# Understanding the Failure of Combination Treatment with the Angiogenesis Inhibitor PTK787 and Chemotherapy in Phase III Trials

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## 1. Abstract:

Antiangiogenic cancer treatments recently achieved a major clinical success with the announcement that Avastin, an antibody specific to vascular endothelial growth factor (VEGF), significantly improves the efficacy of chemotherapy in treating first line colorectal, lung, and breast cancer patients [1, 2]. The synergistic effect of adding Avastin to traditional chemotherapy has been explained by the concept of vascular normalization in which antiangiogenic therapy improves delivery of cytotoxic agents by "normalizing" the morphology and function of aberrant tumor vasculature. Vascular changes induced during normalization include decreased vessel density, diameter, and tortuosity, increased pericyte coverage, decreased permeability, reduced interstitial fluid pressure (IFP), increased oxygen tension ($pO_2$), and improved extravasation of i.v. administered molecules [3, 4].

Following on the success of combination treatment with Avastin, attention turned to PTK787, an antiangiogenic tyrosine kinase inhibitor that blocks signaling from VEGF receptors, as well as receptors for platelet derived growth factor (PDGF) and fibroblast derived growth factor (FGF) [5]. PTK787 demonstrated impressive efficacy in preclinical and Phase I/II trials where it significantly reduced tumor vessel density and in some cases induced tumor regression [6]. However, Phase III trials examining the efficacy of PTK787 in combination with chemotherapy showed no significant improvement in progression free survival (PFS), prompting the discontinuation of one study and leaving the drug's future in doubt [2]. In the wake of this disappointment, is now imperative to understand the mechanisms behind this failure in order to avoid a similar fate with future compounds.

The hypothesis that we propose to test with this grant is that off-target inhibition of PDGF signaling by PTK787 reduces vascular normalization, and therefore clinical efficacy, by blocking recruitment of pericytes to vessel walls. Pericytes play an important role in stabilizing vessel walls during normalization, and the absence of this interaction may contribute to overpruning of tumor vasculature and reduced delivery of cytotoxic agents. To test this hypothesis, we first propose a series of studies in mice to determine if PTK787 administration is able to induce the morphological and functional characteristics of normalization as has been observed with VEGF specific inhibitors. These experiments will include measurements of vessel density, diameter, and tortuosity, pericyte recruitment, vascular permeability, IFP, $pO_2$, and macromolecular extravasation. Secondly, we will identify the molecular basis for different vascular responses to PTK787 and anti-VEGF antibodies, specifically testing the hypothesis that PDGF inhibition is responsible for reduced normalization by administering PDGF inhibitors in conjunction with anti-VEGF antibodies. Finally, we will utilize gene chip hybridization arrays to more broadly probe the molecular changes induced in tumor vasculature by PTK787 administration.

## 2. Specific Aims:

**Specific Aim 1: Determine if PTK787 administration induces normalization of tumor vasculature.** PTK787 will be administered daily to mice containing xenografts of human colorectal cancer cells. The tumor vasculature will be examined for morphological and functional markers of normalization including measurements of vessel density, diameter, and tortuosity, pericyte coverage, vascular permeability, interstitial fluid pressure, oxygen tension, and macromolecular extravasation. These measurements will be repeated at multiple time points to produce a comprehensive kinetic model of PTK787 induced vascular changes.

**Specific Aim 2: Determine if PTK787 induced inhibition of PDGF signaling reduces normalization by blocking pericyte recruitment.** Mice will be administered a PDGF specific inhibitor in conjunction with an anti-VEGF antibody to see if blocking PDGF mediated recruitment of pericytes eliminates the previously observed normalization in response to VEGF specific inhibition. Similarly, PTK787 treated mice will be administered a synthetic analogue of the pericyte inducing factor S1P to determine if improved pericyte recruitment is able to enhance vascular normalization in response to the tyrosine kinase inhibitor.

**Specific Aim 3: Broadly characterize molecular changes induced by PTK787 in the tumor vasculature using a gene chip array.** Tumors from PTK787 treated and control mice will be excised, homogenized, and their mRNA analyzed on a gene chip array containing cDNA for common pro- and anti-angiogenic factors. Statistical analysis will be utilized to determine factors that show significantly altered expression profiles following PTK787 treatment. siRNA or other knockout techniques may then be utilized to determine if selected factors play a functional role in regulating the degree of tumor normalization.

## 3. Background and Significance

### 3.1 Antiangiogenic Therapy

For over 30 years, researchers have attempted to treat cancer by targeting the vascular network that supplies the nutrients and oxygen necessary for continued tumor growth. Tumor vasculature represents an attractive therapeutic target due to its relative genetic stability and homogeneity compared to the highly unstable cancer cells [7]. A variety of antiangiogenic strategies have been attempted, the majority focusing on inhibiting the interaction between vascular endothelial growth factor (VEGF) produced by tumor cells and the related family of VEGF receptors expressed on endothelial cells. This interaction is necessary for endothelial cell proliferation, migration, and survival [8].

Despite the theoretical potential of antiangiogenic therapies, attempts to treat tumors with these agents alone has shown limited clinical efficacy. The failure of antiangiogenic monotherapy can be attributed to the maintenance of viable tumor cells near the outer rim of healthy vasculature, the ability of tumor cells to alter their expression of pro-angiogenic mediators, the extreme potency of VEGF and other factors, and side effects of hypertension, thrombosis, proteinuria,

and hemorrhaging [8, 9]. As a result of these limitations, clinical trials have shifted focus to combining angiogenesis inhibitors with traditional chemo- and radiation therapy.

Antiangiogenic combination therapy recently achieved a major clinical success with the announcement that the addition of Avastin, a monoclonal antibody against VEGF, to the chemotherapeutics irinotecan, fluorouracil, and leucovorin (IFL) increased median survival for patients with first line metastatic colorectal cancer by 5 months when compared to treatment with IFL alone[1]. Similarly, three other Phase III trials demonstrated that the addition of Avastin significantly improves the effectiveness of chemotherapy for treating second line colorectal cancer, first line non-small cell lung cancer (NSCLC), and first line metastatic breast cancer [2]. In all trials, side effects were limited to treatable levels of hypertension and stomach bleeding suggesting that this combination will be a safe and effective therapy in the future [1, 10]

### 3.2 Vascular Normalization

The surprising efficacy of combination treatments with Avastin and traditional chemotherapy has been explained by the idea of vascular normalization, in which antiangiogenic factors are able improve delivery of cytotoxic agents by "normalizing" the tumor vasculature [7, 11]. Tumor vasculature is generally characterized by an abnormal morphology of dilated, leaky, and tortuous vessels produced by aberrant growth factor expression (Figure 1) [9, 12]. Additionally, normal interactions between endothelial cells and pericytes, smooth muscle cells that surround healthy arterioles, venuoles, and capillaries, are frequently absent or disrupted further contributing to vessel leakiness [13]. The hyperpermeability of tumor vessels allows significant leakage of plasma proteins into the tumor interstitium, thereby disrupting the normal oncotic gradient across the vessel wall and increasing the interstitial fluid pressure (IFP). The increased IFP, in turn, reduces the normal hydrostatic gradient across the vessel wall, essentially eliminating convective transport. These changes all contribute to a low and extremely heterogeneous deposition of cytotoxic drugs and other molecules into the tumor tissue, thereby limiting therapeutic efficacy [11].

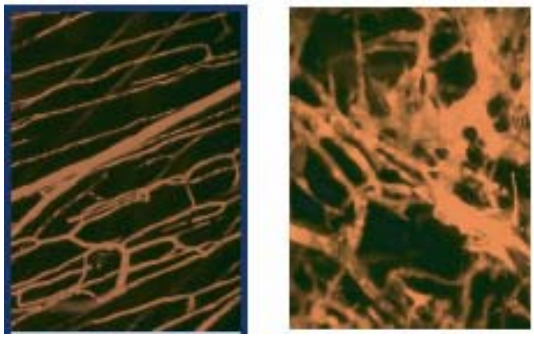

Figure 1: Comparison of blood vessel morphology in normal (left) and tumor (right) vasculature [adapted from ref. 52]

The concept of vascular normalization states that the administration of antiangiogenic agents that inhibit growth factor activity can temporarily transform tumor vasculature to a morphological and functional state resembling normal vasculature. As a result of these changes, there is a window of time in which macromolecules are extravasated into the tumor tissue relatively efficiently and homogeneously. If cytotoxic agents are administered during this window of normalization, therapeutic efficacy may be significantly improved [11]. At the same time, however, continued treatment with angiogenesis inhibitors beyond the normalization window reduces the tumor vasculature to a level that is insufficient for drug delivery, a state referred to as overpruning [7]. Therefore, if combined treatment with angiogenesis inhibitors and

chemotherapeutics is not optimally timed, the antiangiogenic drugs may antagonize rather than improve chemotherapeutic efficacy [14, 15].

A series of mouse studies examining changes in tumor vasculature following inhibition of VEGF signaling support the idea of vascular normalization. Following administration of VEGFR2 blocking antibodies, tumor vessels exhibit a rapid reduction in mean vessel density, diameter, and tortuosity [3, 16] At the same time, there is an increase in endothelial cell coverage by pericytes and a reduction in basement membrane thickness contributing to more stable vessels [3, 4] These stabilized vessels limit macromolecular permeability, which in turn reduces plasma protein extravasation and thereby decreases interstitial oncotic pressure [3, 16] Similarly, there is a decrease in interstitial fluid pressure, thereby inducing a hydrostatic pressure gradient across the vessel wall that contributes to improved transport and perfusion of both macromolecules and small molecule contrast agents into the surrounding tumor tissue [3, 4, 17]**.** Furthermore, increased oxygen transport alleviates hypoxia and improves the efficacy of properly timed radiotherapy [4, 17]. In addition to mouse studies, characteristic markers of normalization including decreased vessel density, reduced IFP, increased pericyte coverage, and increased extravasation of contrast agents have also been observed in human patients following Avastin administration [18] These normalization changes are generally transient, lasting on the order of 5-8 days before the tumor reverts to an abnormal vessel structure with poor transport of molecules. This again stresses the importance of properly timing chemo- or radiation therapy with the optimum window of normalization [19]

### 2.3 PTK787

Following the clinical success of combination therapy with Avastin, interest shifted to other angiogenesis inhibitors, most notably PTK787 (vatalanib). PTK787 is a small molecule, tyrosine kinase inhibitor that blocks signaling from all VEGF, PDGF, and FGF receptors by competitively binding to the ATP active site (Figure 2) [5]. Compared to Avastin, PTK787 offers the advantages of high oral bioavailability, cheaper manufacturing, and reduced immunogenicity [20] Additionally, the ability of PTK787 to block multiple growth factor receptors limits the ability of tumors to escape from anti-angiogenic therapy by switching their angiogenic factors [11] Interest in multi-target inhibitors like PTK787 was also encouraged by preclinical work with other tyrosine kinase inhibitors, which suggested that targeting multiple growth factor receptors may induce greater tumor regression by directly targeting endothelial cells, pericytes, and tumor cells, which express VEGF, PDGF, and FGF respectively [20, 21]. In one particular experiment, administration of SU6668, a tyrosine kinase inhibitor that blocks VEGF, PDGF, and FGF, induced greater tumor regression and necrosis in RIP-TAG mice than a drug inhibiting VEGF alone [22].

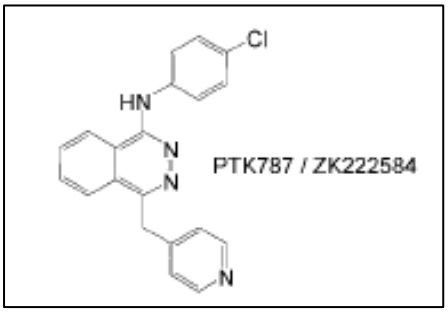


Figure 2: Structue of the tyrosine kinase inhibitor PTK787 [5]

Preclinical trials with PTK787 demonstrated high levels of efficacy and limited toxicity. Daily administration of the drug at doses of 50-100 mg/kg significantly reduced tumor size and almost completely abrogated metastasis in renal carcinoma, prostate, and melanoma models in mice [20, 23, 24]. Additionally, PTK787 administration significantly improved the efficacy of radiation therapy for squamous cell carcinoma and adenocarcinoma xenografts in mice by preventing vascular regrowth after radiation [25, 26]. PTK787 showed similar efficacy in Phase I/II clinical trials as daily doses of the inhibitor at 1000 mg/kg produced an overall response rates of 44-54% when combined with the chemotherapeutic regiment FOLFOX-4 for treating first line colorectal and lung cancer [6, 27, 28]. MRI analysis of patients in these trials showed a significant reduction in mean vessel density (MVD) in treated tumors, which correlated with therapeutic efficacy [24]. Side effects in all Phase I/II trials were limited to medium grade dizziness and proteinuriea with no dose limiting toxicities observed up to 1000 mg [6, 27, 28].

Following this early success, efficacy of PTK787 in combination with chemotherapy was further analyzed in a pair of randomized Phase III trials titled CONFIRM-1 and CONFIRM-2 for treating first and second line colorectal cancer, respectively. Both trials involved daily administration of 1250 mg/kg PTK787 in conjunction with biweekly treatment with the chemotherapeutic combination FOLFOX-4 [29]. Efficacy was compared to a second dosing arm receiving treatment with FOLFOX-4 alone. Although the final results have not been released, preliminary reports indicate that combination treatment with PTK787 and FOLFOX4 shows no significant improvement in progression free survival (PFS) compared to chemotherapy alone. Due to these disappointing results, Novartis recently announced that they will discontinue the CONFIRM-2 trial, leaving the future of PTK787 largely in doubt [30].

### 2.4 Understanding PTK787's Failure

In the wake of disappointing Phase III results for PTK787, attention must now be turned to understanding the reasons for the drug's failure so future compounds may avoid the same fate. Several physicians involved in the trials have suggested that the reduced efficacy of PTK787 compared to Avastin is due primarily to pharmacokinetic differences as Avastin has a half life of 20 days and PTK has a half life of 4-6 hours [2]. As a result, they suggest that the trials be repeated with twice daily dosing of the drug. Although rapid plasma clearance may play a role in limiting therapeutic efficacy, it is unlikely to be the primary cause of PTK787's failure since pharmacokinetic data indicates that an active dose of the drug is maintained in the blood stream throughout the once daily dosing regiment [31]. Instead, we propose that molecular and mechanistic differences between PTK787 and Avastin are responsible for the varied efficacy observed with the two drugs. Specifically, we believe that PTK787 mediated inhibition of PDGF signaling may limit pericyte recruitment to vessel walls, thereby impairing vascular normalization and reducing the delivery of cytotoxic drugs.

PDGF is a paracrine growth factor released by endothelial cells that mediates interactions with adjacent pericytes. These interactions play a vital role in stabilizing blood vessel walls, regulating blood flow, and controlling endothelial cell proliferation, survival, permeability, migration, and maturation [13, 32] In the absence of PDGF, pericyte coverage is nearly completely eliminated producing an array of deleterious morphological and functional consequences including endothelial cell hyperplasia, increased blood vessel diameter,

microaneurysms, and vessel leakiness contributing to edema [32-34]. Similar vessel characteristics are also observed in tumor tissues where normal PDGF signaling between endothelial cells and pericytes is destroyed by abnormally high growth factor levels, as well as mutant isoforms of PDGF lacking retention motifs [35, 36]. As stated above, anti-VEGF angiogenesis inhibitors like Avastin can reverse these problems leading to increased pericyte coverage and normalized vascular function. In contrast, we believe that PTK787 mediated PDGF inhibition may reduce pericyte recruitment, thereby eliminating stabilization of the normalized vessels. Without the stabilizing pericyte interactions, the antiangiogenic activity of PTK787 may overprune the remaining tumor vasculature antagonizing the delivery of chemotherapeutics into the tumor [37]

Although a systematic study of vascular normalization following PTK787 administration has not been undertaken, there are some clues in the literature that PTK787 may reduce vascular normalization by blocking pericyte recruitment as we have proposed. Unlike Avastin treated vasculature which exhibits reduced blood vessel diameters, PTK787 treated vessels take on a significantly dilated morphology with thin endothelial cell walls as measured by immunohistochemistry, corrosion casting, and endoderm perfusion [24, 38]. Secondly, PTK787 treated tumors exhibit significantly reduced extravasation of GdDOTA contrast agents as measured by MRI in mouse studies and Phase I/II trials [31, 38]. Although this measure is somewhat difficult to interpret as it incorporates changes in blood vessel perfusion, vascular transfer rates, and interstitial leakage space, it does suggest that the significant improvement in tumor delivery produced by anti-VEGF therapies is not recapitulated with PTK787 [11, 24, 31, 38]. Finally, although pericyte coverage following PTK787 administration has not been measured, a pair of studies show reduced pericyte coverage of murine tumor vessels following administration of SU6668, which also blocks VEGF, PDGF, and FGF (Figure 3) [22, 39]

Although these studies provide intriguing clues for understanding the failure of PTK787 and the potential role of PDGF inhibition, they are ultimately limited. There has been no comprehensive examination of several of the important hallmarks of normalization including IFP, pO2, and pericyte coverage following PTK787 administration. Additionally, no measurements of PTK787 induced vascular changes have been taken over a comprehensive time course to understand the kinetics of these changes and a potential optimum window of dosing. Finally no study has been undertaken to understand how the different molecular mechanisms of PTK787 and anti-VEGF therapies like Avastin may explain differences in the vascular response, specifically the role of PDGF inhibition. We therefore propose a series of experiments to characterize the ability of PTK787 to normalize tumor vasculature and the molecular basis for these effects. By better understanding the molecular response to PTK787 administration, we should be able to design improved antiangiogenic therapeutics to be used in combination with chemotherapy.

## Research Design and Methods

### 4.0 Specific Aim 1: Determine if PTK787 administration induces normalization of tumor vasculature.

One of the fundamental aspects of our hypothesis for poor efficacy of PTK787 in comparison to Avastin is that the kinase inhibitor is unable to induce significant and stable vascular normalization. In other words, we believe that PTK787 administration induces either no vascular normalization or normalization for such a short time period that the window is too transient to offer any synergism with chemotherapy. In order to examine both of these possibilities, it is imperative that we not only characterize the vascular changes induced by PTK787, but also the kinetics of these changes. Furthermore, it is important that we characterize both morphological changes in the vessels that are important markers of the normalization process, as well as functional changes in molecular transport across the vessel walls that will ultimately determine efficacy in combination therapy.

### 4.1 Mouse and tumor models

Although these normalization studies would optimally be undertaken in human subjects to produce the most relevant model for the Phase III clinical results, the number of time points we propose and the invasive nature of several of the assays necessitate experiments in mice. The similarity of vascular changes induced in humans by Avastin and the response to anti-VEGF antibodies in mice suggest that the mouse system will provide a useful model for the effects of PTK787 [16, 18]. To make the mouse studies as relevant as possible to the CONFIRM-1 and CONFIRM-2 trials for treating colorectal cancer, a human colorectal cancer cell line, LS174T, will be utilized in all experiments. LS174T was also chosen because this cell line has been utilized in prior experiments examining vascular effects of VEGF and VEGFR2 inhibitors, providing a convenient comparison for the changes induced by PTK787 [3, 16].

Two sets of mice will be utilized in the first set of experiments. In the first, tumor xenografts will be grown subcutaneously in dorsal skinfold chambers implanted in severe combined immunodeficient (SCID) mice [3]. These mice will be utilized for taking repeated *in vivo* measurements of vascular dimensions, macromolecular permeability, IFP, and oxygen tension. A second set of mice will have xenografts implanted by subcutaneous injection of LS174T. These mice will be utilized for immunohistochemical measurements of pericyte coverage and molecular extravasation. In all cases, mice will be anesthetized with ketamine/xylazine prior to tumor transplantations, and protocols will follow Massachusetts Institute of Technology guidelines on animal care.

### 4.2 Dosing Schedules

Three dosing arms will be utilized in this experimental section. In the first, PTK787 will be administered intravenously at 50 mg/kg once daily over the course of 21 days beginning 7 days after tumor transplantaion. This dosing schedule is similar to the once daily administration in the CONFIRM-1 and CONFIRM-2 trials, and the 50 mg/kg dose has been shown in several mouse

studies to induce vascular regression without noticeable toxicity [23, 38]. In the second arm, PTK787 will be administered twice daily, again at 50 mg/kg per dose, over the 21 day experiment. This dosing schedule has been included to determine if poor PTK787 efficacy is simply due to pharmacokinetic issues of limited exposure with once daily administration. If the twice a day dosing arm shows considerably greater vascular normalization than the once daily schedule, it will provide evidence that rapid clearance plasma of the drug is responsible for the reduced efficacy, and future clinical trials should utilize twice daily administration. A third arm will be a negative control with daily administration of PBS. The PBS treated mice will be utilized for making size matched comparisons of tumors, since some of the measured vascular changes such as IFP can be altered by tumor size. For all dosing regiments, 21 days has been selected as the experimental time course since previous studies have shown that chronic administration of PTK787 for greater than two weeks is necessary to observe significant tumor regression [38].

### 4.3 Morphological Measures of Vascular Normalization

Animals from all 3 dosing arms will be analyzed for changes in vessel morphology that have been associated with normalization and improved delivery in previous mouse studies [3] [4]. 3-8 mice will be utilized for every experiment/time point to account for heterogeneity among individual animals.

***Vessel Architecture:***
Using the dorsal skinfold chamber, *in vivo* measurements of vessel shape, size, and density will be determined using multiphoton laser scanning microscopy [3]. FITC labeled dextran (m.w. = 2,000,000 kD) will be injected i.v. to specifically label and image blood vessels. Images will be taken on days 1, 3, 6, 10, and 21 after the start of treatment. By taking repeated measurements in this manner, we will be able to quantify acute vascular changes due to PTK787, as well as vascular effects that arise only after chronic treatment. For each tumor, at least 10 images will be taken of different sections with results averaged to account for vessel heterogeneity.

Image analysis software will be utilized to derive quantitative measures of vascular dimensions from the microscopy images. Mean vessel density will be determined by counting the number of vessels within a single frame at x100 magnification. Similarly, average vessel diameter will be measured with scaling tools included in the software. Additionally, the fractal dimension of the vascular network will be calculated by applying a statistical box-counting method to the path of minimum length of vessels across a predetermined optical frame [40]. This value will provide a mathematic description of the degree of tortuosity in the remaining vasculature, with normal vessels exhibiting a dimension of ~1.70 and tumor vessels yielding dimensions of ~1.90 [40]. This is an important measure of tumor vessel morphology since it is often the quality of the vessels rather than the number or size that ultimately determines the efficiency and homogeneity of drug delivery.

***Pericyte Coverage:***
Since our proposed explanation for reduced efficacy with PTK787 administration centers on the inhibition of PDGF mediated pericyte recruitment, it is vital that we fully characterize the degree of pericyte coverage on PTK787 treated and untreated tumor vessels. To achieve this, mice

without the dorsal skinfold localized tumors will be utilized in immunohistochemical measurements of pericyte and endothelial cell co-localizaton. Due to the relative experimental complexity of these measurements compared to the *in vivo* imaging above, only two time points will be utilized. Days 1 and 6 have been selected since they will provide information on the immediate impact of PTK787 treatment, as well as the effect of chronic treatment. At these times, mice will be injected with FITC-conjugated anti-CD31 to label endothelial cells, fixed, and the tumors sectioned into 5 100 μm thick slices. Pericytes will be visualized by staining with a PE-conjugated anti-Desmin monoclonal antibody. Desmin staining has been selected as a pericyte marker, rather than the frequently used smooth muscle actin (SMA), since capillary associated pericytes frequently display desmin expression only [13]. FITC and PE fluorescence will be measured by confocal microscopy, and the degree of endothelial pericyte association will be calculated from co-localization images using the formula [(Number of microvessels w/ pericyte coverage/total number of microvessels)x100] [41]. The measurements will again be averaged over multiple tumor sections to account for heterogeneity.

### 4.4 Functional Markers of Vascular Normalization

Although morphological changes represent convenient markers for normalization, the synergistic action between anti-angiogenic factors and chemotherapy ultimately depends on functional vascular changes that improve delivery of the cytotoxic agents. These changes, which we will quantitatively measure, include decreased permeability, reduced IFP, increased oxygen tension, and improved molecular extravasation.

*Vascular Permeability:*
Using the dorsal skinfold chamber mouse model, vascular permeability of tumor vessels in PTK787 treated and non-treated mice will be determined as described previously [42]. PE-conjugated bovine serum albumin (MW = 60 kD) will be injected into mice i.v. through the tail vein. Following 30 minutes of heart perfusion, the PE fluorescence will be measured in the interstitial space of the tumor tissue using multiphoton laser scanning microscopy. The degree of PE fluorescence in the tumor tissue depends on the amount of labeled BSA that leaks from the tumor vessels, thereby providing a measure of vascular permeability to plasma proteins[42].

***Interstitial Fluid Pressure:***
We will also utilize the dorsal skinfold chamber as a convenient platform for measuring tumor interstitial fluid pressure (IFP) and mean vessel pressure (MVP) *in vivo* using the micropipette technique. In brief, a 23 gauge needle with a side hole 4 mm from the tip will be connected to a pressure transducer through polymer tubing filled with heparynized saline [43]. After anesthetizing and stabilizing the animals, the pipette will be inserted into the tumor tissue, producing pressure readouts from the transducer. Using stereomicroscopy for guidance, pressure measurements will be taken with the pipette tip aligned in the tumor interstitium at a depth of ~1 mm to determine IFP or in the lumen of blood vessels to calculate MVP. The measurements will be taken in at least five locations within each tumor to account for pressure heterogeneity. Due to the relatively non-invasive nature of these measurements, they will again be taken at multiple time points (days 1, 3, 6, 10, and 21 after the start of the treatment) to characterize the kinetic nature of tumor pressure changes. Following these experimental measurements, the

hydrodynamic gradient will be calculated as the difference between MVP and IFP, with a positive value representing a potential gradient for convective transport into the tumor.

***Oxygen Tension:***
The dorsal skinfold chamber mice will also be utilized for measurements of oxygen tension by using phosphorescence quenching microscopy as described previously [44]. In brief, albumin conjugated palladium meso-tetra-(4-carboxyphenyl) porphyrin will be injected i.v. through the tail vein. This reagent provides a useful measure of oxygen levels in the tissue since the lifetime of its phosphorescence signals following activation depends on the level of oxygen in the surrounding environment. 30 minutes after injection of the oxygen probe, the dorsal skinfold chamber will be excited at 540 nm with resulting phosphorescence emissions of >630 nm detected with a photomultiplier tube. The lifetime constant ($\tau$) of these emissions will be calculated which can be related to the oxygen level ($pO_2$) by the equation $pO_2 = 1/k(1/\tau - 1/\tau_o)$ where k is the quenching constant and $\tau_o$ is the lifetime constant in the absence of oxygen [44]. As above, phosphorescence emissions will be measured in at least five locations within the tumor to control for heterogeneity in hypoxia. Since the $pO_2$ level in the tumor depends primarily on the efficiency and homogeneity of oxygen transport from the vasculature, this will provide another convenient, functional measure of the degree of normalization.

***Macromolecular Extravsation:***
Since the ultimate measure of vascular normalization is the improved delivery of chemotherapeutics and other molecules, functional measurements of macromolecular extravasation will be performed. In these measurements, PE-conjugated albumin will be injected along with FITC-conjugated anti-CD31 antibodies through the tail vein of mice 60 minutes prior to fixation and sectioning of tumor slices as described above [3]. FITC and PE fluorescence will be visualized and quantified in each tumor section using a confocal microscope to determine the location of endothelial cells and injected albumin respectively. The degree of extravasation will be calculated by using image analysis software to determine the average PE fluorescence at a series of distances away from a selected point in the vasculature. These average intensity values can be utilized to calculate an average penetration length by fitting to an exponential decay [3]. As stated above for the pericyte immunohistochemistry, extravasation measurements will be performed at two time points of 1 and 6 days to examine the effect of acute and chronic administration.

### 4.4 Potential Concerns and Solutions

As stated above, one important goal of this experimental section is to fully characterize the kinetics of the vascular changes induced by PTK787 administration. With this in mind, we have proposed taking experimental measurements at a number of time points: 1, 3, 6, 10, and 21 days for *in vivo* measurements, and 1 and 6 days for immunohistochemistry measurements. Although these time points were selected to hopefully provide a comprehensive sampling of changes over the 21 day treatment, they are essentially arbitrary since no systematic analysis of the kinetics of PTK787 induced changes has been performed in the past. Therefore, if PTK787 induced vascular regression occurs more rapidly or slowly than we expect, our experimental measurements on the proposed days may not effectively capture the timecourse of vascular changes. To account for this, prior to starting the primary experiment, a small cohort of mice

with implanted dorsal skin folds will be treated with PTK787 and have their tumor vasculature imaged repeatedly over several weeks. These measurements of changes in the vascular architecture should provide a rough estimate of the time course for PTK787 induced changes which can then be utilized in selecting more meaningful experimental time points for the other assays.

## 5.0 Specific Aim 2: Determine if PTK787 induced inhibition of PDGF signaling reduces normalization by blocking pericyte recruitment.

Beyond simply characterizing the vascular changes induced by PTK787 administration, it will also be important to understand how the molecular mechanism of PTK787 activity influences these results in comparison to blocking VEGF or VEGFR2 alone [3, 4, 16]. Specifically, we aim to determine if PTK787's off target inhibition of PDGF signaling is responsible for reducing normalization by blocking pericyte recruitment. To directly address this question, we propose an additional round of mouse experiments with five dosing arms. In the first, treatment with an anti-VEGF antibody will be utilized as a positive control for vascular normalization. In the second, a PDGF specific inhibitor will be administered in conjunction with the anti-VEGF antibody to determine if blocking PDGF activity significantly reduces pericyte recruitment and thereby inhibits functional normalization. In the third, PTK787 will be administered in conjunction with a synthetic analogue of S1P, a lipid signaling molecule that induces pericyte recruitment by interacting with the receptor EDG1, to see if alleviating the PTK787 induced inhibition of pericyte recruitment can increase normalization. Finally, two sets of control mice with S1P or anti-PDGF administration alone will be followed to ensure that the desired effects on pericyte recruitment are achieved and that there are no other aberrant effects from systemically administering these factors. The results from our first round of studies with PTK787 and PBS treated mice will also be utilized in making functional comparisons.

### 5.1 Tumor Model and Time Points

As above, LS174T xenografts in SCID mice will be utilized as the experimental model for normalization. Due to the large number of dosing arms that will be tested in this section, only two time points will be examined for each experimental condition. The first measurement will be taken one day post administration of the respective therapeutics to identify immediate changes induced by the treatments. The second will be taken at day 5, a time at which VEGF or VEGFR2 specific inhibitors have been shown to induce a significant degree of vessel normalization [4, 16]. In contrast, we believe that tumors treated with PTK787 alone will not exhibit signs of normalization at this time due to a lack of vessel stabilization and overpruning. In effect, we should therefore have a set of positive and negative controls to compare with the results of our proposed combination therapies. If the initial round of experiments instead indicates that PTK787 does in fact induce normalization out to 5 days, the selected time points may be changed accordingly.

### 5.2 Dosing

For the positive control arm, the anti-VEGF antibody B20 from Genetech will be utilized. B20 has been selected because it is capable of blocking both human and murine VEGF activity,

thereby inhibiting VEGF contributions from both the implanted tumor cells and surrounding stroma [45]. In this manner, it most accurately recapitulates the ability of Avastin to block VEGF from multiple sources in human patients. Although vascular normalization in response to B20 has not been carefully characterized in mouse models, the consistent normalization by other specific VEGF inhibitors suggests that it should induce a similar response. The antibody will be administered i.v. through the tail vein at a dose of 40 mg/kg on day 1 and day 4 to ensure a consistent plasma level of the antibody as required for efficacy.

For the second arm, an anti-PDGF-B aptamer will be given in conjunction with B20 to specifically inhibit PDGF signaling. The proposed aptamer has been described previously and inhibits PDGF with an affinity of 0.1 nM [46]. Administering this inhibitor alone into mice reduces pericyte coverage and temporarily reduces IFP [46]. The aptamer will be linked to a 40 kD polyethylene glycol chain, creating a molecule with a plasma half life of 8 hours. The aptamer will be administered once daily at a concentration of 25 mg/kg to maintain an effective dose in the blood stream throughout the experimental time course.

In the third arm, PTK787 will be administered in conjunction with the compound FTY720, a synthetic analogue of the lipid signaling molecule S1P [47]. S1P plays an important role in inducing and stabilizing pericyte / endothelial cell interactions by binding to and activating the EDG family of receptors [48-50]. Although the precise mechanism of S1P/EDG signaling is poorly understood, the ability of S1P to induce pericyte migration in the absence of PDGF and the similarity of PDGF / $EDG_1$ knockout mice suggests that the S1P / $EDG_1$ interaction may act downstream of or in parallel to PDGF [51, 52]. Therefore, stimulating the $EDG_1$ receptor may increase pericyte recruitment even in the presence of PTK787 mediated PDGF inhibition. Since the lipid S1P can not be administered systemically, a synthetic analogue FTY720 will be utilized for activating $EDG_1$. FTY720 will be administered once daily at a concentration of 3 mg/kg, a dose that has previously been shown to strengthen interactions between pericytes and endothelial cells in murine lymph nodes [47]. PTK787 will be administered once daily at a dose of 50 mg/kg as described in the first experimental section.

In the final two control dosing arms, the anti-PDGF-B aptamer and FTY720 will be administered individually into mice at the doses described above. These mice will be monitored to ensure that the desired effects on pericyte recruitment are achieved and that there are no other deleterious effects from systemically administering these factors.

### 5.3 Measures of Vascular Normalization

The effect of altering pericyte recruitment in conjunction with anti-angiogenic treatments will be monitored by quantifying the morphological and functional changes induced in tumor vasculature by all 5 dosing regiments. Markers of vascular normalization including vessel architecture, pericyte coverage, IFP, $pO_2$, and molecular extravasation will be analyzed using the general protocols described in sections 4.3 and 4.4. As stated above, these measurements will be made on days 1 and 5 from the beginning of each treatment protocol.

### 5.4 Potential Limitations and Solutions

Due to the pleiotropic nature of signaling molecules involved in regulating angiogenesis, there is always concern that perturbing the expression level of a factor to induce one specific effect (i.e. regulate pericyte recruitment) may also induce a variety of other vasculature changes, thereby complicating experimental interpretations. This is especially true in the case of S1P, or its synthetic analogue FTY720, where the *in vivo* effects of systemically administering these agents on tumor vasculature is essentially unknown. As a result, it is possible that administering FTY720 may not fully induce pericyte recruitment in tumors as we hope, or may induce a variety of other vascular changes that complicate experimental interpretations. By including control mice with FTY720 administration alone, we should gain some insight into these potential off target effects.

If we ultimately determine that FTY720 is not a useful agent for specifically increasing pericyte recruitment, a second option that may be utilized is administration of recombinant Ang-1, a factor known to induce EC-pericyte interactions by signaling through the Tie-2 receptor [52]. Ang-1 may be administered systemically or in a tumor specific manner by transfecting a plasmid containing the Ang-1 gene under control of a tetracycline promoter into tumor cells prior to transplantation. Since Ang-1 also has a variety of vascular effects, including direct interactions with endothelial cells in the absence of pericytes, it would again be important to utilize proper controls to understand the vascular changes [53].

## 6.0 Specific Aim 3: Broadly characterize molecular changes in the tumor vasculature induced by PTK787 administration

Although our primary hypothesis for reduced efficacy of PTK787 administration centers on its direct inhibition of pericyte recruitment by blocking PDGFR, it is likely that other molecular changes in the tumor tissue also contribute to the vascular response. In order to broadly characterize these changes, we will utilize gene chip array technology to probe differences in RNA expression patterns in the tumors of PTK787 treated and untreated mice. By globally probing changes in tumor RNA levels, we will be able to monitor alterations in angiogenic signaling that are the direct result of PTK787 activity, as well as downstream effects due to changing the cellular activity of endothelial cells, pericytes, and tumor cells.

### 6.1 Gene Chip Array

In order to analyze changes in angiogenic signaling, we will utilize the GEArray Q Series Human Angiogenesis Gene Array: AR-SAHS-009 (SuperArray, Bethesda, MD). The Q series is a cDNA array containing 96 genes for factors known to play a significant role in angiogenesis (Figure 4). This array has been utilized previously for examining molecular changes involved in normalization [3]. Of particular interest among the genes included in this array are the endogenous factors Ang1 and Ang2 that stabilize or destabilize vessels, respectively, by interacting with their cognate receptor Tie2 [52]. Vascular normalization induced by anti-VEGFR2 treatment is associated with upregulation of Ang1 and downregulation of Ang2 [3, 4]. These changes should therefore provide a useful comparison for

| | |
|---|---|
| VEGF A-C | Ang1 |
| VEGFR 1,2 | Ang2 |
| PDGF A,B | Tie-2 |
| PDGFR A,B | EDG1 |
| FGF 1-7 | MMP2 |
| FGFR 1-4 | MMP9 |

Figure 4: Subset of genes included in Angiogenesis array

analyzing the molecular response to PTK787.

### 6.2 Experimental Protocol

Gene chip analysis of PTK787 mediated vascular response will be performed on day 3 following the start of pharmacological dosing. Day 3 has been selected as a median time which is likely to occur after the drug has had sufficient exposure to induce a vascular response, but before the vasculature is completely destroyed. Tumors will be excised from sacrificed mice at this time and size matched between PTK787 treated and non-treated controls. The tumor tissues will be homogenized and lysed, followed by isolation of total RNA using the acid-phenol extraction method and the Qiagen Poly(A)$^+$ RNA purification system [54]. The RNA extracted from treated and control mice will be labeled with Cy3- or Cy5- dUTP, respectively, by a performing reverse transcription with the reverse transcriptase SuperScript and an oligo(dT) primer. The two labeled RNA pools will be mixed and competitively hybridized to the cDNA array [55].

The relative levels of treated and untreated RNA hybridized to each cDNA spot will be quantified using a laser reader with excitation pulses at 532 and 635 nm wavelengths for Cy3 and Cy5, respectively. By comparing the relative intensity of emissions for each wavelength at a given spot, relative mRNA levels between the two treatments will be calculated. Genes represented by cDNA spots having normalized intensity ratios outside of a 95% confidence interval will be considered differentially expressed following PTK787 administration [55]. To confirm that differences in mRNA expression correspond to variations in the level of protein production, Western blots for selected proteins will be performed on tumor lysates from treated and untreated mice. Differentially expressed angiogenic factors identified in this manner may ultimately be probed for functional effects on vascular normalization using additional mouse studies.

### 6.3 Potential Limitations and Solutions

As with any cDNA array application, there is ultimately a question of whether variations in transcription levels of different genes actually correspond to important functional effects. For instance, a gene may show significantly altered mRNA levels following PTK787 administration due to secondary interactions with other proteins, without playing any important role in the vascular response. In order to distinguish between functionally important changes in gene expression and secondary fluctuations, siRNA knockdowns will be performed on identified genes in conjunction with PTK787 treatment. If eliminating expression of a particular gene by siRNA alters the morphological or functional vascular response to PTK787, then it is likely that this gene is playing an important functional role in the vascular changes.

## 7.0 Conclusion

The impressive success of Avastin in combination with chemotherapy has elicited significant excitement for the future for antiangiogenic therapies. However, the surprising failure of PTK787 in similar trials shows that there is still much to be learned about the mechanism and optimum administration of these agents. In the case of PTK787 and other multi-target tyrosine kinase inhibitors, the effect of the compounds on vascular morphology and function may be especially difficult to understand given the large number of potential off target effects. In this

grant proposal, we put forward a number of experimental protocols that should not only characterize the vascular response to PTK787 administration, but also improve our understanding of the molecular mechanisms responsible for these changes. By understanding these molecular mechanisms, we should be able to design improved antiangiogenic therapies in the future.

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