Original Article
Optimizing dosage regimen for FP3 based on vascular normalization window monitored by ratio of Ang-1 to Ang-2

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Abstract: Vascular normalization explained the successful efficacy of combined antiangiogenic and cytotoxic therapy. The optimal dosage regimen of anti-VEGF therapy to achieve a maximized efficacy and a minimized toxicity response needs further investigations based on vascular normalization monitoring. FP3 (also referred to as Conbercept, KH902 or Fusion protein III, developed by KanghongBiotechnology, China) is a novel anti-VEGF agent, which has been demonstrated to have a stable effect on antiangiogenesis as well as on vascular normalization. A patient-derived colorectal cancer xenograft model was established. Dose-escalation study of FP3 (7.5, 15, 30 and 60 mg/kg) combined with CPT-11 was performed to discover the optimal dosage regimen. Serum Ang-1 and Ang-2 expression were detected by ELISA. A potential correlation between drug responses and Ang-1/Ang-2 ratio were analyzed. The PDX model was evaluated as FP3-sensitive. FP3 (15 mg/kg, i.v.qw) combined with CPT-11 was found to be the optimal dosage regimen. All dosages of FP3 groups showed an ascending Ang1/Ang2 ratio after drug administration, while ascending velocity was less significant in 7.5 and 15 mg/kg FP3 groups than that in 30 and 60 mg/kg FP3 groups. The Ang1/Ang2 ratio was shown somewhat higher in 15 mg/kg FP3 compared with that in 7.5 mg/kg FP3 group despite of no statistical significance. In our study, the optimal dosage regimen for FP3 in combination with CPT-11 was discovered. The ratio of Ang-1/Ang-2 was demonstrated as an effective surrogate maker for vascular normalization, although further confirmations are needed.

Keywords: Patient-derived xenograft model, colorectal cancer, vascular normalization, anti-VEGF therapy, optimal dosage regimen, Ang-1/Ang-2 ratio

Introduction

Several clinical trials have demonstrated the clinical benefits of anti-angiogenic agents on cancer over the past few years [1, 2]. Vascular endothelial growth factor-A, (VEGF-A, usually named as VEGF), an important proangiogenic growth factor, is known to play an important role in angiogenic processes through both direct and indirect mechanisms [3]. VEGF inhibitors, including anti-VEGF monoclonal antibodies (Bevacizumab), VEGF-binding proteins (such as Aflibercept), and VEGFR tyrosine kinase inhibitors (such as Regorafenib), have been validated effective at inhibiting angiogenesis in many tumors [4, 5]. Bevacizumab conferred survival benefit as a combination therapy (although not for monotherapy) [6-8]. However, it seems paradoxical that destroying the vasculature would severely depress the delivery of oxygen and agents to the solid tumor. Jain RK firstly raised the potential hypothesis for the success of combined therapies that anti-VEGF therapies "normalized" the architecture and function of existing vasculature, resulting in enhanced delivery of concurrently administered drugs [9-11]. The normalization window, defined as a period of time when blood flow and oxygen-supplying transitorily increases, was dose and time dependent [12]. Therefore, more studies regarding the degree and length of vascular normalization window will be critical for optimiz-
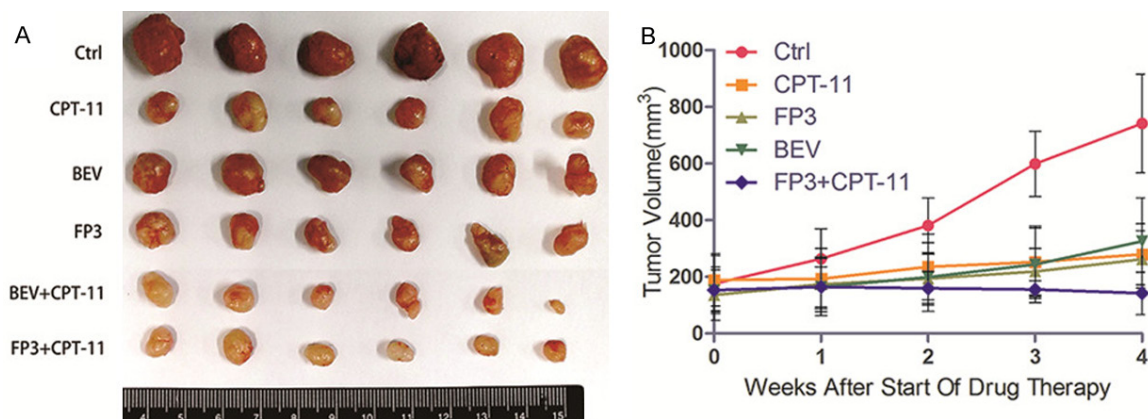
Figure 1. Efficacy evaluation of FP3 based on a colon cancer PDX model. A. Anti-tumor-growth ability evaluation by endpoint tumor volumes showed the sensitivity of the PDX model to both single FP3 and combined with CPT-11 treatment. B. Response curve of FP3 in the PDX model of colorectal cancer. Anti-tumor-growth ability of single FP3 and FP3 in combination with CPT-11.

Table 1. Average TGI of multisteptreatment groups in FP3 preliminary evaluation (%)

CPT-11	67.50461794
FP3 (20)	59.43733925
BEV (20)	46.67878413
FP3 (20)+CPT-11	80.75035137
BEV (20)+CPT-11	77.00048396

ing the efficacy of combined anti-VEGF and cytotoxic therapy.

One of the challenges is to identify suitable surrogate markers for monitoring changes in the architecture and function of the vasculature [9]. Angiopoietin-1 (Ang-1) and its natural antagonist angiopoietin-2 (Ang-2) are among the leading growth factors involved in the maturation, maintenance, and remodeling of the tumor vasculature [13, 14]. Although the regulatory effect of Ang-1 and Ang-2 on tumor angiogenesis remains controversial, increasing studies have shown that Ang-1/Ang-2 ratio is related with the balance of pro- and antiangiogenic processes in most malignancies [15, 16]. Vascular normalization will occur when the imbalance of pro- and antiangiogenic molecules has been corrected [9]. The normalized vasculature appears as less tortuous and less dilated vessels, covered by pericytes more widely [6, 17]. The main producer of Ang-1 is pericytes, whereas Ang-2 is produced predominantly endothelial cells [18]. Therefore, the upregulation and balance of Ang-1/Ang-2 ratio, indicating more extensive pericytes coverage, might be potential surrogate markers for vascular normalization.

Patient-derived xenografts (PDXs), so-called Avatar models [19], have been increasingly widely used for cancer research in recent years, with the greatest advantage of its ability to better predict clinical tumor response [20]. Accumulating evidences indicate that PDX is an reliable cancer research tool for understanding of mechanisms of drug resistance, drug screening and personalized medicine applications [21]. [Aparicio S, 2015 #21; Chen W, 2016 #89] APDX model which was relatively sensitive to anti-VEGF therapies will be a priority selection for vascular normalization research.

A Novel VEGF-trapFP3 (also referred to as Conbercept, KH902 or Fusion protein III), which is engineered by fusing the 2nd extracellular domain of Flt-1 (VEGF receptor 1) and the 3rd and 4th extracellular domain of KDR (VEGF receptor 2) to the Fc portion of human immunoglobulin G1 [22, 23]. In the previous studies, our research group had showed that FP3 has an antitumor efficacy in PDXs of gastric carcinoma and colorectal cancer [23-26], as well as an effect in normalizing vasculature [27].

In this study, we established a patient-derived colorectal cancer xenograft model, which was demonstrated as FP3-sensitive, thus reliable for vascular normalization research. Different drug responses were compared among groups established in line with multistep dosages scheme of FP3 (7.5, 15, 30 and 60 mg/kg) combined with CPT-11 groups, and ELISA expressions of Ang1 and Ang2 were evaluated at different time points, such as 0, 7, 14, 21 and 28 days after treatment initiation. A rela-
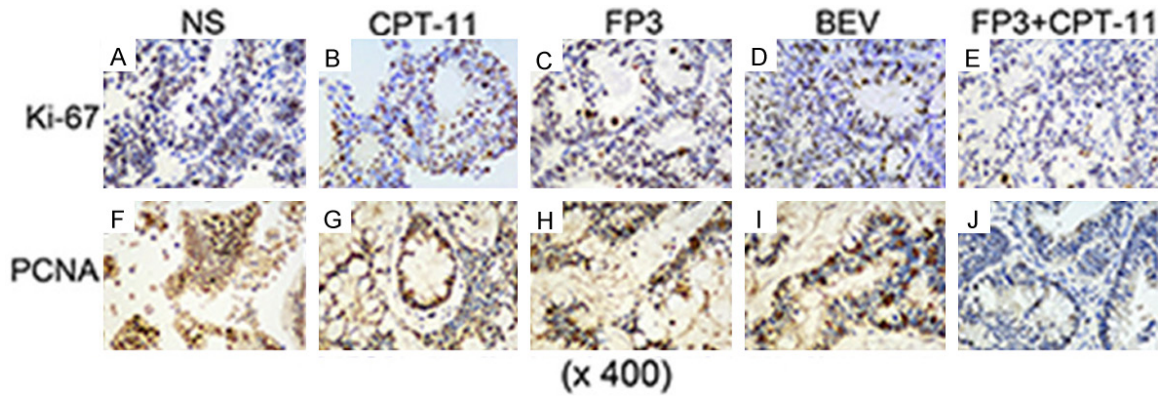
Figure 2. Immunohistochemical expressions of ki-67 and PCNA to evaluate the ability of anti-tumor-growth in different treatment groups.
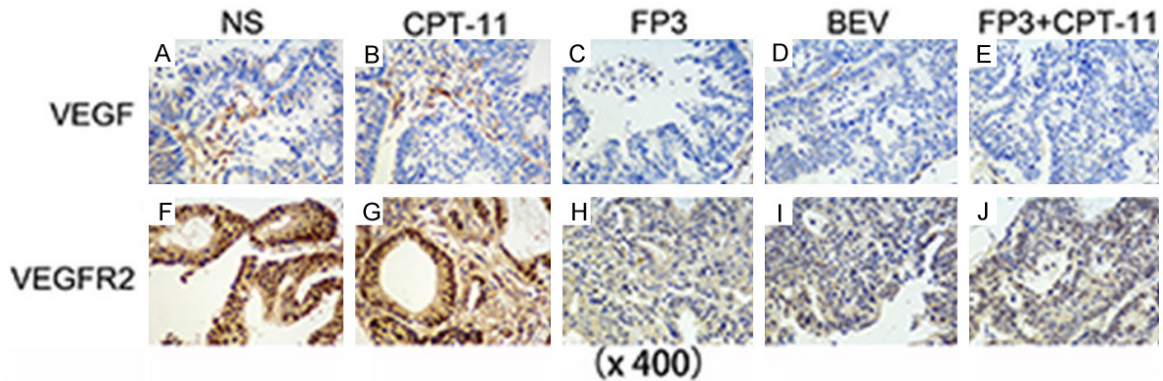

Figure 3. Immunohistochemical expressions of VEGF and VEGFR2 to evaluate the ability of anti-angiogenesis in different treatment groups.
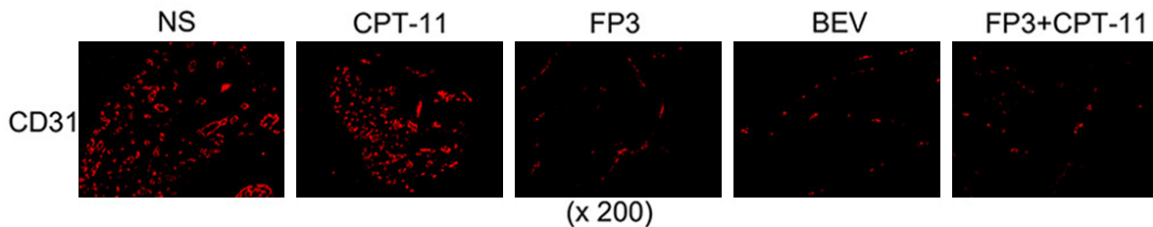

Figure 4. Vasculature density changes examined by angiography with immunostaining for endothelial cells (using anti-CD31 antibody; Magnification=200).

tionship was explored between the drug responses and serum Ang1/Ang2 expressions.

Materials and methods

Reagents and drugs

FP3 was provided by Kanghong Biotechnology, Inc. BEV (bevacizumab) was kindly provided by Department of Chemotherapy, the 1st Affiliated Hospital, School of Medicine, Zhejiang University. CPT-11 (Irinotecan HClT rihydrate) was purchased from Dalian Melun Biology Technology Co. The antibodies against CD31, α-SMA, VEGF, VEGFR2, ki67, PCNA were purchased from Abcam.

Patient and tumor tissues

Colon Tumor (diagnosed as mucinous adenocarcinoma, T3N0M0) tissues were obtained at surgery from a 55-y-old female patient, without
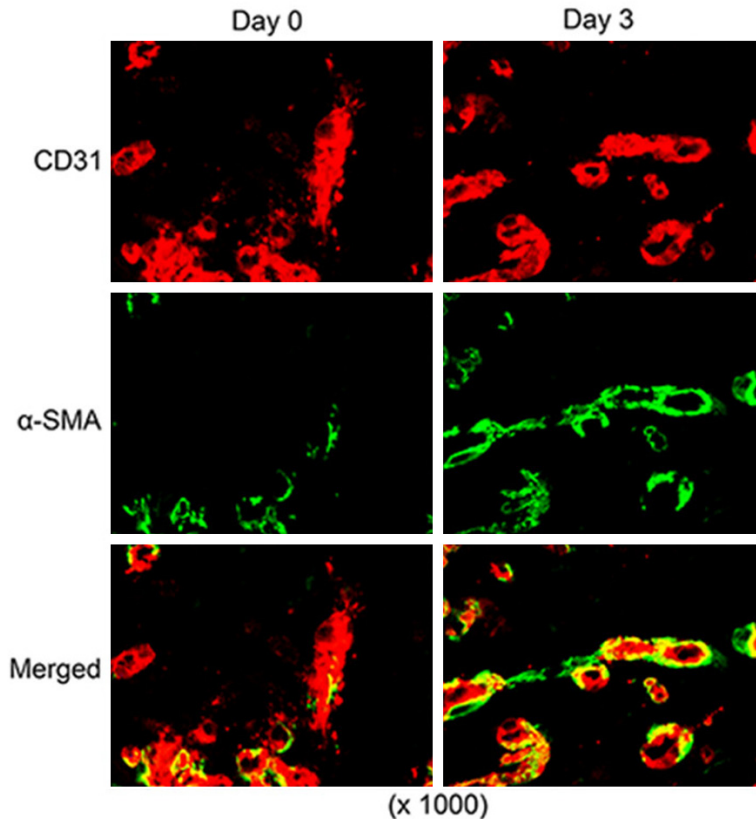
Figure 5. Normalizdvasculature examined by angiography with immunostaining for endothelial cells (using anti-CD31 antibody; magnification=1000) and pericytes (using anti-α-SMA antibody; magnification=1000).

diameter respectively. Tumors were then harvested, minced and re-implanted as described above for passaging. At each generation, tumors were harvested and stored in liquid nitrogen for further use. The usage of experimental animals was according to the Principles of Laboratory Animal Care (NIH #85-23, 1985 version). All animal studies were according to the Institutional Animal Care and Use Committee of Zhejiang University, and the approval ID was SYXK(ZHE)2005-0072.

Treatment protocol

From the 3rd generation, PDX tumors were permitted to grow to a volume of 150-200 mm^3, then mice were randomized (6 mice with tumors per group and housed in per rearing cage; While 10 mice with tumors per group in the dose-escalation study of FP3 combined with CPT-11, among which 5-6 mice per group were needed for serum for ELISA examination). Then dosing was administrated by intravenous injection once per week for FP3 or BEV/bevacizumab and by intraperitoneal injection once per week for CPT-11/Irinotecan HCl Trihydrate (dosage details were shown in the Results section) for 4 weeks. Mice were weighed for signs of toxicity and tumor size was evaluated once per week. TGI (Relative tumor growth inhibition) was calculated using the following formula: (1-T/C)%, where T means the relative tumor volume of the treated mice, and C means the relative tumor volume of the control mice.

Immunofluorescence

Selected mice with similar tumor volume were anesthetized with chloral hydrate (5%, 0.2 ml/20 g) injected intramuscularly on day 30 (while on day 0 and 3 for observation of tumor vascular normalization after FP3 treatment). The vasculature was perfused with 4% paraformaldehyde. Then, xengraft tumor was harvested and stored in fixative for 2 hours at 4°C.

radio chemotherapeutic treatment before surgery. Informed consent was signed by the patient, while the study was according to the ethics board approval of the 1st Affiliated Hospital, School of Medicine, Zhejiang University.

Establishment of PDX model

BALB/c nude mice (3-to-4-week-old, female) were purchased from Shanghai Slaccas Laboratory Animal and housed in SPF laboratory animal rooms at laboratory animal center of Zhejiang University. Mice were acclimated to new environments for at least 3 days before use. Surgical tumor tissues were cut into pieces of 3 to 4 mm and transplanted within 30 min s.c. to mice. Additional tissues were snap-frozen and stored at -80°C until use. Animals were monitored periodically for their weight with an electronic balance and tumor growth with a Vernier caliper twice every week. The tumor volume was calculated as formula V=LD × (SD)^2/2, where V represents the tumor volume, LD and SD are the longest and the shortest tumor
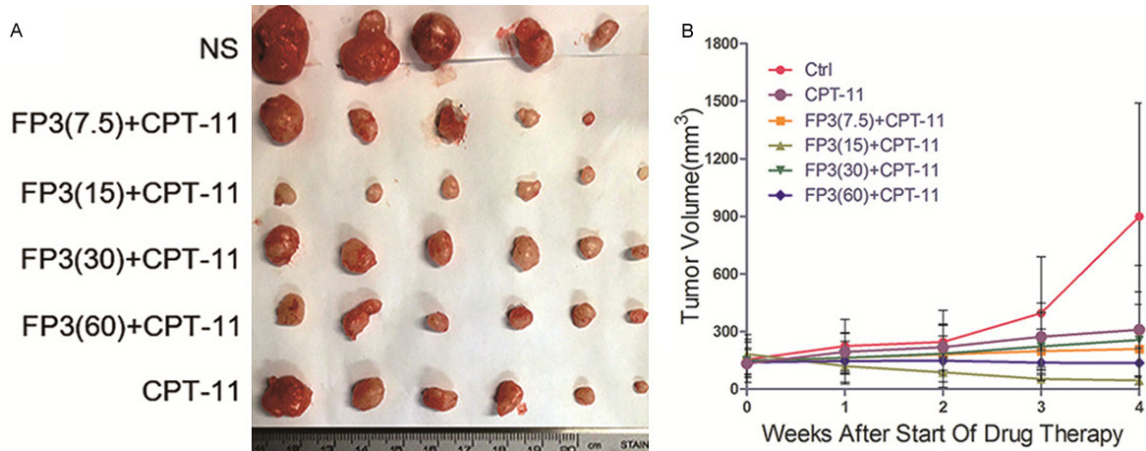
Figure 6. Discovery of the optimaldosage regimen forFP3 combination therapy. A. Anti-tumor-growth ability evaluation by endpoint tumor volumes showed multistep dosages of FP3 combined with CPT-11 groups. B. Response curve of multistep dosages of FP3 combined with CPT-11.

Table 2. Average TGI of multistepFP3 dosages in combination with CPT-11 (%)

FP3 (7.5)+CPT-11	75.24117216
FP3 (15)+CPT-11	95.72920319
FP3 (30)+CPT-11	68.70258714
FP3 (60)+CPT-11	83.23096142
CPT-11	60.04704447

After PBS rinse and infiltration with 30% sucrose overnight, tissues were embedded in OCT and then frozen for cryostat sectioning. Then, the cryostat sections were fixed by acetone for 10 min. After that, slides were washed in PBS and dried for several times. After blocking nonspecific antibody binding, two primary antibodies (CD31 and α-SMA) were added on the slides overnight at room temperature. The signal was amplified for one hour with fluorescent secondary antibodies. All slides were counterstained with DAPI (Invitrogen). Tissue sections were photographed using Olympus BX51 Fluorescence Microscope.

Immunohistochemistry

Specimen were fixed by 10% neutral formalin, then embedded in paraffin, sectioned (5 μm thick) and placed on slides for marker analysis. Sections were incubated with the primary antibodies overnight at 4°C, after blocking nonspecific antibody bindings. The streptavidin-biotin peroxidase complex method (Lab Vision) was used for Immunohistochemistry. The slides were photographed using an Olympus BX60 (Olympus).

Enzyme-linked immunosorbent assay (ELISA)

Xengrafts tumor serum was obtained from CPT-11 combination treatment groups at different time points during treatments (details were shown in the results section). Concentration of serum Ang1 and Ang2 were evaluated by ELISA according to the manufacturer's protocol (Multi Sciences Biotech).

Statistical analysis

Results were presented as mean ± SD. Calculation and statistics were performed with Excel 2010 (Microsoft) and GraphPad Prism 5 (GraphPad Software). One-way ANOVA were used to analyze the significance of differences among groups. $P<0.05$ was considered statistically significant.

Results

APDX model reliable for vascular normalization study

To test whether the CRC PDX model we established were sensitive to the anti-VEGF therapies, anti-tumor-growth ability of FP3 were firstly evaluated. Since tumors volume reached 150-200 mm^3, injections of FP3 (20 mg/kg), BEV (bevacizumab, 20 mg/kg), CPT-11 (Irinotecan, 5 mg/kg) and saline were given i.v. once
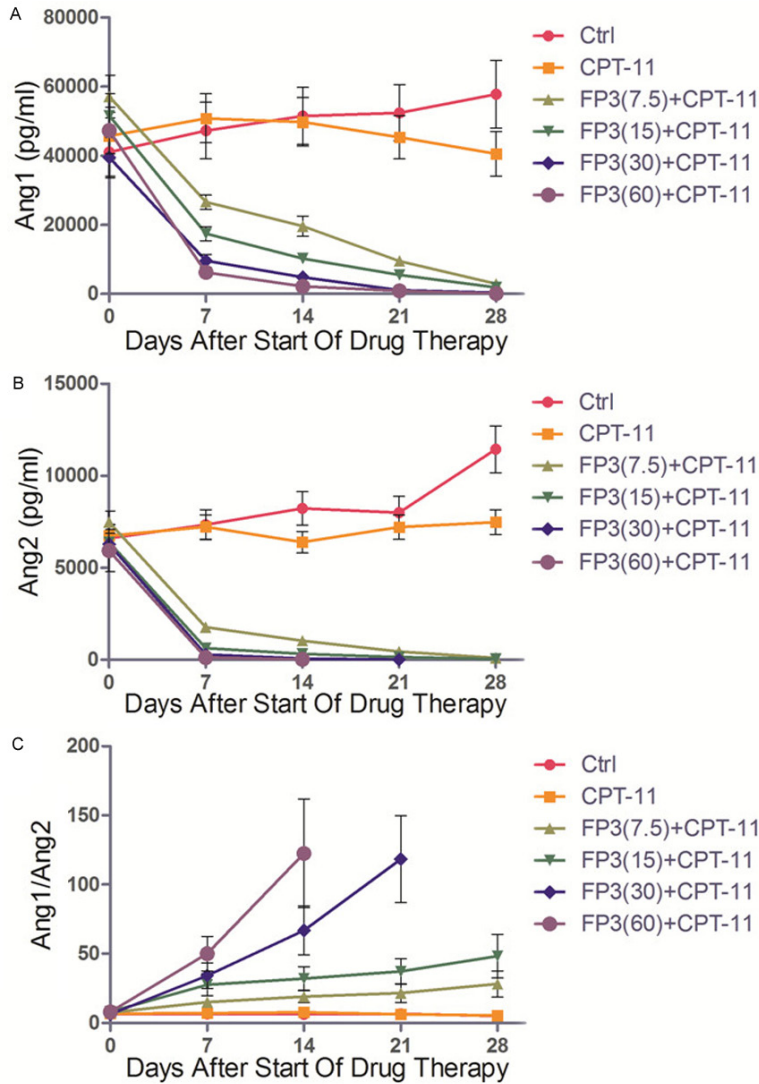
Figure 7. ELISA results of serum Ang1 and Ang2 on day 0, 7, 14 and 28 in multistep dosages of FP3 combined with CPT-11.

Then, anti-angiogenesis ability of FP3 was evaluated by immunohistochemical expressions and immunofluorescence of CD31. In both single FP3 group and FP3 combined with CPT-11 group, VEGF/VEGFR2 expressions and vasculature density were significantly suppressed (**Figures 3** and **4**).

Further, normalized vasculatures were investigated by immunofluorescence. Vascular normalization was observed on the third day since FP3 injection. The normalized vasculature appears as less tortuous and less dilated vessels, covered by pericytes more widely (**Figure 5**).

Discovery of the optimal dosage regimen forFP3 combination therapy

To discover the optimal therapeutic scheme for FP3, antitumor efficacy of FP3 were compared among groups established in line with multistep dosages scheme of FP3 (7.5, 15, 30 and 60 mg/kg) combined with CPT-11 (5 mg/kg). In the CPT-11 combination groups, the best effect of FP3 were observed in 15 mg/kg FP3 group (P<0.05, compared with 7.5 or 30 mg/kg groups; P>0.05, compared with 60 mg/kg group) (**Figure 6**, TGI values were shown in **Table 2**).

To evaluate the toxicity response, we compared mice weight among groups. No significant different loss of weight was found in multistep dosages scheme of FP3in combination with CPT-11 (Supplementary Figure 1).

Variation of vascular normalization along with dose and time changes

To investigate whether the optimal therapeutic scheme forFP3 were based on the different degree or window length of vascular normaliza-

per week for 28 days. Harvested tumors were measured. Then, TGI (relative tumor growth inhibition) was calculated as per the following formula: (1-T/C)%. We found that the TGI of single FP3 treatment on this model reached 59.4% (P<0.05), slightly higher than single BEV group, though without statistical significance (**Figure 1**; **Table 1**). For a combination therapy with FP3 and CPT-11, the PDX model was also evaluated as sensitive. The FP3 combined with CPT-11 treatment received a better tumor inhibition (TGI=80.8%) effect than eiher single FP3 or CPT-11 (**Figure 1**). Suppressed expressions of both ki-67 and PCNA were seen in the single FP3-treated group and more significantly in FP3+CPT-11 group (**Figure 2**).

Table 3. ELISA results of serum Ang1 and Ang2 in different treatment groups (n=3)

	pg/ml	Day 0	Day 7	Day 14	Day 21	Day 28
	Ang1	41032.12±6954.59	47250.5±8208.56	51438.58±8238.4	52390.8±8109.79	57774.68±9792.32
Ctrl	Ang2	6588.54±732.06	7337.43±815.27	8218.74±913.19	7989.63±887.73	11431.82±1270.2
	Ang1	45723.66±6824.43	50792.23±7080.93	49729.72±7022.35	45289.1±6259.57	40523.28±6448.25
CPT-11	Ang2	6729.37±611.76	7213.17±655.74	6390.42±580.94	7202.45±654.76	7471.03±679.18
	Ang1	57081.56±6204.52	26544.89±2085.31	19590.12±2929.36	9491.62±1211.69	2920.67±337.46
FP3(7.5)+CPT-11	Ang2	7474.04±607.65	1767.53±143.7	1025.27±83.35	439.53±35.73	103.53±8.41
	Ang1	51580.32±6367.94	17406.05±2048.89	10204.49±1212.81	5487.26±607.44	1820.67±214.77
FP3(15)+CPT-11	Ang2	6398.04±457.01	586.26±45.14	318.03±22.71	147.31±10.52	37.72±2.69
	Ang1	39482.71±5892.94	9539.39±1923.79	4820.18±710.43	1308.33±159.83	331.04±45.41
FP3(30)+CPT-11	Ang2	6271.24±591.63	278.27±26.25	92.037±6.79	18.76±0.82	n.d.
	Ang1	47241.82±6756.55	6203.82±717.27	2189.25±354.11	928.78±112.85	131.04±15.74
FP3(60)+CPT-11	Ang2	5928.4±1140.08	123.75±23.79	17.86±3.43	n.d.	n.d.

Data were presented as mean ± SD. Abbreviations: n.d.: No detect; Ang: angiopoietin.

tion along with dose and time, ELISA expressions of Ang1 and Ang2 were performed using xengrafts tumor serum samples obtained (6 hours after drug injection) from CPT-11 combination treatment groups (3 wells for per serum sample) at different time points, such as 0, 7, 14, 21 and 28 days after treatment initiation, respectively (**Figure 7**; **Table 3**). Expressions of both Ang1 and Ang2 were suppressed in each group (**Figure 7A**, **7B**), and moresubstantial decreases were observed in two groups with relatively high dosage of FP3 (30 and 60 mg/kg). In the terms of Ang1/Ang2 ratio, all dosages of FP3 groups showed aascending after drug administration, while ascending velocity were more significant in 30 and 60 mg/kg FP3 groups; In contrast, 7.5 and 15 mg/kg FP3 treatment groups maintained the molecular balance between angiopoietins Ang-1 and Ang-2 (**Figure 7C**; **Table 3**).

Discussion

In this study, different drug responses were compared among groups with multistep dosages of FP3 (7.5, 15, 30 and 60 mg/kg) combined with CPT-11 therapy. Interestingly, the 15 mg/kg FP3 group was conferred a prominent efficacy among groups combined with CPT-11 (P<0.05). No significant different loss of weight was found in multistep dosages scheme of FP3 in combination with CPT-11. Therefore, dosage 15 mg/kg of FP3 was considered as the optimal dosage in combination of CPT-11 treatment. However, what's the reasons underlying the abnormal response of FP3 (15 mg/kg) combined with CPT-11? The answer to how anti-VEGF therapies should be combined with other therapeutics rely on the efficacy weight distri-

bution of both antivascular effects and vascular normalizing [28]. Higher dosages of anti-VEGF therapy was thought to have a stronger antivascular effect, however, which failed to explain the results in our study: in CPT-11 combined groups, lower FP3 dosage 15 mg/kg showed a better anti-tumor growth effect than higher dosages of FP3, especially the 30 mg/kg group (P<0.05). Therefore, we confirmed the predominant role of vascular normalization of FP3 when combined with CPT-11. We supposed that the 15 mg/kg FP3 might generate a higher degree of vascular normalization or a longer window.

To verify this hypothesis, ELISA expressions of serum Ang1 and Ang2 were evaluated at different time points, such as 0, 7, 14, 21 and 28 days after treatment initiation. Expressions of both Ang1 and Ang2 were suppressed in each group (**Figure 7A**, **7B**), and more substantial decreases were observed in two groups with relatively high dosage of FP3 (30 and 60 mg/kg). Several studies reported that anti-VEGF therapies could downregulated Ang-1 and Ang-2 [29-31]. The antivascular effects of anti-VEGF therapies on endothelial cells downregulated Ang-2 expression, whereas the downregulation of Ang-1 was indirect, thus leading to the upregulation of the Ang1/Ang2 ratio [32]. In this study, all dosages of FP3 groups showed a ascending Ang1/Ang2 ratio after drug administration.

Interestingly, ascending velocity of Ang1/Ang2 ratio were less significant in 7.5 and 15 mg/kg FP3 groups than that in 30 and 60 mg/kg FP3 groups, probably indicating a longer vascular normalization. The Ang1/Ang2 ratio were

shown somewhat higher in 15 mg/kg FP3 compared with 7.5 mg/kg FP3 group despite of no statistical significance, possibly indicating a higher degree of vascular normalization than the latter. Therefore, by exploring the potential relationship between the drug responses and serum Ang1/Ang2 expressions, we discovered the potential answer to explain why the 15 mg/kg FP3 showed the best anti-growth efficacy in CPT-11 combined therapy groups.

This study demonstrated the role of vascular normalization in anti-VEGF therapy combined with chemotherapeutic agents. The optimal dosage regimen for FP3 in combination with CPT-11 was discovered. The ratio of Ang-1/Ang-2 was demonstrated as an effective surrogate maker for vascular normalization, although further confirmations are needed. In fact, whether the optimal dose-time scheme varies along with different anti-VEGF therapies or tumor types remains unclear. More effective predictive biomarkers for vascular normalization need to be further discovered.

Acknowledgements

Disclosure of conflict of interest

None.

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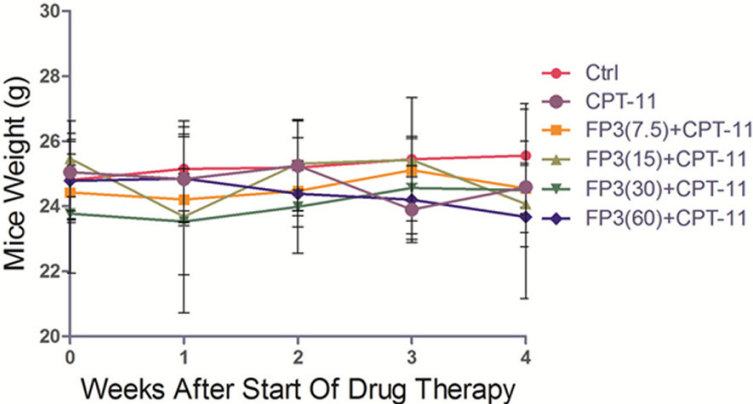
Supplementary Figure 1. Mice body weight changes in multistep dosages of FP3 combined with CPT-11.