Dual Targeting of Angiopoietin-2 and VEGF Signaling for the Treatment of Glioblastoma

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Dual Targeting of Angiopoietin-2 and VEGF Signaling for the Treatment of Glioblastoma

A dissertation presented

by

Teresa Erin Peterson

to

The Division of Medical Sciences

in partial fulfillment of the requirements

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Dual Targeting of Angiopoietin-2 and VEGF Signaling for the Treatment of Glioblastoma

Abstract

Glioblastoma (GBM) is the most common and aggressive brain tumor in humans. Because GBM is highly angiogenic, the anti-vascular endothelial growth factor (VEGF) antibody bevacizumab has now become the standard of care for treatment of recurrent GBM. However, GBMs rapidly become refractory to standard anti-VEGF therapy. It was previously demonstrated that vessel normalization and subsequent reduction of intracranial vasogenic edema accounts for a majority of the benefit from inhibiting VEGF-signaling in GBM. Also, ectopic over-expression of Ang-2 compromises the benefits of anti-VEGF receptor (VEGFR) treatment in mice and showed that circulating levels of Ang-2 rebound after an initial decrease in GBM patients treated with anti-VEGFR agents. This thesis examines the hypothesis that combined inhibition of VEGF and Ang-2 signaling can improve survival in murine models of GBM, and investigates potential underlying mechanisms.

The efficacy of cediranib, a pan-VEGFR tyrosine kinase inhibitor, combined with MEDI3617, an anti-Ang-2 neutralizing antibody, was tested in two orthotopic models of GBM (U87 and Gl261). Combination therapy improved survival in both models beyond that of the monotherapy arms and of control IgG by delaying Gl261 growth and increasing U87 necrosis. Combination therapy increased perivascular cell coverage in both tumor models and led to a more mature, normalized tumor vasculature than that in the tumors treated with cediranib alone. Importantly, combination therapy was as effective at controlling edema as cediranib. Vascular normalization with VEGF-blockade has been shown to convert an immunoinhibitory tumor
microenvironment to an immunostimulatory one. Improved vessel normalization resulting from combined Ang-2 and VEGFR inhibition was associated with an increase in the number of M1-like (anti-tumor) tumor associated macrophages (TAMs) compared to the cediranib-treated tumors. Inhibition of TAM recruitment with an anti-colony stimulating factor-1 (CSF1) neutralizing antibody compromised the survival benefit of anti-Ang-2/VEGFR combination therapy.

The work described here provides new insight into a potential therapeutic strategy for the treatment of GBM and should inform of the design of clinical trials on this therapeutic combination.
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# TABLE OF CONTENTS

Abstract .................................................................................................................................................. iii

Acknowledgements .................................................................................................................................... v

Table of Contents ..................................................................................................................................... vii

## CHAPTER 1 ................................................................................................................................. 1

Clinical Significance of Glioblastoma ................................................................................................. 2
Conventional Therapies in GBM ......................................................................................................... 5
Angiogenesis .......................................................................................................................................... 6
VEGF ..................................................................................................................................................... 10
Anti-angiogenic Therapy ...................................................................................................................... 14
Anti-angiogenesis in GBM .................................................................................................................... 20
Angiopoietin-2 ..................................................................................................................................... 23
Ang-2 in Non-CNS Cancers ................................................................................................................. 27
Ang-2 in GBM ..................................................................................................................................... 28
Targeting Ang-2 .................................................................................................................................. 35
Tumor-associated Macrophages (TAMs) .............................................................................................. 35
Conclusions and Perspectives .............................................................................................................. 38
References ............................................................................................................................................. 40

## CHAPTER 2 ................................................................................................................................. 59

ABSTRACT ......................................................................................................................................... 63
Introduction ........................................................................................................................................... 65
Materials and Methods ....................................................................................................................... 66
Results ....................................................................................................................................... 72
Discussion .................................................................................................................................. 109
Conclusions ................................................................................................................................. 116
Acknowledgements ..................................................................................................................... 117
References .................................................................................................................................... 118

CHAPTER 3 ................................................................................................................................... 124

Summary ....................................................................................................................................... 125
TAMs and GBM Progression ......................................................................................................... 125
Vascular Normalization and TAMs ............................................................................................... 127
Tie-2 Expressing Monocytes/Macrophages (TEMs) .................................................................... 128
Role of Ang-2 in Promoting Tumor Invasion ................................................................................ 130
Clinical Implications and Future Directions .............................................................................. 133
Conclusions .................................................................................................................................. 136
References .................................................................................................................................... 137

APPENDIX ..................................................................................................................................... 142

PlGF Inhibition in GBM .................................................................................................................. 143
References .................................................................................................................................... 151
CHAPTER 1

Introduction
Clinical Significance of Glioblastoma

Glioblastoma (GBM), also known as a grade IV astrocytoma, is the most common malignant primary brain tumor with an estimated 10,000 cases diagnosed each year in the US\(^1\,^2\). GBMs can be classified as either primary or secondary, depending on how they have developed. Primary GBMs develop \textit{de novo}, without evidence of a less malignant precursor lesion, whereas secondary GBMs develop via the degeneration of lower-grade astrocytomas. Primary GBMs account for approximately 90% of all GBM cases\(^3\) and are typically found in older patients (mean age = 62), while secondary GBMs are much less common and tend to develop in younger patients (mean age = 45)\(^4\,^5\). In both cases, males have a higher tumor incidence than females. While they are largely indistinguishable histologically, primary and secondary GBMs differ in their genetic and epigenetic signatures. Primary GBMs commonly have epidermal growth factor receptor (EGFR) overexpression, PTEN mutations, and/or loss of chromosome 10, whereas secondary GBMs are found to often have mutations in isocitrate dehydrogenase-1 (IDH1) and/or \textit{TP53} and loss of 19q. IDH1 in particular has become a key molecule distinguishing primary and secondary GBMs\(^6\), with greater than 80% of secondary GBMs having mutations in it compared to only 5% of primary GBMs.

GBMs are found primarily in the cerebral hemispheres but have also been found much less commonly in the brainstem and the spinal cord, usually having developed from lower-grade astrocytomas. These tumors are characterized by the presence of anaplastic, malignant cells surrounding small areas of necrotic tissue (aka pseudopalisading necrosis) and by the presence of hyperplastic blood vessels (Figure 1-1)\(^7\,^9\). They are also extremely invasive tumor, invading both
**Figure 1-1: Representative H&E of human GBM.** Representative H&E image of a human GBM with areas pseudopalisading necrosis and microvascular proliferation highlighted with red arrows.
Figure 1-1 (Continued)
locally into the nearby brain parenchyma and more distally throughout the brain, making it extremely difficult to treat, as complete surgical resection is nearly impossible.

There are four distinct molecular subtypes of GBM: Classical, Mesenchymal, Proneural, and Neural\(^1\). Each GBM subtype can be characterized by a distinct genetic signature. Classical GBMs are driven by EGFR overexpression, along with frequent loss of chromosome 10 and CDKN2A. Mesenchymal GBMs are characterized by alterations in NF1 and deletions in PTEN, and have increased expression of TNF and NF-κB pathways resulting in increased necrosis and associated inflammation. Proneural GBMs are driven primarily by alterations in platelet-derived growth factor receptor α (PDGFRA) or point mutations in IDH1. TP53 mutations and loss of heterozygosity (LOH) is also common in this GBM subtype. Secondary GBMs are often found to be of the proneural subtype. The neural subtype gene expression signature is very similar to that of the normal brain, with enrichment for markers commonly found in neurons, as well as astrocytes and oligodendrocytes. Given the vast heterogeneity within GBMs, it is critical that future therapies target these distinct subtypes or utilize an alternative approach that will treat all GBMs, regardless of subtype.

**Conventional Therapies in GBM**

GBM has an incredibly poor prognosis with a 2-year survival rate of less than 10% and a 5-year survival rate of less than 5%. Treatment choice is dependent on tumor size and location within the brain, and on the age and relative health of the patient. For most newly diagnosed GBM (nGBM) patients, the standard of care includes maximal safe surgical resection followed by a combination of radiation and chemotherapy with temozolomide (TMZ)\(^1\). Unfortunately, even after surgical resection there is often tumor remaining within the brain that continues to
grow in distal sites due to the invasive nature of GBM cells, thus making cytotoxic therapies a necessity. Even with this combined therapeutic approach, the prognosis for patients with GBM is very poor with an overall median survival (OS) of 14.7 months\textsuperscript{11}. Patients with recurrent GBM (rGBM) suffer even worse survival outcomes, with progression free survival after 6-months (PFS6) reaching only 10-25\% in patients receiving the standard chemotherapeutic regimen\textsuperscript{12-14}.

In addition to surgery, radiation, and chemotherapy, patients with GBM are often treated with glucocorticosteroids, like dexamethasone, to combat tumor-associated vasogenic edema and related neurological deficits. Edema is the result of fluid accumulation within the brain parenchyma due to the breakdown of the blood brain barrier (BBB) and, left untreated, can lead to increased intracranial pressure, compression of the brain, and significant morbidity. The secretion of vascular endothelial growth factor (VEGF) and other angiogenic growth factors facilitates the breakdown of the BBB and instigates the growth of new vessels to support rapid growth. Though dexamethasone is very efficient at relieving edema, its use is often associated with a number of serious adverse effects that can significantly impact patient quality of life, including steroid myopathy, gastrointestinal bleeding, infections, and mood changes\textsuperscript{15,16}.

**Angiogenesis**

Tumors acquire blood supply via multiple mechanisms: angiogenesis (sprouting new vessels from existing vessels), co-option, intussusception, vasculogenesis, vascular mimicry and trans-differentiation of cancer cells into endothelial cells (Figure 1-2)\textsuperscript{17}. Angiogenesis is the physiological process by which new blood vessels sprout from the pre-existing vasculature. Unlike vasculogenesis, the generation of new blood vessels *de novo* from angioblasts or circulating endothelial progenitor cells (EPCs), which occurs primarily during embryogenesis,
**Figure 1-2: Mechanisms of vessel formation.** Vessel formation commonly occurs via (A) sprouting angiogenesis, (B) vasculogenesis via the recruitment of endothelial progenitor cells (EPCs), (C) or intussusception, whereby vessels split into daughter vessels. Less common forms of tumor vessel formation include (D) vessel co-option of existing healthy vessels, (E) vascular mimicry, whereby tumor cells line vessels and function as endothelial cells, (F) transdifferentiation of cancer stem-like cells into endothelial cells. Figure adapted from 17.
Figure 1-2 (Continued)
angiogenesis is responsible for the majority of blood vessels created during development, after injury, following exercise, and in disease.

There are two main forms of angiogenesis: sprouting angiogenesis and intussusceptive angiogenesis. Sprouting angiogenesis, as the name suggests, occurs when endothelial cells sprout out of the existing vascular network begin to proliferate and form new vessels. This often occurs in response to pro-angiogenic stimuli and is a major source of new vessels in previously avascular areas. Intussusceptive angiogenesis is the formation of new vessels via the splitting of existing vessels and the expansion of the vascular network more locally. Both forms of angiogenesis can occur in parallel and nearly everywhere in the body. The tumor vasculature can also be formed by other mechanisms, though they are less well understood. They can take over the existing vasculature as they expand into the healthy tissue surrounding them\(^\text{18}\). Tumor cells have also been found lining vessels and it has been proposed that they mimic endothelial cells and contribute to vessel function\(^\text{19}\).

Finally, there is evidence that cancer stem-like cells may be able to transdifferentiate and generate endothelium\(^\text{20}\). Although still somewhat controversial\(^\text{21}\), GBM stem cells (GSCs) have been described in both pre-clinical and clinical GBMs and are often located within the perivascular niche\(^\text{22-25}\). GSCs can promote angiogenesis through the production of VEGF and bi-directional interaction with the endothelium\(^\text{24,26-28}\). Additionally, the generation of endothelial cells from GSCs has been described as a major contributing factor towards to the formation of new blood vessels in GBM\(^\text{20,29,30}\). GSCs have also been shown to transdifferentiate into pericytes and promote neovascularure formation, vessel stability, and function\(^\text{31}\).

Angiogenesis is a complex process that requires the cooperation of multiple cellular processes to occur. First, in order to begin generating new vessels, the endothelial cells in the
existing vasculature must degrade the surrounding basement membrane and extracellular matrix (ECM) to allow for cellular migration. Endothelial cells then begin to migrate towards the angiogenic stimulus and proliferate to create a new vessel sprout. Tubes are then formed and support cells, including pericytes and vascular smooth muscle cells, are recruited to the nascent vessel. Finally, the new tubule is interconnected to the existing vessel network (anastamosis).\textsuperscript{32}

Angiogenesis occurs in response to the metabolic demands of the surrounding tissue; and tissue oxygenation is a key regulator of much of the angiogenesis that occurs in healthy tissues. Most cells within the body are found no more than 100-200 µm from the nearest blood vessel to allow for adequate oxygen and nutrient diffusion.\textsuperscript{33} When oxygen levels decrease, tissues become hypoxic and signal for the activation of pro-angiogenic signals and suppression of anti-angiogenic signals, leading to the formation of new vessels. As the newly formed vessels perfuse the tissue, oxygen levels increase – due both to the increase vascular surface area and decreased diffusion distance – and these signals return to normal. Many pro-angiogenic growth factors and their receptors are regulated directly (via hypoxia-inducible factor-1 (HIF-1) and other transcriptional factors) or indirectly by hypoxia in poorly perfused tissues. These include, but are not limited to, VEGF\textsuperscript{34-36} and its receptors VEGF receptor 1 (VEGFR1) and VEGFR2\textsuperscript{37,38}, placental growth factor (PIGF)\textsuperscript{39}, basic fibroblast growth factor (bFGF)\textsuperscript{40}, transforming growth factor beta (TGFβ)\textsuperscript{41}, and angiopoietin-1 (Ang-1), Ang-2, and their receptor Tie-2\textsuperscript{42,43}.

**VEGF**

VEGF, also known as VEGF-A and first identified as vascular permeability factor (VPF) in 1983\textsuperscript{44-46}, is a secreted glycoprotein that has long been known to be a key regulator of angiogenesis. Since its identification, several additional family members have been described –
VEGF-B\textsuperscript{47}, VEGF-C\textsuperscript{48}, VEGF-D\textsuperscript{49}, and PlGF\textsuperscript{50,51} – though it remains the most critical family member for angiogenesis. Both VEGF-/- and VEGF +/- mice have severe vascular defects and are embryonic lethal, underscoring the biological importance of VEGF\textsuperscript{46,52}. A wide range of cell types secrete VEGF, including vascular smooth muscle cells, macrophages, and tumor cells\textsuperscript{53}. In humans, six splice variants of VEGF exist, with VEGF-A\textsubscript{165} being the most abundant and biologically active form of VEGF\textsuperscript{54}. VEGF binds to the extracellular immunoglobulin-like domain of homo- or hetero-dimeric transmembrane tyrosine kinase receptors VEGFR1 (Flt-1 in mice)\textsuperscript{55} and VEGFR2/KDR (Flk-1 in mice)\textsuperscript{56}, as well as their co-receptors\textsuperscript{57,58} neuropilin 1 (Nrp-1) and Nrp-2\textsuperscript{59}.

The majority of the angiogenic activity of VEGF occurs as a result of its interactions with VEGFR2\textsuperscript{56,60}. In adults, VEGFR2 is expressed primarily on vascular endothelial cells\textsuperscript{61}. The binding of VEGF to VEGFR2 induces receptor dimerization\textsuperscript{62} and autophosphorylation\textsuperscript{63,64}, which stimulates endothelial cell survival, proliferation, migration, and permeability (Figure 1-3). VEGFR2 mediates survival by activating PI3K, which phosphorylates Akt\textsuperscript{65,66} and inhibits the activation of pro-apoptotic proteins Bcl2-associated death promoter (BAD) and caspase 9\textsuperscript{67,68}. VEGF also induces the expression of anti-apoptotic proteins, such as Bcl2\textsuperscript{69}. Activation of PI3K/Akt also promotes vascular permeability by stimulating endothelial nitric oxide synthase (eNOS) mediated generation of nitric oxide (NO)\textsuperscript{70}. Migration can also be stimulated by VEGFR2-induced PI3K activation\textsuperscript{71} and subsequent actin reorganization as well as by FAK phosphorylation\textsuperscript{72}. VEGFR2 also induces proliferation via activation of the MAPK/Erk pathway in a PKC-dependent manner\textsuperscript{63,73}. Together, these processes are critical for the promotion of angiogenesis.
**Figure 1-3: Schematic of the VEGFR-2 signaling pathways.** VEGF binds to VEGFR-2 on endothelial cells and induces VEGFR-2 dimerization and autophosphorylation. VEGFR-2 activation leads to activation of downstream signaling pathways leading to changes in cell survival, proliferation, migration, and vessel permeability. Figure adapted from 74.
Figure 1-3 (Continued)
**Anti-angiogenic Therapy**

Angiogenesis is a vital process throughout development, growth, and wound healing but is also essential during tumor development and often marks the transition from a dormant to a malignant state. In order to grow, tumors begin to stimulate the formation of a new vascular network, as they require dedicated oxygen and nutrient supplies to sustain abnormal growth\(^7\). They do this by tipping the balance of pro- and anti-angiogenic molecules in the tumor in favor of angiogenesis. This “angiogenic switch” is a critical step that enables tumor expansion, local invasion, and dissemination\(^7\)\(^-\)\(^8\).

Tumors provide a favorable environment for angiogenesis – VEGF is highly expressed in a number of tumor types, including GBM\(^9\). Hypoxia is a common feature of solid tumors and drives the production of VEGF\(^3\)\(^4\)\(^,\)\(^7\)\(^9\) and other pro-angiogenic factors within tumors. VEGF expression can also be upregulated by tumor acidosis\(^8\), mutated oncogenes\(^8\)\(^1\)\(^-\)\(^4\), hormones\(^8\), stress, and growth factors. High expression of VEGF causes tumor blood vessels to grow unchecked and abnormally. Tumor microvessels are often dilated and tortuous with excessive branching and distorted patterns of interconnection leading to heterogeneous vessel distribution (Figure 1-4). Vessels are often leaky due to the weakening of cell-cell junctions by VEGFR2 activation and an absence or disconnection of perivascular cells. Additionally, the basement membrane surrounding these vessels is often abnormally thick or thin, depending on the tumor type. These structural abnormalities lead to heterogeneous blood flow and perfusion, increased interstitial hypertension, and vascular collapse. Drug delivery and efficacy can thus be extremely varied throughout the tumor as the penetration of cytotoxic drugs may be limited by the spatial heterogeneity and function of the vasculature and radiotherapy relies on oxygen for efficacy\(^8\)\(^6\).
Figure 1-4: Optical frequency domain imaging (OFDI) image of U87 GBM tumor vessels.

Depth-projected vasculature of a mouse brain bearing an orthotopic U87 xenograft GBM tumor in the upper left cerebral hemisphere of the brain. The tumor has a much higher microvessel density that the surrounding brain. The vessels are also more dilated, tortuous, heterogeneous, and branched then the capillaries in the brain. Depth is denoted by color – yellow is more superficial and red is deeper. Scale bar = 500µm. Figure adapted from 87.
In 1971, Dr. Judah Folkman proposed that inhibition of angiogenesis could be an effective therapeutic strategy for cancer. It was hypothesized that anti-angiogenic therapy would cause widespread vascular regression within tumors and effectively starve the tumors to death. Indeed, early pre-clinical studies seemed to support this hypothesis in numerous murine cancer models treated with an anti-VEGF monoclonal antibody. Unfortunately, clinical trials exploring the anti-tumor effects of VEGF-inhibition as a monotherapy were underwhelming and showed little to no OS benefit. However, when combined with chemotherapy, anti-VEGF therapy significantly improved PFS and OS compared to chemotherapy alone. These results were contradictory to the theory that anti-angiogenic agents would have anti-vascular effects, as chemotherapy relies on efficient tumor blood flow for effective delivery.

In 2001, Dr. Rakesh Jain proposed the “vascular normalization” hypothesis as an alternative to the starvation hypothesis in order to help explain these clinical findings. As illustrated in Figure 1-5, instead of causing rampant vascular pruning, anti-angiogenic therapy causes some vessel pruning and the reversion of the remaining tumor vasculature to a state that is closer to the normal vasculature, thus resulting in a normalized tumor microenvironment that allows for more efficient drug and oxygen delivery. In healthy tissue, pro- and anti-angiogenic molecules are in balance to maintain the existing vasculature. When tumors grow, they shift the balance towards a pro-angiogenic state, which leads to the aggressive formation of new, abnormal vessels. Anti-angiogenic therapy can help restore the balance of angiogenic factors. This shift results in a more homogeneous, functional, orderly network of vessels that can reduce the heterogeneity in the blood flow in different regions throughout the tumor. Also, anti-angiogenic therapy can improve contact between endothelial cells and the perivascular cells that stabilize the vessels, resulting in a more mature and less leaky vessel, which in turn should
Figure 1-5: Schematic of vascular normalization in response to anti-angiogenic therapy. (A) The tumor vasculature is abnormal, both structurally and functionally. The use of anti-angiogenic agents improves vessel structure and function, resulting in a vasculature that more closely resembles the normal tissue. Excessive vascular pruning due to high doses or prolonged use of anti-angiogenic agents may lead to aggressive vessel pruning and the development of a hypoxic microenvironment and vasculature that results in inadequate delivery of drugs and oxygen. (B) Two-photon images of the dynamics of vascular normalization after anti-VEGFR2 therapy. The left image shows normal skeletal muscle vessels, followed by images of human colon carcinoma in mice at days 0, 3, and 5 after therapy. (C) Diagram depicting the changes in pericytes (red) and basement membrane (blue) during normalization. (D) These vasculature changes may be the result of changes in the balance between pro- and anti-angiogenic factors within the tumor tissue. Figure adapted from 101.
Figure 1-5 (Continued)
reduce vascular permeability and intratumoral fluid pressure. Additionally, increased blood flow should help alleviate hypoxia within the tumors and prevent further angiogenic stimuli. Normalization of the tumor vasculature, therefore, should make the tumor more susceptible to chemotherapy, radiation, and immunotherapy.

**Anti-angiogenesis in GBM**

GBM is one of the most vascular and angiogenic tumors. Intra-tumoral levels of VEGF and VEGFR2 are correlated with the histological grade of glioma\textsuperscript{102,103}. The high degree of vascular proliferation, permeability, and expression of pro-angiogenic growth factors, including VEGF, found in GBM makes it a very attractive target for anti-angiogenic therapy\textsuperscript{104}. GBM blood vessels are structurally abnormal: they are disorganized and tortuous with increased vessel diameters, decreased perivascular cell coverage, and thicker basement membranes than that of normal brain vessels\textsuperscript{9,101,105-110}. GBMs are also extremely hypoxic, making them highly resistant to radiotherapy\textsuperscript{111}.

A pre-clinical study of anti-angiogenic therapy in GBM done in our lab found that inhibition of VEGF signaling, through the selective inhibition of VEGFR2, was able to transiently normalize vessels and improve overall survival\textsuperscript{105}. Winkler et al. showed that anti-VEGFR2 therapy temporarily increased pericyte coverage and decreased basement membrane thickness, mean vessel density, and vessel diameter of tumor vessels during a “window of normalization (Figure 1-6)”. These changes in vessel structure led to reduced hypoxia and enhanced response to radiation therapy during the normalization window\textsuperscript{105}. Further pre-clinical studies by Kamoun et al. showed that a major part of the benefit of normalization in GBM comes
Figure 1-6: Anti-angiogenic therapy produces a normalization window. The use of anti-angiogenic agents leads to transient vessel normalization. This window of normalization is characterized by changes in vascular morphology, pericyte coverage, and basement membrane thickness. During the window Ang-2 levels decrease, but levels rebound as tumors escape from therapy. Figure adapted from 105.
Figure 1-6 (Continued)
from the transient reduction of brain edema that occurs as a result of improved vessel function and reduced leakiness, rather than from direct anti-tumor growth effects\textsuperscript{112}.

Clinical studies in rGBM patients have also found that transient vessel normalization is accompanied by a reduction in tumor-associated edema and the need for corticosteroids, thus greatly improving quality of life\textsuperscript{113,114}. However, despite a prolongation of PFS, the majority of GBM patients treated with agents targeting the VEGF pathway eventually relapse. To date, no trial has shown an OS benefit with anti-VEGF monotherapy. Resistance is thought to be mediated in part by the alteration and adaptation of VEGF-independent angiogenic, growth, and survival programs in response to therapy\textsuperscript{115} as well as the recruitment of pro-angiogenic bone marrow derived cells (BDMCs) to facilitate revascularization and invasion. Additionally, GSCs may be a source of resistance to anti-VEGF therapy as they are able to promote VEGF-independent angiogenesis, possibly through transdifferentiation into endothelial cells and/or pericytes\textsuperscript{17,20,22,29-31}.

A recent study of tumor autopsy tissues from rGBM patients treated with therapies targeting VEGF or VEGFR shows abnormally high levels of Angiopoietin-2 (Ang-2)\textsuperscript{116}. Additionally, in both clinical and pre-clinical GBM studies, Ang-2 levels are temporarily decreased following VEGF-pathway inhibition, but then rebound as tumors become resistant to anti-VEGF therapy. These data collectively suggest that Ang-2 may be a part of the escape mechanism used by GBM in response to anti-VEGF therapy\textsuperscript{105,113}.

**Angiopoietin-2**

Ang-2 is a member of the angiopoietin family of angiogenic growth factors. Also included in this family are Ang-1, Ang-4, angiopoietin-like protein 1 (ANGPTL-1, also known
as Ang-3), ANGPTL-2, ANGPTL-3, ANGPTL-4, ANGPTL-5, ANGPTL-6, and ANGPTL-7. The angiopoietins form homomeric higher-order multimers\textsuperscript{117} (three to six proteins or more) and signal through two related tyrosine kinase receptors: Tie-1 and Tie-2/TEK. The core components of the Ang-Tie signaling system are Ang-1, Ang-2, and Tie-2.

Ang-1 is expressed by perivascular cells and acts as an agonist of the Tie-2 receptor. Tie-2 is expressed primarily on endothelial cells but has also been found on hematopoietic stem cells and subsets of monocytes/macrophages. Both Ang-1 and Tie-2 are essential for proper vessel development during embryogenesis; mice deficient of either Ang-1\textsuperscript{118} or Tie-2\textsuperscript{119,120} have very similarly severe cardiac and vascular defects which lead to embryonic lethality, suggesting that Ang-1 activation of Tie-2 mediates remodeling and stabilization of developing vessels. Transgenic overexpression of Ang-1 enhances vessel formation and promotes vessel enlargement\textsuperscript{121}. Interestingly, vessels exposed to Ang-1 are resistant to leakage induced by VEGF\textsuperscript{122,123}.

Ang-1 promotes vessel maturation and stabilization by stimulating Tie-2 phosphorylation and promoting interactions between endothelial cells, perivascular cells, basement membrane, and the surrounding matrix (Figure 1-7)\textsuperscript{124}. Ang-1 binding to Tie-2 stimulates Tie-2 auto-phosphorylation and activation of multiple downstream signaling pathways, including the PI3K/AKT and ERK pathways. Tie-2 activation promotes endothelial cell survival by inhibiting pro-apoptotic proteins, including BAD, procaspase-9, and survivin\textsuperscript{67,125-127}. Tie-2 also promotes endothelial cell survival through the activation of eNOS\textsuperscript{128}. ERK activation leads to endothelial cell proliferation and migration\textsuperscript{124,129,130}. Finally, Tie-2 inhibits inflammation by preventing NF-κB-mediated expression of inflammatory molecules such as intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1), and others\textsuperscript{131-134}. 

24
Figure 1-7: Schematic of the Tie-2 signaling pathways. Ang-1 binds to Tie-2 on endothelial cells and induces Tie-2 dimerization and autophosphorylation. Tie-2 activation leads to activation of downstream signaling pathways leading to changes in endothelial cell proliferation, migration, survival, and local inflammation. Ang-2 is a competitive inhibitor of Ang-1 and normally functions as a Tie-2 antagonist but can function as a partial agonist under certain circumstances. Vascular endothelial protein tyrosine phosphatase (VEPTP) dephosphorylates activated Tie-2 and prevents downstream signaling. Ang-2 can also signal changes in ERK1/2 independent of Tie-2 via integrin α5β1. Figure adapted from\textsuperscript{135}. 
Figure 1-7 (Continued)
Ang-2 is a competitive inhibitor of Ang-1 and can prevent Tie-2 signaling leading to vessel destabilization. Ang-2 is predominantly expressed by endothelial cells at sites of vascular remodeling but can also be expressed by some smooth muscle cells. Constitutive Ang-1-Tie-2 signaling maintains vessel stability and quiescence. Disruption of this association by Ang-2 facilitates endothelial activation and responsiveness to pro-angiogenic stimuli. Ang-2 mediated vessel destabilization can be either pro- or anti-angiogenic. In the presence of VEGF, Ang-2 stimulates pericyte dissociation from the endothelium, allowing for existing vessels to sprout and form new blood vessels. In the absence of VEGF, Ang-2-induced loss of pericyte coverage stimulates vessel regression\(^1\). It has also been suggested that Ang-2 is also capable of stimulating Tie-2 activity under certain conditions. High levels of Ang-2 can activate Tie-2 phosphorylation under stress conditions and act as an endothelial cell survival factor\(^{18,136,137}\).

**Ang-2 in Non-CNS Cancers**

Both Ang-1 and Ang-2 have been implicated as playing a role in tumor angiogenesis and are highly expressed in the tumor vasculature\(^{138}\). High expression of Ang-2 is correlated with metastasis, malignancy, and poor prognosis in various types of solid tumors including hepatocellular carcinoma\(^{139}\), breast cancer, metastatic melanoma, and lung cancer\(^{140}\). Additionally, the balance between Ang-1 and Ang-2 can be predictive of disease state and progression in some cancers, such as ovarian cancer\(^{141}\). Increased expression of Ang-1 and Ang-2 in pre-clinical models has also been shown to be a part of the tumor “angiogenic rescue” in response to inhibition of VEGF-mediated angiogenesis and contributes to the acceleration of metastasis\(^{142-146}\). By shifting the balance between pro- and anti-angiogenic molecules, specifically
Ang-1 and Ang-2, tumors may be able to reactivate angiogenesis and facilitate further tumor growth\textsuperscript{147,148}.

In order to determine the role of the angiopoietins in non-CNS cancers, a number of groups have studied the effects of genetic and pharmalogical inhibition or overexpression of Ang-1 and/or Ang-2 in a variety of tumors, as shown in Table 1-1. Depending on the tumor type, level of expression or inhibition, and location of the implantation (orthotopic or subcutaneous (SC)), Ang-1 and Ang-2 could be either pro-angiogenic or anti-angiogenic. Additionally, these effects can be either pro-tumorigenic or anti-tumorigenic. For example, over-expression of Ang-2 in Lewis Lung Carcinoma (LLC) decreased tumor angiogenesis, slowed tumor growth, and increased tumor cell apoptosis\textsuperscript{149}. In contrast, a later study in LLC found that inhibition of Ang-2 using a knockout mouse system was able to slow tumor growth and promote vessel maturation\textsuperscript{150}. This conflicting body of work suggests that the role of the angiopoietins is highly context and dose dependent and may differ in different tissues and tumor types.

**Ang-2 in GBM**

The role of Ang-2 in the development and progression of GBM is extremely complex due to temporal and spatial differences in expression. Ang-2 mRNA is highly expressed in hyperplastic tumor blood vessels but is not normally found in vessels in the brain\textsuperscript{8,18,138}. Ang-2 expression is often found to be upregulated in human and pre-clinical GBMs\textsuperscript{18,151,152} and the local production of Ang-2 has been identified as an early marker of glioma- and glioblastoma-induced neovascularization\textsuperscript{8,138}. Additionally, the increasing ratio of Ang-1/Ang-2 has been shown to positively correlate with survival\textsuperscript{153} and vascular normalization\textsuperscript{113}.
Table 1-1: Effects of Ang-2 inhibition or over-expression in non-CNS tumors

<table>
<thead>
<tr>
<th>TUMOR TYPE/SITE</th>
<th>EXPRESSION/ INHIBITION</th>
<th>ANGIOGENESIS</th>
<th>OUTCOME</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lewis Lung carcinoma - SC</td>
<td>Inhibition – knockout mouse</td>
<td>No change</td>
<td>Slower tumor growth; decreased vessel diameter at later stages; increased maturation; increased perfusion</td>
<td>150</td>
</tr>
<tr>
<td>Lewis Lung carcinoma - SC</td>
<td>Expression</td>
<td>Decreased</td>
<td>Decreased tumor growth; increased apoptosis of tumor cells and ECs; decreased maturation</td>
<td>149</td>
</tr>
<tr>
<td>MT-ret melanomas - SC</td>
<td>Inhibition – knockout mouse</td>
<td>Decreased</td>
<td>Slower tumor growth; decreased vessel diameter</td>
<td>150</td>
</tr>
<tr>
<td>Colon cancer cell (HCT116) - SC</td>
<td>Expression - Adenovirus</td>
<td>Decreased</td>
<td>Tumor growth and angiogenesis inhibition; microvessel regression</td>
<td>154</td>
</tr>
<tr>
<td>Colorectal (HT29) – SC</td>
<td>Expression</td>
<td>Increased</td>
<td>Increased tumor growth; increased tumor proliferation</td>
<td>148</td>
</tr>
<tr>
<td>Colo205 – SC</td>
<td>Inhibition – Peptide-Fc protein</td>
<td>Decreased</td>
<td>Tumor growth delay; normalized vessels</td>
<td>155</td>
</tr>
<tr>
<td>HCC – SC</td>
<td>Expression – Inducible</td>
<td>No change</td>
<td>No change in tumor development; co-expression with VEGF increased tumor growth, angiogenesis, and MMP-2 and -9 activity</td>
<td>156</td>
</tr>
<tr>
<td>HCC (HuH-7) – IP near liver bed</td>
<td>Expression</td>
<td>Increased</td>
<td>Increased tumor growth; intratumoral hemorrhage; intraperitoneal bleeding</td>
<td>157</td>
</tr>
<tr>
<td>B16F10 melanomas - SC</td>
<td>Inhibition – knockout mouse</td>
<td>No change</td>
<td>Slower tumor growth; small decrease in vessel diameter; increased maturation</td>
<td>150</td>
</tr>
<tr>
<td>MCF7, MDA-MB231</td>
<td>Inhibition – Ab (3.19.3)</td>
<td>Decreased</td>
<td>Tumor growth delay</td>
<td>158</td>
</tr>
<tr>
<td>MMTV-PyMT, RIP1-Tag2</td>
<td>Inhibition – Ab (3.19.3)</td>
<td>Decreased</td>
<td>Tumor growth inhibition and reduced metastasis; increased hypoxia; impaired TEM-vascular association</td>
<td>159</td>
</tr>
<tr>
<td>LLC, B16F10 – SC, CT-26-luc, OVCA – orthotopic; MMTV-PyMT</td>
<td>Inhibition of VEGF and Ang-2 by Double Anti-Angiogenic Protein (DAAP)</td>
<td>Decreased</td>
<td>Tumor growth delay; increased vessel pruning; increased tumor hypoxia; decreased metastasis in melanoma model; enhanced efficacy with cytotoxic agents</td>
<td>160</td>
</tr>
<tr>
<td>Lewis Lung Carcinoma – SC</td>
<td>Expression – Inducible</td>
<td>Increased</td>
<td>No change in tumor growth; increased microvessel density; immature vessels (little pericyte coverage); increased EC apoptosis, hypoxia, and hemorrhage; increased TEM infiltration</td>
<td>161</td>
</tr>
</tbody>
</table>

SC = subcutaneous
<table>
<thead>
<tr>
<th>TUMOR TYPE/SITE</th>
<th>EXPRESSION/INHIBITION</th>
<th>ANGIOGENESIS</th>
<th>OUTCOME</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon – SC (Colo205, LoVo), Renal - SC (786-0, A498), Liver - SC (PLCPRF/5), Lung – SC (Calu6), Ovarian – SC (HeyA8)</td>
<td>Inhibition – Ab (MEDI3617)</td>
<td>Decreased</td>
<td>Reduced tumor angiogenesis; increased hypoxia; tumor growth inhibition; enhanced efficacy chemotherapy or bevacizumab</td>
<td>162</td>
</tr>
<tr>
<td>Colo205-SC</td>
<td>Inhibition – CovX Body (CVX-060)</td>
<td>Decreased</td>
<td>Tumor growth inhibition; reduced microvessel density; reduced TEM infiltration; enhanced efficacy in combination with anti-angiogenic or cytotoxic agents</td>
<td>163</td>
</tr>
<tr>
<td>Colo205 – SC, KPL-4 breast cancer - orthotopic</td>
<td>Inhibition – Ab (LC06/LC08)</td>
<td>Decreased</td>
<td>Tumor growth inhibition and reduced metastasis; increased tumor necrosis; vessel normalization</td>
<td>164</td>
</tr>
<tr>
<td>Colo205 – SC</td>
<td>Inhibition – Ab (LC06/LC08)</td>
<td>Decreased</td>
<td>Decreased tumor growth; reduced vessel density; increased tumor apoptosis</td>
<td>165</td>
</tr>
<tr>
<td>Colo205 – SC, A431 – SC, PC3 – SC</td>
<td>Inhibition – Ab (REGN910)</td>
<td>Decreased</td>
<td>Tumor growth inhibition; reduced vessel density; reduced EC proliferation and enhanced EC apoptosis; enhanced efficacy in combination with aflibercept</td>
<td>166</td>
</tr>
<tr>
<td>Colo205 - SC</td>
<td>Inhibition – peptibody (L1-7(N))</td>
<td>Decreased</td>
<td>Reduced tumor growth; reduced vascular sprouting; enhanced efficacy when combined with anti-VEGF</td>
<td>167</td>
</tr>
<tr>
<td>A431- SC, Colo205 - SC</td>
<td>Inhibition – peptibody (L1-7(N))</td>
<td>Decreased</td>
<td>Tumor growth inhibition and/or regression; reduced EC proliferation</td>
<td>168</td>
</tr>
<tr>
<td>Colo205-SC</td>
<td>Inhibition of Ang-2/VEGF - CovX Body (CVX-241)</td>
<td>Decreased</td>
<td>Tumor growth delay; decreased microvessel density; enhanced efficacy when combined with chemotherapy</td>
<td>169</td>
</tr>
<tr>
<td>KPL-4, MDA-MB-231, MCF-7, E0771, Colo2015 - SC, MC38, SUDHL4, H460M2 - SC, Calu-3, Panc-1, PC-3, RXF-486, HCC07-0409Av7, GC23-0909, N87</td>
<td>Inhibition of Ang-2 and VEGF – Cross-MAb (A2V)</td>
<td>Decreased</td>
<td>Tumor growth delay; decreased vessel density; increased pericyte coverage; no change in tumor apoptosis, proliferation, or necrosis; enhanced efficacy when combined with chemotherapy</td>
<td>170</td>
</tr>
</tbody>
</table>

SC = subcutaneous
To investigate the role of Ang-2 in GBM tumor angiogenesis and progression, a number of groups have over-expressed or inhibited Ang-2 in GBM tumors. Table 1-2 summarizes the results of these studies. As in non-CNS tumors, the role of Ang-2 in tumor angiogenesis is not entirely clear. In an orthotopic human xenograft model of GBM, U87, over-expression of Ang-2 led to disrupted vessel formation and tumor growth delay\textsuperscript{151}. In the same model, however, another study found that Ang-2 over-expression stimulated tumor growth and led to a more invasive phenotype\textsuperscript{152}. Other overexpression models have shown similar increases in angiogenic activity, which may promote tumor growth\textsuperscript{18,17}. In line with these findings, one study showed that Ang-2 inhibition immediately following tumor implantation decreased angiogenesis and tumor growth, which lead to an increase in survival\textsuperscript{172}. These data, in addition to the fact that a high Ang-2/Ang-1 ratio is correlated with increased angiogenesis and tumor growth in patients, suggest that Ang-2 is a potential therapeutic target in GBM.

To study the role of Ang-2 in promoting the angiogenic escape from anti-VEGF therapy, Chae et al. engineered a U87 cell line ectopically over-expressing Ang-2. Alone, Ang-2 over-expression had no effect on tumor growth or overall survival compared to mock-transfected U87 tumors. However, U87-Ang-2 tumors did have a much higher microvascular density, increased vessel diameter, and decreased pericyte coverage. When these tumors were treated with an anti-VEGFR2 antibody, ectopic expression of Ang-2 destabilized tumor vessels and increased vessel permeability, resulting in impaired edema control, and compromised the survival benefits of therapy (Figure 1-8)\textsuperscript{171}. These data suggest that Ang-2 may be a good therapeutic target for GBM in combination with VEGF-signaling inhibition.
Table 1-2: Effects of Ang-2 inhibition or over-expression in GBM

<table>
<thead>
<tr>
<th>TUMOR TYPE</th>
<th>EXPRESSION/ INHIBITION</th>
<th>ANGIOGENESIS</th>
<th>OUTCOME</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>U87MG</td>
<td>Expression</td>
<td>Increased vascular density</td>
<td>No changes in tumor growth or permeability</td>
<td>171</td>
</tr>
<tr>
<td>U87MG</td>
<td>Expression</td>
<td>Increased at invasive front</td>
<td>More invasive tumor with MMP2 expression</td>
<td>152</td>
</tr>
<tr>
<td>U87MG</td>
<td>Expression - Adenovirus</td>
<td>Decreased</td>
<td>Tumor growth delay</td>
<td>151</td>
</tr>
<tr>
<td>GS9L</td>
<td>Expression</td>
<td>Decreased vessel length</td>
<td>Tumor growth delay; increased survival; increased vascular perfusion; increased tumor hypoxia</td>
<td>173</td>
</tr>
<tr>
<td>GS9L</td>
<td>Expression – Inducible</td>
<td>Decreased</td>
<td></td>
<td>174</td>
</tr>
<tr>
<td>C6</td>
<td>Expression</td>
<td>Increased at tumor periphery</td>
<td>Increased vessel cooption</td>
<td>18</td>
</tr>
<tr>
<td>Gl261</td>
<td>Inhibition with L1-10 peptide-Fc fusion protein – begun immediately after implantation</td>
<td>Decreased vessel diameter and vascular density</td>
<td>Tumor growth delay; increased survival</td>
<td>172</td>
</tr>
</tbody>
</table>
Figure 1-8: Ang-2 over-expression compromises the survival benefit of anti-VEGFR2 therapy. U87 tumors were engineered to ectopically over-expressing Ang-2. Mice were treated with control IgG (dotted lines) or the anti-VEGFR2 antibody DC101 (solid lines). In wild-type U87, survival was significantly increased with anti-VEGFR2 therapy but this survival benefit was compromised in U87-Ang-2 tumors. Figure adapted from 171.
Figure 1-8 (Continued)
**Targeting Ang-2**

There are a number of Ang-2 inhibitors currently under development and in clinical trials for the treatment of multiple tumor types. Table 1-3 summarizes the inhibitors that are currently, or were formerly, being pursued for therapeutic use. There are two main types of inhibitors being investigated – peptide-based inhibitors and monoclonal antibodies. Trebananib (AMG386, Amgen), a peptide-FC fusion construct (peptibody) that targets both Ang-2 and Ang-1, is by far the most advanced of these inhibitors with over 30 active clinical trials, including 4 phase III trials in ovarian cancer (NCT01493505, NCT01204749, NCT01281254) and post-menopausal osteoporosis (NCT01575834). Additionally, three antibodies are in clinical development – MEDI3617 (MedImmune, NCT01248949, NCT02141542), REGN910 (Sanofi, NCT01688206, NCT02141295), and the Ang-2-VEGF-A CrossMab RO5520985 (Roche, NCT01271972, NCT01688960). Pre-clinically, each of these inhibitors has shown promising results in multiple therapeutic settings, though they have yet to be evaluated in GBM.

**Tumor-associated Macrophages (TAMs)**

Tumor-associated macrophages (TAMs) represent a major class of bone marrow-derived cells (BMDCs) in tumors and have been shown to influence tumor growth, both in terms of progression and regression. In mice, macrophages are distinguished from other BMDCs by the expression of CD11b, F4/80, and CSF-1 receptor (CSF-1R) and the absence of Gr1. Macrophages are a main component of the innate immune system and are called to tissues to “sample” the environment and provide an appropriate immune response. Macrophages can decide to do one of three things – fight (e.g. pathogen response), fix (e.g. wound-healing response), or call in reinforcements (e.g. T cells in response to “foreign” stimuli).
Table 1-3: Ang-2 inhibitors

<table>
<thead>
<tr>
<th>INHIBITOR</th>
<th>TARGET</th>
<th>TYPE</th>
<th>CLINICAL TRIALS (TUMOR TYPE)</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEDI3617 (Medimmune)</td>
<td>Ang-2</td>
<td>Human mAb</td>
<td>NCT01248949 (advanced solid tumors, advanced recurrent ovarian tumors), NCT02141542 (unresectable Stage III or IV melanoma)</td>
<td>162</td>
</tr>
<tr>
<td>3.19.3 (AstraZeneca)</td>
<td>Ang-2</td>
<td>Human mAb</td>
<td></td>
<td>158</td>
</tr>
<tr>
<td>CVX-060 (Pfizer)</td>
<td>Ang-2</td>
<td>CovX-Body</td>
<td>NCT00879684 (advanced solid tumors), NCT00982657 (advanced renal cell carcinoma (RCC)), NCT01225510 (rGBM), NCT01441414 (metastatic RCC)</td>
<td>163</td>
</tr>
<tr>
<td>CVX-241 (Pfizer)</td>
<td>VEGF, Ang2</td>
<td>CovX-Body</td>
<td>NCT01004822 (advanced solid tumors)</td>
<td>169</td>
</tr>
<tr>
<td>DAAP (KAIST)</td>
<td>VEGF, Ang-2</td>
<td>Chimeric decoy receptor</td>
<td></td>
<td>160</td>
</tr>
<tr>
<td>AMG386/trebananib (Amgen)</td>
<td>Ang-1, Ang-2</td>
<td>Peptide-Fc fusion protein (Peptibody)</td>
<td>31 studies currently active.</td>
<td>168</td>
</tr>
<tr>
<td>AMG780 (Amgen)</td>
<td>Ang-2</td>
<td>mAb</td>
<td>NCT01137552 (advanced solid tumors)</td>
<td>175</td>
</tr>
<tr>
<td>LC06 (Roche)</td>
<td>Ang-2</td>
<td>Human mAb</td>
<td></td>
<td>164,165</td>
</tr>
<tr>
<td>LC08 (Roche)</td>
<td>Ang-1, Ang-2</td>
<td>Human mAb</td>
<td></td>
<td>164,165</td>
</tr>
<tr>
<td>RO5520985/A2V CrossMab (Roche)</td>
<td>VEGF, Ang-2</td>
<td>Bi-specific bivalent human mAb</td>
<td>NCT01688206 (advanced or metastatic solid tumors), NCT02141295 (metastatic colorectal cancer)</td>
<td>170</td>
</tr>
<tr>
<td>REGN910 (Sanofi)</td>
<td>Ang-2</td>
<td>Human mAb</td>
<td>NCT01271972 (solid tumors), NCT01688960 (advanced solid tumors)</td>
<td>166</td>
</tr>
</tbody>
</table>

**Bold** = no longer under development
TAMs are an extremely heterogeneous cell population that can be distinguished into two major phenotypic populations – anti-tumoral M1 (or classically activated) TAMs and pro-tumoral M2 (or alternatively activated) TAMs – though TAMs often fall into a spectrum between extremes depending on tumor type and micro-environmental triggers\textsuperscript{176,177}. M1 and M2 macrophages were so named because they promote Th1 (fight) and Th2 (fix) responses, respectively\textsuperscript{178,179}, however they have also been found to have a variety of T-cell and B-cell independent functions.

Classically activated M1 macrophages encourage tissue inflammation and are usually considered to play an anti-tumoral role in tumors. They are polarized by LPS and IFN\textgreek{y} and secrete high levels of IL-12 and low levels of IL-10. M1 TAMs can also be distinguished from M2 TAMs by the expression of inducible nitric oxide synthase (iNOS)\textsuperscript{180,181}. The production of reactive oxygen species (ROS) and nitric oxide (NO) mediate the cytotoxic activities of M1 TAMs.

In malignant tumors, TAMs are often skewed towards the M2 phenotype in tumors and are mostly pro-tumoral, promoting tumor cell survival, proliferation, migration, and angiogenesis\textsuperscript{182-186}. M2 macrophages are distinguished from classically activated TAMs by the expression of interleukin-10 (IL-10), arginase-1, TGF-\textbeta, and mannose receptor complex 1 (MRC1)\textsuperscript{187-190}. One mechanism by which M2 TAMs stimulate tumor growth is through the facilitation of tumor angiogenesis. TAMs promote angiogenesis through the secretion of growth factors such as VEGF, PDGF, TGF\textbeta, and FGF, as well as other angiogenesis-enhancing enzymes including matrix metalloprotease-9 (MMP-9) and thymidine phosphorylase (TP)\textsuperscript{191-193}. For example, stimulation of macrophages with CSF1 leads to the upregulation of VEGF and
promotes angiogenesis\textsuperscript{194}. M2 TAMs also directly promote tumor cell proliferation and resistance to apoptosis by secreting growth factors such as EGF and FGF\textsuperscript{183,195}.

Given that TAMs often function in a pro-tumor manner, a number of studies have been carried out to determine how blocking TAMs affects tumor growth and survival. Macrophage depletion experiments have been shown to decrease tumor growth and metastasis in multiple pre-clinical models\textsuperscript{196-198}. Reprogramming the tumor immune environment is also a recent trend in therapeutic research. TAMs, by nature, display extreme plasticity and it has been shown that TAMs can be “re-educated” from an M2 phenotype to an M1 phenotype\textsuperscript{199}. Pre-clinical studies blocking TAM function or re-polarizing TAMs in multiple tumor models have shown promising survival benefits\textsuperscript{200-203}.

Conclusions and Perspectives

The use of anti-angiogenic therapeutic agents targeting the VEGF pathway in GBM have had mixed successes in clinical trials, despite promising pre-clinical results. Anti-VEGF agents normalize tumor vessels and temporarily relieve intracranial edema. In patients, as in mice, there appears to be a window of time during which anti-angiogenic therapy is most effective. Resistance is mediated in part by the up-regulation of alternative angiogenic pathways. Ang-2 is decreased during the window of normalization and rebounds as tumors escape from therapy, making it an attractive target for combination with anti-VEGF therapies. My thesis work will test the hypothesis that combined inhibition of Ang-2 and VEGF signaling promotes survival in GBM beyond that of anti-VEGF monotherapy. In Chapter 2, I will detail our work looking at the effects of combined inhibition on survival and tumor growth in two orthotopic murine models of GBM. We also determined how combined inhibition affects the tumor vessels and intratumoral
edema. Finally, we observed changes in the macrophage population and assessed whether they might play a role in promoting survival during combined inhibition of Ang-2 and VEGF signaling. In Chapter 3, I will further discuss the significance of these findings in the context of the field. Unanswered questions and future directions for this project will also be explored.
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CHAPTER 2

Results
**Author Contributions**

TEP, NDK, YH, CTF, KM, WSK, GS, MD, TVK, MS, JG performed experiments and analyzed results. TEP and AM performed statistical analyses. TEP, NDK, and RKJ designed experiments. LX, DGD, DF, and CCL advised on experimental design. TEP wrote the article and this thesis. All authors edited the article. S. Roberge assisted in tumor implantation and cranial window preparation. P. Huang managed the mouse colony and generated mice for the paper. E. Ager, A. Batista, S. Goel, C. Kesler, and K. Jung provided valuable discussions and experimental assistance.
At the time of submission of this thesis, much of the work presented in this chapter has been submitted as a manuscript entitled:

**Dual Targeting of Ang-2 and VEGF Receptors Normalizes Glioblastoma Vasculature and Prolongs Survival via Tumor Associated Macrophages**

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Running Head
Anti-Ang-2/VEGFR Combination Therapy Improves Survival in GBM
ABSTRACT

**Purpose:** Glioblastomas (GBMs) rapidly become refractory to standard anti-vascular endothelial growth factor (VEGF) therapy. We previously demonstrated that ectopic over-expression of Angiopoietin-2 (Ang-2) compromises the benefits of anti-VEGF receptor (VEGFR) treatment in mice and that circulating Ang-2 levels in GBM patients rebound after an initial decrease following cediranib (a pan-VEGFR tyrosine kinase inhibitor) administration. Here we tested whether dual inhibition of VEGFR/Ang-2 improved survival in murine models of GBM.

**Methods:** We tested the efficacy of cediranib, MEDI3617 (an anti-Ang-2 neutralizing antibody) or cediranib + MEDI3617 dual therapy versus control IgG in two orthotopic models of GBM (Gli261 and U87). We examined tumor growth and vascularization using intravital microscopy, MRI, and optical frequency domain imaging, evaluated tumor-associated macrophage (TAM) recruitment/phenotype using flow cytometry, and blocked TAM infiltration using an anti-colony-stimulating factor-1 (CSF-1) antibody.

**Results:** Following anti-VEGFR/Ang-2 dual therapy, microvessel perivascular cell coverage was increased in both models, which was associated with an extended window of vascular normalization beyond cediranib. Dual therapy improved survival over monotherapies by delaying Gli261 growth and increasing U87 necrosis, effectively reducing viable tumor burden. The improvements in vascular structure and survival were associated with an increased proportion of anti-tumor TAMs in Gli261. Inhibition of TAM recruitment with anti-CSF1 compromised the survival benefit of dual therapy.

**Conclusions:** Dual inhibition of VEGFR and Ang-2 can improve survival in preclinical GBM models by reducing tumor burden in part via increased anti-tumor TAMs. This approach may
represent a potential therapeutic strategy to overcome the limitations of anti-VEGFR therapy in GBM patients.
Introduction

Glioblastoma (GBM) is the most aggressive primary brain tumor in adults. Based on promising phase II trials, bevacizumab (Avastin®), an anti-vascular endothelial growth factor (VEGF) monoclonal antibody, was approved by FDA in 2009 as salvage therapy for recurrent GBM (rGBM)\(^1\)\(^-\)\(^7\). However, two randomized phase III trials of bevacizumab with chemoradiation in newly-diagnosed GBM patients failed to demonstrate overall survival benefit despite prolongation of progression-free survival\(^8\)\(^,\)\(^9\). Inhibition of VEGF-signaling can alleviate vascular abnormality (e.g. vascular hyper-permeability and heterogeneous blood flow) in GBM (“normalization”) and subsequently reduce intracranial edema, accounting for a major part of the benefit in animal models\(^10\)\(^,\)\(^11\). Similarly, cediranib therapy induces transient vessel normalization in rGBM patients, effectively reducing edema and the requirement for corticosteroids, thus improving quality of life\(^12\)\(^,\)\(^13\). Unfortunately, resistance to anti-VEGF therapy develops rapidly. The prevailing hypothesis is that modulation of other angiogenic, growth, and survival programs in response to VEGF-signaling blockade induces resistance\(^1,\)\(^6\)\(^,\)\(^7\)\(^,\)\(^14\). Thus, despite the initial promise of anti-angiogenic therapy, there is an urgent need to devise novel combinations with other therapeutics to increase survival in GBM\(^6\).

Angiopoietin-2 (Ang-2) is often upregulated in GBM\(^15\)\(^-\)\(^17\) where it is thought to play a role in tumor angiogenesis\(^15,\)\(^18,\)\(^19\). Ang-2 is a member of the angiopoietin family of angiogenic growth factors that signals primarily through the tyrosine kinase receptor Tie-2. Ang-1 and Ang-2 play important, often complementary roles in maintaining normal vasculature through the modulation of vessel stability\(^20\). Ang-2-mediated vessel destabilization can be either pro- or anti-angiogenic in a context-dependent manner\(^15,\)\(^21\)\(^-\)\(^23\). Recent data indicate a potential role for increased Ang-2 expression as an escape mechanism to anti-VEGF therapy. In both clinical and preclinical GBM
studies, Ang-2 levels temporarily fall following VEGF-pathway inhibition, but later rebound as tumors become resistant to therapy\textsuperscript{10,12}. Ectopic expression of Ang-2 in a GBM animal model compromised the survival benefit of VEGF-signaling inhibition by impairing vessel normalization and edema control\textsuperscript{24}. Moreover, tumor autopsy tissues from rGBM patients treated with anti-VEGF therapy have abnormally high levels of Ang-2\textsuperscript{25} and the Ang-1/Ang-2 ratio positively correlates with survival\textsuperscript{26} and vascular normalization\textsuperscript{12}. Therefore, we hypothesized that dual inhibition of VEGF and Ang-2 signaling could prolong the window of normalization or normalize vessels to a greater extent and thereby enhance the survival benefit of anti-VEGF therapy.

In this study, we combined an Ang-2 neutralizing monoclonal antibody that targets both human and mouse Ang-2, MEDI3617 (MedImmune, Gaithersburg, MD)\textsuperscript{27}, with cediranib (AstraZeneca Pharmaceuticals, Cheshire, UK) and assessed effects of dual therapy in two orthotopic models of GBM in mice: GL261 (a hypovascular murine GBM cell line) and U87 (a highly angiogenic human GBM cell line).

**Materials and Methods**

**Animal Models and Cell Lines**

GL261 cells (NCI Repository, Bethesda, MD) were cultured in Neurocult\textsuperscript{TM} NS-A Proliferation Medium (Stemcell Technologies, Vancouver, Canada) as non-adherent neurospheres. U87 cells (ATCC, Manassas, VA) were cultured in DMEM with 10% FBS. Cell lines were authenticated by IDEXX laboratories (Westbrook, ME). Both cell lines were stably transduced with green-fluorescent protein (GFP) or discosoma red fluorescent protein (dsRed) using a retroviral construct.
For most studies, cranial windows were implanted into nude or C57Bl/6 mice as previously described\textsuperscript{28} and mice were allowed to recover one week before tumor implantation. Briefly, U87-GFP fragments (0.2 - 0.3 mm diameter) were then implanted into the left cerebral hemisphere 0.4 - 1 mm deep. A Gl261-dsRed cell suspension (500,000 cells) in Neurocult\textsuperscript{TM} was injected stereotactically 1.5mm deep.

For the anti-CSF1 survival experiments, Gl261-GFP tumors were implanted into C57Bl/6 mice through a small cranial “door” (made by cutting a 3-sided square in the skull to allow temporary access to the brain during injection) instead of using cranial windows.

All animal procedures followed Public Health Service Policy on Humane Care of Laboratory Animals guidelines and were approved by the Massachusetts General Hospital (MGH) Institutional Animal Care and Use Committee. MGH is accredited with AAALAC and the NIH Office of Laboratory Animal Welfare (A3596-01).

Pre-treatment Tumor Size Monitoring

For survival, growth, histological, and edema studies, U87-GFP and Gl261-dsRed tumors were monitored daily by fluorescent intravital microscopy and optical frequency domain imaging (OFDI)\textsuperscript{29} until the tumors reached an average diameter of 2.5 mm or 2.0 mm, respectively, at which point treatment began (defined as day 0). For the anti-CSF1 survival experiments, Gl261-GFP tumors were allowed to grow for 21 days, at which point the tumors were approximately 2.0 mm in diameter, before treatment started.

Treatment Protocols
Mice were treated with control IgG (10mg/kg, MedImmune) + 1% Tween; MEDI3617 (10mg/kg) + 1% Tween; control IgG + cediranib (6mg/kg); cediranib + MEDI3617; anti-CSF1 (20mg/kg, BD Biosciences 557858, San Jose, CA) or MEDI3617 + cediranib + anti-CSF1. Cediranib dissolved in 1% Tween was administered by oral gavage daily. MEDI3617 was administered by intraperitoneal (i.p.) injection twice weekly. Anti-CSF1 was administered i.p. weekly. For survival studies, mice were humanely euthanized when they became moribund or displayed severe neurological symptoms.

**In vitro Viability Assay**

GL261-dsRed tumor spheres were dissociated to form a single-cell suspension. 5x10^3 cells were plated per well in 96 well plates in triplicate, to be read at 24, 48, and 72 hours after addition of the stimulant. Cells were treated with IgG (1µg/ml), 0.1% Tween, recombinant mouse VEGF (rmVEGF), 10ng/ml), cediranib (4.5ng/ml), rmVEGF + cediranib, rmAng-2 (1µg/ml), MEDI3617 (1µg/ml), rmAng-2 + MEDI3617, rmVEGF + rmAng-2, cediranib + MEDI3617, rmVEGF + rmAng-2 + cediranib + MEDI3617, anti-CSF1 (1µg/ml), anti-CSF1 + cediranib + MEDI3617, and anti-CSF1 + rmVEGF + rmAng-2, cediranib + MEDI3617. Plates were incubated at 37°C/5%CO₂ then viability, inferred from ATP content measurement, was read using the Cell-Titer Glo Luminescent Cell Viability Assay (G7570, Promega, Madison, WI).

**Western Blotting**

Protein lysates were generated from tumors treated with IgG, MEDI3617, cediranib, or cediranib + MEDI3617. Untreated HUVECs acted as a positive control for Tie-2 and HUVECs treated with Ang-2 acted as a positive control for pTie-2 staining. Tumors or cells were lysed
with RIPA buffer (Boston Bioproducts, Ashland, MA) supplemented with protease inhibitors (Complete Ultra, Roche, Basel, Switzerland) and phosphatase inhibitors (Phostop, Roche). Protein from each sample (50µg total lysate) was immunoprecipitated using Protein A Sepharose beads (GE Healthcare Lifesciences, Pittsburgh, PA) conjugated to an anti-Tie-2 antibody (c20, Santa Cruz Biotechnology, Santa Cruz, CA) or IgG antibody (“Mock IP”). Western blotting was performed on an SDS-PAGE electrophoresis system using an 8% Tris gel, followed by transfer to a PVDF membrane. The membrane was blotted with an antibody against phospho-tyrosine (EMD Millipore, Billerica, MA) before development, antibody stripping, and blotting with a total Tie-2 antibody.

**Histology and Immunostaining**

Brains with tumors were collected from U87-bearing mice at days 3 and 8 after treatment initiation and from GL261-bearing mice at days 14 and 21. Samples were snap-frozen and OCT (Tissue-Tek, Torrance, CA)-embedded. Samples were sectioned every 10µm, incubated with primary antibodies (see below) overnight at 4°C before incubation with fluorescently-conjugated secondary antibodies (1:200, Jackson ImmunoResearch, West Grove, PA), and imaged using an Olympus FV1000 Laser Scanning Confocal Microscope (Olympus America Inc., Melville, NY). Necrotic areas were excluded from analysis. Microvessel density, perivascular cell coverage of vessels, basement membrane coverage, proliferation, apoptosis, and macrophage densities were assessed using a semi-automatic in-house MATLAB (Mathworks, Natick, MA) segmentation algorithm. Basement membrane thickness was assessed using ImageJ (NIH, Bethesda, MD).

Primary antibodies included: CD31 (endothelial cells, 1:200, MAB1398Z, EMD Millipore, Billerica, MA), Desmin (perivascular cells, 1:500, AF3844, R&D Systems,

Additional tissue was collected on day 6 from mice bearing U87 or day 20 from mice bearing Gl261 tumors for histological analyses in paraffin and 5 μm sections were stained with hematoxylin and eosin (H&E) to determine the extent of necrosis.

**MRI-based Measurement of Tumor Volume**

T2-weighted Rapid Acquisition with Relaxation Enhancement (RARE) images were acquired on a 9.4 Tesla MRI scanner (Bruker Biospin, Billerica, MA) as previously described. Tumor volumes were determined from the T2 hyperintense regions of the brain.

**Water Content Analysis by Dry/Wet Weight Analysis**

Edema in the tumor, ipsilateral hemisphere, and contralateral hemisphere was assessed, immediately following sacrifice, using dry/wet weigh analysis as previously described to determine the water content of the tumor. Briefly, tissues were weighed immediately after dissection and dried in a 100C oven for up to one week. They were weighed throughout the drying period until a final dry weight was established. The water content of the tissues was calculated using the formula:

\[
\text{Water content} = \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}}
\]

**Optical Frequency Domain Imaging Analysis of Tumor Growth**
Mice bearing cranial windows were assessed every other day beginning on day 0 of treatment using a custom-built OFDI microscope as previously described\textsuperscript{29}. Tumor growth was assessed from structural images generated using ImageJ.

**Flow Cytometry**

On day 10 of treatment animals were anesthetized, PBS-perfused, and tumors were collected. Single-cell suspensions were blocked with rat anti-mouse CD16/CD32 mAB (BD Biosciences) and stained with the following monoclonal anti-mouse antibodies: FITC-CD4, PE-Cy7-CD4, FITC-CD8a, PE-CD8a, APC-CD8a, PE-CD45, PE-Cy7-CD45, APC-Gr1, APC-Cy7-CD11b, FITC-CD11c, APC-CD11c, APC-Cy7-CD11c, (BD Biosciences), FITC-F4/80, PE-F4/80, APC-F4/80 (eBioscience, San Diego, CA), and FITC-MRC1, PE-MRC1 (AbD Serotec). 7-Amino-actinomycin D (7AAD) (eBioscence) was used to gate viable cells for flow analysis. Appropriate fluorochrome-conjugated isotype-matched control IgGs were employed. Flow cytometry data was acquired on an LSRII flow cytometer (BD Biosciences) and were analyzed with FACSDiva software (BD Biosciences).

**Statistical Analysis**

Data are expressed as mean ± standard error of the mean (SEM). All statistical tests were two-sided and results were considered statistically significant at p<0.05. ROUT was used to determine if outliers were present. Survival studies were assessed using nonparametric log-rank tests with Bonferroni post-hoc correction for multiple comparisons. Tumor growth curves were analyzed by linear regression – linearity holds because the residuals were randomly distributed around the zero. Statistical analyses for all other experiments were preformed using unpaired t-
tests or, when more than two groups were assessed, by ANOVA followed by Holm-Sidak *post-hoc* tests for multiple comparisons. Statistical analyses were performed using the statistical software Prism (GraphPad Software Inc. San Diego, CA).

**Results**

*Dual anti-VEGFR/Ang-2 therapy has superior vascular normalizing effects in Gl261 tumors compared to cediranib monotherapy.*

To determine the effects of cediranib + MEDI3617 dual therapy on GBM vasculature, we assessed vessel morphology. In Gl261, we focused our analysis on day 14 and 21 after beginning therapy to identify effects from prolonged dual therapy. Tumor microvessel density (MVD) and perivascular cell coverage were higher in the dual therapy group than in cediranib-treated tumors at day 14 but there were no differences in the extent of basement membrane (BM) coverage or thickness (Figure 2-1A-D). By day 21, dual therapy-treated tumors were structurally normalized compared to cediranib-treated tumors, with significantly higher BM coverage (Figure 2-1A-D). The vessels in the dual therapy-treated tumors had a MVD, perivascular cell coverage, and BM coverage that more closely resembled that of the normal brain than the cediranib-treated vessels (Figure 2-1E-F).

*Dual anti-VEGFR/Ang-2 therapy controls vasogenic edema as effectively as cediranib monotherapy.*

Given that dual therapy induced structural vessel normalization in Gl261 tumors, we next investigated if these changes could translate to improved edema control. In addition to determining intratumoral edema, we assessed edema in both the ipsilateral hemisphere (to
Figure 2-1: Dual cediranib + MEDI3617 therapy extends normalization in viable tissue compared to cediranib therapy alone. Gli261 tissues were collected from mice treated with cediranib (red) or dual cediranib + MEDI3617 therapy (blue) at days 14 or 21 after beginning treatment. Sections were stained for CD31 (vessels), either desmin (perivascular cells) or collagen IV (basement membrane, BM), and DAPI (nuclei). Both MVD (A) and perivascular cell coverage (B) were higher in the dual therapy-treated tumors than in cediranib-treated tumors at days 14 and 21 while BM coverage (D) was higher only at day 21. There was no significant difference in BM thickness between groups (C). (E) Representative images of CD31 (green)/Desmin (red) staining in the normal brain and control, cediranib, and dual therapy-treated tumors on day 21. (F) Representative images of CD31 (green) /collagen IV (red) staining in the normal brain and control, cediranib, and dual therapy-treated tumors. Error bars represent standard error of the mean. *p < 0.05 compared to control unless otherwise indicated. Scale bars = 50 µm.
Figure 2-1 (Continued)
measure peri-tumoral edema) and the contralateral hemisphere (as a control for global edema changes). Both cediranib and dual therapy decreased edema in the ipsilateral and contralateral hemispheres compared to control, though only cediranib was statistically significant (Figure 2-2A,B dual therapy p = 0.052). There was no difference in edema between treatment groups within the tumor (Figure 2-2C). These data suggest that edema control is not the major benefit of improved normalization in dual therapy-treated tumors compared to cediranib-treated tumors.

_Dual anti-VEGFR/Ang-2 therapy increases M1-like TAMs compared to cediranib monotherapy._

We recently demonstrated that tumor vascular normalization leads to efficient immune cell recruitment and their polarization to an anti-tumor phenotype\(^3\). Based on these data we assessed whether immune cell composition might also be altered by dual therapy. We found a trend towards increased CD11b\(^+\)F4/80\(^+\) TAMs within Gl261 tumors treated with dual therapy compared to control tumors (Figure 2-3A p = 0.062). While the increase in total proportion of TAMs did not reach statistical significance, the percentage of CD11c\(^+\)MRC1\(^-\) M1-like TAMs in dual therapy-treated tumors was significantly higher than in cediranib monotherapy-treated tumors (p = 0.0017) (Figure 2-3B). Conversely, there was no change in the percentage of CD11c\(^-\) MRC1\(^+\) M2-like TAMs in any treatment group (Figure 2-3C), leaving the overall M1/M2 TAM composition shifted towards an anti-tumor M1-like phenotype in the dual therapy treated-tumors. In contrast, cediranib tended to decrease M1-like TAMs, shifting the balance of M1/M2 TAMs towards a pro-tumor phenotype (Figure 2-3D). We detected no significant change in the proportion of other immune cell populations (CD8\(^+\) T cells, CD4\(^+\) T cells, and CD11b\(^+\)Gr1\(^+\) myeloid-derived suppressor cells) (Figure 2-4).
Figure 2-2: Dual cediranib + MEDI3617 therapy promotes edema control in Gl261. Gl261-bearing mice (n = 5-7) were treated with control (green), cediranib (red), or dual therapy (blue). Water content (edema) was measured on day 10 after treatment start by wet-dry weight ratio in the contralateral hemisphere (Contra, A), ipsilateral hemisphere (Ipsi, B), and tumor (C). Cediranib monotherapy significantly decreased water content in both the contralateral (p = 0.0294) and ipsilateral (p = 0.0234) hemispheres. Dual therapy also decreased water content compared to control-treated tumors, though it did not reach statistical significance (contralateral p = 0.0949, ipsilateral p = 0.052). There was no significant difference in edema in any of the tissues treated with dual therapy compared to cediranib. Error bars represent standard error of the mean. *p < 0.05 compared to control unless otherwise indicated.
Figure 2-2 (Continued)
Figure 2-3: Dual cediranib + MEDI3617 therapy increases M1-like TAMs in Gl261 tumors.

Gl261-bearing mice were treated with control (n = 7), MEDI3617 (n = 7), cediranib (n = 7), or dual therapy (n = 8). Tumors were harvested on day 10 after beginning treatment. After disassociation, the single cell suspensions were stained with CD11b, F4/80, MRC1, and CD11c antibodies for flow cytometric analysis of macrophage populations within the tumors. (A) The percentage of CD11b^+F4/80^+ TAMs in dual therapy-treated was increased compared to control tumors, but this failed to reach significance (p = 0.062). (B) There was a significant increase in the percentage of CD11c^−MRC1^− M1-like TAMs in the dual therapy group compared to the cediranib group (†p = 0.0017), while M1-like TAMs in cediranib tumors were also decreased compared to control tumors (*p = 0.0389). (C) There was no significant difference between any of the groups in the percentage of CD11c^−MRC1^+ M2-like TAMs. (D) There was a significant decrease in the ratio of M1/M2 TAMs in the cediranib-treated tumors compared to both control (*p = 0.0047) and dual therapy-treated tumors (†p = 0.0022). Error bars represent standard error of the mean.
Figure 2-3 (Continued)
Figure 2-4: Dual cediranib + MEDI3617 therapy has no effect on granulocyte or T cell recruitment to GL261 tumors. GL261 tumors were collected from mice treated with control (n = 7), MEDI3617 (n = 7), cediranib (n = 7), or dual cediranib + MEDI3617 therapy (n = 8) at day 10 after beginning treatment. After disassociation, the resulting single cell suspensions were stained with CD45, Gr1, CD4, and CD8 antibodies for flow cytometric analysis of myeloid and lymphoid populations within the tumors. There was no difference between any treatment group in the percentages of (A) CD45⁺ cells, (B) CD45⁻Gr1⁺ granulocytes, (C) CD4⁺ T lymphocytes, or (D) CD8⁺ T lymphocytes. Error bars represent standard error of the mean.
Figure 2-4 (Continued)
Dual anti-VEGFR/Ang-2 therapy delays GBM progression beyond cediranib therapy alone.

Given the changes in vessel normalization and TAM phenotype seen with dual therapy, we next examined tumor size and growth rate following treatment using Optical Frequency Domain Imaging (OFDI) (Figure 2-5). Indeed, GBM tumors treated with either cediranib or dual therapy grew significantly slower than control tumors. Moreover, dual therapy-treated tumors grew slower than cediranib-treated tumors (Figure 2-6A). This sustained decrease in growth resulted in significantly smaller tumors near the median survival date of cediranib-treated tumors (day 20) in the dual therapy group compared to the cediranib group (Figure 2-6C). We next examined the extent of necrosis but saw no significant difference between the dual therapy and cediranib monotherapy-treated tumors in GBM (Figure 2-6D, 2-7). Thus, dual therapy delayed GBM progression by slowing tumor growth rate, resulting in smaller viable tumor volume compared to cediranib monotherapy-treated tumors. Analysis of tumor apoptosis and proliferation did not reveal significant changes (Figure 2-8), suggesting alternative mechanisms are responsible for the reduced tumor growth.

We also studied how these inhibitors affect tumor viability in vitro in order to determine if there might be any direct cytotoxic effects on the tumor cells. Stimulation of the cells with Ang-2 and/or VEGF or inhibition of cells with MEDI3617 and/or cediranib did not affect cell viability (Figure 2-9). We also checked the phosphorylation status of Tie-2 in orthotopically grown tumors treated with control, MEDI3617, cediranib, or cediranib + MEDI3617 and found that there was very little Tie-2 in GBM tumors (Figure 2-10). We confirmed that GBM, as well as U87, tumor cells do not express Tie-2 or Ang-2 by qPCR. Additionally, none of the treatment groups induced Tie-2 expression or phosphorylation (Figure 2-10). These data suggest that the change in tumor viability observed after combined cediranib + MEDI3617 therapy is not due to
Figure 2-5: Representative images of OFDI tumor size. Representative images of tumor growth over time, as monitored by OFDI. Tumors are delineated with dashed lines. Scale bar = 1mm.
Figure 2-5 (Continued)
Figure 2-6: Dual cediranib + MEDI3617 therapy reduces tumor growth and enhances survival in Gl261 tumors compared to cediranib therapy alone. Mice bearing Gl261 tumors were treated with control (green), MEDI3617 (orange), cediranib (red), or dual therapy (blue). (A) Tumor growth rate was monitored with OFDI and the maximum tumor area at each timepoint was determined using ImageJ. There was a significant difference in the rate of growth of tumors treated with dual therapy compared to both control-treated (**p < 0.0001) and cediranib-treated tumors (†p = 0.0076). (B) Both cediranib (n = 13) and MEDI3617 (n = 13) monotherapies led to significantly higher overall median survival (24 days) than the control (20 days, n = 12, cediranib *p = 0.017, MEDI3617 *p = 0.011). Dual therapy (n = 13) led to a significantly higher median survival (38 days) than control (**p < 0.0001) and cediranib-treated tumors (†p = 0.002). (C) There was a significant difference in the volume of dual therapy-treated tumors compared to cediranib-treated tumors at day 20 as measured by MRI (†p = 0.0089). (D) There was no change in the extent of necrosis at day 20 (p = 0.1071). Error bars represent standard error of the mean.
Figure 2-6 (Continued)

A

Percent Change in Area (vs D0)

Days

B

Percent Survival

Days

C

Day 20

Tumor volume (mm³)

cediranib  cediranib + MEDI3617

D

Day 20

Necrosis (% Total Area)

cediranib  cediranib + MEDI3617

*  †  ‡  ***
Figure 2-7: Representative images of necrosis in Gl261 and U87. (A) Representative image of H&E staining to highlight necrosis found in Gl261 tumors at day 20. (B) Representative image of early necrosis exhibiting diffuse hypoxic ischemic changes in U87 tumors at day 6.
Figure 2-7 (Continued)

A  Gl261 Necrosis

B  U87 Early Necrosis - Diffuse Hypoxic Ischemic Changes
Figure 2-8: Dual cediranib and MEDI3617 therapy does not affect tumor apoptosis or proliferation. Gl261 (A, B) and U87 (C, D) tissues were collected from mice treated with control (green), MEDI3617 (orange), cediranib (red), or dual therapy (blue) at various time-points after beginning treatment. Sections were stained for cleaved caspase 3 (apoptosis) or Ki67 (proliferation, and DAPI (nuclei). In Gl261, neither apoptosis (A) nor proliferation (B) were altered by dual therapy at days 14 or 21. Similarly, dual therapy in U87 had no effect on apoptosis (C) or proliferation (D). Error bars represent standard error of the mean.
Figure 2-8 (Continued)
Figure 2-9: Effects of Ang-2 and VEGF stimulation and/or inhibition on Gl261 tumor cells

*in vitro*. Gl261 cells were treated for 24, 48, or 72 hours with IgG (1µg/ml), 0.1% Tween, recombinant mouse VEGF (rmVEGF), 10ng/ml), cediranib (4.5ng/ml), rmVEGF + cediranib, rmAng-2 (1µg/ml), MEDI3617 (1µg/ml), rmAng-2 + MEDI3617, rmVEGF + rmAng-2, cediranib + MEDI3617, rmVEGF + rmAng-2 + cediranib + MEDI3617, anti-CSF1 (1µg/ml), anti-CSF1 + cediranib + MEDI3617, and anti-CSF1 + rmVEGF + rmAng-2, cediranib + MEDI3617. There was no significant change in tumor cell viability with any treatment group.
Untreated, IgG, Tween, VEGF, cediranib, Ang-2, MEDI3617, VEGF + cediranib, Ang-2 + MEDI3617, Ang-2 + MEDI3617, Ang-2 + MEDI3617, aCSF1, aCSF1 + VEGF, Ang-2 + cediranib + MEDI3617, VEGF + Ang-2 + MEDI3617, aCSF1 + VEGF + Ang-2 + cediranib + MEDI3617, aCSF1 + VEGF + Ang-2 + cediranib + MEDI3617

Cell Viability

**Figure 2-9 (Continued)**
Figure 2-10: Tie-2 expression and phosphorylation in GL261 tumors. Protein lysates were generated from tumors treated with Control IgG, MEDI3617, cediranib, or dual cediranib + MEDI3617. Untreated HUVECs acted as a positive control for Tie-2 and HUVECs treated with Ang-2 acted as a positive control for pTie-2 staining. 50ug from each sample was immunoprecipitated to enrich for Tie-2 and blotted with a phospho-tyrosine (pTyr) antibody. (A) Western blots of Tie-2 (left) and pTyr (right). (B) 160kDA (Tie-2) bands from Tie-2 and pTyr blots. Both untreated and Ang-1 stimulated HUVECs expressed high levels of Tie-2, while GL261 tumors expressed significantly lower levels. Ang-1 stimulation induced Tie-2 phosphorylation in HUVECs. There was little to no Tie-2 phosphorylation observed in the GL261 tumors, regardless of treatment group.
Figure 2-10 (Continued)
direct anti-tumor activity of the therapeutics.

**Dual anti-VEGFR/Ang-2 therapy extends survival beyond that of cediranib therapy alone.**

We next assessed whether the changes in tumor growth rate translated into a survival benefit. As expected based on our previous experience in GBM, treatment with cediranib alone significantly increased survival of Gl261-bearing mice compared to control (median 24 days vs. 20 days). MEDI3617 treatment also increased median survival (24 days). Strikingly, dual therapy significantly prolonged survival compared to monotherapy arms and control (38 days). (Figure 2-6B, Table 2-1).

**Survival benefit of dual anti-VEGFR/Ang-2 therapy is mediated by anti-tumor TAMs.**

The prominent shift in TAM number/phenotype prompted us to assess the functional role of TAMs in the response of Gl261 tumors to dual therapy. To this end, we used an anti-CSF1-neutralizing antibody to deplete recruitment of TAMs. CSF1 inhibition decreased the TAM population by 50% without altering the phenotype of the infiltrating TAMs (Figure 2-11). Additionally, CSF1 inhibition did not affect tumor cell viability in vitro (Figure 2-9). While CSF1-inhibition had no effect on survival, when combined with the dual therapy it significantly compromised the therapeutic benefits of this treatment (median 10 days vs. 17 days, Figure 2-12). CSF1-blockade had no significant effect on any of the other treatment groups (Figure 2-13). These data are consistent with the observation that dual therapy leads to increased recruitment and polarization of M1-like anti-tumor TAMs and that these TAMs are responsible, at least in part, for the additional survival benefit seen with dual therapy over cediranib monotherapy.
Table 2-1: Median survival of U87 and Gl261 mice. In both tumor models, the dual therapy had significantly higher survival than control treatment (Gl261: 38 days vs. 20 days, U87: 26 days vs. 5 days, ***p < 0.0001). In Gl261, both cediranib and MEDI3617 monotherapies led to significantly higher overall median survival (24 days) than the control (cediranib *p = 0.017, MEDI3617 *p = 0.011). In U87, overall median survival with cediranib treatment (13 days) was significantly higher than the control (***p < 0.0001) and MEDI3617 alone also significantly increased survival compared to control (7 days; *p = 0.014). In both tumor models, dual therapy had significantly higher survival compared to either monotherapy (Gl261: cediranib †p = 0.002 MEDI3617 †p = 0.0012; U87: †p < 0.0001).
Table 2-1 (Continued)

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<tr>
<td>MEDI3617</td>
<td>10</td>
<td>24*†</td>
</tr>
<tr>
<td>cediranib</td>
<td>10</td>
<td>24*†</td>
</tr>
<tr>
<td>MEDI3617 + cediranib</td>
<td>11</td>
<td>38***</td>
</tr>
</tbody>
</table>
**Figure 2-11: Extent of macrophage inhibition by CSF1-blockade.** Mice bearing Gl261 tumors were treated with control (n=3, green), anti-CSF1 (αCSF1, n=4, green checkered), dual cediranib + MEDI3617 (n=2, blue), dual therapy + anti-CSF1 (n=3, blue checkered). (A) The addition of anti-CSF1 significantly decreased the percentage of TAMs within the tumor when combined with either control or dual therapy to similar extents. (B) The addition of anti-CSF1 had no effect of the percentage of M1-like TAMs recruited to the tumor. Data are expressed as a percentage of TAMs normalized to the average of the relevant groups not treated with anti-CSF1. Error bars represent standard error of the mean. *p < 0.05.
Figure 2-11 (Continued)
Figure 2-12: Inhibition of macrophage recruitment by CSF1-blockade ablates the survival benefit of dual cediranib + MEDI3617 therapy. Mice bearing Gl261 tumors were treated with control (n = 16, solid green), anti-CSF1 (n = 18, dotted green), dual cediranib + MEDI3617 therapy (n = 28, solid blue), or anti-CSF1 + dual therapy (n = 26, dotted blue). Anti-CSF1 had no effect by itself on overall median survival compared to control (7 days vs. 6 days). When combined with dual therapy, however, anti-CSF1 significantly reduced overall median survival compared to dual therapy alone (10 days vs. 17 days, *p = 0.003). Dual therapy with and without combined anti-CSF1 treatment led to significant increases in overall median survival compared to control treated mice (p = 0.0035 and p < 0.0001 respectively). These data confirm the role of M1-like macrophages as essential mediators of the survival benefits observed with dual therapy.
Figure 2-12 (Continued)
Figure 2-13: Animal survival after treatment with cediranib and/or MEDI3617 with or without inhibition of macrophage recruitment by CSF1-blockade. Mice bearing GL261 tumors were treated with control (n = 16, solid green), anti-CSF1 (n = 18, dotted green), MEDI3617 (n = 6, solid orange), anti-CSF1 + MEDI3617 (n = 7, dotted orange), cediranib (n = 12, solid red), anti-CSF1 + cediranib (n = 12, dotted red), dual therapy (n = 28, solid blue), or anti-CSF1 + dual therapy (n = 26, dotted blue). The addition of anti-CSF1 had no effect on overall median survival when combined with control, MEDI3617, or cediranib compared to each therapy alone. When combined with dual therapy, however, anti-CSF1 significantly reduced overall median survival compared to dual therapy alone (10 days vs. 17 days, *p = 0.003). Dual therapy with and without combined anti-CSF1 treatment led to significant increases in overall median survival compared to control treated mice (p = 0.0035 and p < 0.0001 respectively). These data confirm the anti-tumoral macrophages as essential mediators of the survival benefits observed with dual therapy.
Figure 2-13 (Continued)
Dual anti-VEGFR/Ang-2 therapy induces vascular normalization and increases survival compared to cediranib alone in the U87 xenograft model.

Finally, in order to determine if the effects of dual therapy were isolated to the Gl261 GBM model or if they could be applied generally across GBM subtypes, we analyzed the effects of dual therapy on vessel normalization, tumor growth, and survival in the U87 human xenograft. Vessels were analyzed at days 3 and 8 after treatment initiation to determine the effects of therapy during the previously defined window of normalization (day 3) and as the window is closing (day 8). Tumors treated with either cediranib alone or dual therapy had a significant decrease in the MVD and BM thickness at day 3 compared to control tumors (Figure 2-14A, C). There was no difference between treatment groups in BM coverage (Figure 2-14D). Perivascular cell coverage was significantly higher in dual therapy-treated tumors compared to vessels from control- and cediranib-treated tumors, suggesting that dual therapy resulted in a vasculature that more closely resembles the normal vessels in the brain (normalization) (Figure 2-14B, E, F). These features were maintained in both the cediranib- and dual therapy-treated tumors at day 8. Unfortunately, by day 8 most of the control mice had to be sacrificed due to poor health and could not be analyzed. MEDI3617 monotherapy induced some features of normalization, but in an inconsistent manner; while there was no change in the MVD of MEDI3617-treated tumors, BM thickness was decreased compared to control tumors at day 3 and perivascular cell coverage was increased at day 8 (Figure 2-14A-C). Thus, as in Gl261, vascular normalization was observed in dual therapy-treated U87 tumors compared to cediranib-treated tumors. We also found that, similar to Gl261, this vessel normalization with dual therapy did not translate into improved edema control beyond cediranib monotherapy (Figure 2-15).
Figure 2-14: Dual cediranib + MEDI3617 therapy improves vessel normalization, increases tumor necrosis, and prolongs survival in U87 compared to cediranib monotherapy. U87 tissues were collected from mice treated with control (green), MEDI3617 (orange), cediranib (red), or dual cediranib + MEDI3617 therapy (blue) at days 3 or 8 after beginning treatment. Sections were stained for CD31, either desmin or collagen IV (BM), and DAPI. Both MVD (A) and BM thickness (C) were significantly decreased at day 3 by both cediranib and dual therapy compared to control, and remained low at day 8. In the dual therapy-treated tumors, perivascular cell coverage was also significantly higher on day 3 compared to control tumors (B). There was no significant difference in BM coverage between groups (D). (E) Representative images of CD31 (green)/Desmin (red) staining in the normal brain and control, cediranib, and dual therapy-treated tumors. (F) Representative images of CD31 (green)/collagen IV (red) staining in the normal brain and control, cediranib, and dual therapy-treated tumors. (G) There was no difference in tumor volume between dual therapy and cediranib-treated tumors at day 7 as measured by MRI. (H) There was a significant increase in ischemic hypoxic changes (“early necrosis”) in the dual therapy-treated tumors compared to cediranib-treated tumors at day 6 (↑p = 0.0297). (I) Both cediranib (n = 10) and dual therapy (n = 11) treated mice had a significantly higher overall median survival (26 days and 13 days, respectively) compared to control-treated mice (5 days, ***p < 0.0001). Error bars represent standard error of the mean. *p < 0.05 compared to control unless otherwise indicated. Scale bars = 50 µm.
Figure 2-14 (Continued)
Figure 2-15: Dual cediranib + MEDI3617 therapy promotes edema control in U87. U87-bearing mice (n = 5-8) were treated with control (green), MEDI3617 (orange), cediranib (red), or dual therapy (blue). Water content (edema) was measured on days 3, 5, and 10 after treatment start by wet-dry weight ratio in the contralateral hemisphere (Contra, A), ipsilateral hemisphere (Ipsi, B), and tumor (C). Cediranib monotherapy significantly decreased water content compared to control in all tissues measured at day 3 (contra *p = 0.0061, ipsi **p = 0.0004, tumor **p = 0.0002). Cediranib monotherapy also decreased edema on day 5 compared to control in both the contralateral (**p = 0.0002) and ipsilateral (**p = 0.0007) hemispheres. Dual therapy also decreased edema on day 3 (contra *p = 0.0370, ipsi **p = 0.0004, tumor ***p = 0.0001) and day 5 (contra and ipsi ***p < 0.0001) compared to control. There was no significant difference in edema in any of the tissues treated with dual therapy compared to cediranib except, unexpectedly, for an increase in water content of the contralateral hemisphere at day 10 (*p = 0.0126). Error bars represent standard error of the mean. *p < 0.05 compared to control unless otherwise indicated.
Figure 2-15 (Continued)
We next tested whether dual therapy affected U87 tumor growth and survival as it did in Gl261. Interestingly, there was no difference in the tumor volume of U87 tumors treated with either cediranib monotherapy or dual therapy (Figure 2-14G), suggesting that tumor growth inhibition is not a chief cause of the observed survival benefit beyond that of cediranib alone. Similar results were found using MRI to monitor U87 tumor volume (Figure 2-16). This is consistent with our previous study in the same tumor model\textsuperscript{11}. There was, however, a striking increase in diffuse hypoxic ischemic changes (i.e. early necrosis, Figure 2-7) by day 6 in the dual therapy-treated tumors compared to both control and cediranib-treated tumors (Figure 2-14H) leaving a decreased viable tumor burden in dual therapy-treated tumors. As seen in Gl261, there was no significant change in tumor apoptosis or proliferation (Figure 2-8), however, there was an increase in the percentage of F4/80\textsuperscript{+} TAMs in U87 tumors treated with dual therapy similar to that seen in Gl261 (Figure 2-17). Also as in Gl261, the change in tumor burden with dual therapy resulted in a significant increase in mouse survival (median 26 days) compared to cediranib (13 days), MEDI3617 (7 days), or control-treated mice (5 days, Figure 2-14I, Table 2-1).

**Discussion**

We report here that combined inhibition of VEGFRs and Ang-2 significantly improves the extent of vessel maturation and normalization in two models of GBM and effectively reduces edema to a similar extent as cediranib monotherapy. Dual therapy is associated with either reduced tumor growth, in Gl261, or increased tumor necrosis, in U87. While the reasons for these differences are unclear, these data show that dual therapy could have benefits in GBM via alternative mechanisms of action in tumors that are either hypovascular (Gl261) or hypervascular (U87) compared to the normal brain. In both models, there was a significant increase in survival
Figure 2-16: Dual cediranib + MEDI617 therapy does not affect tumor growth in U87. (A) Mice bearing U87 tumors were treated with control (green), MEDI3617 (orange), cediranib (red) or dual therapy (blue). Tumor size was monitored using T2 MRI and the volume at each timepoint was calculated using MATLAB and ImageJ. There was no significant difference in the growth of any of the treatment groups. (B) Representative T2-weighted images of dual therapy-treated tumor growth over time. Tumors are delineated with dashed lines. Error bars represent standard error of the mean.
Figure 2-16 (Continued)

A  

U87 Tumor Volume

B  

Day 0  |  Day 2  |  Day 5  
---|---|---

Day 7  |  Day 14  |  Day 21  

---|---|---

Tumor Volume (mm$^3$)
Figure 2-17: Dual cediranib + MEDI3617 therapy increases TAM recruitment of U87 tumors. U87 tumors were collected from mice treated with control, MEDI3617, cediranib, or dual therapy at days 3 and 8 after beginning treatment. Sections were stained for F4/80 (macrophages) and DAPI. (A) TAMs in tumors treated with dual therapy were significantly increased compared to MEDI3617 treated tumors (*p = 0.0014) and there was a trend towards an increase compared to cediranib-treated tumors (p = 0.0533). (B,C) Representative image of F4/80/DAPI staining in MEDI3617- (B) and dual therapy-treated (C) tumors. Error bars represent standard error of the mean.
Figure 2-17 (Continued)

A

Macrophage Density

\[ \frac{F4/80^+}{\text{Total Area}} \]

\begin{align*}
\text{Day 3} & : & \text{Control} & : 10 \pm 2 & & \text{MEDI3617} & : 15 \pm 3 & & \text{cediranib} & : 12 \pm 1 \\
\text{Day 8} & : & \text{Control} & : 20 \pm 4 & & \text{MEDI3617} & : 25 \pm 5 & & \text{cediranib} & : 18 \pm 2 \\
\end{align*}

\[ * \]

B

C

F4/80/DAPI

MEDI3617

F4/80/DAPI

cediranib + MEDI3617
with dual therapy compared to monotherapy arms. In Gl261, we found that combined blockade of VEGFR and Ang-2 led to an increase in the proportion of anti-tumor M1-like TAMs. This mechanism was critical for the efficacy of this regimen in immunocompetent mice, as blockade of TAM infiltration compromised the survival benefit of dual therapy.

Our findings are consistent with previous pre-clinical reports of combined VEGF and Ang-2 signaling inhibition in a variety of subcutaneous and orthotopic non-CNS tumor models. However, unlike in non-CNS models, we found little survival benefit of anti-Ang-2 monotherapy in GBM models, with only a small increase in survival in both tumor models. This may reflect the greater dependence of GBM angiogenesis on VEGF. We show that Ang-2 inhibition alone leads to modest improvement in vessel normalization only and had no effect on intracranial edema or tumor growth. Additionally, in U87 tumors the ectopic overexpression of Ang-2 has little effect on the tumor vasculature or survival, but is able to impair the ability of VEGF-signaling inhibition by preventing normalization. These data suggest that Ang-2 may be more relevant as a therapeutic target in GBM in the context of VEGF pathway inhibition.

In U87 we found a significant increase in the amount of early necrosis, indicative of diffuse hypoxic ischemic changes, in tumors treated with dual therapy compared to both control- and cediranib-treated tumors. Interestingly, these differences occurred without a corresponding change in apoptosis or proliferation, as measured by cleaved caspase 3 and Ki67 immunohistochemical staining, respectively. Similarly, a study using the bispecific antibody Ang-2-VEGF-A CrossMab (A2V), which targets both Ang-2 and VEGF, found that A2V treatment reduced tumor growth and improved survival without activating caspase 3 (apoptosis) or altering pHis3 (proliferation), suggesting that dual inhibition of VEGF and Ang-2 may affect
tumor growth through caspase-independent apoptosis mechanisms or through non-apoptotic pathways, such as autophagy.

Previously, we have shown that the inhibition of VEGF during tumor growth leads to vascular normalization, which can positively affect survival in both preclinical models of GBM\textsuperscript{10,11,24} and clinically\textsuperscript{41-44}, even when tumor growth is unaffected. Recent clinical data results suggest that improved vessel function in both newly diagnosed and rGBM patients during anti-VEGF therapy correlates with significantly longer survival than those whose tumor blood perfusion does not go up\textsuperscript{41-44}. In U87, anti-angiogenic therapy-induced vascular normalization is associated with decreased vessel diameter and MVD, increased perivascular cell coverage or proximity to vessels, and decreased BM thickness, ultimately resulting in a tumor vasculature that is more mature, better functioning, and more homogenously distributed throughout the tumor\textsuperscript{10,11,24}. However, GL261 differs from U87 in vascularity and other characteristics and our results regarding vascular normalization, tumor growth, and necrosis appear to reflect these differences. In GL261, dual therapy increased perivascular cell coverage but did not reduce MVD. However, in both the U87 and GL261 model, cediranib + MEDI3617 combined therapy resulted in a vasculature that more closely resembled the surrounding brain. In GL261 this was achieved without a reduction in MVD while the more vascularized U87 model required reduction in MVD to attain the same endpoint (i.e. $\sim 300$ vessels per mm$^2$). The resulting extension of vascular normalization with dual therapy beyond that of cediranib monotherapy warrants further studies in combination with radiation or chemotherapy, as in Riedemann et al.\textsuperscript{45}, as improvements in the tumor vasculature often improve the delivery and efficacy of other therapeutics.
Vascular normalization can enhance the influx of immune effector cells into the tumor and prolong survival of tumor-bearing mice that receive an active immunotherapy\(^7\). TAM infiltration is an important determinant of tumor progression and response to therapy in GBM and other tumors\(^46\)\(^-\)\(^48\). In many malignant tumor settings, including GBM, TAMs are skewed towards a pro-tumor M2-like phenotype and can be directly involved in the promotion of tumor angiogenesis and progression through the secretion of molecules such as VEGF and MMP-9\(^47\)\(^,\)\(^49\)\(^-\)\(^56\). Reprogramming the tumor immune environment is a promising therapeutic strategy, with pre-clinical studies blocking TAM function or re-polarizing TAMs showing promising survival benefits in multiple tumor models\(^54\)\(^,\)\(^57\)\(^-\)\(^59\). We have previously shown that “normalizing doses” of anti-VEGF therapy can enhance the recruitment of TAMs in pre-clinical GBM models and that the number of TAMs is correlated with poor survival in GBM patients, confirming their potential as a therapeutic target in GBM\(^11\)\(^,\)\(^60\). A recent pre-clinical study in GBM found that survival could be enhanced by shifting TAM phenotype away from M2\(^48\). Here, we show a shift in TAMs towards an M1-like phenotype after combined inhibition of VEGF- and Ang-2-signaling and demonstrate that they are responsible, at least in part, for the added survival benefit of combination therapy over VEGF signaling inhibition alone. These results also warrant studies assessing the therapeutic potential of combination with novel immunotherapeutics in GBM models.

**Conclusions**

Dual blockade of Ang-2 and VEGFR leads to an extension of vascular normalization and a shift in TAM phenotype and/or recruitment compared to anti-VEGFR alone. These changes are associated with delayed GBM progression and increased survival. This dual targeting approach
may be an effective way to overcome the rapid resistance of GBM to anti-VEGF monotherapy in patients with GBM.

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References


CHAPTER 3

Discussion
Summary

The work presented in this thesis provides new insight into the potential of using dual inhibition of Ang-2 and VEGF as a therapeutic strategy for the treatment of GBM. Combined inhibition of Ang-2 and VEGFR significantly increased survival compared to monotherapy arms in two orthotopic GBM mouse models – U87 and GL261. Increased survival after combination therapy was associated with a reduction in tumor viability. In U87, this was due to increased tumor necrosis and in GL261 this was due to reduced tumor growth. In both tumor models, combination therapy improved vessel maturity and normalization compared to monotherapy-treated tumors but did not contribute to further edema control beyond that of anti-VEGFR therapy alone. Improved vessel normalization was, however, associated with an increase in the number of M1-like (anti-tumor) TAMs in combination treated tumors compared to anti-VEGFR treated tumors. Blocking TAM infiltration with an anti-CSF1 antibody compromised the survival benefit of combined anti-Ang-2 and anti-VEGFR therapy, revealing a critical role for TAMs in promoting survival during combination therapy.

TAMs and GBM Progression

The results presented in this thesis show that M1-like TAMs play a key role in promoting survival during combined inhibition of Ang-2 and VEGFR. TAMs in GBM are believed to be comprised of two cell types – microglia, which are the resident macrophage-like cell in the brain, and bone marrow-derived macrophages, which are recruited to the tumor from the circulation. Microglia and macrophages have largely overlapping marker profiles and to date there is no definitive way to distinguish them in humans\(^1\). Therefore they will be referred to here
collectively as TAMs. Many studies have reported that TAM infiltration is associated with the histological grade and vessel density\(^2\)\(^{-5}\). TAMs in GBM are extremely variable morphologically\(^6\) and appear to be more common in mesenchymal GBMs than in other subtypes of GBM\(^7\). TAMs make up approximately 30% of cells within GBMs and are actively recruited to the tumor through the secretion of cytokines, chemokines, and other proteins\(^4\)\(^{,8\text{-}11}\). Similarly, the results presented here in both Gl261 and U87 suggest that TAMs make up a large percentage of the tumor bulk.

Recently, Lu-Emerson et al. showed that the number of TAMs is correlated with poor survival in GBM patients treated with anti-VEGF therapies\(^12\). Similarly, a pre-clinical study in murine models of GBM found that anti-VEGF therapy enhanced the recruitment of TAMs\(^13\). Kamoun et al. showed that anti-VEGFR therapy decreases F4/80\(^+\) cells during the window of normalization, but as the tumor escapes, the percentage of CD11b\(^+\) cells in the tumor increased significantly, suggesting that there is a VEGF-independent mechanism for recruitment of TAMs. However, neither study analyzed the phenotype of the TAMs recruited, making it difficult to say what these TAMs might have been doing. Given that the TAMs were associated with poor survival and the end of the normalization window, they likely play a role in the escape from anti-VEGF therapies observed in GBM models.

Here we showed that VEGFR therapy reduces the percentage of M1-like macrophages in Gl261 tumors, resulting in an M2-skewed immune tumor environment. The addition of Ang-2 inhibition of anti-VEGFR therapy reversed this and increased the percentage of M1-like TAMs, leaving the resulting tumor microenvironment more immunostimulatory and anti-tumor. A recent pre-clinical study by Pyontec et al. also found that GBM survival could be enhanced by shifting TAM phenotypes away from an M2-like state\(^14\). TAMs were targeted using a small-molecule
inhibitor against CSF-1R in GBM mouse models. CSF-1R blockade led to tumor regression and increased survival. Interestingly, anti-CSF-1R therapy did not deplete TAMs in the tumors, but rather reduced the expression of M2-like macrophage markers and impaired their tumor-promoting function. In contrast to this study, there was no change in TAM phenotype with anti-CSF1 therapy alone and there were significant decreases in TAM accumulation. This is perhaps due to the fact that we targeted the circulating ligand and not the receptor and thus did not affect downstream signaling as completely as we might have with a receptor antagonist. Similar to what was found in Pyonteck et al., the results presented here show that M1-like TAMs can improve survival in GBM. These results warrant further studies to determine how VEGFR inhibition blocks M1-like macrophage recruitment and/or polarization and how combined inhibition with anti-Ang-2 relieves this blockade and promotes anti-tumor activity in TAMs.

Vascular Normalization and TAMs

Vascular normalization, in addition to having potential direct effects on tumor growth and survival, also affects the influx of immune effector cells into the tumor. Because a large number of TAMs are derived from circulating monocytes, especially in GBM, improvements in vascular function could facilitate TAM infiltration. Hamzah et al. found that deletion of regulator of G-protein signalling 5 (Rgs-5) leads to vascular normalization, which results in prolongation of survival in tumor-bearing mice via recruitment of circulating immune cells\textsuperscript{15}. A recent study by Farsaci et al. showed that vascular normalization due to treatment with anti-angiogenic tyrosine kinase inhibitors decreased TAMs and other BMDCs while promoting survival in murine colon and breast cancer models\textsuperscript{16}. 
Vascular normalization has also been linked to TAM polarization. Rolny et al. over-expressed histidine-rich glycoprotein (HRG) in fibrosarcoma, pancreatic, and breast tumor cells and found that tumors grew slower, metastasized less, had a more normalized vasculature, and were better perfused. Though TAM accumulation was only slightly increased with HRG over-expression, they observed reduced expression of M2-like markers and increased expression of M1-like markers\textsuperscript{17}. Similarly, Huang et al. found that vascular normalization following anti-VEGR2 therapy polarizes TAMs away from an M2-like phenotype to an M1-like phenotype and facilitates T cell infiltration and potentiates vaccine therapy\textsuperscript{18}. The results presented here similarly show that vascular normalization correlates with changes in TAM recruitment and macrophage polarization. It is not clear yet, however, whether these TAMs are promoting normalization or whether they are being recruited into the tumor by therapy-induced normalization. Further studies will be needed to determine how TAMs affect normalization, and vice versa, and to try to find the molecular mechanisms behind the change in TAM polarization with combined anti-Ang-2 and anti-VEGFR therapy.

**Tie-2 Expressing Monocytes/Macrophages (TEMs)**

Recently, a subset of M2 TAMs have been found to be particularly important in facilitating tumor angiogenesis – those that highly express Tie-2. In circulation, Tie-2-expressing monocytes/macrophages (TEMs) represent approximately 20% of circulating monocytes\textsuperscript{19,20}. TEMs have been found in human tumors, including in kidney, colon, pancreatic, and lung cancers\textsuperscript{19}, as well as in mouse models\textsuperscript{21}. Ablation of these TEMs in mice showed that this subpopulation of TAMs are more important for tumor angiogenesis than Tie-2\textsuperscript{+} macrophages\textsuperscript{21,22}. De Palma et al. developed a mouse model in which proliferating Tie-2\textsuperscript{+} could be selectively
killed by ganciclovir (GCV). Administration of GCV early during tumor growth led to smaller, less vascular tumors than those of untreated mice\textsuperscript{21}. Interestingly, ablation of TEMs did not completely inhibit TAM recruitment or accumulation in tumors, suggesting that TEMs are a distinct monocytic subpopulation of TAMs.

TEMs can be distinguished from Tie-2 negative TAMs by the production of COX2, MMP-9, VEGF, and MRC-1 in addition to the expression of Tie-2\textsuperscript{23}. TEMs are often aligned along the surface of blood vessels. Inhibition of Ang-2 or Tie-2 in the PyMT and RIP1-TAG models of breast and pancreatic cancers leads to decreased vessel association by TEMs and inhibition of angiogenesis\textsuperscript{24}. Injection of TEMs into subcutaneously grown U87 tumors led to significantly more vascularized tumors than those injected with Tie-2\textsuperscript{-}macrophages\textsuperscript{19}.

The exact mechanism for their recruitment is unknown, however studies have shown that Ang-2 can stimulate the migration of TEMs \textit{in vitro} through the activation of Tie-2 downstream signaling\textsuperscript{19,20}. Additionally, inhibition of Ang-2 resulted in reduced tumor angiogenesis, tumor growth, and TEM infiltration\textsuperscript{25}. These data suggest that Ang-2 may be responsible for attracting TEMs to the tumor vasculature, as Ang-2 levels are high at sites of tumor vessel growth. Furthermore, it was recently shown that tumor-derived Ang-2 can stimulate TEMs to become more pro-angiogenic and pro-tumorigenic\textsuperscript{23}. Therefore, inhibition of Ang-2 could inhibit the recruitment and function of TEMs, further hampering tumor growth and survival. The M2 marker used here for flow cytometry, MRC1, has been used as a surrogate marker for TEMs\textsuperscript{23} and the results presented here show that the number of MRC1\textsuperscript{+} M2-like macrophages does not change with anti-Ang-2 therapy. Additionally, isolated TAMs from Gl261 expressed little to no Tie-2 as observed by quantitative real-time PCR (data not shown). These data suggest that TEMs
are not involved in Gl261 progression or response to therapy, though further studies are necessary to fully understand their involvement.

**Role of Ang-2 in Promoting Tumor Invasion**

In a subset of GBM patients, VEGF-inhibition has been implicated in the promotion of GBM invasion\textsuperscript{26-29}. This is thought to be due, in part, to the modulation of other angiogenic programs. In addition to its roles in promoting tumor growth and angiogenesis, Ang-2 has been implicated as playing a role in promoting GBM invasiveness\textsuperscript{30,31}. Ang-2 is upregulated in the invasive areas of the tumor and correlates with increased invasion in GBM patients\textsuperscript{32-34}. In a pre-clinical mouse model, it was shown that Ang-2 expression by tumor cells stimulates MMP-2 secretion along with increase invasion\textsuperscript{32}. Ang-2 bound to $\alpha_v\beta_1$ integrins and stimulated MMP-2 production through FAK-mediated activation of ERK1/2 and JNK\textsuperscript{35}. Also, in a model of breast cancer metastasis, it was found that Ang-2 activated integrin signaling and led to increased invasion and metastasis in a Tie-2-independent manner\textsuperscript{36}. These data suggested that the inhibition of Ang-2 may reduce tumor invasion, which is a chronic problem in GBM.

To address the role of Ang-2 in promoting invasion following anti-VEGF therapy, Gl261-dsRed tumor tissues collected at the end of the survival study were stained with DAPI and imaged using confocal microscopy. To assess single-cell invasion, the distance of the dsRed-labeled tumor cells to the main body of the tumor was calculated using ImageJ. There was no significant difference between any of the treatment groups in the number of tumor cells that had migrated away from the main tumor body (Figure 3-1). In addition to analyzing single-cell invasion into the brain parenchyma, the invasiveness of the tumor edge was analyzed using an in-house MATLAB program to determine the tortuosity of the edge. There was a trend towards
Figure 3-1: Analysis of distal (single cell) invasion away from the main tumor body in Gl261. Tissues from mice treated with (a) control (green), (b) MEDI3617 (orange), (c) cediranib (red), or (d) dual cediranib + MEDI3617 therapy (blue) were collected at survival endpoint and the distance of dsRed-labeled cells from the main tumor body was calculated using ImageJ. Error bars represent standard error of the mean.
Figure 3-1 (Continued)

Single Cell Invasion

- Control
- MEDI3617
- cediranib
- cediranib + MEDI3617

Distance from main tumor body (µm)

Number of Cells
an increase in local invasion in the anti-VEGFR treated tumors (p=0.08) compared to the other treatment groups (Figure 3-2), suggesting that the combination of Ang-2 and VEGFR inhibition may prevent VEGF-mediated increases in invasion. Further study in tissues collected at the same timepoint after treatment start, as well as in vitro mechanistic studies, will be needed to further determine how Ang-2 might be involved in promoting GBM invasion.

Clinical Implications and Future Directions

SEQUENCE AND DOSING OF COMBINED INHIBITORS

It will be important to determine the correct sequence and dosing of Ang-2 and VEGF inhibitors before proceeding onto clinical trials. One of the main drawbacks of anti-VEGF therapies is that treatment is only effective for a short period of time – the “normalization window” – before resistance mechanisms are triggered\textsuperscript{37-42}. Additionally, Ang-1 and Ang-2 expression levels may be altered by additional therapeutics. For example, Ang-2 levels were transiently decreased during the normalization window in pre-clinical models of GBM\textsuperscript{43} while Ang-1 levels increased\textsuperscript{39}. Similar results have also been seen in rGBM patients\textsuperscript{44}. Future studies will be required to find the correct staging and dosing for combined anti-Ang-2 and anti-VEGF therapeutics in order to maximize the clinical benefits.

COMBINATION WITH CHEMOTHERAPY AND RADIATION

It has been suggested that anti-angiogenic therapies will be most effective in tumors when combined with chemotherapy or radiation. Studies in non-CNS settings with combining anti-Ang-2 or anti-Ang-2/VEGF and cytotoxic therapies have shown enhanced anti-tumor effects compared to any individual therapy\textsuperscript{25,45-48}. Currently, our lab is studying the effects of anti-Ang-2 and anti-VEGF therapy in combination with lomustine in GBM. Early results are promising and
Figure 3-2: Analysis of local (edge) invasion in Gli261. Tissues from mice treated with (a) control, (b) MEDI3617, (c) cediranib, or (d) dual cediranib + MEDI3617 therapy were collected at survival endpoint and the invasiveness (tortuosity) of the tumor edge was calculated using MATLAB. Representative images of cediranib- and dual therapy-treated tissues are also shown. Error bars represent standard error of the mean.
Figure 3-2 (Continued)
point to changes in the tumor vasculature allowing for enhanced delivery of cytotoxic therapy to GBM cells. We are also looking at the effects of radiation on combined therapy and expect that vascular normalization by combined Ang-2 and VEGFR inhibition will confer a greater survival benefit when combined with radiotherapy.

**Conclusions**

Dual blockade of Ang-2 and VEGFR is able to delay GBM progression and increase survival in murine models. These benefits are associated with improved vascular normalization and a phenotypic shift in TAMs as a result of increased M1-like macrophage recruitment and/or switching from M2-like TAMs. The results presented in this thesis provide new insight into the potential of dual Ang-2 and VEGFR inhibition.
References


APPENDIX
**PIGF Inhibition in GBM**

In addition to investigating the role of Ang-2 in GBM tumor progression, we also looked into placental growth factor (PIGF) as a potential alternative to anti-VEGF therapy for GBM. PIGF is a member of the VEGF family of pro-angiogenic growth factors. Though structurally very similar to VEGF, PIGF shares only 42% sequence identity with VEGF. PIGF signals primarily through VEGFR1, though we recently discovered that PIGF is capable of signaling through neuropilin-1 (Nrp-1) in a VEGFR1-independent manner. PIGF was first discovered in human placenta but appears to be redundant in health as PIGF homozygous knockout mice (PIGF-/-) grow and reproduce normally with little differences in vascular function during physiological conditions. Consistent with this theory, PIGF expression is not detected in most healthy tissues, with the exception of the thyroid. It should be noted that PIGF may have functions in embryogenesis unrelated to vascular development that have yet to be discovered, such as neuronal growth and guidance through interactions with Nrp-1.

In many disease conditions, PIGF is upregulated compared to normal physiological levels. Additionally, PIGF-/- mice display abnormal vascular responses in response to induced pathologies. During ischemia, for example, PIGF-/- mice show a reduced ability to respond to damage through angiogenesis and pro-angiogenic macrophage recruitment. Recombinant PIGF administration reverses revascularization defect and restores macrophage mobilization. In human thyroid tumors, for example, downregulation of PIGF and upregulation of VEGF is associated with increased malignancy. On the other hand, upregulation of PIGF in some breast, lung, colorectal, and gastric tumor cases is correlated with more aggressive disease or reduced survival. There is conflicting evidence, however, about the prognostic value of PIGF in
human tumors because of the variability of data collected from human tumors and mouse tumor models.

In medulloblastoma (MB), a pediatric brain tumor, we found that PIGF plays a key role in tumor growth and progression and inhibition of PIGF or Nrp-1 leads to tumor growth inhibition and tumor regression$^3$. Figure A-1 is a schematic of the key findings of our work in MB$^{12}$. However, the role of PIGF in GBM angiogenesis and progression remains relatively unstudied, though high levels of PIGF are expressed in GBM tumors$^{13}$. Kerber et al. showed that PIGF-over-expressing GBM tumors grow larger in mice than those that don’t express PIGF. They also found that active VEGFR1 was required for growth. Additionally, PIGF induced angiogenesis, in part through the recruitment of pro-angiogenic macrophages$^{14}$. Given these data and our own experience in MB, we hypothesized that inhibition of PIGF in GBM would promote survival in GBM and vascular normalization.

In order to test this hypothesis, mice were implanted with U87-GFP or Gl261-dsRed tumors as described in Chapter 2. Tumors were treated three times per week with 25mg/kg i.p. of an anti-PIGF antibody (5D11D4, Thrombogenics) or IgG and mice were monitored for tumor growth using fluorescent intravital microscopy. Unexpectedly, we found no significant difference between survival in mice treated with either anti-PIGF or IgG in either of the U87 (Figure A-2A) or Gl261 (Figure A-3) tumor models. We also saw no difference between treatment groups in the rate of growth in the U87 model (Figure A-2B). Given the lack of survival benefit, we did not pursue PIGF inhibition further and instead focused solely on Ang-2 inhibition. Interestingly, these results were predictive of later phase I clinical trial results with the human version of our anti-PIGF antibody, TB-403 (RO5323441, Roche), where they found no significant added benefit of anti-PIGF in GBM when added to bevacizumab (NCT01308684)$^{15}$. 
Figure A-1: Schematic of PlGF activity in MB. Medulloblastoma growth is initially stimulated by autocrine PlGF signaling. As tumors progress they secrete sonic hedgehog (Shh) which stimulates the activated granule cells to produce more PlGF and further encourage tumor growth. PlGF signaling through Nrp-1 leads to ERK1/2 phosphorylation in a VEGFR-1-independent manner and promotes tumor cell survival. This lead to increased tumor growth and metastasis.

Figure adapted from 12.
Figure A-1 (Continued)
Figure A-2: PI GF inhibition has no effect on U87 tumor growth or survival. Mice bearing U87 tumors were treated with control IgG (n=4, green) or anti-PI GF (n=5, blue). (A) Anti-PI GF therapy did not improve survival compared to IgG treatment. (B) Tumor growth was monitored by fluorescent intravital microscopy and tumor volumes were calculated based on the assumption that U87 tumors grow as an ellipsoid. There was no difference between the treatment groups in tumor growth. Error bars represent standard error of the mean.
Figure A-2 (Continued)

A

U87 Survival

Percent survival

Days

0 2 4 6 8 10

0 50 100

IgG
anti-PIGF

B

U87 Tumor Volume

Volume (mm$^3$)

0 50 100 150 200 250

0 2 4 6 8 10

Day
Figure A-3: PIGF inhibition has no effect on survival in mice bearing Gl261. Mice bearing Gl261 tumors were treated with control IgG (n=4, green) or anti-PIGF (n=5, blue). (A) Anti-PIGF therapy did not improve survival compared to IgG treatment.
Figure A-3 (Continued)

**Gi261 Survival**

- **IgG**
- **anti-PIGF**

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Days

Percent survival

0 10 20 30 40
References


