

## Differential Antitumor Activity of Aflibercept and Bevacizumab in Patient-Derived Xenograft Models of Colorectal Cancer

Marielle Chiron<sup>1</sup>, Rebecca G. Bagley<sup>3</sup>, Jack Pollard<sup>4</sup>, Parminder K. Mankoo<sup>4</sup>, Christophe Henry<sup>1</sup>, Loïc Vincent<sup>2</sup>, Catherine Geslin<sup>1</sup>, Nina Baltes<sup>5</sup>, and Donald A. Bergstrom<sup>4</sup>

### Abstract

The recombinant fusion protein aflibercept (ziv-aflibercept in the United States) binds VEGF-A, VEGF-B, and placental growth factor (PlGF). The monoclonal antibody bevacizumab binds VEGF-A. Recent studies hypothesized that dual targeting of VEGF/PlGF is more beneficial than targeting either ligand. We compared activity of aflibercept versus bevacizumab in 48 patient-derived xenograft (PDX) colorectal cancer models. Nude mice engrafted subcutaneously with PDX colorectal cancer tumors received biweekly aflibercept, bevacizumab, or vehicle injections. Differential activity between aflibercept and bevacizumab, determined by mouse (m), human (h), VEGF-A, and PlGF levels in untreated tumors, was measured. Aflibercept induced complete tumor stasis in 31 of 48 models and bevacizumab in 2 of 48. Based on statistical analysis, aflibercept was more active than bevacizumab in 39 of 48 models; in 9 of 39 of these models, bevacizumab was considered inactive. In 9 of 48 remaining models, aflibercept and bevacizumab had similar activity. Tumor levels of hVEGF-A (range 776–56,039 pg/mg total protein) were ~16- to 1,777-fold greater than mVEGF-A (range 8–159 pg/mg total protein). Tumor levels of mPlGF (range 104–1,837 pg/mg total protein) were higher than hPlGF (range 0–543 pg/mg total protein) in 47 of 48 models. Tumor cells were the major source of VEGF; PlGF was primarily produced by tumor stroma. Because tumor levels of hVEGF-A were far greater than mVEGF-A, bevacizumab's inability to bind mVEGF-A is unlikely to explain higher and more consistent aflibercept activity. Neutralizing PlGF and VEGFR-1 activation may be a factor and should be investigated in future studies. In these colorectal cancer PDX models, aflibercept demonstrated greater antitumor activity than bevacizumab. *Mol Cancer Ther*; 13(6); 1636–44. ©2014 AACR.

### Introduction

The recognition that tumors require vasculature to grow, and that the VEGF pathway is a major mediator of tumor angiogenesis, created an opportunity for the development of new therapies that benefit patients with cancer (1). The VEGF pathway comprises 5 ligands, VEGF-A, -B, -C, -D, and placental growth factor (PlGF), some of which may have more than 1 isoform (2, 3). The receptors for these molecules are VEGF receptors (VEGFRs)-1, -2, and -3 with ligand-specific binding affin-

ities (4, 5). In combination with chemotherapy, antiangiogenic drugs targeting the VEGF pathway have shown clinical benefit in colorectal, non-small cell lung cancer, and other solid tumors (6, 7).

VEGF, originally termed "vascular permeability factor," was shown to be a major driver of tumor angiogenesis in what soon became a rapidly expanding field of investigation (1, 8). Shortly thereafter, PlGF was encoded and characterized from a human placenta cDNA library (9). Similar to VEGF, PlGF is a secreted, N-glycosylated, dimeric protein, and shares 53% amino acid homology with VEGF (10). The angiogenic activity of PlGF was suggested in culture and in transgenic mice engineered to overexpress PlGF (9, 11). Physiologic and pathologic roles for PlGF were also suggested from preclinical models of cancer, ischemia, inflammation, and wound healing (12). These studies additionally revealed synergy between PlGF and VEGF.

Higher levels of PlGF in tumor tissue compared with normal tissue have been detected in multiple cancers, including colorectal, breast, gastric, and lung (13–17). Interestingly, circulating levels of PlGF were increased in patients with renal cell, prostate, or colorectal carcinoma who were treated with bevacizumab or sunitinib,

**Authors' Affiliations:** <sup>1</sup>Sanofi Oncology, Translational & Experimental Medicine; <sup>2</sup>Sanofi Oncology, Pharmacology, Vitry-sur-Seine, France; <sup>3</sup>Sanofi Oncology, Clinical Development; <sup>4</sup>Sanofi Oncology, Translational & Experimental Medicine, Cambridge, Massachusetts; and <sup>5</sup>Oncotest GmbH, Freiburg, Germany

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**Corresponding Author:** Marielle Chiron, Sanofi Oncology, Translational and Experimental Medicine, 13 Quai Jules Guesde, 94403 Vitry-sur-Seine, France. Phone: 33-1-58-93-20-97; Mobile: 33-6-88-80-54-80; Fax: 33-1-58-93-34-71; E-mail: [Marielle.Chiron@Sanofi.com](mailto:Marielle.Chiron@Sanofi.com)

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which target the VEGF pathway (18–21). The value of targeting PIGF in cancer was demonstrated with an anti-PIGF antibody that inhibited tumor growth and metastasis in multiple tumor models and also enhanced the efficacy of chemotherapy (22). Subsequent studies demonstrated that the efficacy of the anti-PIGF antibody strongly correlated with the tumor cell expression of VEGFR-1, the receptor for PIGF, implicating an autocrine/paracrine role of PIGF in tumor cell survival (23,24).

Further evidence correlating VEGFR-1 and the progression of disease was observed in patient-derived xenograft (PDX) models of melanoma, where VEGFR-1 expression on malignant tumor cells was associated with a more aggressive tumor-initiating cell population (25). Other studies with the CT26 colorectal cancer syngeneic mouse model showed that blockade of both VEGFR-1 and VEGFR-2 was required to prevent the vascularization and growth of micrometastases (26). These results suggest that dual targeting of VEGF/PIGF or VEGFR-1/VEGFR-2 pathways would be beneficial in the clinical setting, as the development of metastases, rather than the growth of the primary tumor, results in a poorer prognosis in many human cancers.

For the treatment of colorectal cancer, several antiangiogenic drugs have emerged in the clinic and have been approved by the U.S. Food and Drug Administration (FDA). The monoclonal antibody bevacizumab was the first antiangiogenic agent approved to treat human cancers; it binds directly to soluble human VEGF-A (hVEGF-A; refs. 27–29). The small molecule regorafenib inhibits the VEGFR tyrosine kinase signaling pathways (30) and was approved for treatment of metastatic colorectal cancer (mCRC) by the FDA in early 2013 for use as third- or fourth-line treatment based on results of the CORRECT trial [A Randomized, Double-blind, Placebo-controlled Phase III Study of Regorafenib Plus BSC Versus Placebo Plus BSC in Patients With Metastatic Colorectal Cancer (CRC) Who Have Progressed After Standard Therapy; refs. 31 and 32]. Based on an overall survival benefit observed in the phase III VELOUR trial [A Multinational, Randomized, Double-blind Study, Comparing the Efficacy of Aflibercept Once Every 2 Weeks Versus Placebo in Patients With Metastatic Colorectal Cancer (MCRC) Treated With Irinotecan/5-FU Combination (FOLFIRI) After Failure of an Oxaliplatin Based Regimen; ref. 33], aflibercept in combination with FOLFIRI (irinotecan, 5-fluorouracil, and leucovorin) was approved by the FDA and the European Medicines Agency for patients with colorectal cancer that is resistant to or has progressed following an oxaliplatin-containing regimen (34).

The molecular structure and mechanism of action of aflibercept is distinct from other antiangiogenic agents. Aflibercept is a fusion protein comprising the key domains of hVEGFR-1 and hVEGFR-2 fused to the Fc portion of IgG<sub>1</sub> (35). Aflibercept has a unique mechanism of action, neutralizing both hVEGF-A and -B and mouse VEGF-A (mVEGF-A) and -B, in addition to human and mouse PIGF (hPIGF, mPIGF), in contrast to bevacizumab,

which only binds to hVEGF-A (36). Aflibercept binds hVEGF-A with a greater affinity (50- to 100-fold higher) than bevacizumab and has a faster association rate. This translates into a more potent inhibition of VEGF-A-induced receptor activation and endothelial cell migration induced by aflibercept than bevacizumab (36).

To test the potential difference in activity between aflibercept and bevacizumab, we used 48 PDX colorectal cancer models. These are models in which engraftment of patient-derived tumors is performed directly into recipient mice, as opposed to injection of cultured cancer cell lines into the animals. PDX models have been shown to better mimic human disease in terms of histology, and genetic and molecular heterogeneity, as well as clinical response (37,38). The 48 models created were of colorectal cancer that originated from adenocarcinoma primary tumors or metastases to the liver, skin, lung, or other sites. After expansion, tumors were treated with either agent or vehicle control. In addition, the production of mVEGF-A, hVEGF-A, and mPIGF and hPIGF proteins was quantified prior in the control groups from whole tumor lysates to determine whether the human malignant cells or the murine host tissue or stroma were the major source of VEGF-A within the tumors.

## Materials and Methods

### *In vivo* evaluation

**Animal tumor models.** Colorectal cancer tumors were freshly excised from primary or metastatic sites of 48 patients and engrafted and passaged subcutaneously into female NMRI nu/nu mice (Harlan) at Oncotest, creating 48 PDX models. Only healthy animals were selected to enter testing procedures. All experiments were approved by local authorities, and were conducted according to all applicable international, national, and local laws and guidelines.

For each model, mice bearing tumors of 50 to 400 mm<sup>3</sup> were treated with aflibercept 25 mg/kg s.c. ( $n = 6-8$ ) in the nape of the neck, bevacizumab 25 mg/kg ( $n = 6-8$ ) i.v., or vehicle (aflibercept buffer) s.c. ( $n = 6-8$ ). Unless stated otherwise, mice received 6 treatments of aflibercept, bevacizumab, or vehicle 2 times per week for 3 weeks.

The dosing regimens of aflibercept and bevacizumab were chosen based on prior results generated in mice, showing the dose of aflibercept 25 mg/kg produced optimal VEGF blockade and antitumor activity and that aflibercept has a shorter circulating time in mice than antibody (35, 39, data not shown).

### Genomic characterization of PDX models

PDX tumors from each of the 48 models were subjected to genomic characterization (Supplementary Fig. S1). Fifty percent of the models (24/48 models) were *KRAS* mutant (75% of metastatic origin, 25% from primary tumors), 77% were *TP53* mutant (37/48 models: 86% of metastatic origin, 14% from primary tumors), 17% were *PIK3CA* mutant (7/48 models: 87.5% of metastatic origin, 12.5%

from primary tumors), and 8% were *BRAF* mutant (4/48 models: 50% of metastatic origin, 50% from primary tumors). These results are consistent with published reports comparing the genomic profiles of primary and mCRC tumors (40–43). Previous and independent studies have demonstrated through use of a panel of tumor models established from cell lines that the status of *KRAS*, *BRAF*, *PIK3CA*, and *PTEN* did not significantly influence the response to aflibercept (44, data not shown). Similar findings have been reported for bevacizumab (45).

**Tumor measurement.** Absolute tumor volume (ATV) was determined by 2-dimensional measurement with a caliper. Tumor volumes were calculated according to the equation  $(a \times b^2) \times 0.5$ , where "a" represents the largest tumor diameter and "b" represents the perpendicular tumor diameter. Median absolute deviation (MAD), defined as the median of the absolute deviations from the data's median, was calculated. Group median ATV values  $\pm$  MAD were then used for drawing growth curves. ATVs were also used for treatment evaluation. Changes in tumor volume for each treated (T) and control (C) group were calculated for each animal by subtracting the tumor volume on a specified observation day from the tumor volume on the day of first treatment (staging day). Median  $\Delta T$  was calculated for the treated group and median  $\Delta C$  for the control group. The ratio  $\Delta T/\Delta C$  was calculated and expressed as a percentage:  $\Delta T/\Delta C = (\text{median } \Delta T/\text{median } \Delta C) \times 100$ . The  $\Delta T/\Delta C$  values  $\leq 0\%$  were indicative of a complete stasis of the tumor or a decrease in tumor volume, respectively. Snap-frozen tumors were collected at the end of the treatment phase, that is, 24 hours after the final therapy or when tumors reached a large volume.

**Measurement of ligands and receptor.** Resected frozen tumors were placed into 2 mL Precellys tubes with ceramic beads (Bertin Technologies, Montigny Le Bretonneux, France). Lysis buffer (Triton 1%, NaCl 0.1 mol/L, Tris HCl, pH 7.4 10 mmol/L, EDTA 1 mmol/L, EGTA 1 mmol/L, NaF 1 mmol/L,  $\text{Na}_4\text{P}_2\text{O}_7$  20 mmol/L, glycerol 10%, 1 mmol/L Na vanadate and protease inhibitors) was added to each tube at approximately 1 mL per 150 mg of tumor. Tumors were mechanically disrupted on a Precellys 24 homogenizer (Bertin Technologies). Samples were centrifuged at 13,000 rpm for 15 minutes. Total protein in the supernatant was quantified using the Pierce BCA optical protein assay (Pierce and Warriner).

For the proteomic assessment of tumor lysates, 8 biologic replicates were analyzed for each model. In the reading plate, standards were run in triplicate and samples in duplicate. mVEGF and mPIGF were assessed using the ELISA DuoSet Kit from R&D Systems, Inc. Lysates were processed and analyzed as described in the manufacturer's instructions. Absorbance values were read using a microplate reader set to 450 nm. hVEGF and hPIGF were assessed using MILLIPLEX MAP angiogenesis panel I from EMD Millipore Corporation. hVEGFR-1 was assessed using Bio-Plex Pro Human Cancer Biomarker Panel 1 (Bio-Rad Laboratories, Inc.). For both methods,

samples were diluted in lysis buffer to obtain a concentration of 500  $\mu\text{g}/\text{mL}$  of total protein. Samples were processed and analyzed according to the manufacturer's instruction. The assays were read on the Bio-Plex Suspension Array System. The standard curves were optimized automatically by the software (Bio-Plex Manager) and verified manually. The Bio-Plex Manager software was used to calculate analyte concentrations and only measurements that showed a coefficient of variability of  $<20\%$  were included for further analysis.

### Statistical analysis

Tumor volumes are expressed as median  $\pm$  MAD. Statistical differences in final tumor volumes between treatment groups were evaluated using Microsoft Excel 2010. Comparisons between the changes in ATVs from initial tumor volume to the terminal endpoint ( $V_t - V_0$ ) were made using Student *t* tests.  $P < 0.05$  was considered statistically significant.

### Results

Of the tumor specimens that were implanted into mice, 47 of 48 were adenocarcinomas (Supplementary Table S1). Nine of the original patients' tumors were primary tumors, 39 were obtained from metastatic sites, and 1 from recurrent disease.

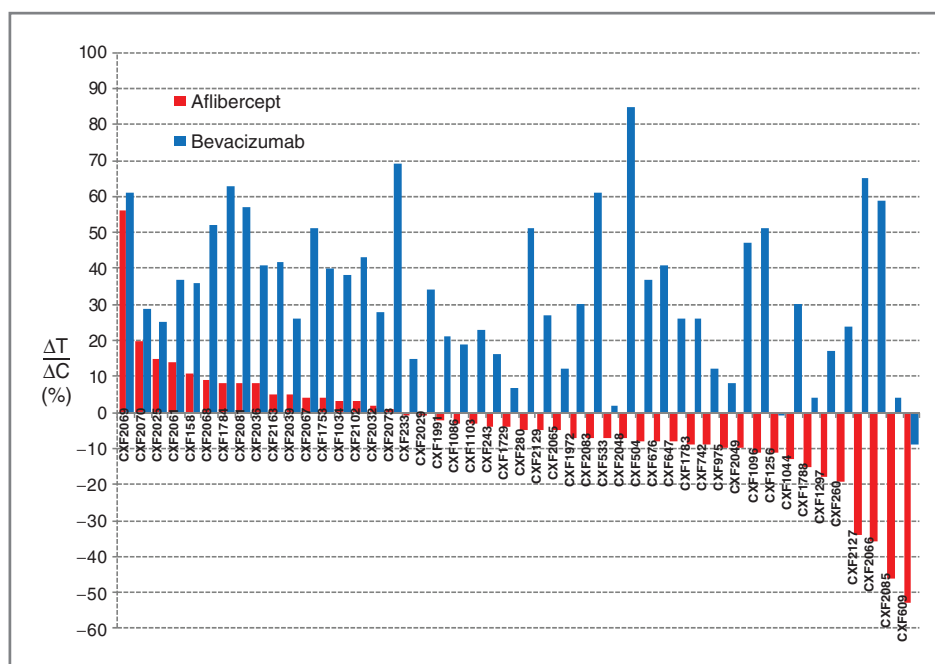
### Tumor growth inhibition studies

**Induction of complete tumor stasis.** One method to compare antitumor activity is induction of complete tumor stasis in PDX models. In these studies, the  $\Delta T/\Delta C$  values varied from 56% to  $-53\%$  for aflibercept and from 85% to  $-9\%$  for bevacizumab. Aflibercept induced complete tumor stasis in 31 of 48 PDX models ( $\Delta T/\Delta C \leq 0$ ) and bevacizumab in 2 of 48 PDX models (Fig. 1). Furthermore, aflibercept demonstrated complete tumor stasis in 15 of 31 PDX models that had activating mutations in *KRAS* (data not shown).

**Induction of tumor growth inhibition.** Another method to compare tumor growth inhibition induced by the 2 agents was to compare final tumor volumes ( $V_t - V_0$ ) after the 3-week treatment schedule in PDX models. For a given PDX model, differences in tumor volume between the aflibercept and bevacizumab groups were considered statistically significant when the *P* values by *t* test were  $<0.05$ . This analysis divided PDX models into 2 phenotypes: phenotype A, where tumor volumes were statistically different between the aflibercept and bevacizumab groups, and phenotype B, where there was no significant difference between the 2 treatment groups. Tumor growth curves representing phenotypes A and B are shown in Fig. 2.

**Phenotype A exhibited by 39 of 48 PDX models.** Model CXF2163 (Fig. 2) illustrates a subset (30/39) of phenotype A in which aflibercept and bevacizumab were both active. Aflibercept treatment resulted in final median tumor volume of  $324 \pm 60 \text{ mm}^3$ , representing a 79% tumor growth inhibition versus control ( $1,573 \pm 226 \text{ mm}^3$ ;  $P <$

**Figure 1.** Aflibercept induced complete tumor stasis more frequently in PDX models compared with bevacizumab. Tumor growth inhibition ( $\Delta T/\Delta C$ ) induced by aflibercept (red) and bevacizumab (blue) was plotted for 48 PDX models. Mice bearing subcutaneous patient-derived colorectal cancer tumors were treated with aflibercept (subcutaneous injection, 2 $\times$ /week, 25 mg/kg), vehicle (subcutaneous injection, 2 $\times$ /week), or bevacizumab (intravenous injection, 2 $\times$ /week, 25 mg/kg) for a total of 3 weeks. Tumor measurements were recorded twice per week. Relative changes of tumor volume in response to treatment were determined after the 3-week treatment schedule in 48 colorectal cancer PDX models.



0.05). In contrast, bevacizumab treatment resulted in a final median tumor volume of  $799 \pm 29 \text{ mm}^3$ , representing a 49% tumor growth inhibition versus control ( $P < 0.05$ ). Aflibercept tumor growth inhibition exceeded that of bevacizumab ( $P < 0.05$ ).

Model CFX2048 (Fig. 2) illustrates a subset (9/39) of phenotype A. Aflibercept treatment resulted in a final median tumor volume of  $122 \pm 20 \text{ mm}^3$ —a 69% tumor growth inhibition versus control ( $387 \pm 99 \text{ mm}^3$ ;  $P < 0.05$ ) on day 18 postrandomization. Bevacizumab treatment resulted in a final median tumor volume of  $352 \pm 142 \text{ mm}^3$ , which is not statistically significant compared with control ( $P > 0.05$ ) but significantly different from aflibercept ( $P < 0.05$ ). This illustrates the second subset of phenotype A in which aflibercept is active and bevacizumab is inactive.

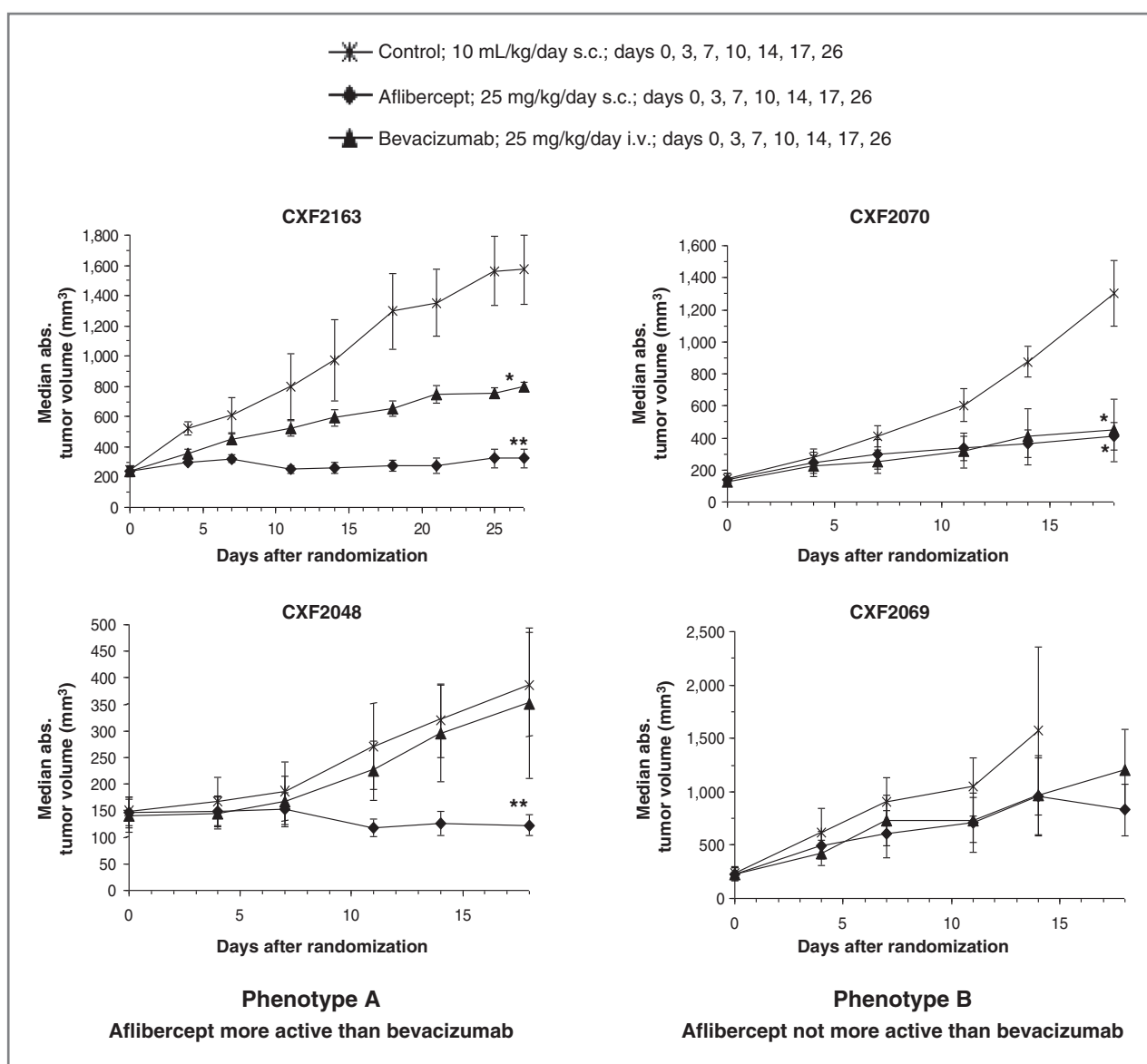
**Phenotype B exhibited by 9 of 48 PDX models.** In phenotype B, there was no statistically significant difference in tumor volumes between the aflibercept and bevacizumab groups. Among these 9 models, both drugs were active (i.e., statistically different from control) in 8 models and both drugs were inactive in only 1 model.

Model CXF2070 illustrates phenotype B in which both drugs were active (Fig. 2). Aflibercept and bevacizumab treatments resulted in final median tumor volumes of  $410 \pm 86 \text{ mm}^3$  and  $447 \pm 195 \text{ mm}^3$ , respectively, versus  $1,303 \pm 203 \text{ mm}^3$  in the control group. The activity of aflibercept and bevacizumab generated 68% and 66% of tumor volume, respectively, which were statistically significantly different from control ( $P < 0.05$  for both drugs) but not from each other ( $P \geq 0.05$ ). By contrast, in the CXF2069 model (phenotype B; Fig. 2), neither aflibercept nor bevacizumab resulted in a reduction in tumor growth compared with control (all  $P$  values  $\geq 0.05$ ). Tumor growth

curves and statistical data for all 48 PDX colorectal cancer models in which aflibercept and bevacizumab were evaluated are shown in Supplementary Figs. S2 and S3 and Table S2. There were no overt signs of toxicity or loss of body weight  $>10\%$  in any of the treatment groups in any of the models.

**Activity of bevacizumab not correlated with human and mouse VEGF-A ratio.** To investigate the mechanism of differential activity of aflibercept versus bevacizumab that was observed in the majority of the PDX colorectal cancer models, tumors from vehicle-treated mice were collected and analyzed for protein levels of mVEGF-A, the ligand that bevacizumab has no affinity for, and hVEGF-A, the ligand for both drugs. Tumor levels of hVEGF-A ( $776\text{--}56,039 \text{ pg/mg}$  of total protein) were  $\sim 16\text{--}1,777$ -fold greater than mVEGF-A ( $8\text{--}159 \text{ pg/mg}$  of total protein) in the PDX (Fig. 3A and B). In all 48 models, the tumor levels of hVEGF-A were in excess of mVEGF-A. In 80% of the models, the levels of hVEGF were more than 100-fold higher than mVEGF, indicating that human tumor cells are the major source of VEGF within the tumor mass. The ratio of human/mouse VEGF was not correlated with the activity of bevacizumab (Fig. 3C).

**PIGF and VEGFR-1 highly expressed in some PDX models.** To further explore the angiogenic properties of the PDX colorectal cancer models, the protein levels of mPIGF, hPIGF, and hVEGFR-1 in tumors were also measured. Tumor levels of mPIGF ( $104\text{--}1,837 \text{ pg/mg}$  of total protein) were higher than hPIGF ( $0\text{--}543 \text{ pg/mg}$  of total protein) in 47 of 48 models (Fig. 4A). A high level of PIGF (mPIGF combined with hPIGF)  $>521 \text{ pg/mg}$  was observed in 25% of PDX models. This threshold corresponds to PIGF level higher than concentration observed in nontumoral and tumoral breast cancer

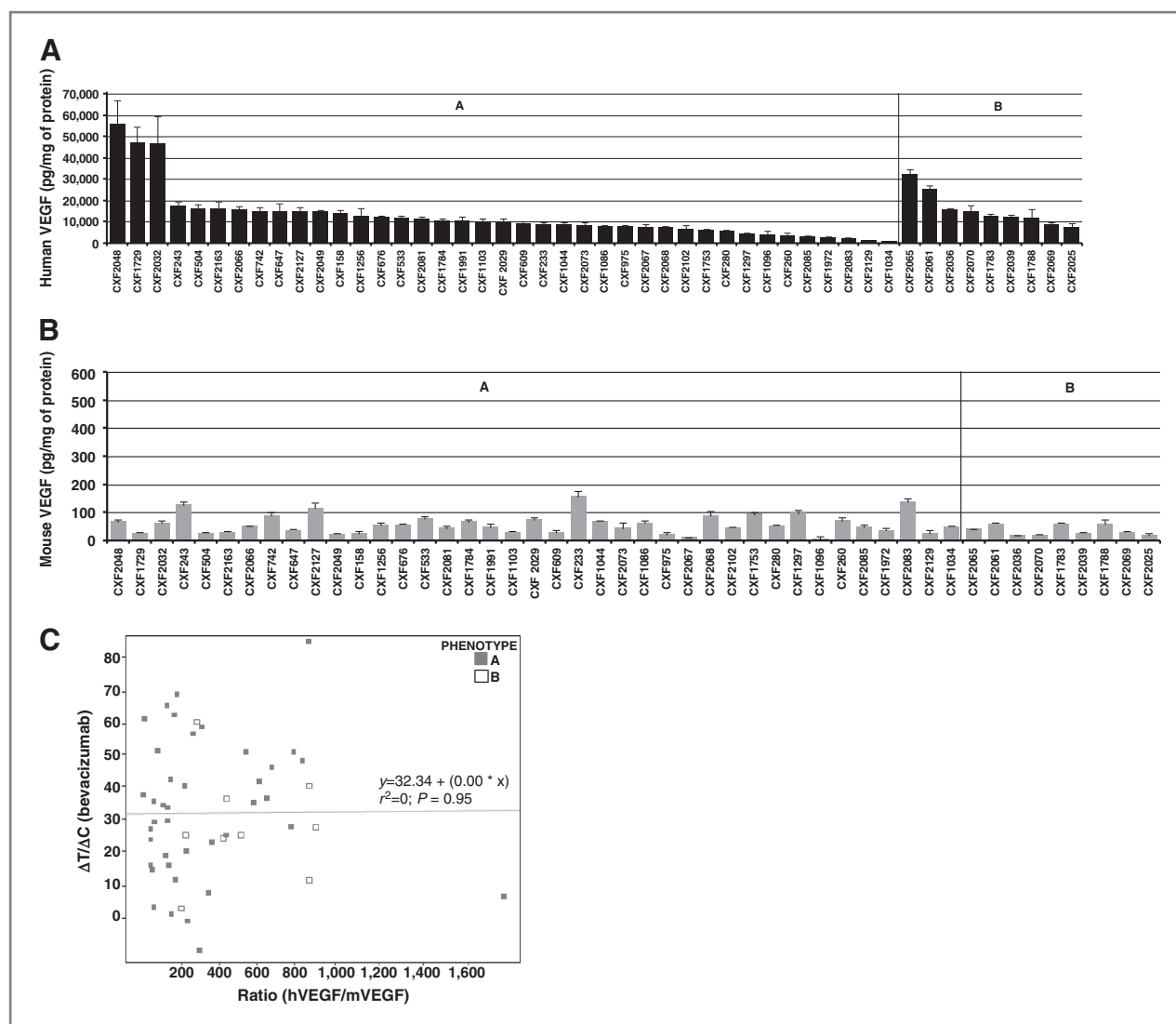


**Figure 2.** Tumor growth inhibition induced by aflibercept occurred more frequently in PDX models compared with bevacizumab. Tumor growth curves were plotted for aflibercept, bevacizumab, or control groups during the 3-week treatment period. For a given PDX model, differences in tumor volumes between aflibercept and bevacizumab were considered statistically significant when  $P$  values by pairwise  $t$  test were  $<0.05$ . This analysis divided the PDX models into 2 phenotypes: phenotype A illustrated by CXF2163 and CXF2048 models, where tumor volumes were statistically different between aflibercept and bevacizumab groups (39/48 models), and phenotype B illustrated by CXF2070 and CXF2069 models, where there was no significant difference between the 2 groups (9/48 models). \*, tumor growth different from control ( $P < 0.05$  by  $t$  test). \*\*, tumor growth different from bevacizumab ( $P < 0.05$  by  $t$  test). Error bars represent MAD.

tissues (median of 31 and 70 pg/mg, respectively; ref. 17) and above the range of concentration measured in placenta (median of 300 pg/mg) considered as a PlGF high expressing tissue. In the PDX models characterized as phenotype A (aflibercept activity  $>$  bevacizumab activity), 12 of 39 models had a combined mPlGF and hPlGF content of  $>521$  pg/mg. mPlGF and hPlGF levels were  $<521$  pg/mg in the 9 models categorized as phenotype B where there was no differential activity. Although these differences between phenotype A and B models are not

statistically significant, they suggest a trend. The highest levels of hVEGFR-1, the receptor for both VEGF-A and PlGF, were observed in the models of phenotype A (Fig. 4B). However, when statistical analysis was applied, there was not a single ligand or receptor that could significantly be associated to a phenotype or that could explain the difference of response to both agents; thus, these results should be viewed as hypothesis generating.

The patient-derived tumors that were used to generate the xenograft models were either primary or metastatic



**Figure 3.** Antitumor activity of bevacizumab in PDX models was not correlated with the ratio of human and mouse VEGF-A of the models. Protein levels of hVEGF-A (A) and mVEGF-A (B) were measured in 6 to 8 tumors from each of the 48 PDXs. Models were classified in 2 phenotypes (A and B). Phenotype A represents models in which aflibercept was active and bevacizumab was not active, whereas phenotype B represents models in which the activity of aflibercept and bevacizumab were similar. In both A and B, models were ranked according to hVEGF-A concentration. Error bars, standard error of the mean. C, scatter plot of hVEGF-A/mVEGF-A against tumor growth inhibition ( $\Delta T/\Delta C$ ) for bevacizumab. The ratio hVEGF:mVEGF was not correlated with tumor growth inhibition expressed as  $\Delta T/\Delta C$ . Each point represents a different PDX model. This representation depicts the entire 48 colon PDX collection classified as phenotype A or B.

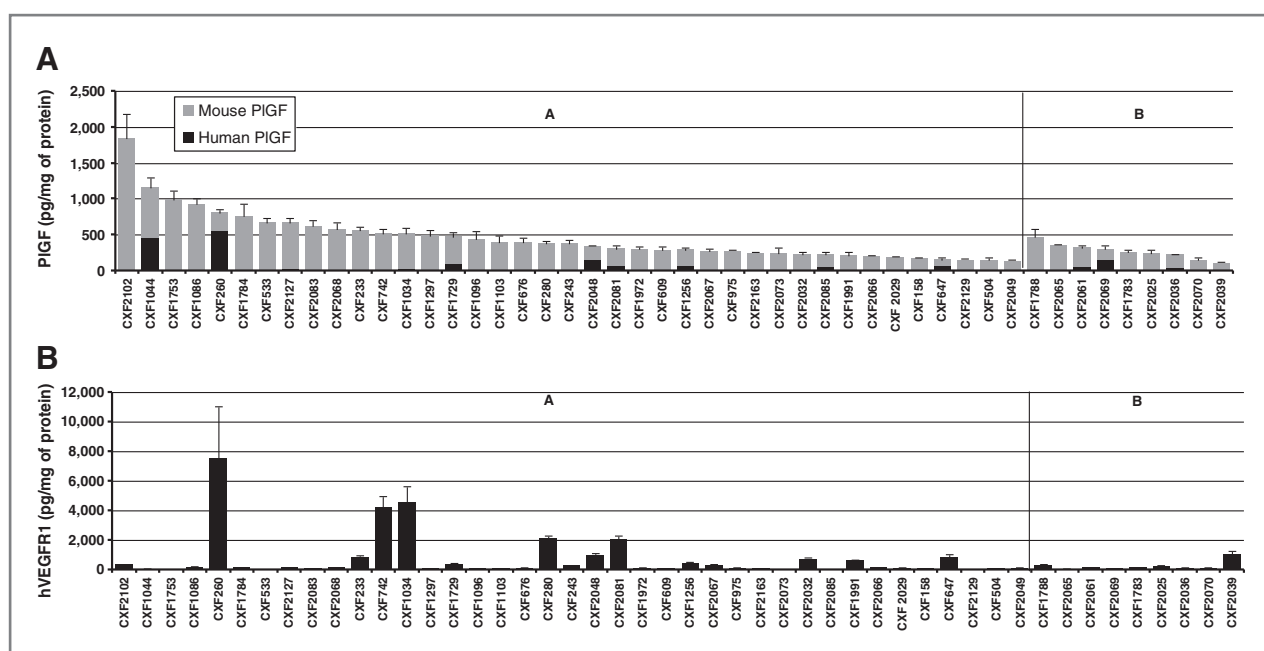
tumors (Supplementary Table S1). In examining the distribution of the 48 colorectal cancer PDXs, we observed that all the primary tumors (8/8) belonged to phenotype A (aflibercept activity > bevacizumab; Supplementary Fig. S4). However, the lower number of tumors from primary versus metastatic sites precludes the ability for a more rigorous analysis.

## Discussion

The activity of aflibercept and bevacizumab in preclinical models of colorectal cancer was compared across a spectrum of 48 colorectal cancer PDX models, which are

clinically relevant to this indication based on the mutational status of the tumors and derivation of the xenografts from colorectal cancer specimens of both primary tumors and metastases from several anatomical sites. These models are relevant to patient response not only because they represent the underlying genetics of mCRC but also because the responses were not driven by mVEGF.

Results indicate that aflibercept was more active in a greater number of colorectal cancer PDX models than bevacizumab. Based on statistical differences of the tumor volumes at endpoint, the activity of aflibercept was greater than that of bevacizumab in 39 of 48 models (phenotype A). Antibodies such as bevacizumab have longer



**Figure 4.** PIGF and VEGFR-1 were highly expressed in some PDX models. Protein levels of human and mouse PIGF (A) were measured in all 48 PDXs by Luminex technology and ELISA. Sum of hPIGF (black) and mPIGF (gray) is represented as stacked histogram. Protein levels of hVEGFR-1 (B) were measured by Luminex technology. Models were classified in 2 phenotypes (A and B). Phenotype A represents models in which aflibercept was active and bevacizumab was not active, whereas phenotype B represents models in which the activity of aflibercept and bevacizumab were similar. In both A and B, models were ranked according to total human + mouse PIGF concentration. Error bars, standard error of the mean.

circulation times in mice than Fc fusion proteins and aflibercept in particular (35); therefore, exposure to bevacizumab should be equivalent or superior to aflibercept exposure in mice. Even so, the activity of bevacizumab was less than that of aflibercept in these models. This is consistent with other preclinical findings showing aflibercept to be more potent than bevacizumab in several types of preclinical studies (36, 44, 46).

Xenograft tumors consist of human malignant cells and host stromal and inflammatory cells. Our evaluation of the angiogenic output of VEGF-A and PIGF by both the human and murine components of the tumors revealed several key findings. The first was that the protein levels of hVEGF greatly exceeded mVEGF; this is relevant as hVEGF is the target for both aflibercept and bevacizumab. In addition, the result suggests that in these colorectal cancer PDX models, the tumor growth is driven more by the VEGF-A produced by the malignant cells rather than the stromal cells. Furthermore, the enhanced relative activity of aflibercept relative to bevacizumab could not solely be attributed to mVEGF-driven angiogenesis given that hVEGF levels were in vast excess. One caveat is that these levels were measured at the outset of tumor growth and the relative levels may have changed during the course of treatment. Ideally, the studies should be performed using anti-VEGF antibodies that inhibit both stroma- and tumor-derived VEGF to more closely mirror the clinical situation.

The levels of hVEGF-A were also greater than those of mPIGF and hPIGF—ligands for VEGFR-1, and to which

aflibercept, but not bevacizumab, binds. With rare exception, the protein levels of mPIGF were greater than hPIGF, indicating that, unlike the production of VEGF-A, the stromal compartment plays a critical role in the development of the colorectal cancer PDX tumors. The applicability of VEGFR-1 and PIGF as biomarkers for aflibercept has not yet been determined; analysis of this is ongoing in both preclinical models and clinical samples. However, it is our observation that the colorectal cancer PDX models that were more sensitive to aflibercept trended toward higher levels of VEGFR-1 and total PIGF. For example, colorectal cancer PDX models (CXF742, CXF1034, CXF260) with highest levels of VEGFR-1 ( $\geq 4,000$  pg/mg of protein) also had high levels of total PIGF ( $\sim 500$  pg/mg of protein), suggesting autocrine signaling in the malignant cells (24). Thus, the ability of neutralizing PIGF could allow for inhibition of both malignant and stromal cells. This concept is supported in part by preclinical results generated by Yao and colleagues (24), demonstrating that the greater efficacy of neutralizing PIGF was observed in xenograft models in which the tumor more strongly expressed VEGFR-1. Elevated serum levels of PIGF before disease progression have been observed in mCRC and renal cancer patients treated with bevacizumab and other antiangiogenic compounds, implicating upregulated PIGF as a possible resistance mechanism (18, 21, 47). The ability to predict when to switch to an alternative antiangiogenic therapy before a patient's disease progresses could be valuable in optimizing the sequential dosing involving aflibercept.

Hypothesizing clinical outcome from preclinical models remains an ongoing effort in the field of oncology to allow further investigation and validation in clinical studies. Proper selection and characterization of preclinical models may not only provide guidance for identifying the patient population that is most likely to benefit from a given therapy but also for selection of the therapeutic agent that will provide the most benefit when more than 1 option is available. The strategy of incorporating PDXs into the drug development scheme rather than relying solely on traditional xenografts that use cancer cell lines grown in culture is expected to be a step forward in bridging the gap from lab bench to bedside. Preclinical studies have demonstrated that the response of PDXs to standard of care better mimics clinical outcome to therapies than do traditional xenografts. This may be attributed to stromal involvement, molecular and genetic heterogeneity, and lack of clonal selection that occurs from long-term passaging of cells *in vitro* (48, 49).

However, the activity of aflibercept in 43 of 48 (89.6%) of the colorectal cancer PDXs utilized here suggests that the use of aflibercept therapy in combination with chemotherapy may result in different clinical outcomes than that of bevacizumab. Furthermore, as many of the xenograft tumors were derived from mCRC tumors, there is the suggestion that aflibercept may have activity in retarding the progression of later-stage disease. Also, as aflibercept demonstrated robust activity in colorectal cancer xenografts in the absence of chemotherapy, the concept of a single-agent, antiangiogenic regimen as

"maintenance" therapy post-chemotherapy merits clinical investigation (50).

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Authors' Contributions

**Conception and design:** M. Chiron, R.G. Bagley, J. Pollard, P.K. Mankoo, L. Vincent, N. Baltes, D.A. Bergstrom

**Development of methodology:** M. Chiron, P.K. Mankoo, C. Henry, C. Geslin, N. Baltes

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** C. Henry, C. Geslin, N. Baltes

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** M. Chiron, R.G. Bagley, J. Pollard, P.K. Mankoo, C. Henry, L. Vincent, C. Geslin, D.A. Bergstrom

**Writing, review, and/or revision of the manuscript:** M. Chiron, R.G. Bagley, J. Pollard, P.K. Mankoo, C. Henry, N. Baltes, D.A. Bergstrom

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** M. Chiron, P.K. Mankoo, C. Henry, N. Baltes

**Study supervision:** M. Chiron, P.K. Mankoo, C. Henry, N. Baltes

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