**Preclinical Development**

**Aflibercept Exerts Antivascular Effects and Enhances Levels of Anthracycline Chemotherapy In vivo in Human Acute Myeloid Leukemia Models**

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**Abstract**

We examined whether potent vascular endothelial growth factor (VEGF) blockade mediated by aflibercept, a decoy VEGF receptor (VEGFR) 1/2 moiety with stronger affinity for VEGF than bevacizumab, resulted in antileukemia effects and enhanced the efficacy of systemic chemotherapy. The efficacy of aflibercept alone and in combination with doxorubicin was evaluated in human VEGF-expressing acute myeloid leukemia (AML) cell lines and primary cells xenotransplanted into immunodeficient mice. Aflibercept reduced primary VEGF/VEGFR-positive AML colony formation growth in vitro and inhibited AML xenograft growth up to 93% in association with antiangiogenic and antiproliferative effects, hypoxia, and VEGF sequestration in multiple models. High VEGF-A expression by AML cells promoted in vivo xenograft growth and aflibercept sensitivity. Aflibercept therapy slowed disease progression in two systemic human AML xenograft models and reduced peripheral leukemia disease in a primary relapsed AML model in NOD/SCID/IL2Rγnull mice. Combination aflibercept and doxorubicin enhanced antitumor effects in local xenograft models. Sequential aflibercept followed by doxorubicin resulted in progressive anthracycline accumulation in marrow and extramedullary AML sites and resulted in 2-fold higher drug levels 24 hours after administration. In contrast, tissues (tumor, plasma, marrow) treated with chemotherapy only showed progressive drug clearance over time. Combination aflibercept and doxorubicin also resulted in vascular narrowing, decreased vessel number, and perivascular apoptosis. These data suggest that inefficient drug delivery by leukemia-associated vasculature may mediate chemoresistance and support further clinical evaluation of combination aflibercept and anthracycline therapy in refractory/relapsed AML patients. Mol Cancer Ther; 9(10); 2737–51. ©2010 AACR.

**Introduction**

Acute myeloid leukemia (AML) represents a heterogeneous group of neoplasms characterized by unchecked rapid growth of undifferentiated hematopoietic cells. Standard upfront AML therapy, consisting of high-dose chemotherapy and stem cell transplantation, has remained unchanged since the 1980s, with overall survival rates of 20% to 30%. Angiogenesis and paracrine interactions mediated by vascular endothelial growth factor (VEGF) isoforms are known key mediators of leukemia growth. Heterogeneous production of VEGF ligands [VEGF-A,-B,-C, and -D, and placental growth factor (PIGF)] by bone marrow stroma and AML cells promote vessel formation and secretion of proinflammatory and leukemogenic growth factor, further supporting tumor growth (1, 2). Bone marrow biopsies from AML patients and preclinical studies have shown the close interaction between leukemia cells and vascular endothelium, with enhanced marrow vessels correlating with increased blasts, higher VEGF-A levels, and poorer overall outcome (2, 3). Elevated VEGF-A levels in patient sera and primary AML cells are also predictive of poor outcome independent of other clinical factors (3–5). A small subset of AML cells aberrantly express VEGF receptors (VEGFR-1/-2/-3) that function in autocrine VEGF/VEGFR loops to promote antiapoptotic effects, chemoresistance, and extramedullary egress (6). It has been suggested that these VEGF/VEGFR-positive leukemia cells represent malignant transformation of early normal VEGF/VEGFR-positive hematopoietic progenitors, which similarly rely on VEGF/VEGFR pathways for survival (7).

Antileukemia effects following VEGF blockade with murine anti-VEGFR antibodies (8), VEGF antisense gene transfection (9), soluble antagonists (10), and small
VEGF-A protein sequestration

VEGF-A (human, mouse) protein production by AML cells (1 x 10^6) was measured in cell-free supernatants following 24-hour incubation in serum-free RPMI media containing vehicle (PBS) or aflibercept (1–100 nmol/L) using reagents provided in a commercial enzyme-linked immunosorbent assay (ELISA; R&D Systems). Experiments were repeated at least three times.

Primary AML clonogenic assays

Viable primary AML cells (2 x 10^5) with VEGF-A expression were plated in triplicate in human methylcellulose complete media containing recombinant human stem cell factor 50 ng/mL, recombinant human granulocyte-macrophage colony stimulating factor 10 ng/mL, recombinant human interleukin-3 10 ng/mL, and recombinant erythropoietin 3 U/mL (HSC003, R&D Systems). No exogenous VEGF-A was added to this culture media. Control (PBS) or aflibercept (0.1–100 nmol/L) were added to the media. Mean (± SE) leukemia colony formation units (CFU) were quantified after 10 to 14 days using an inverted microscope (10).

Leukemia xenograft 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 female severe combined immunodeficient mice (SCID)/SCID mice were sublethally irradiated with 2.5 cGy and inoculated with 10 x 10^6 AML cells s.c. under the skin as described previously (22). The two largest perpendicular axes of s.c. tumors (l, length; w, width) were measured three times weekly. Tumor volume (TV, mm^3) was calculated as TV = 4/3 l w r^2, where r = (l + w)/4. When tumor volumes approached 100 mm^3, the mice were divided into groups of 5 to 10 mice. 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 x 10^6 cells into irradiated SCID mice. P-glycoprotein mediated drug efflux was confirmed in cells prior to and following in vivo propagation (23). 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 cm^2 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.

For the primary AML xenogeneic experiment, primary mononuclear marrow aspirate cells (10 x 10^6) from a 54-year-old patient with relapsed CD45+CD34+CD38+ AML were injected via tail vein into sublethally irradiated

Materials and Methods

Reagents and primary samples

Aflibercept (VEGF Trap) was provided through a collaborative agreement with National Cancer Institute-Cancer Therapy Evaluation Program (CTEP) and Sanofi-Aventis/Regeneron Pharmaceuticals. All patients provided Institutional Review Board (IRB)-approved informed consent for procurement and cryopreservation of cells in our institute’s tissue bank. These specific studies were also approved under a separate IRB protocol. AML samples consisting of ficolled cryopreserved or fresh bone marrow or peripheral blood mononuclear cells were obtained from adults with pathologically confirmed AML between 1997 and 2006.
8-week-old NOD/SCID/IL2Rγnull mice (Jackson Laboratory) as described previously (24), under IRB- and IACUC-approved protocols. Peripheral blood, bone marrow, and spleen AML engraftment was confirmed three months later by flow cytometric evaluation of hCD45+hCD38+ expression in peripheral blood, spleen, and marrow sites. Harvested unsorted cells from these animals were then injected (10 × 10⁶ cells/mouse) into irradiated secondary recipient mice. AML engraftment and complete blood cell analyses were monitored by peripheral blood testing at 2- to 3-week intervals until week 16. At this time, the mice were divided into two experimental groups (n = 5) and treated with control or aflibercept (25 mg/kg i.p.) for 15 days, at which time the animals were sacrificed due to clinical disease progression.

**In vivo treatment regimens**

Treatment in these xenograft models consisted of control (PBS), aflibercept (5–25 mg/kg s.c./i.p. twice weekly; ref. 17), bevacizumab (10 mg/kg i.p. three times weekly; ref. 25), maximally tolerated dose (MTD) cytarabine (100 mg/kg i.p. five times per week; ref. 26), or doxorubicin (3 mg/kg i.p. once during week 2; ref. 27). Treatment in systemic models consisted of control (PBS), aflibercept (5–25 mg/kg s.q./i.p. twice weekly; ref. 17), bevacizumab (10 mg/kg i.p. three times weekly; ref. 25), sunitinib 40 mg/kg p.o. daily (28), and sorafenib 60 mg/kg p.o. daily (29). PBS was used as the control for aflibercept due to prior data showing equivalency of this vehicle with the Fc fragment of human immunoglobulin in preclinical models. Due to the development of antihuman antibodies in SCID mice, aflibercept therapy in all SCID mouse models was limited to three weeks; other agents were administered continuously unless otherwise indicated.

Experiments evaluating doxorubicin levels in mice were done in sublethally irradiated SCID mice simultaneously injected with 10 × 10⁶ luciferase-transfected HEL and HL60/VCR AML cells via tail vein and s.c. routes. Mice confirmed to have leukemia engraftment by bioluminescent imaging were divided into equivalent groups. Animals were treated with control (PBS) or aflibercept (25 mg/kg i.p. twice a week) for one week followed by a single 30 mg/kg i.p. doxorubicin dose. At designated time points (1–30 hours) following doxorubicin injection, mice were anesthetized for retro-orbital bleeds followed by euthanasia. Excised tumors and normal tissues were placed in cryomolds (Tissue-Tek) with embedding medium (optimum cutting temperature compound) and frozen in liquid nitrogen or fixed overnight in formalin-free zinc fixative (BD Biosciences PharMingen) or 10% formalin followed by paraffin embedding.

**Determination of doxorubicin levels**

Total doxorubicin concentration in s.c. leukemia tumors, bone marrow, liver, and plasma was determined using ultra high-performance liquid chromatography (UPLC) as previously described (30). The separation method was carried out on a Waters Nova-Pak C18 column equipped with Bondapak C18 guard column with mobile phase consisting of 20% acetonitrile and 80% triethylamine acetate. Doxorubicin levels were detected by fluorescence with excitation at 370 nm and emission at 510 nm. Doxorubicin concentration was assessed in s.c. leukemia xenografts, bone marrow, liver, and plasma of animals treated with PBS followed by doxorubicin as compared with aflibercept followed by doxorubicin. Uptake of autofluorescent doxorubicin drug was also measured by fluorescent microscopy in s.c. leukemia xenografts at 1 to 24 hours following doxorubicin injection. Frozen tumors were cut into approximately 5- to 10-µm-thick sections and evaluated for fluorescent tissue intensity in an average of three maximum intensity projection images with a resolution of 0.23 μm under a ×63 objective lens of Leica confocal microscope as previously described (31). Images were obtained and digitized under identical acquisition variables. AUC (μs), the area under the plasma concentration-time curve from time zero to infinity, and AUC (μs) the area under the plasma concentration versus time curve from time zero to the last measured time point, were both calculated using the linear trapezoidal rule.

**Statistical analysis**

Results of xenograft experiments are shown as mean TV or tumor weight (TW) ± SE at each time point for groups of 5 to 10 mice. Statistical evaluation of differences in mean values was done using two-tailed Student’s t-test analysis with unequal variance. All P values < 0.05 were considered significant. Log-rank tests were used to calculate the statistical significance of the difference in Kaplan-Meier survival curves. These analyses were done using the GraphPad Prism software program (GraphPad Software).

Additional Materials and Methods, including primary AML results, transfection experiments, primers, and immunohistochemical staining, are provided in Supplementary Data.

**Results**

**VEGF receptor expression is associated with poor outcome in normal karyotype AML samples**

Although VEGF isoforms and VEGFR have been previously identified in AML patient samples (32–35), few studies have examined the prognostic impact of concomitant VEGF/VEGFR expression in AML subsets. Normal karyotype AML constitutes up to half of all new AML diagnoses with intermediate prognosis. We assessed expression of two VEGF ligands (VEGF-A and -C) and two VEGFR (VEGFR-1 and -2) by quantitative PCR (Q-PCR) in diagnostic marrow samples from 91 consecutive older adult normal karyotype AML patients (median age, 66 years; range, 21–87) treated at our institute. Levels were expressed relative to normal bone marrow controls set...
Figure 1. Aflibercept (Aflib) inhibits primary leukemia cell growth and reduces human AML growth in multiple xenograft models. A, aflibercept sequesters human (h) VEGF-A in the serum-free supernatants of human AML cell lines incubated with vehicle (PBS) or aflibercept (1–100 nmol/L) for 48 hours. B, aflibercept inhibits primary leukemia colony formation growth of VEGF/VEGFR-1/2-expressing primary AML cells from three individual patients. Cells were plated in triplicate in complete methylcellulose (MC) plates in the presence of PBS or aflibercept (0.1–100 nmol/L) for 10 to 14 days. The number of leukemia mean colony forming units (CFU-L) ± SE is shown. C, aflibercept treatment reduced local tumor growth in three human AML xenograft models, as shown by differences in mean volumes (± SE), of control- (PBS) versus aflibercept-treated (25 mg/kg) xenografts (n = 6-10 mice/group).
equal to 1. VEGF-A was overexpressed in most samples (median 1.35). Coexpression of VEGF receptors (i.e., VEGFR1+R2+R3) was found in a third of samples (27 of 84, 32%). VEGF-A significantly correlated with VEGFR-1, whereas VEGF-C correlated with VEGFR-1 and -2 (Supplementary Table S1). Significant factors for overall and event-free survival by univariate analysis were age, achievement of complete remission, and VEGFR-2 (Supplementary Fig. S1). These results suggest that autocrine VEGF-A/VEGFR-mediated loops in AML cells may confer therapy resistance and/or increased relapse risk.

**Aflibercept sequesters VEGF-A protein produced by leukemia cells**

Three human AML cell lines (HEL, HL60/VCR, and ML-2) expressing VEGF-A and VEGFR by Q-PCR (Supplementary Fig. S2) were selected for further studies. HL60/VCR expressed up to 10-fold higher VEGF-A mRNA levels than normal marrow. ML-2 expressed high VEGFR-1, and HEL expressed high VEGFR-2 levels. Aflibercept (1–100 nmol/L) treatment led to sequestration of up to 85% to 96% of AML-secreted VEGF-A measured in serum-free cell supernatants (Fig. 1A). Aflibercept also led to sequestration of murine VEGF-A produced by the murine lymphoma cell line A-20 (results not shown).

**Aflibercept inhibits primary leukemia colony formation growth**

Next, we examined aflibercept treatment on growth of primary VEGF-A/VEGFR-positive AML cells in methylcellulose colony-formation unit (CFU-L) assays with hematopoietic colony factor support. Here, aflibercept (0.1–100 nmol/L) significantly reduced CFU-L growth in multiple individual patient samples. Of interest, CFU-L inhibition was noted to occur following minimal aflibercept exposure (0.1 nmol/L) and was not always dose dependent (Fig. 1B). The effects of aflibercept treatment on *in vitro* HL60/VCR and ML-2 proliferation and viability were also evaluated under serum-free conditions with no significant inhibition noted (Supplementary Fig. S3). The failure of aflibercept to inhibit VEGF/VEGFR autocrine pathways in established cell lines versus primary AML cells in CFU-L assays may reflect differences in the culture conditions and/or cell materials.

**Aflibercept inhibits tumor growth in multiple human AML models with evidence of antivascular and antiproliferative effects**

We then evaluated the effects of *in vivo* aflibercept (5-25 mg/kg) therapy on local VEGF/VEGFR-positive AML xenografts established with HEL, ML-2, and HL60/VCR human AML cells in SCID mice. Due to prior reports of development of antihuman antibodies in SCID mice, aflibercept therapy was limited to three weeks duration. In all three AML models, aflibercept consistently reduced tumor growth with differences noted after as little as one treatment week. After 14 days, TVs were reduced by 56% to 90%: PBS 552 ± 174 versus aflibercept 245 ± 76 mm³ for HEL (*n* = 10/group; *P* < 0.05), PBS 2,701 ± 192 versus aflibercept 460 ± 55 mm³ for ML-2 (*n* = 15/group; *P* < 0.05), PBS 2,317 ± 321 versus aflibercept 229 ± 31 mm³ for HL60/VCR (*n* = 10/group; *P* < 0.05). By day 21 to 30, all vehicle-treated mice required sacrifice per institute guidelines due to tumor size (Fig. 1C). At this time, aflibercept-treated xenografts weighed 78% to 94% (*P* = 0.0001–0.0142) less than controls, with markedly less gross vascularization on gross appearance (Supplementary Fig. 4A and B). In general, leukemia-bearing mice treated with aflibercept lived twice as long as control mice before requiring sacrifice for large tumor volumes (data not shown). The efficacy of aflibercept in Pgp+ HL60/VCR AML cells (23) was consistent with a MDR-independent mechanism of action. Because
Aflibercept could theoretically block VEGF/VEGFR promoting normal hematopoiesis, serial complete blood cell counts were done on all control- and aflibercept-treated mice at intermittent intervals with no significant differences noted (Supplementary Table S2).

Immunohistochemistry revealed confluent sheets of AML cells with high Ki67 expression in control-treated tumors as compared with aflibercept-treated xenografts, which showed large areas of avascular necrosis with residual Ki-67-positive AML cells. High VEGF-A sequestration and increased hypoxia, via carbonic anhydrase IX (CAIX) staining, was seen. Angiogenesis, as reflected by the mean number of CD31-positive blood vessels, was significantly reduced in all aflibercept-treated tumors (Fig. 1D, Supplementary Fig. 4C).

**In vivo leukemia growth and sensitivity to aflibercept therapy correlate with VEGF-A expression by AML cells**

In contrast to our in vitro cell line data, we noted that VEGFR-positive AML xenografts established with HL60/VCR cells expressing the highest VEGF-A levels responded best to in vivo aflibercept therapy. Therefore, we asked whether VEGF-A expression by VEGFR-positive AML cells predicted for in vivo aflibercept response. To examine this, we selected a VEGF-positive HEL subclone (designated tHEL) expressing much lower constitutive VEGF-A expression than parental HEL cells. This clone was stably transfected with a pcDNA3.1/human VEGF-A construct followed by selection for additional subclones (denoted tHEL-O/E #1–10) exhibiting 3.9 to 7.7 fold higher constitutive VEGF-A expression than parental HEL cells. This clone was stably transfected with a pcDNA3.1/human VEGF-A construct followed by selection for additional subclones (denoted tHEL-O/E #1–10) exhibiting 3.9 to 7.7 fold higher constitutive VEGF-A expression than parental HEL cells. This clone was stably transfected with a pcDNA3.1/human VEGF-A construct followed by selection for additional subclones (denoted tHEL-O/E #1–10) exhibiting 3.9 to 7.7 fold higher constitutive VEGF-A expression than parental HEL cells. This clone was stably transfected with a pcDNA3.1/human VEGF-A construct followed by selection for additional subclones (denoted tHEL-O/E #1–10) exhibiting 3.9 to 7.7 fold higher constitutive VEGF-A expression than parental HEL cells. This clone was stably transfected with a pcDNA3.1/human VEGF-A construct followed by selection for additional subclones (denoted tHEL-O/E #1–10) exhibiting 3.9 to 7.7 fold higher constitutive VEGF-A expression than parental HEL cells. This clone was stably transfected with a pcDNA3.1/human VEGF-A construct followed by selection for additional subclones (denoted tHEL-O/E #1–10) exhibiting 3.9 to 7.7 fold higher constitutive VEGF-A expression than parental HEL cells. This clone was stably transfected with a pcDNA3.1/human VEGF-A construct followed by selection for additional subclones (denoted tHEL-O/E #1–10) exhibiting 3.9 to 7.7 fold higher constitutive VEGF-A expression than parental HEL cells. This clone was stably transfected with a pcDNA3.1/human VEGF-A construct followed by selection for additional subclones (denoted tHEL-O/E #1–10) exhibiting 3.9 to 7.7 fold higher constitutive VEGF-A expression than parental HEL cells. This clone was stably transfected with a pcDNA3.1/human VEGF-A construct followed by selection for additional subclones (denoted tHEL-O/E #1–10) exhibiting 3.9 to 7.7 fold higher constitutive VEGF-A expression than parental HEL cells. This clone was stably transfected with a pcDNA3.1/human VEGF-A construct followed by selection for additional subclones (denoted tHEL-O/E #1–10) exhibiting 3.9 to 7.7 fold higher constitutive VEGF-A expression than parental HEL cells. This clone was stably transfected with a pcDNA3.1/human VEGF-A construct followed by selection for additional subclones (denoted tHEL-O/E #1–10) exhibiting 3.9 to 7.7 fold higher constitutive VEGF-A expression than parental HEL cells. This clone was stably transfected with a pcDNA3.1/human VEGF-A construct followed by selection for additional subclones (denoted tHEL-O/E #1–10) exhibiting 3.9 to 7.7 fold higher constitutive VEGF-A expression than parental HEL cells. This clone was stably transfected with a pcDNA3.1/human VEGF-A construct followed by selection for additional subclones (denoted tHEL-O/E #1–10) exhibiting 3.9 to 7.7 fold higher constitutive VEGF-A expression than parental HEL cells. This clone was stably transfected with a pcDNA3.1/human VEGF-A construct followed by selection for additional subclones (denoted tHEL-O/E #1–10) exhibiting 3.9 to 7.7 fold higher constitutive VEGF-A expression than parental HEL cells.

### Table 1. Results of Q-PCR and ELISA for human VEGF-A in luciferase-transfected control (tHEL luciferase) and VEGF-A–transfected (tHEL-O/E clone 10) human AML (HEL) clones showing overexpression of VEGF-A in the latter cells

<table>
<thead>
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<th>hVEGF-A Q-PCR</th>
<th>Fold change</th>
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<tr>
<td>tHEL Luciferase</td>
<td>1.0</td>
</tr>
<tr>
<td>tHEL-O/E Clone 10</td>
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<tr>
<th>hVEGF-A ELISA</th>
<th>hVEGF (pg/mL)</th>
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<tr>
<td>tHEL-O/E Clone 10</td>
<td>904</td>
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Aflibercept prolongs survival in a systemic multidrug-resistant human leukemia model with marrow and blood involvement

We then examined the effects of VEGF inhibition in a systemic human AML model established with HL60/VCR-luciferase cells (23). This is an aggressive AML disease model with rapid disease progression in marrow, central nervous system, liver, spleen, and ovarian sites, resulting in animal euthanasia usually within 20 to 25 days of inoculation. To determine the relative efficacy of aflibercept as compared with other VEGF/VEGFR inhibitors in clinical usage, we treated systemic HL60/VCR-luciferase-engrafted mice with PBS, vehicle, aflibercept, sunitinib, sorafenib, and bevacizumab, and followed mice for time to morbidity. Due to prior reports of anti-human antibody development in SCID mice, aflibercept therapy was limited to three weeks (CTEP/Regeneron information), whereas other agents were administered continuously unless otherwise indicated. MTDs of the other inhibitors were determined based on prior literature review (see Materials and Methods) and limited dose escalation experiments in our AML systems (data not shown). For instance, sorafenib was first administered at 90 mg/kg daily, which was subsequently reduced to 60 mg/kg and then to 30 mg/kg due to evidence of treatment-related toxicity potentially limiting overall efficacy at the higher doses. No difference in treatment outcomes was noted among these three sorafenib doses. Median survival of each treatment group was 22 days for control (PBS; n = 25), 21.5 days for vehicle (n = 10), 20 days for sunitinib (n = 9), 26 days for sorafenib (n = 20), 34 days for bevacizumab (n = 10), and 39 days for aflibercept (n = 15). Although aflibercept, bevacizumab, and sorafenib all significantly prolonged survival over PBS- and vehicle-treated controls, aflibercept (25 mg/kg) resulted in statistically superior survival compared with the other agents at their respective doses in this particular leukemia model (Fig. 3A, Supplementary Fig. S5A). Aflibercept-treated mice lived longer than controls or bevacizumab-treated animals (P < 0.0001 and P = 0.0038, χ² test) with evidence of reduced disease burden in multiple sites and slower disease advancement as reflected by bioluminescent imaging (Fig. 3B and C). Similar data were obtained following aflibercept versus control treatment in a second systemic human AML (tHEL-luciferase) xenograft model with disease in marrow and extramedullary areas (Supplementary Fig. S5B).

Aflibercept exerts antileukemia effects in a primary human AML in vivo model

Next, we examined the effects of aflibercept in a primary AML xenograft model established by secondary passage of relapsed CD45+CD34+CD38+ AML cells in irradiated NOD/LtSz-scid IL2Rnull mice. The patient's disease had
previously relapsed following allogeneic stem cell transplantation and two induction chemotherapy regimens. Secondary mouse recipients of human AML cells confirmed to have florid human disease based on peripheral blood flow cytometric analysis were treated for two weeks with control (PBS) or aflibercept (25 mg/kg i.p.) followed by sacrifice. As shown in Fig. 3D, human AML disease burden, as measured by peripheral flow cytometry for hCD38-positive cells, was significantly decreased in aflibercept-treated animals versus controls.

**Combination aflibercept and doxorubicin chemotherapy enhances antileukemia effects**

The fact that bevacizumab has been proven to be most clinically effective for solid tumor therapy in combination with chemotherapy led us to further examine the *in vivo* efficacy of aflibercept in combination with cytarabine or doxorubicin (substituted for anthracyclines daunorubicin, idarubicin, and mitoxantrone) used in standard AML induction. We first compared the effects of PBS, aflibercept (5 and 25 mg/kg twice per week) and bevacizumab (5 mg/kg IP three times per week) in local ML-2 xenografts (25).

Both agents exerted significant antitumor effects, with aflibercept (5–25 mg/kg) treatment somewhat better than bevacizumab in tumor weight reduction (*P* = 0.0029–0.0033) and antiangiogenic effects (Supplementary Fig. S6A), which was similar to the results in the HL60/VCR systemic model. Aflibercept monotherapy (25 mg/kg) also compared favorably with MTD cytarabine or doxorubicin in local HL60/VCR and ML-2 xenograft models. Although aflibercept plus cytarabine led to no improved antileukemic activity over single-agent therapy (Supplementary Fig. 6B), concomitant aflibercept and doxorubicin seemed to enhance antitumor effects (Fig. 4A). Leukemia xenografts treated with aflibercept plus doxorubicin showed increased tumor ablation with areas of central necrosis surrounded by rims of proliferating leukemia cells. These effects were consistent with direct and indirect effects affecting AML cells, not just decreased tumor blood flow. Combination aflibercept and doxorubicin also induced more hypoxia (via CAIX staining) than did any other treatment (Fig. 4B). Although similar experiments assaying aflibercept and cytotoxic agents were done in systemic leukemia xenografts using these and 50% reduced doses, all animals treated with combination (but not single agent) succumbed to systemic toxicities of uncertain cause 10 to 14 days after treatment initiation (not shown).

**Aflibercept alters leukemia vascular response to doxorubicin and results in enhanced antiangiogenic effects and higher drug levels**

To explain these enhanced antileukemia effects, we next sought to determine if vascular effects mediated by aflibercept altered *in vivo* doxorubicin delivery to leukemia disease sites and if these effects were site specific. Sublethally irradiated SCID mice engrafted with luciferase-transfected HL60/VCR or HEL AML cells in s.c. and marrow sites were treated with vehicle (PBS) or aflibercept (25 mg/kg i.p. twice per week x 6 doses) and sacrificed on day 42.
(25 mg/kg) for two doses over one week followed by a single MTD dose of doxorubicin (30 mg/kg). Tissues were harvested up to 30 hours later for evaluation of drug and host effects (see schema, Fig. 5A). As compared with PBS-treated controls, tumor vessels in aflibercept-treated marrow and local xenograft disease sites were reduced in diameter at initiation of doxorubicin. Extramedullary tumor vessels subsequently underwent further involution whereas marrow
vessels became progressively dilated over the duration of doxorubicin treatment, potentially due to eradication of other supporting marrow stromal and hematopoietic cells. Sequential aflibercept- and doxorubicin-treated mice showed fewer vessels at both initiation and end of chemotherapy consistent with antiangiogenic effects (Fig. 5B and C, Supplementary Fig. S7A–D). Combination treatment also resulted in areas of increased perivascular apoptosis (Supplementary Fig. 7E) and enhanced tumor necrosis consistent with local tumor responses (shown in HL60/VCR-luciferase xenografts, Fig. 5D), although there were no overall significant changes in tumor volumes over this short time frame (Supplementary Fig. S8).

We then measured doxorubicin levels in various tissues of HL60/VCR-luciferase engrafted mice and found that anthracycline levels in extramedullary and marrow sites accumulated over time in aflibercept-pretreated animals, resulting in up to 2-fold higher anthracycline concentrations after 24 hours. In contrast, doxorubicin levels measured in tissues from animals pretreated with PBS instead of aflibercept showed gradual drug clearance over time (with the exception of doxorubicin levels 6 hours after

Figure 4. Aflibercept enhances the effects of doxorubicin (Dox) chemotherapy in a human AML xenograft model. Mean tumor volumes and weights (±SE) of local human AML xenografts engrafted in SCID mice and treated with control (PBS), aflibercept (25 mg/kg i.p. twice per week × 6 doses), doxorubicin (3 mg/kg i.p. for one dose), or combination aflibercept and doxorubicin therapy (n = 5–10 mice/group) over two weeks are shown. A, aflibercept monotherapy (25 mg/kg) was more effective than doxorubicin in reducing HL60/VCR xenograft growth. Combination aflibercept and doxorubicin treatment resulted in improved antileukemia effects. B, immunohistochemistry of tumors in each treatment group showed differences in overall tumor burden/necrosis (H&E), proliferation (Ki-67), hypoxia (CAIX), and VEGF-A production.

A

B

Control

H&E

Ki67

Dox

CAIX

VEGF-A

Aflibercept

Control

Aflibercept

Aflibercept + Dox

Aflibercept + Dox

Aflibercept + Dox

Figure 4. Aflibercept enhances the effects of doxorubicin (Dox) chemotherapy in a human AML xenograft model. Mean tumor volumes and weights (±SE) of local human AML xenografts engrafted in SCID mice and treated with control (PBS), aflibercept (25 mg/kg i.p. twice per week × 6 doses), doxorubicin (3 mg/kg i.p. for one dose), or combination aflibercept and doxorubicin therapy (n = 5–10 mice/group) over two weeks are shown. A, aflibercept monotherapy (25 mg/kg) was more effective than doxorubicin in reducing HL60/VCR xenograft growth. Combination aflibercept and doxorubicin treatment resulted in improved antileukemia effects. B, immunohistochemistry of tumors in each treatment group showed differences in overall tumor burden/necrosis (H&E), proliferation (Ki-67), hypoxia (CAIX), and VEGF-A production.
aflibercept treatment in the liver; Fig. 6A and B). Results in a systemic HEL-luciferase AML model were similar, including doxorubicin clearance in normal cardiac tissue (Supplementary Table S3).

Total doxorubicin exposure, also known as area under the curve or AUC, in the tissues of doxorubicin-only-treated versus combination-treated animals showed slightly higher exposure of liver tissues to doxorubicin following aflibercept. In contrast, total doxorubicin (AUC) in plasma, marrow, and s.c. leukemia tumors was approximately the same over time. As aflibercept-treated s.c. xenografts in general had similar to somewhat

Figure 5. Sequential aflibercept and doxorubicin therapy alters vascular morphology and enhances antiangiogenic effects in extramedullary and marrow leukemia disease sites. A, experimental schema showing pretreatment of human AML (HL60/VCR-luciferase) engrafted SCID mice with vehicle (PBS) or aflibercept (25 mg/kg) for one week prior to single-dose doxorubicin (30 mg/kg) administration. Tissues were harvested up to 30 hours later. Sequential aflibercept followed by doxorubicin versus doxorubicin treatment alone resulted in morphologic alterations in marrow sinusoidal vessels (as determined by VEGFR-3 staining; magnification, ×10; B) and extramedullary tumor vessels (as determined by CD31+ staining, magnification, ×4; C).
smaller tumor volumes than controls, these data imply higher drug penetration into s.c. leukemia sites mediated by aflibercept (Fig. 6C). Fluorescent microscopic examination of \textit{in vivo} anthracycline uptake in s.c. leukemia tumors further confirmed this finding (Fig. 6D).

**Discussion**

AML cells utilize VEGF ligands and VEGFR to promote growth, chemotherapy resistance, and angiogenesis. Despite this, prior trials of VEGF inhibitors in AML have been of modest clinical efficacy, potentially due to limited VEGF blockade mediated by these agents and the inability of VEGF/VEGFR-directed therapy alone to control rapidly proliferating heterogeneous AML cells.

Our data in primary AML samples showing poor outcomes associated with VEGFR-2 expression led us to evaluate the preclinical antileukemia effects of aflibercept (VEGF Trap), a decoy VEGFR-1/-2 moiety with a higher reported affinity for VEGF-A and a broader spectrum of VEGF ligand inhibition than bevacizumab (17). Experiments done in primary AML colony formation assays, local and systemic AML xenografts, and a primary AML stem cell model confirmed that aflibercept exhibits potent \textit{in vitro} and \textit{in vivo} activity against VEGF/VEGFR-positive AML cells. Although \textit{in vivo} response to aflibercept correlated with levels of VEGF-A produced by AML cells, inhibition of VEGF ligands secreted by marrow stromal and other microenvironment cells also likely play a significant role in the observed antileukemic effects. By immunohistochemical staining, we noted decreased vascularization, increased hypoxia, and VEGF sequestration in the local leukemia microenvironment following aflibercept therapy, consistent with this agent's previously described effects on solid tumor-associated angiogenesis (36). Because immunodeficient murine hosts were used for human AML engraftment, the effects of \textit{in vivo} VEGF inhibition on VEGF/VEGFR-positive host immune cells, such as dendritic cells, macrophages, and monocytes, were not formally assessed here. Similarly, we were unable to evaluate inhibition of VEGFR-1/positive hematopoietic and endothelial progenitor cell migration into "premetastatic" niches, as reported in solid tumor models (37), due to the presence of VEGFR-1-positive AML cells. Our data in systemic leukemia models did show delayed disease progression and reduction of primary peripheral leukemia disease consistent with extramedullary disease effects. The results of aflibercept-mediated inhibition on primary AML growth in colony formation assays and in NOD/SCID/IL-2rynull mice also implicate a potential role for VEGF/VEGFR inhibition on leukemia stem cell biology (1). Further detailed studies of this are needed.

Several features distinguishing aflibercept from other clinical VEGF inhibitors are of particular relevance in hematologic malignancies. Unlike multitargeted receptor tyrosine kinase VEGF inhibitors (i.e., sorafenib, sunitinib), aflibercept is highly specific for VEGF ligands binding VEGFR-1/-2 and exhibits no known cross-reactivity with other kinase-mediated receptors (17, 38). Aflibercept would therefore be expected to induce fewer toxicities related to "off target" effects but also would not inhibit other receptor kinases known to be constitutively active in AML cells and potentially representing alternate clinical targets, i.e., inhibition of FLT3 mutated AML by sorafenib (39, 40). As none of the cell lines used here contained known FLT3 mutations (data not shown), our results should be interpreted only in the context of FLT3 wild-type AML. Unlike bevacizumab, aflibercept is composed of entirely human sequences and has not induced anti-VEGF antibodies or antibody-ligand immune complexes (41). Despite the importance of VEGFR-1/-2 to normal hematopoietic recovery, we noted no negative effects of aflibercept on hematologic parameters in our models (42), and hematologic toxicities have not been extensively reported in other aflibercept-treated cancer patients (18).

As anti-VEGF monotherapy has been shown to be clinically effective in only a subset of human cancers, we then showed enhanced antileukemic effects of aflibercept and doxorubicin chemotherapy in our AML models. Combinatorial regimens of bevacizumab and anthracyclines have improved outcomes in solid tumor models and patients (43, 44), and encouraging clinical responses were reported in a phase I trial of bevacizumab and anthracycline chemotherapy in refractory/relapsed AML (15). Our results show that combination effects of aflibercept were agent dependent, as no improvement in chemotherapy response to aflibercept (Fig. 6C). Fluorescent microscopic examination of \textit{in vivo} anthracycline uptake in s.c. leukemia tumors further confirmed this finding (Fig. 6D).
Interstitial fluid pressure leading to improved intratumoral perfusion and systemic chemotherapy delivery (25, 45). Theoretically, the rapid in vivo clearance of cytarabine (<1 hour) as compared with doxorubicin (with a half-life of several hours) may have rendered it less amenable to aflibercept-mediated vascular changes and hence reduced its efficacy in our AML models. Although leukemia marrow vasculature has been shown to function similarly to other tumor vasculature in vivo (46), data showing vascular normalization following VEGF inhibition in leukemia models, and showing differential effects in different organs involved with the same tumor cells, are lacking (25, 45). Based on our assumption that VEGF-mediated effects on leukemia vasculature may be context dependent, we examined aflibercept treatment effects in marrow and extramedullary sites and found that aflibercept treatment led to progressive increases in anthracycline drug levels up to 24 hours after administration. This was associated with increased apoptosis, decreased microvessel density, and local necrosis. The observed differential effects of aflibercept on patterns of doxorubicin accumulation and vascular changes in marrow versus s.c. tumor locations support the site-specific heterogeneity of leukemia vasculature and in vivo doxorubicin pharmacokinetics (47). Although p-glycoprotein expression by human HL60/VCR cells may theoretically have enhanced intracellular drug levels in this model (23), we noted similar results in a second (HEL) AML model lacking multidrug efflux protein expression. Calculations of doxorubicin tissue exposure (AUC) in different tissues imply potentially improved drug penetration in smaller aflibercept-treated local xenografts but do not explain the higher anthracycline levels found in aflibercept-treated marrow sites. Therefore, it is possible that other aflibercept-mediated effects, such as increased drug retention in poorly vascularized marrow regions, may be responsible.

These data support a potential role for leukemia vasculature in mediating both chemoresistance and hematologic toxicity in AML patients via alterations...
in systemic drug delivery and/or retention. Higher doxorubicin levels achieved following sequential aflibercept and doxorubicin therapy also may explain the increased toxicities noted following concomitant aflibercept and doxorubicin treatment in systemic leukemia models. Because SCID mice used in these experiments are known to be significantly more susceptible to doxorubicin toxicity than are immunocompetent mice, this may not necessarily reflect human toxicities (48). Anthracycline dose intensification has been shown to be well tolerated and to improve outcomes in newly diagnosed AML patients <65 years of age (49). Ongoing trials of aflibercept and chemotherapy in heavily pretreated solid tumor patients have also reported few severe cytopenias (19, 20, 50). Nevertheless, given these results and the fact that effects observed in mouse vasculature may not truly reflect human vasculature, clinical evaluation of aflibercept with anthracycline chemotherapy in AML patients is warranted. Moreover, the potential of aflibercept and other antivascular agents to alter chemotherapy drug delivery and overcome therapy resistance in acute leukemia should be further explored.
Disclosure of Potential Conflicts of Interest

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