Research Article

Development of a Preclinical PK/PD Model to Assess Antitumor Response of a Sequential Aflibercept and Doxorubicin-Dosing Strategy in Acute Myeloid Leukemia

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Abstract. Timing of the anti-angiogenic agent with respect to the chemotherapeutic agent may be crucial in determining the success of combination therapy in cancer. We investigated the effects of sequential therapy with the potent VEGF inhibitor, aflibercept, and doxorubicin (DOX) in preclinical acute myeloid leukemia (AML) models. Mice were engrafted with human HL-60 and HEL-luciferase leukemia cells *via* S.C. and/or I.V. injection and treated with two to three doses of aflibercept (5–25 mg/kg) up to 3–7 days prior to doxorubicin (30 mg/kg) administration. Leukemia growth was determined by local tumor measurements (days 0–16) and systemic bioluminescent imaging (days 0–28) in animals receiving DOX (3 mg/kg) with or without aflibercept. A PK/PD model was developed to characterize how prior administration of aflibercept altered intratumoral DOX uptake. DOX concentration–time profiles were described using a four-compartment PK model with linear elimination. We determined that intratumoral DOX concentrations were 6-fold higher in the aflibercept plus DOX treatment group *versus* DOX alone in association with increased drug uptake rates (from 0.125 to 0.471 ml/h/kg) into tumor without affecting drug efflux. PD modeling demonstrated that the observed growth retardation was mainly due to the combination of DOX plus TRAP group; 0.00794 *vs.* 0.0043 h^{–1}. This PK/PD modeling approach in leukemia enabled us to predict the effects of dosing frequency and sequence for the combination of anti-VEGF and cytotoxic agents on AML growth in both xenograft and marrow, and may be useful in the design of future rational combinatorial dosing regimens in hematological malignancies.

KEY WORDS: acute myeloid leukemia; aflibercept; doxorubicin; pharmacokinetics/pharmacodynamics; VEGF TRAP.

INTRODUCTION

Extensive research has suggested a clinical advantage of combining anti-angiogenic agents, specifically vascular endothelial growth (VEGF) inhibitors, with chemotherapeutic agents to enhance antitumor efficacy in many solid cancers (1,2). The beneficial therapeutic effects of combination VEGF inhibition and chemotherapy have been attributed, in part, to “vascular normalization,” anti-VEGF/VEGFR-mediated decreases in tumor vessel permeability and interstitial fluid pressure leading to improved intratumoral perfusion and systemic chemotherapy delivery (3,4). Two recent studies have called into question the normalization effects of anti-VEGF therapy. Despite obvious differences in these reports (one involving lung cancer patients and the other using a preclinical breast xenograft model), both

sets of investigators demonstrated decreased tumor perfusion and no evidence of improved chemotherapy drug delivery following anti-VEGF antibody therapy (5,6). These results highlight the need for further clinical and preclinical investigations into the potential advantages of such a combinatorial approach, particularly in different tumor types. To date, there have been few studies evaluating the combination of anti-angiogenic and chemotherapy drugs in the treatment of hematological malignancies, and no detailed pharmacokinetic/pharmacodynamic (PK/PD) models of this therapeutic approach in blood cancers.

Acute myeloid leukemia (AML) is an aggressive hematopoietic malignancy characterized by the unchecked proliferation of undifferentiated blasts. Standard chemotherapy for AML has not changed since the 1980s and results in overall cure in only 20–30% of patients, highlighting the need for novel therapeutic approaches. Bone marrow biopsies from AML patients have shown the close proximity of leukemia cells to vascular endothelium and demonstrated correlations between increased marrow blood vessel density and leukemic burden (7). Given the high levels of the pro-angiogenic factor vascular endothelial growth factor in AML patients, multiple studies have focused on VEGF inhibition as a means of inhibiting marrow angiogenesis in leukemia. However,

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despite promising preclinical results in AML models, clinical trials of VEGF inhibitors in relapsed/refractory AML patients have largely been disappointing with only transient anti-leukemic effects (8).

The work described in this paper builds on our prior preclinical results demonstrating that the combination of the potent VEGF inhibitor, aflibercept, (also known as VEGF Trap) with the anthracycline drug, doxorubicin, increased anti-leukemic activity *in vivo* as compared with single-agent therapy in human AML xenograft models (9). Aflibercept is a novel decoy receptor bearing VEGF receptor (VEGFR-1/2) moieties with a reported higher binding affinity for VEGF-A than bevacizumab (10). Based on the drug's inherent chemical properties, it is presumed that aflibercept will bind to VEGF-A as well as other VEGF family ligands (VEGF-C, placental growth factor, neuropilin-1/2). The subsequent ligand-receptor complex will undergo receptor-mediated endocytosis followed by target-mediated drug disposition. Recently, a mechanistic model for the target-mediated drug disposition of aflibercept has been published (11). Currently, aflibercept was recently approved for the treatment of metastatic colorectal cancer in combination with FOLFIRI, and is currently undergoing clinical trials for various other cancer types (12–15). In the clinic, it appears that concentrations, which would saturate the disposition of aflibercept have been exceeded even at the lowest dose, making its elimination from the serum apparently linear (15,16).

Doxorubicin is a widely used anthracycline agent for the treatment of hematological malignancies which is metabolized in the liver to doxorubicinol (17). This agent was selected for further preclinical evaluation in combination with aflibercept in our AML models due to its close relation to daunorubicin, a standard agent used in upfront AML therapy. It has also been reported that the broad spectrum resistance of AML cells to multiple anti-cancer agents can be predicted by doxorubicin due to its multifactorial mode of action (18). Moreover, the auto-fluorescent properties of doxorubicin allow it to be semi-quantitatively localized with respect to tumor vasculature in tumor tissues, a method previously used to show the overall poor penetration of systemic drug into solid tumor xenografts (19). The upfront AML drug, cytarabine, was not selected for further combinatorial studies as our prior work demonstrated that concomitant aflibercept and cytarabine treatment did not improve anti-leukemic activity, probably due to the relative inefficacy of cytarabine monotherapy to reduce AML growth in our models and/or the very short *in vivo* half-life of this drug (9).

By incorporating information from several dosing regimens in preclinical AML models, here we used a PK/PD modeling approach to determine the most effective *in vivo* combination of aflibercept and doxorubicin therapy and explore the underlying mechanisms responsible for the observed anti-leukemic effects of combination therapy. Because effective drug levels and anti-vascular effects of this combination may differ based on differences in tumor propagation in hosts with solid tumor *versus* hematological cancers, we examined the PK/PD effects of treatment in both localized (subcutaneous) and systemic (marrow) human AML disease sites in murine xenotransplantation models (20).

MATERIALS AND METHODS

Chemicals

Aflibercept (VEGF Trap) was provided through a collaborative agreement with National Cancer Institute-Cancer Therapy Evaluation Program (CTEP) and Sanofi-Aventis/Regeneron Pharmaceuticals. Doxorubicin, doxorubicinol, and the internal standard, daunorubicin, were purchased from Sigma (St. Louis, MO).

Cell Lines and Culture

Human AML (HEL, HL60) cells were purchased from American Tissue Culture Collection (Manassas, VA) and were grown in humidified incubators (37°C, 21% O₂, 5% CO₂) in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 4.5 g/L glucose, 1.5 g sodium bicarbonate, 1 U/ml penicillin/streptomycin, 10 mM HEPES, and 1 nM sodium pyruvate. Cell lines underwent stable transfection with pGL4 luciferase reporter vector (Promega, Madison, WI) followed by clonal selection as previously described (9).

Animal Models

All animal experiments were carried out under an institute-approved Institutional Animal Care and Use Committee protocol. For cell line xenograft experiments, 8-week-old (20–25 g) (Department of Laboratory Research, Roswell Park Cancer Institute (RPCI) (Buffalo, NY)) female severe combined immunodeficient (SCID) mice were sublethally irradiated with 2.5 cGy and inoculated with 10×10^6 AML cells s.c. under the skin as described previously (9). The two largest perpendicular axes of s.c. tumors (*l*, length; *w*, width) were measured three times weekly. Tumor volume (TV, mm³) was calculated as $TV = 4/3 \pi r^3$, where $r = (l + w)/4$. When tumor volumes approached a minimum of 100 mm³, the mice were divided into groups of four to five mice for each treatment regimen. In a separate experiment, systemic bioluminescent human leukemia xenograft models were established by stable transfection of Pgp-expressing HL60/VCR and HEL (human AML) cells with pGL4 luciferase reporter vector (Promega) followed by tail vein injection of 10×10^6 cells into irradiated SCID mice. Tumor distribution and burden was evaluated weekly by serial whole body noninvasive bioluminescent imaging (Xenogen IVIS 50 System, Calipers Life Science) following i.p. D-luciferin injection (75 mg/kg). Dorsal and ventral images were acquired from each animal at each time point. Data were expressed as photon emission=photons per second per cubic centimeter per steradian and were quantified using Living Imaging Software (Calipers Life Sciences). Mice were monitored three times per week for moribund signs (*i.e.*, weight loss >20%, hind-limb paralysis, respiratory distress) prompting sacrifice.

PK/PD Studies

Various dosing regimens of aflibercept (TRAP) and doxorubicin (DOX) treatment were employed to gain a better understanding of the synergistic interactions of the

two drugs. Schematic representation of the different regimens can be found in Fig. 1. For pharmacokinetic studies, doxorubicin was administered via i.p. injection alone at a single dose of 30 mg/kg, and following two doses of 5 mg/kg of aflibercept given on days 0 and 3 or three doses of 25 mg/kg of aflibercept given on days 0, 4, and 7 ($n=3-4$ /group). For both groups treated with aflibercept, DOX was administered on day 7 only. Animals were sacrificed within 24 h to determine DOX levels in plasma and various tissues. Based on the PK results, aflibercept was administered three times at 25 mg/kg prior to DOX administration for the pharmacodynamic antitumor efficacy studies and a lower dose of doxorubicin (3 mg/kg) was administered to alleviate adverse effects of the drug that are more apparent at higher doses when mice are evaluated for more than 24 h. Tumor measurements and total bioluminescence (tumor burden) were evaluated for up to 16 and 28 days post-dose of DOX, respectively. Also, a PBS control group (no aflibercept or DOX) of at least five animals was monitored for tumor growth. Each treatment group evaluating antitumor efficacy was composed of up to five mice at the start of treatment.

Doxorubicin Measurements in Various Tissues

At sacrifice, animals were weighed, and then euthanized by carbon dioxide asphyxiation followed by cervical dislocation. Blood was drawn via retro-orbital bleed prior to euthanasia into syringes using heparin as an anticoagulant. Plasma was prepared by centrifugation ($2,000\times g$ for 15 min at 4°C) and frozen at -20°C until further analysis. Organs (bone marrow, liver, and subcutaneous tumor) were harvested for additional analysis. Femurs of mice were flushed with 2 ml saline using a 25-gauge needle inserted into the acetabulum. Suspension cells underwent red blood cell lysis, pelleted, and stored at -80°C prior to PK analysis. The excised tissues were weighed and frozen in liquid nitrogen immediately after sacrifice and stored at -80°C until homogenization. Doxorubicin plasma and tissue samples ($n=3-4$ /timepoint) were collected at serial

times of 0, 0.5, 1, 2, 3, 6, and 24 h after single-dose administration of doxorubicin at the maximal tolerated dose (30 mg/kg) (9). Doxorubicin was determined by a validated HPLC assay with fluorescence detection previously described, with a lower limit of quantitation of 5 ng/mL (9). Samples were extracted with a liquid/liquid method using chloroform/isopropanol. Drug concentrations in tissues were determined using a plasma standard curve with a dynamic range of 5 to 1,000 ng/mL, with inter- and intra-day variability of less than 15%. Drug concentrations obtained in bone marrow were normalized per million cells and then each tissue (*i.e.*, tumor, liver, and bone marrow) was converted to ng/g by assuming a tissue density of 1 g/mL.

Pharmacokinetic Modeling

A base PK model describing doxorubicin concentrations in serum and collected tissues was developed (Fig. 2a). The model was comprised of 4 compartments, with a first order intraperitoneal absorption rate constant into the central serum compartment. The doxorubicin is then distributed into various tissues including the liver, tumor, and bone marrow. Elimination of the drug was modeled to occur from the serum, tumor, and liver compartment as tissue elimination may be a result of metabolism of doxorubicin within these tissues, as well as lymphatic elimination from the tumor. The rate constants were coded as clearance and volume terms, including absorption. Subsequently, the presence of concomitant administration of aflibercept was tested on various system pharmacokinetic parameters as a treatment effect, including both the tumor uptake and efflux of doxorubicin, CL_{Tup} or CL_{Teff} , to determine whether the increased intratumoral concentrations observed were the result of increased uptake or decreased efflux of doxorubicin in the tumor. Additional PK models were tested for the inclusion of a remainder compartment for other tissues, as well as presence or absence of a elimination clearance term from tumor, and the need for a transfer clearance from the

Fig. 1. Schema for pharmacokinetic (top panel) and pharmacodynamic (bottom panel) experiments

Fig. 2. a Pharmacokinetic model for doxorubicin (Dox) in SCID mice after intraperitoneal (i.p.) administration is shown. **b** Pharmacodynamic model for leukemia (tumor) growth after doxorubicin administration is shown

peritoneum to serum, but neither of these models improved the overall goodness-of-fit of the data.

Equations for PK model are provided here:

$$\frac{dA_{ip}}{dt} = -CL_{ip} \times \frac{A_{ip}}{V_{ip}} \quad (1)$$

$$\frac{dC_{serum}}{dt} = \frac{(CL_{ip} \times A_{ip} + CL_{liver} \times C_{liver} + CL_{marrow} \times C_{marrow} + CL_{Teff} \times C_{tumor}) - (CL_{liver} + CL_{marrow} + CL_{Tup} + CL_{Tout}) \times C_{serum}}{V_{serum}} \quad (2)$$

$$\frac{dC_{tumor}}{dt} = \frac{CL_{Tup} \times C_{serum} - CL_{Teff} \times C_{tumor} - CL_{Tout} \times C_{tumor}}{V_{tumor}} \quad (3)$$

$$\frac{dC_{liver}}{dt} = \frac{(CL_{liver} \times C_{serum} - CL_{liver} \times C_{liver} - CL_{Lout} \times C_{liver})}{V_{liver}} \quad (4)$$

$$\frac{dC_{marrow}}{dt} = \frac{(CL_{marrow} \times C_{serum} - CL_{marrow} \times C_{marrow})}{V_{marrow}} \quad (5)$$

The change in CL_{Tup} or CL_{Teff} of doxorubicin was estimated based on the presence or absence of aflibercept, as well as the dose intensity of the drug.

C_{tissue} represents the concentration of doxorubicin in the tissue indicated in the subscript. V_{tissue} represents the volume of each of the tissue compartments modeled. CL_{serum} represents the elimination clearance from the central serum

compartment, CL_{liver} represents clearance from the liver, CL_{Tup} and CL_{Teff} represents the uptake or efflux from the tumor, respectively, while CL_{Tout} represents elimination clearance out of the tumor. CL_{Lout} represents metabolic clearance out of the liver. CL_{liver} and CL_{marrow} represent the distribution clearances from these compartments to and from the central compartment.

Initial estimates of organ blood flow rates were obtained from the literature (21–24) and scaled to the experimental animal weights. The organ volumes were determined by weight (assuming a density of 1 g/mL) for tumor, bone marrow, and liver. The organ volumes, except the peritoneum, were fixed to their measured tissue weights during the model estimation process to reduce the number of parameters being estimated. The values were 100, 0.42, 1.92, and 0.93 mL/kg for serum, bone marrow, tumor, and liver, respectively. Data obtained from the PK study were treated as replicate animals sampled as a single individual with averaged tissue volumes.

The equations for plasma and all tissues were solved simultaneously to estimate the model parameters using a naïve data averaging approach with maximum likelihood estimation method (MLEM) implemented in ADAPT 5 (25) with a proportional error variance model for residual error $\text{Var}(Y) = (\text{Slope}_{var} \cdot Y + \text{Intercept})^2$. Model selection, comparison, and the choice of final structural models, was guided using the likelihood ratio test, the precision of the parameter estimates, and diagnostic plots (observed concentrations vs. individual and population predicted concentrations, weighted residuals vs. predicted time and concentrations). The inter-animal variability in pharmacokinetic parameters was assumed to be log-normally distributed. A change in $2 \cdot \log$ likelihood of at least 3.84 ($\alpha < 0.05$, 1df) was used to define statistical significance for addition or deletion of a single parameter during the model building process. Model discrimination was accomplished according to the “rule of parsimony” based on Akaike’s information criterion (26).

Covariate Effects. Evaluation of the presence or absence of aflibercept in combination with doxorubicin treatment was treated as a covariate effect. Relationships between all PK parameters and the presence of aflibercept were visually assessed for trends by plotting the PK parameter estimates *versus* this categorical covariate.

Pharmacodynamic Modeling of Antitumor Efficacy

PD modeling of leukemia growth data was performed in a sequential manner. It was assumed the doxorubicin pharmacokinetics were linear between the two dose groups employed in the PK (doxorubicin 30 mg/kg) and PD (doxorubicin 3 mg/kg) experiments. As a result, the PK parameters were fixed to the estimates from the PK study and used for the PD modeling. Data from animals who were administered vehicle (PBS), doxorubicin alone, and the combination of aflibercept (25 mg/kg) plus doxorubicin (3 mg/kg) therapy were modeled simultaneously. To accommodate for differences in the metrics of tumor growth in the xenograft and systemic tumor growth models, separate PD models were built for each endpoint to describe the relationship between drug concentration and tumor growth inhibition (Fig. 2b). The inherent differences between the two PD models were that the intratumoral xenograft concentrations of doxorubicin drove cell kill in the xenograft model, while the bone marrow doxorubicin concentrations drove cell kill in the systemic model. On inspection of the data, we observed some inter-animal variability in the initial whole bioluminescence index (BLI) in the systemic tumor models among the treatment groups compared with control (PBS). In order to account for the BLI differences, we included an effect on the estimate of the initial tumor weight among the treatment groups of PBS, DOX alone, and DOX plus TRAP. The model used was a previously published model by Simeoni *et al.* (Fig. 2), with the modification that the tissue concentrations (*i.e.*, tumor or bone marrow) rather than plasma concentrations drove cell kill (27). The equations of the PD models are given below.

For xenografts, x_1 was modeled using the equation

$$\frac{dx_1}{dt} = \frac{L0 \cdot x_1}{\left[1 + \left(\frac{L0}{LT} \cdot w\right)^{\frac{1}{\varphi}}\right]^{\frac{1}{\varphi}}} - k2 \cdot C_{\text{tissue}} \cdot x_1 \quad (6)$$

$$\frac{dx_2}{dt} = k_2 \cdot C_{\text{tissue}} \cdot x_1 - k_1 \cdot x_2 \quad (7)$$

$$\frac{dx_3}{dt} = k1 \cdot (x_2 - x_3) \quad (8)$$

$$\frac{dx_4}{dt} = k1 \cdot (x_3 - x_4) \quad (9)$$

For xenograft tumor modeling, $C_{\text{tissue}} = C_{\text{tumor}}$, while for the systemic tumor,

$$C_{\text{tissue}} = C_{\text{bonemarrow}} \quad (10)$$

The total tumor load was expressed as a summation of the various tumor compartments.

$$Y = x_1 + x_2 + x_3 + x_4 \quad (11)$$

The initial conditions for x_1 was set to w_0 (baseline tumor weight or BLI), while the rest of the compartments were set at 0. L0 represents the exponential rate of tumor growth, while L1 represents the rapid zero order linear rate of tumor growth. K1 represents the transit rate between the tumor kill compartments, while k2 is a second order rate constant which drives cell kill.

The pharmacodynamics were assessed using a population analysis using a mixed effects modeling approach with the equations for the assessment of doxorubicin's effect on tumor growth being solved simultaneously to estimate the model parameters using MLEM implemented in ADAPT 5 (25) with a proportional error variance model $\text{Var}(Y) = (\text{Slope}_{\text{var}} \cdot Y)^2$. Similar to the PK model development, the PD model selection, comparison, and the choice of final structural models were evaluated using the same criteria as the PK model did. The inter-animal variability in pharmacodynamic parameters was assumed to be log-normally distributed.

RESULTS

Intratumoral Concentrations of Doxorubicin Increased Following Aflibercept Treatment

The pharmacokinetics of doxorubicin from all three treatment groups (DOX alone, DOX+TRAP (days 0, 3), and DOX+TRAP (days 0, 4, and 7)) were modeled simultaneously, to investigate the effect of dosing frequency and sequential administration of aflibercept on the uptake or efflux clearance of doxorubicin. The PK model characterizes the data in serum and each tissue reasonably well, with minimal over- and under-estimation of the observed doxorubicin concentrations in liver (Fig. 3). DOX concentrations in bone marrow were predicted well except for minimal overestimation at the 30-h post-dose timepoint. Serum doxorubicin concentrations declined bi-exponentially with time, with or without concomitant administration of aflibercept (Fig. 3). The PK parameters were all estimated reasonably well, with the inter-study variability all less than 38% except for CL_{ip} , which had a %CV of 98 due to the minimal amount of samples collected during the transfer of doxorubicin from peritoneum to serum (Table I). Development of the PK model warranted inclusion of not only clearance from the serum, but also liver and tumor clearance, indicative of drug metabolism and lymphatic clearance, respectively. Doxorubicin concentrations in both liver and tumor peaked at about 4–6 h, while bone marrow drug concentrations continued to rise up to 24 h post-dose. The estimated elimination clearances from the liver (CL_{Lout}) and tumor (CL_{Tout}) were similar (0.122 vs. 0.09, respectively) (Table I). In addition, the distribution clearance of DOX to the liver was approximately 10,000-fold higher compared to that of bone marrow (1.83 vs. 1.92×10^{-4} ml/h/kg), supportive of the fact that the liver is highly vascularized and the main metabolizing organ for DOX. The uptake clearance of doxorubicin (CL_{Tup}) from the serum into the

Fig. 3. Observed and predicted doxorubicin concentrations *versus* time plots when drug was administered alone or following prior aflibercept treatment. Blue represents DOX alone (30 mg/kg), red represents DOX plus two prior doses of aflibercept (5 mg/kg), green represents DOX plus three prior doses of aflibercept (25 mg/kg)

subcutaneous xenograft tumor increased approximately 3-fold (0.125 for DOX alone vs. 0.471 mL/h/kg for DOX+3 doses of 25 mg/kg of aflibercept) (Table I). The same trend did not occur following only two doses of aflibercept administered before doxorubicin. Additionally, the estimates of the efflux clearance of doxorubicin from the tumor into the serum were not altered among different treatment schedules.

Aflibercept dosed at 25 mg/kg i.p. on days 0, 4, and 7 increased the concentration of doxorubicin in all tissues, as well as plasma, where the predicted AUC₂₄ was 6,164 ng h/mL (DOX alone) vs. 4,724 ng h/mL (DOX+TRAP; 0 and 3) vs. 8,635 ng h/mL (DOX+TRAP; 0, 4, and 7) (Table II). The most significant increase in tissue concentration was seen in the subcutaneous AML xenograft tumors where the average maximal concentration increased 9-fold from the doxorubicin treated alone group (Fig. 3b). Model-predicted tumor exposure of doxorubicin was increased 6-fold in the DOX+TRAP (days 0, 4, and 7) group compared to DOX alone (Table II).

Relationship Between Tumor Growth Inhibition and Doxorubicin Concentrations Following Concomitant Doxorubicin and Aflibercept Administration

Following successful completion of the PK model, a PK/PD model was developed to ascertain the temporal

relationship between the time course of DOX concentrations and antitumor efficacy, with or without aflibercept. The PK/PD model was developed sequentially. The PK parameters were fixed to their mean parameter estimates. Goodness-of-fit plots illustrate that the PD models for both tumor volume and total BLI, as a measure of tumor burden, characterize the antitumor effect by doxorubicin (Figs. 4 and 5). The PD parameters were all estimated reasonably well (Tables III and IV). In addition, the parameters for the PD model were not significantly altered between treatment groups. Most importantly, the rate of cell kill (K2) remained relatively constant between treatment groups, indicative of cell eradication solely by DOX (2.4×10^{-5} and 2.2×10^{-5} ng/mL/h for the subcutaneous and systemic leukemia tumor models, respectively; Tables III and IV). Comparing the different treatment groups, a 1.5-2-fold decrease in tumor growth in the TRAP plus DOX group was observed (0.00794 (PBS) vs. 0.00632 (DOX alone) vs. 0.0043 (DOX+TRAP)) (Table III). One plausible reason for this increase in efficacy is mainly due to the increased intratumoral doxorubicin concentrations among the treatment groups, associated with the three doses of aflibercept causing increased uptake of DOX into tumor (Fig. 4). A similar approach was used linking bone marrow AML growth inhibition, using total bioluminescence as a representation of total tumor burden, to the PK of

Table I. Pharmacokinetic Model Parameters of Doxorubicin After Intraperitoneal (i.p.) Administration With and Without Concomitant Administration of Aflibercept

PK parameter	Estimate	%C.V. ^a
CL _{ip} (ml/h/kg)	9.45	97.6
CL _{out} (ml/h/kg)	53.4	16.4
CL _{D, liver} (ml/h/kg)	1.83	5.74
CL _{D, BM} (ml/h/kg)	1.92×10^{-4}	29.3
CL _{Teff, tumor} (ml/h/kg)	7.12×10^{-2}	50
CL _{Lout, liver} (ml/h/kg)	0.122	6.57
CL _{Tout, tumor} (ml/h/kg)	9.09×10^{-2}	37.6
CL _{Tup, tumor} (effect of dox alone) (ml/h/kg)	0.125	
CL _{Tup, tumor} (effect of 2 doses of aflibercept) (ml/h/kg)	0.0734	
CL _{Tup, tumor} (effect of 3 doses of aflibercept) (ml/hg/kg)	0.471	
V _{ip} (ml/kg)	6.13×10^{-3}	25.2
V _c (serum) (ml/kg)	100 fixed	
V _{BM} (ml/kg)	0.42 fixed	
V _{tumor} (ml/kg)	1.92 fixed	
V _{liver} (ml/kg)	0.93 fixed	

Cl_{ip} absorption clearance from intraperitoneal to serum, *Cl_{out}* elimination clearance from serum, *CL_D* distribution clearances for liver and bone marrow (BM), *CL_{Lout}* and *CL_{Tout}* elimination clearances from liver and tumor, *CL_{Tup}* and *CL_{Teff}* uptake and efflux clearances to and from tumor, *V* volumes of distribution for intraperitoneum, serum, bone marrow, tumor, and liver

^aCoefficient of variation of the estimate; reflects inter-study variability

doxorubicin (Fig. 5). While the difference was modest, the first order tumor growth rate, *L₀*, was decreased from 0.0167 to 0.0127 (DOX alone vs. DOX+TRAP) (Table IV). Elevated levels of doxorubicin in bone marrow could, in part explain this alteration in the tumor growth doubling time of 1.7 vs. 2.3 days. Of note, each treatment group lost 1–2 animals at day 21 post-dose of DOX, suggesting that there was an increased tumor burden due to the systemic tumor.

DISCUSSION

In preclinical solid tumor xenograft models, administration of an anti-VEGF agent has been reported to enhance chemotherapeutic drug delivery by normalizing aberrant tumor vasculature and inducing changes in intratumoral pressures such that challenges to drug uptake are transiently removed (4,28). Improved clinical outcomes following the addition of the anti-VEGF antibody (bevacizumab) to standard chemotherapy in many cancer types have been attributed to this vascular normalization effect. However, to date, there have been few studies combining anti-angiogenic drugs with chemotherapy drugs for the treatment of hematological malignancies, and no prior published data on PK/PD modeling of anti-VEGF therapeutic strategies in acute myeloid leukemia. We have previously shown that combination therapy with the potent VEGF inhibitor, aflibercept, and doxorubicin increased antitumor activity in preclinical AML xenograft models as compared with single-agent therapy (9). Although prior preclinical PK/PD models have been developed to predict the synergistic effects of anti-angiogenic

agents and chemotherapy, these studies were all performed in solid tumor xenografts and therefore are of uncertain relevance in hematological malignancies (29–31). It is currently unclear whether tumor vasculature in blood cancers can be “normalized” by anti-VEGF therapy or whether other effects of aflibercept therapy, such as impaired drug efflux, were responsible for the enhanced efficacy of combination therapy (32).

Here, our objectives were: (1) to demonstrate whether differences existed between the PK of doxorubicin administered concomitantly with or following aflibercept therapy in preclinical leukemia models; (2) and to develop a PK/PD model to link these PK changes to potential changes in the efficacy of leukemia growth inhibition by increased tumor uptake of doxorubicin in a systemic model of leukemia.

As the effects of aflibercept on tumor drug clearance have not previously been reported in any preclinical models (1,32), we first specifically assessed two doxorubicin drug clearances (uptake and efflux) in localized leukemia xenografts by taking a naïve data averaging population PK approach and estimating the two as distinct rates, based on prior studies of the pharmacokinetics of doxorubicin, in mice (33,34). Our model indicated that tumor uptake drug clearance is more strongly affected than drug efflux by three doses of the anti-vascular agent aflibercept administered prior to chemotherapy. These data are consistent with prior vascular normalization data in other studies (4,28). In contrast with prior reports, we did not detect statistically any changes in tumor drug efflux following aflibercept therapy accounting for the observed effects (32,35).

Smaller differences in doxorubicin drug levels were detected in the bone marrows of systemically engrafted leukemia mice following aflibercept, as shown by the marrow area under the curves in mice treated with doxorubicin alone as compared with doxorubicin and three doses of aflibercept treatment. To some degree, this may simply reflect the lower detectable concentrations of doxorubicin within the bone marrow than those in localized xenografts and thus minimal enhanced efficacy in this systemic tumor model. Another explanation is the presence of both injected human AML cells and diverse host murine cell populations within the marrow space, all of which may process chemotherapeutic drugs at different rates (36).

Although modest, the observed increases in marrow doxorubicin levels following aflibercept nevertheless could result in clinically significant anti-leukemic activity. The bone marrow is the primary site of disease in AML, and in our prior work, it was noted that markedly enhanced marrow necrosis in leukemia-engrafted mice treated with aflibercept and doxorubicin as compared with doxorubicin treatment alone (9). Leukemia expansion within the bone marrow space has been associated with decreased marrow blood flow which can markedly limit the exposure of leukemia cells to systemic chemotherapy (37). In leukemic rats, much less of the anthracycline drug, daunomycin, was found in the bone marrow as compared with other leukemia-infiltrated (but better perfused) organs or healthy non-leukemic rat marrows, even though leukemic cells took up more daunomycin *in vitro* than normal marrow cells (38–40). Here, we noted greater than a 100-fold lower doxorubicin concentration in leukemia-infiltrated bone marrow than subcutaneous AML xenografts

Table II. Comparison of Model-Predicted $C_{\max}$ and AUC_{24} in Plasma and Tissues Among the Treatment Groups

		PK parameter	Estimate
Serum	DOX alone	$C_{\max}$ ($\mu\text{g/mL}$)	2.14
		AUC_{24} ($\mu\text{g} \times \text{h/mL}$)	5.84
	DOX+aflibercept (5 mg/kg)	$C_{\max}$ ($\mu\text{g/mL}$)	1.58
		AUC_{24} ($\mu\text{g} \times \text{h/mL}$)	4.63
	DOX+aflibercept (25 mg/kg)	$C_{\max}$ ($\mu\text{g/mL}$)	2.13
		AUC_{24} ($\mu\text{g} \times \text{h/mL}$)	8.59
Tumor	DOX alone	$C_{\max}$ ($\mu\text{g/mL}$)	0.178
		AUC_{24} ($\mu\text{g} \times \text{h/mL}$)	2.93
	DOX+aflibercept (5 mg/kg)	$C_{\max}$ ($\mu\text{g/mL}$)	0.0873
		AUC_{24} ($\mu\text{g} \times \text{h/mL}$)	1.39
	DOX+aflibercept (25 mg/kg)	$C_{\max}$ ($\mu\text{g/mL}$)	0.898
		AUC_{24} ($\mu\text{g} \times \text{h/mL}$)	18.1
Liver	DOX alone	$C_{\max}$ ($\mu\text{g/mL}$)	4.90
		AUC_{24} ($\mu\text{g} \times \text{h/mL}$)	74.2
	DOX+aflibercept (5 mg/kg)	$C_{\max}$ ($\mu\text{g/mL}$)	3.82
		AUC_{24} ($\mu\text{g} \times \text{h/mL}$)	51.3
	DOX+aflibercept (25 mg/kg)	$C_{\max}$ ($\mu\text{g/mL}$)	6.22
		AUC_{24} ($\mu\text{g} \times \text{h/mL}$)	111
Bone marrow	DOX alone	$C_{\max}$ ($\mu\text{g/mL}$)	0.00234
		AUC_{24} ($\mu\text{g} \times \text{h/mL}$)	0.0423
	DOX+aflibercept (5 mg/kg)	$C_{\max}$ ($\mu\text{g/mL}$)	0.0025
		AUC_{24} ($\mu\text{g} \times \text{h/mL}$)	0.0369
	DOX+aflibercept (25 mg/kg)	$C_{\max}$ ($\mu\text{g/mL}$)	0.00462
		AUC_{24} ($\mu\text{g} \times \text{h/mL}$)	0.0789

DOX doxorubicin, $C_{\max}$ maximum estimated doxorubicin concentration, AUC_{24} estimated doxorubicin exposure over 24 h

or the liver and serum of systemically engrafted leukemic mice, consistent with the marrow as a pharmacologic sanctuary. Dose intensified anthracycline chemotherapy, specifically daunorubicin 90 mg/m^2 , in AML patients younger than 65 years old has been shown to be well tolerated and was associated with higher response rates than conventional dose daunorubicin (45 mg/m^2). Review of the literature suggests that higher peak exposure levels of daunorubicin administered over one cycle, rather than cumulative anthracycline dose, accounts for the improved AML outcomes (41,42). Other strategies to improve marrow anthracycline delivery have been also associated with potential clinical benefit. CPX-351, a liposome formulation of cytarabine and daunorubicin co-encapsulated at a synergistic 5:1 M ratio, was found to improve marrow delivery and drug uptake in marrow leukemia cells relative to conventional chemotherapy and resulted in promising remission rates in refractory and relapsed AML patients (43).

In an attempt to generate a more detailed, mechanistic PK/PD model to describe the potential effects of sequential aflibercept and doxorubicin therapy in human AML xenograft models, we made certain assumptions. Our PK experiments were done with doxorubicin given as a single nearly lethal dose of 30 mg/kg followed by euthanasia of animals up to 30 h later in order to facilitate measurements of bone marrow drug concentrations. In contrast, our PD experiments were performed with doxorubicin administered at 3 mg/kg , which is the maximally tolerated weekly dose for SCID mice, to allow for leukemia growth response measurements over a longer period of time (44). This assumption was necessary to link our PK model to our PD model. Data from several

experiments was also consolidated for analysis using this PK/PD model. To take advantage of all the data, we used a naïve data averaging population approach in ADAPT 5, which allowed us to incorporate more physiologically meaningful parameters with the data we have. Upon successful model development, our goal was to test the hypothesis of whether the uptake or efflux of doxorubicin from the tumor site was enhanced following aflibercept pre-treatment. To accommodate the two different doxorubicin-dosing levels for the PK and PD experiments, we used a sequential approach. The estimates of PK of doxorubicin were fixed to their estimated values for each treatment group, and PD modeling was then performed. Ideally, one would want to simulate several scenarios of aflibercept and doxorubicin therapy. However by measuring doxorubicin in several tissues, including the marrow, we were able to directly demonstrate that sequential dosing as well as dosing frequency of a vascular normalizing agent may lead to increased delivery of a cytotoxic drug to the site of action. Lastly, while there may be potential synergistic effects of the two drugs, here we assumed that the primary effect of aflibercept in these models occurred via binding to VEGF within the tumor microenvironment, resulting in alterations in the tumor vasculature. This strategy, in part was supported by the greatest improvement in the antitumor effect only in the treatment group that was administered three doses of 25 mg/kg aflibercept prior to doxorubicin administration, thus supporting that the tumor growth delay is mainly due to the chemotherapeutic drug. This assumption does not take into account any direct induction of cell death or stasis by aflibercept and does not include data on aflibercept concentrations in tumor and serum sites.

Fig. 4. a Goodness-of-fit plots for observed and predicted tumor volume, independent of dosing regimen. **b** Observed and predicted tumor growth curves of subcutaneous leukemia xenograft tumors following doxorubicin alone as compared with sequential aflibercept and doxorubicin treatment. *Green symbols* represent observed and predicted (10th–50th percentile) tumor growth with PBS, *blue symbols and lines* represent observed and predicted (10th–50th percentile) tumor growth with DOX alone, while *red symbols and lines* represent observed and predicted (10th and 50th percentile) tumor growth with DOX plus three doses of aflibercept

Overall, the PK/PD model characterizes the data reasonably well with minimal overestimation of DOX marrow levels at the last timepoint post-dose. This overestimation relates to at most a 10% increase in total DOX exposure in marrow. The implications on the anti-leukemic effects for the systemic tumor would be minor, and thus the overall conclusions between the minor differences in the treatment group are still valid. Although there are other PD models that could have been attempted, the Simeoni (27) transit compartmental system that was used seemed to adequately characterize both pharmacodynamics endpoints, although it characterized

Fig. 5. a Goodness of fit plots for observed and predicted total BLI (tumor burden), independent of dosing regimen. **b** Observed and predicted tumor growth curves of leukemia cells within bone marrow disease sites following with doxorubicin treatment alone as compared with aflibercept prior to doxorubicin treatment. *Blue symbols and lines* represent observed and predicted (10th–50th percentile) tumor growth with doxorubicin alone, while *red symbols and lines* represent observed and predicted (10th and 50th percentile) acute leukemia growth after sequential aflibercept and doxorubicin treatment

the antitumor effects of the xenograft tumor better. One of the potential limitations of this PD model is that it did not account for potential mechanistic differences in how DOX exhibited its cytotoxic effects on the proliferation rate of the xenograft tumor or systemic tumor. The current model accounts for the temporal delay between PK and PD responses empirically through the use of transit compartments. Other PD models have been developed where there are some differences in how the drug interacts with the tumor in the promotion of cell death (45,46). The drug either interacts directly with the

Table III. Mean (%CV) Pharmacodynamic Model Parameters for Subcutaneous Xenograft Leukemia Growth Inhibition

PD mean parameter estimates (%CV ^a) for subcutaneous tumor model					
Treatment	L0 (h ⁻¹)	K2 (ng/mL/h)	K1 (h ⁻¹)	L1 (mm ³ /h)	W0 (mm ³)
PBS	0.00794 (9.17)	2.37×10 ⁻⁵ (1.03)	0.00273 (1.18)	233 (0.45)	216 (26.4)
DOX alone	0.00632 (15.4)	2.36×10 ⁻⁵ (0.74)	0.00274 (0.54)	234 (0.7)	188 (47)
DOX+VEGF TRAP (25 mg/kg)	0.0043 (18.4)	2.38×10 ⁻⁵ (2.74)	0.00272 (0.33)	232 (0.57)	145 (39.7)
Residual variability		21.7%			

PD pharmacodynamics, DOX doxorubicin, VEGF TRAP aflibercept, PBS phosphate-buffered saline, L0 exponential tumor growth rate, L1 tumor linear growth rate, K2 tumor cell-killing rate, K1 tumor cell transfer rate between compartments, W0 baseline tumor volume

^a Coefficient of variation of the population estimate reflects the inter-animal variability

cancer cells causing a cascade events leading to cell killing or the drug binds to receptor causing a signal transduction process, leading to the killing effect that exerts through the transduction cascade rather than directly upon the cycling cells. In addition, an alternative approach for further model building would involve linking aflibercept PK to modulation of the tumor vasculature, thus causing changes in doxorubicin uptake in local xenograft and marrow disease sites. Overall, all these PD models may be attempted in the future to provide improvement in the fit of the tumor volume and BLI data.

Similar to data in solid tumor models, we observed differences in uptake of doxorubicin between treatment groups highlighting a potential therapeutic time window as a key factor affecting leukemia responses following combination anti-VEGF and chemotherapeutic strategies (4,28,32). In our studies, the intratumoral drug uptake clearance did not change markedly following two doses of low-dose aflibercept (5 mg/kg) administered 4 days prior to doxorubicin. On the other hand, drug uptake was significantly enhanced in leukemia-engrafted animals treated with three doses of high-dose aflibercept (25 mg/kg) administered 7 and 4 days prior to and on the same day of doxorubicin, indicating that there may be not only be a dose-dependent process, but also a time-dependent process on the increase in uptake of doxorubicin into tumor sites. In accord with this, cyclophosphamide pre-treatment in leukemic rats increased accumulation of daunomycin in bone marrow sites, presumably due to improved marrow perfusion (40). Anti-VEGF agents have previously been clinically explored in refractory and relapsed AML patients. Despite limited activity of single-agent VEGF inhibition (47–50), combined anti-angiogenic therapy and cytotoxic chemotherapy was associated with some clinical

responses (51,52). Our preclinical data demonstrating that sequential VEGF inhibition followed by chemotherapy can selectively enhance drug uptake in leukemia disease sites with improved anti-leukemic activity, provide a compelling rationale for future clinical development of this approach in AML patients.

CONCLUSIONS

Our PK/PD model in its current stage gives insight into how an anti-vascular agent can play a role in the improvement of delivery of a cytotoxic agent and clearly can predict changes in doxorubicin concentrations resulting from aflibercept pre-treatment. This model also provides significant data supporting that intratumoral uptake of doxorubicin to the tumor site in both local and marrow disease sites is being altered by aflibercept and is responsible for the observed anti-leukemia activity. This effect was highly dependent on the timing and doses of aflibercept therapy with respect to doxorubicin. Given these PK/PD data, further in-depth studies characterizing the specific aflibercept-mediated changes in the leukemia marrow microenvironment responsible for the enhanced drug uptake (*i.e.*, vascular function, marrow perfusion, acute cytokine release, direct anti-leukemic effects) are warranted (53). Future adaptations of this PK/PD model will enable us to determine the optimal dosing and sequence regimens of alternative anti-vascular and chemotherapy combinations for cancers in the preclinical setting prior to proceeding to clinical trials. Importantly, this model will also allow us to evaluate the effects of other biological agents on marrow chemotherapy delivery as a novel means of optimizing tumor growth inhibition in hematological malignancies.

Table IV. Mean (%CV) Pharmacodynamic Model Parameters for Leukemia Growth Inhibition Within Bone Marrow Disease Sites

PD mean parameter estimates (%CV ^a) for systemic tumor model					
Treatment	L0 (h ⁻¹)	K2 (ng/mL/h)	K1 (h ⁻¹)	L1 (×10 ⁷ mphotons/s/h)	W0 (×10 ⁷ mphotons/s)
DOX alone	0.0167 (54.8)	2.15×10 ⁻⁵ (1.9)	0.00274 (1.6)	0.142 (2.3)	2.4 (78.7)
DOX+VEGF TRAP (25 mg/kg)	0.0127 (78.4)	2.14×10 ⁻⁵ (1)	0.00272 (1.6)	0.141 (4.9)	3.02 (70.1)
Residual variability		42.1%			

PD pharmacodynamics, DOX doxorubicin, VEGF TRAP aflibercept, PBS phosphate-buffered saline, L0 exponential tumor growth rate, L1 tumor linear growth rate, K2 tumor cell-killing rate, K1 tumor cell transfer rate between compartments, W0 baseline bioluminescence (systemic tumor burden)

^a Coefficient of variation of the population estimate reflects the inter-animal variability

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