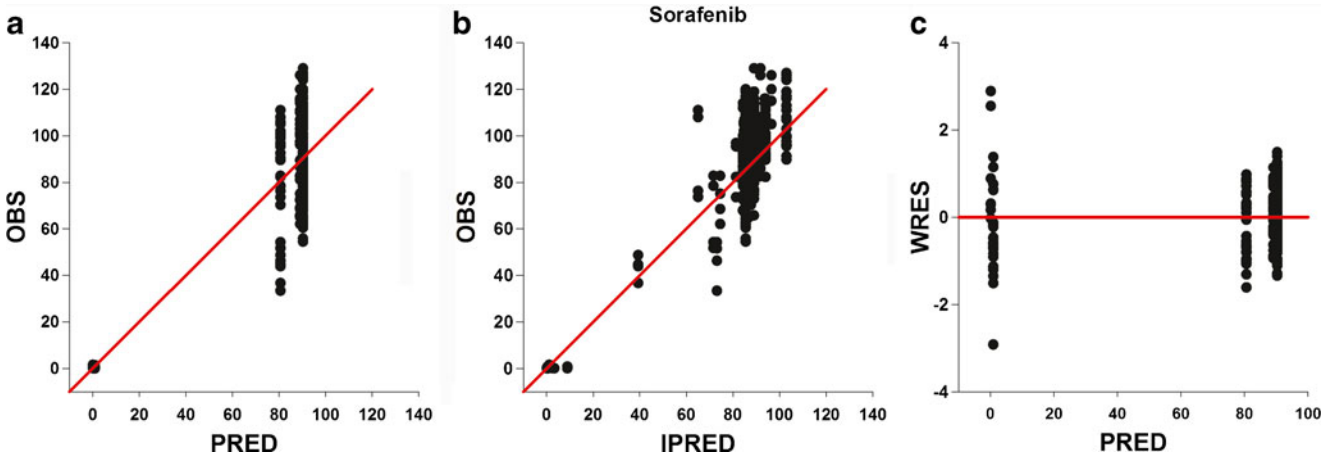

Fig. 4 Goodness-of-fit plots for modeling inhibition of lymphocyte proliferation by sorafenib. (**a**) Observed vs. population predicted responses; (**b**) observed vs. individual predicted responses; (**c**) weighted residuals vs. predicted responses.

17.5 mg (2). The capability of everolimus to inhibit mTOR, which is involved in the PI3k-Akt signaling pathway, has been exploited in oncology to target cancer cell survival and proliferation mechanisms.

Sorafenib is a multikinase inhibitor that inhibits MAPK, and it arrests cells in the G0-G1 stage of the cell cycle. Inhibition of the MAPK pathway is known to constrain activation of T cells (17). Sorafenib is also postulated to induce apoptosis in lymphocytes by a MAPK- independent mechanism (5,6). Sorafenib causes suppression of T-lymphocyte proliferation at cancer therapeutic doses of 400 mg twice daily (6).

Everolimus and sorafenib in combination are under investigation for the treatment of a variety of solid tumors. Given their mechanistically-independent potential for effects upon lymphocyte function, these drugs together could have immunosuppressive effects at clinically-used doses, thus increasing the possibility of infections. Toxicity such as lymphopenia of grade 3 or higher has been noted in 5% of patients undergoing combination therapy using everolimus (or rapamycin) and sorafenib, which has proved fatal in a rare case (18,19). Therefore an assessment of the drug combination on lymphocyte proliferation could yield clinically-relevant insight into immunosuppression. The effect of gender on drugs effect on lymphocyte proliferation was also assessed in this study. Prior studies have shown gender differences in the potency of rapamycin (sirolimus), with the drug being more potent in males (9,14). In the data reported here, everolimus was more potent in females. The gender difference in potency has been attributed previously to differential expression of transporters such as P-gp that are responsible for drug efflux from cells, and which are known to play a role in the transport of everolimus (20,21). However there are no known studies elucidating the exact mechanism of the gender differences.

Sorafenib has low aqueous solubility, which hinders *in vitro* studies designed to investigate the effects of high, therapeutically-relevant drug concentrations. The effect of sorafenib on lymphocyte proliferation has not been assessed extensively, but one study suggested that it causes complete and irreversible inhibition of lymphocyte proliferation (6). Sorafenib at clinically-approved doses of 400 mg administered twice daily was reported to result in peak plasma concentrations of 15 μM (22). Therefore, we included

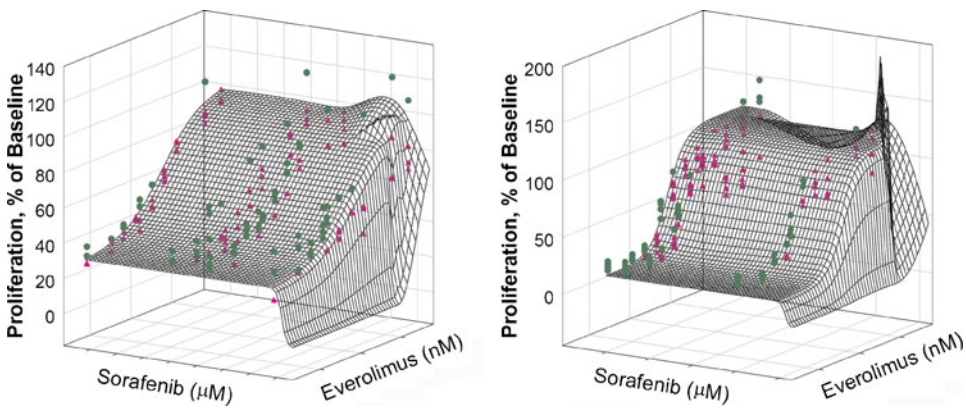

Fig. 5 Interaction surface showing model prediction fit (Eq. 3) indicating antagonistic interaction ($\Psi > 1$) of everolimus and sorafenib for inhibition of lymphocyte interaction for a typical female (*left*) and male (*right*) subject. Closed triangles (*pink*) identify data below the surface, and open circles (*green*) show data above the surface.

Table II Population Parameter Estimates for Combination Drug Effects

	Estimate	%RSE	Inter-individual variability (%)	%RSE
R_0 (%)	106	4.0	9.8	25.1
ψ	1.15	7.4	17.1	68.8
Residual error (%)	21.8	16.7	–	–

concentrations in the range of 15 μM in these studies. We observed marked inhibition of lymphocyte proliferation in the range of 10 to 20 μM, and the concentration- response curve was very steep. Therefore it was not possible to include a wider range of concentrations in the region in which the greatest concentration-dependent differences in proliferation were observed. There was also large variability in the data in the range of the steep concentration-response, to which the poor aqueous solubility of sorafenib at >20 μM may contribute. The relatively sparse reliable data for high concentrations of sorafenib resulted in the bias observed in fittings of the lymphocyte proliferation effects of sorafenib .

Clinically approved doses of everolimus in oncology are 5–10 mg per day, which are significantly higher than those employed in organ transplantation. Everolimus administered at 5 mg/day in patients was reported to produce a C_{max} of 33.4 nM (23). Therefore we included concentrations of 0.5–400 nM in this study to assess the inhibition of everolimus over a wide range of concentrations. A concentration-dependent effect on lymphocyte proliferation was observed over this range of everolimus concentrations.

We performed simulations with the population mean estimates obtained in our study to predict the degree of lymphocyte proliferation resulting from these drugs at their maximal plasma concentrations as single agents or in combination. Simulations with the model suggest that the C_{max} concentration of 33.4 nM everolimus (clinical C_{max} at dose 5 mg/day) would reduce lymphocyte proliferation to 33% of maximal in females and 41.5% in males. At a dose of 10 mg/day, everolimus was reported to produce a C_{max} of 64 nM (23), a concentration that would reduce lymphocyte proliferation to 30% of control in females and 34% in males, based on simulations with the parameters obtained in this study. Sorafenib alone at 400 mg twice daily was reported to produce a C_{max} of 15 μM in humans (22), which simulations suggest would reduce the lymphocyte proliferation to 10% of control. Therefore, if the two drugs were combined at their C_{max}, the lymphocyte proliferation response would be reduced to approximately 10% of maximal. The simulations thus suggest that there exists considerable potential for appreciable immunosuppression in patients if efficacy studies require dosing of both agents at the higher range of clinically-employed doses.

The drug combination experiments yielded rich data in the concentration ranges necessary to evaluate the nature and intensity of drug-drug interactions. The value obtained for the interaction parameter Ψ was greater than 1, which indicates a slight antagonistic interaction. This is a key positive finding in terms of combination therapy, in that everolimus and sorafenib together would not be expected to mediate supra-additive immunosuppression. Diagnostic plots supporting the analysis of the combinations show good model fitting and unbiased population- and individual estimates. Both drugs exert their effects by inhibiting the cell cycle at consecutive phases. The mild *in vitro* antagonism observed may arise from sorafenib arrest of cells in the G0–G1 phase thus rendering fewer cells to transition into the S phase in which everolimus exerts its inhibitory effects. Thus fewer cells would be susceptible to the full inhibitory potential of everolimus. In ongoing clinical studies, the two drugs are being used concurrently, and therefore our study design did not consider sequential treatments.

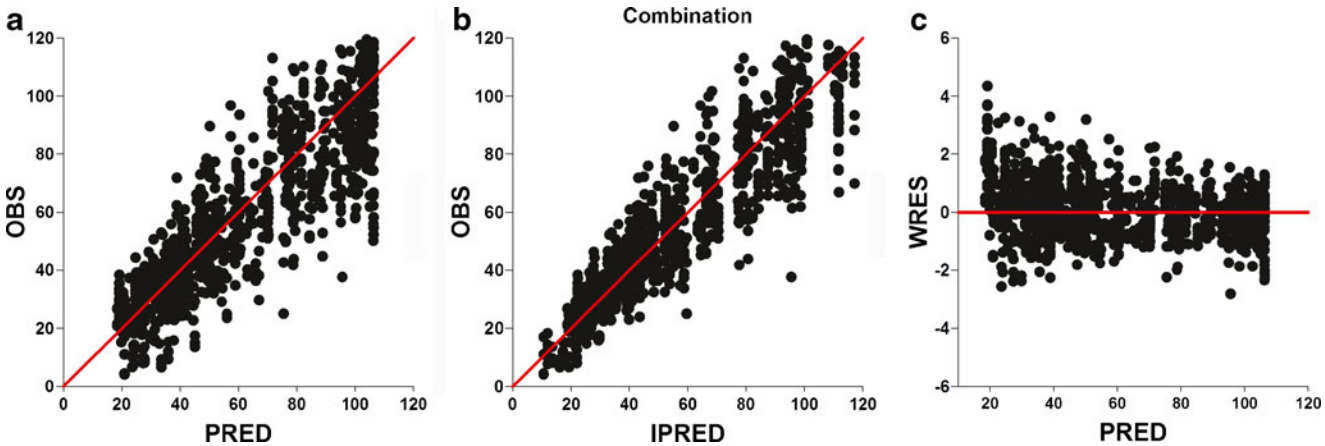

Fig. 6 Goodness-of-fit plots for modeling inhibition of lymphocyte proliferation by combinations of everolimus and sorafenib. (**a**) Observed vs. population predicted responses; (**b**) observed vs. individual predicted responses; (**c**) weighted residuals vs. predicted responses.

Population analysis was employed in the studies reported here because it allows estimation of mean values for parameters in the population studied, as well as their associated variability. The effects of the two drugs on lymphocyte proliferation, alone and in combination, were obtained using blood obtained from a small number of individual donors, thus raising the possibility that inter-individual variability could influence the results. To our knowledge, a population analysis approach has not been used previously to analyze these types of drug interactions. This approach has the advantage that it permits characterization of the entire dataset. It allows pooling of information from the various individuals, thus compensating for sub-optimal data in the overall data set from each individual, for example, where drug solubility may have limited acquisition of data for the highest concentrations of sorafenib. A further benefit of the population approach is illustrated by the fact that it enabled identification of covariates such as gender in the response to everolimus.

In conclusion, both everolimus and sorafenib inhibit lymphocyte proliferation in a concentration- dependent manner. Everolimus was significantly more potent in females. Quantitative analysis of drug interaction suggested modest antagonism at clinically relevant concentrations. The finding of less than additive interaction of the drugs is a positive finding, in that a supra-additive, synergistic interaction would adversely impact clinical deployment of this drug combination in oncology. Nonetheless, the reduction of lymphocyte proliferation, which was close to additive, suggests that vigilance is appropriate in oncology applications because both drugs would be expected to contribute to the overall immunosuppression experienced by patients undergoing therapy, if dosed at the higher range of clinically-used concentrations.

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