Molecular heterogeneity and angiogenesis in glioblastoma

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Chapter 11

Dual inhibition of Angiopoietin-2 and VEGFA-signaling in glioblastoma xenografts

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Abstract

Due to the highly vascularized nature of the malignant brain tumor glioblastoma (GBM) anti-angiogenic therapy could potentially be beneficial for this disease. Therapeutic targeting of single angiogenic factors has resulted in marginal improvements, and the exploitation of alternate angiogenic signaling pathways has been suggested to mediate therapy refractoriness. In the current study we have therefore therapeutically inhibited the ligands, Angiopoietin-2 (Ang-2) and VEGFA, alone or in combination and have examined the effects of this approach on *in vivo* xenograft growth, vascularization parameters and invasive growth. Additionally, effects of treatment discontinuation were also assessed. We found that although tumor growth was unaffected, Ang-2 and VEGFA inhibition resulted in expected reduced CD34 (endothelial) and α-SMA (pericyte) expression levels, respectively. L1-10-mediated Ang-2 inhibition also introduced increased necrosis in mouse xenografts that resembled guillande necrosis. Bevacizumab-discontinued xenografts tended to grow slightly more invasively, but vascular integrity was unaffected by the applied treatments. In conclusion, although vascular parameters were physiologically affected by L1-10 and/or Bevacizumab treatment, *in vivo* GBM xenograft growth was unaffected.
Introduction

Glioblastoma (GBM) is a highly malignant brain tumor that unexceptionally results in short survival. Standard of care comprises of maximal safe surgical resection, followed by chemoradiation [1-3]. The high degree of tumor vascularization [4] has encouraged extensive exploration of anti-angiogenic therapeutic approaches for these malignant brain neoplasms, but results have been disappointing in GBM [5]. Accelerated approval was granted to Bevacizumab in 2009 for recurrent GBMs based on durable objective radiological responses in two Phase II trials [6,7]. As add-on treatment to current standard of care in the upfront setting, however, Bevacizumab is not indicated, because it did not result in durable responses and only marginal increases in progression-free survival in two independent Phase III clinical trials [8,9].

Bevacizumab is a humanized monoclonal antibody against vascular endothelial growth factor A (VEGFA, [10]) and was developed as a potential anti-tumor agent due to its critical involvement in angiogenesis. When tumors progress they outgrow the originally available nutrient and oxygen supply, rendering neoangiogenesis a necessary process for continued tumor progression [11,12]. In tumor angiogenesis and also in gliomas, VEGFA has been centrally implicated [13,14], can be induced by hypoxia [15,16], and protein expression correlates with glioma grade and degree of vascularization [17].

Therapeutic targeting of VEGFA-signaling in GBM has however been associated with the development of resistance mechanisms [18]. The upregulation of factors from alternate signaling pathways can confer this resistance, and particularly Angiopoietin-2 has been identified as a mediator with VEGFA-targeting therapies. In vivo Ang-2 has been shown to diminish the survival benefit from VEGF receptor inhibition [19], and to mediate vascular permeabilization and macrophage recruitment [20]. Clinically Ang-2 has been suggested as a mediator of resistance following Bevacizumab treatment [21]. For these reasons, targeting of both VEGFA and Ang-2 has been explored in vivo in GBM xenograft models. These studies have pointed out that the addition of Ang-2 inhibition enhances the anti-tumor effects observed with VEGFA inhibition alone, survival was extended significantly as well [22,23].

In response to VEGF inhibition enhanced invasive growth has also been reported in vivo [24-27] and in GBM patients treated with Bevacizumab [27,28]. These findings could illustrate the initial response that is observed with anti-angiogenic therapy, but the subsequent induction of invasion represents unintended malignant disease progression. Therefore, in this study we have examined the effects of dual inhibition of VEGFA and Ang-2 in an in vivo GBM model and xenografts collected during the dosing period and after treatment discontinuation are compared in parallel. To this end, the effects of the therapies were assessed on tumor growth, vascularization parameters, invasion, necrosis and hypoxia.
Methods

Cell culture and treatments

GSC23 cells were maintained in DMEM/F12 (Lonza, Verviers, Belgium) supplemented with 2% B27 (Life Technologies, Bleiswijk, the Netherlands), 20 ng/ml bFGF and EGF (Life Technologies) and 1% pen/strep (Lonza). For in vivo imaging purposes the cells were transduced with pCignal lenti-CMV-luc particles to express luciferase (SA Biosciences, Frederick, MO) and transduced cells were selected through antibiotic selection with puromycin.

For the inhibition of Ang-2 and VEGFA-signaling drugs against the respective ligands were utilized. Ang-2 was inhibited with an Fc-fusion protein that was kindly provided by Amgen (Cambridge, MA), while VEGFA was inhibited with the commercially available monoclonal antibody Bevacizumab.

Chorioallantoic membrane (CAM) assay

Fertilized chicken eggs, obtained from het Anker B.V. (Ochten, the Netherlands) were cultured in an egg incubator (38°C, 60% humidity) for 3 days with continuous movement to prevent attachment of the yolk sac to the eggshell. Then the eggs were kept in a steady position and a small punch was created in the top of the egg so that an air sac could form. Then the eggs were left undisturbed for another 4 days, and the holes in the eggs were slightly enlarged to expose the membrane for treatment application. A volume of 500 µl antibody solution containing 400 ng/ml L1-10 and/or 500 ng/ml Bevacizumab was added to the newly formed CAM using 15 viable eggs per treatment condition. The eggs were then further incubated for 2 days, after which the membranes were excised and photographed. The microvessel density was analyzed on 5 representative images per CAM using Aperio Imagescope software version 12.1.0 and the data was averaged per CAM.

In vivo mouse study

The in vivo study was designed in a way that both the direct treatment effects could be explored as well as the rebound that has been described in patients after treatment withdrawal. Therefore 50 female 8-week-old nude (Foxn1nu) mice were engrafted intracranially with 2.0×10⁵ GSC23 cells through previously implanted guide-screws [29]. After the mice were given 2 weeks recovery time, twice weekly treatment for a period of 4 weeks was commenced with L1-10 (4 mg/kg converted to single doses of 0.1 mg) and/or Bevacizumab (5 mg/kg, converted to single doses of 0.08 mg). After the dosing period 6 mice were sacrificed per group (termination time point T₁), and another 6 mice were left without treatment for exploration of the rebound effect. The second group of mice was terminated 10 days after
Treatment cessation (termination time point $T_2$).

Prior to termination the mice were injected intravenously in their tail veins with 50 µl of pimonidazole hydrochloride (15 mg/ml in PBS, Hypoxyprobe, Inc., Burlington, MA) and 50 µl of high molecular weight FITC-dextran (30 mg/ml in PBS, MW 2000 kDa, Sigma-Aldrich). The mice were euthanized 30 minutes post-injection through CO$_2$ inhalation. The mouse brains were collected and fixed in 10% formalin solution for 48 hours. Thereafter tissues were processed, embedded in paraffin and sections were prepared for downstream immunohistochemical analyses. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the M.D. Anderson Cancer Center.

**In vivo imaging**

Tumor growth kinetics were monitored throughout the study, first confirming tumor cell engraftment on the day of implantation, and subsequently prior to treatment initiation, right after treatment cessation and prior to the first termination time point ($T_1$) and also the second termination time point ($T_2$). The *in vivo* bioluminescent images were obtained using an *in vivo* bioluminescence imaging system (Ivis® 200, Perkin Elmer, Waltham, MA). Tumor visualization was achieved through intraperitoneal injection with 200 µl of D-Luciferin sodium salt (15 mg/ml in PBS, Gold biotechnology, Olivette, MO) and scanning of the mice after 15 minutes incubation. During all imaging sessions 30 seconds exposure times were used and the images were analyzed with Living Image software version 4.5 (Perkin Elmer).

**Immunohistochemistry**

For IHC purposes mouse brain tissue sections were deparaffinized and rehydrated in a graded ethanol series. For the H&E staining slides were incubated with hematoxylin for 10 minutes, rinsed in water, incubated in Eosin for 2 minutes and dehydrated. Additionally, specific staining with antibodies was performed against CD34 (MEC14.7, Abcam, Cambridge, UK), FITC (Abcam), Nestin (10C2, Santa Cruz, Heidelberg, Germany) and pimonidazole (4.3.11.3, Hypoxyprobe). For the CD34, FITC and Nestin stainings heat-induced antigen retrieval using 10 mM sodium citrate buffer (pH 6.0) was performed, while pimonidazole staining required 0.1% protease pre-treatment for 30 minutes. Endogenous peroxidase was blocked with 0.3% H$_2$O$_2$ for 30 minutes and subsequently the sections were incubated with primary antibodies. Incubation with secondary and tertiary peroxidase-labeled antibodies was performed for signal enhancement and enzyme linking. Antigen expression was then visualized with 3,3'-diaminobenzidine and nuclei were counterstained with hematoxylin.
**Histological evaluation and morphometrical analyses**

Morphometrical analyses were all performed in Aperio Imagescope software version 12.1.0 (Leica Biosystems, Nussloch, Germany). The level of vascularization in the mouse tumors was assessed by quantification of the positive pixel fraction. The vital tumor area was selected and the level of vascularization was expressed as the percentage of CD34-positive pixels within the pixel count for vital tumor area. The measurement for vascular integrity was obtained through area quantification of FITC-positive pixels within the vital tumor area and was also expressed as a percentage. The level of invasion was quantified in a rim of approximately 10 cell layers adjacent to the tumor bulk. In this specific area the percentage of Nestin-positive pixels was expressed in comparison to the selected area of approximately 10 cell layers in width. Lastly, necrotic and hypoxic tissue areas were both quantified in relation to total tumor area and expressed as a percentage. Necrosis was identified on H&E-stained sections, while hypoxia was scored in those areas where tumor nuclei were stained positively.

**Statistical analyses**

Statistical analyses were performed using Graphpad Prism version 5 (Graphpad Software Inc, San Diego, CA). Multiple group comparisons were determined through One-way ANOVAs or Kruskal-Wallis tests depending on the data distribution, and they were followed up by Bonferroni or Dunn’s post-hoc tests, respectively. *P*-values <0.05 were considered significant and exact two-sided *P*-values are reported.

**Results**

**L1-10 and Bevacizumab reduce capillary formation in the developing CAM**

The effect of dual inhibition of Ang-2 and VEGFA was assessed through *in vivo* xenograft studies using orthotopic tumor cell engraftment. But first, the effect of the inhibitors was assessed in an *in ovo* angiogenesis assay. CAMs of developing fertilized eggs were stimulated in the time window that capillary development in this membrane takes place and 2 days after application of the inhibitors the membranes were excised from the eggs, photographed and capillary density was examined. The focus in this experiment was specifically on the developing microvasculature and for that reason larger vessels were excluded from the quantification (Figure 1A). The single treatments with L1-10 or Bevacizumab alone both resulted in reduced capillary density in the CAMs of respectively 25 and 27% (Figure 1B), while the mean reduction in capillary density in the dual-treated membranes was insignificant. In the CAM model, both inhibitors as single agents were able to reduce capillary development, but combination treatment did not provide indications for synergistic anti-angiogenic effects.
L1-10 and/or Bevacizumab do not reduce GBM xenograft growth

Inhibition of Ang-2 and/or VEGFA was then assessed further with in vivo orthotopic xenograft models in mice. Female nude mice were implanted with GBM cells and tumor growth was monitored throughout the study as indicated in Figure 2A. The study was commenced with 49 mice and each group was randomly assigned 12 or 13 (control group) mice prior to the start of dosing. The experimental design prescribed dosing for 4 weeks, after which half of the groups would be sacrificed, and the 6 mice left of each group would be sacrificed after an additional tumor development window of 10 days was allowed. Three mice were found dead and could not be included in the analyses (1 control mouse, 1 Bevacizumab-treated mouse and 1 L1-10 plus Bevacizumab-treated mouse). Additionally, several mice presented with symptoms that required early euthanasia and these mice were also excluded from further analyses (2 control mice, 6 L1-10-treated mice, 2 Bevacizumab-treated mice and 2 L1-10 plus Bevacizumab-treated mice). One mouse was excluded from further study due to lack of tumor engraftment which belonged to the control group. This finally resulted in the inclusion of 9 mice in the control group (5 T1, 4 T2), 6 L1-10-treated mice (all T1), 10 Bevacizumab-treated mice (6 T1, 4 T2) and 9 L1-10 plus Bevacizumab-treated mice (5 T1, 4 T2). The imaging results of the in vivo bioluminescence are displayed in Figure 2B. The quantification of these images indicated that there was possibly a slight trend for reduced tumor growth with Bevacizumab-treatment at day 37 right after the dosing schedule was finished (Figure 1C), but this trend was no longer present 10 days post-treatment cessation.
Figure 2. L1-10 and Bevacizumab treatment ultimately does not slow in vivo GBM growth. Mice were intracranially implanted with GSC23 cells and subsequently dosed intraperitoneally with L1-10 and/or Bevacizumab (A). Half of the study groups were terminated right after finishing the 4-week long dosing schedule (T1), while the others were sacrificed 10 days after treatment cessation (T2). Bioluminescent images of mice throughout the study that are included in downstream analyses are depicted in the second panel (B). The quantification of the bioluminescent images hinted towards a trend for reduced tumor growth right after the dosing schedule (T1) in Bevacizumab treatment alone or in combination with L1-10, but this trend was no longer present 10 days after treatment cessation at T2 (C). Horizontal lines represent median values.

Vascularization levels are reduced following L1-10 and Bevacizumab treatment

To examine how the applied treatments impacted vascularization levels and vascular function the FFPE tissue was assessed with CD34 endothelial staining (Figure 3A) and FITC-extravasation (Figure 3D) from the circulation was quantified. First, the level of CD34 staining
in relation to the total tumor area was quantified, and compared between the treatment groups at the termination time points. The CD34 level was significantly different between groups at T1, but due to variation the post-hoc tests were insignificant (Figure 3B). The data indicate that the largest reduction in vascularization was achieved with Bevacizumab-treatment, and also combination treatment with L1-10 seemed to reduce vascularization levels when the mice were on treatment. Interestingly, the reduction in vascularization level in the combination-treated mice was no longer maintained after the treatment was abrogated and median CD34 expression were similar to the control group level at T₂. Overall, the extent of vascularization could be correlated with total tumor size (Figure 3C, r = 0.7461, P<0.001).

Figure 3. Vascularization levels are temporarily reduced by Bevacizumab treatment, but vessel integrity remains unaltered. Through CD34 staining (A, scale bar = 100 µm) the level of vascularization were determined in relation to total vital tumor area in the mouse xenografts. At T1 differences between groups were present and likely Bevacizumab can be held responsible for the reduction in CD34-positivity in the single Bevacizumab treatment and the combination setting with L1-10 (B). At T2 no differences were detected between groups. The level of vascularization was positively associated with the 2D measurement of tumor size (C). Vascular integrity was examined through staining for FITC extravasation (D, scale bar = 100 µm). No differences were observed at both time points between groups (E) and the extent of FITC extravasation was not associated with the level of vascularization (F). The extent of pericyte coverage was assessed with staining for α-SMA (G, scale bar = 100 µm). At T1 a trend for enhanced α-SMA staining was observed with L1-10 treatment (H), but a strong association between the α-SMA positive pixel count and the CD34 positive pixel count suggests that relative pericyte coverage for unaffected (I). Horizontal lines represent median values.
Vascular integrity or vascular leakiness was next assessed with staining for FITC-extravasation. Since the mice were perfused with FITC-labeled high molecular weight dextran, staining for this marker outside vasculature could here be interpreted as increased vascular leakiness. The quantification of this staining did not indicate any changes in response to the treatments that were applied (Figure 3E), and the extent of vascular leakiness was unrelated to the extent of vascularization (Figure 3F).

Pericytes can function as vascular supporting cells and enhanced coverage of vessels with pericytes is considered a marker for vascular maturation and functionally thought to decrease vascular permeability. Interestingly, staining for pericytes with α-SMA (Figure 3G) presented a trend for increased pericyte expression in L1-10-treated tumors (Figure 3H), while vascular leakiness was not affected as assessed with the FITC staining described above. The correlation between the α-SMA positive pixel count and the CD34 positive pixel count (SR = 0.7027, P<0.001) also suggests that pericyte coverage was not different between groups (Figure 3I). The fact that the outliers in the plot are equally spread over all treatment condition strengthens this conclusion.

**Bevacizumab discontinuation potentially induced tumor invasiveness**

Clinical study reports have speculated on the potential induction of enhanced invasive tumor growth following Bevacizumab-treatment in patients. In this study, we have assessed this malignant tumor behavior through staining for human Nestin and subsequent quantification of human tumor cells in a 10-cell layer thick rim adjacent to the tumor border (Figure 4A).

![Figure 4](image.png)

*Figure 4. Invasive growth of GBM xenografts is unaffected by L1-10 and Bevacizumab treatment.* The extent of invasive growth was measured on mouse brains with xenografts stained for Nestin (A, scale bar = 200 µm). The level of invasion was similar at T1, while at T2 a trend for increased invasive growth in tumors treated with Bevacizumab could be observed (B). A strong correlation between the selected invasion area and the tumor border length along which invasion was quantified confirmed that areas were selected consistently in the different mouse samples (C). Horizontal lines represent median values.

The data from this analysis show substantial variation, especially at T1, and do not provide any indication for differences in invasive tumor behavior when mice are on anti-angiogenic treatment with either L1-10 and/or Bevacizumab (Figure 4B). However, at T2 a decrease in variation was observed, and a trend for increased invasiveness in Bevacizumab-treated mice was seen. The combination-treatment with L1-10 and Bevacizumab showed
more variation and on average resulted in comparable invasion levels to the control group. Technically we were able to show that the analyzed areas in all tumors were relatively similar and the total analyzed area in pixels was strongly associated with the tumor border length along which the invasion was quantified (Figure 4C). The results from this analysis strengthen the suspected enhanced invasiveness following Bevacizumab discontinuation.

L1-10 induces necrosis in GBM xenografts

Angiogenesis is often considered a malignant process through which tumors can attempt to satisfy increased nutrient and oxygen requirements, and anti-angiogenic therapies could from this perspective be intended to prevent the provision of these increased requirements. The starvation of tumor cells in this paradigm should then result in necrosis and eventually result in tumor regression. We have looked at the levels of necrosis and hypoxia with this paradigm in mind. Necrosis was quantified on H&E sections and especially guillande necrosis could be observed in several mouse xenografts (Figure 5A).

Figure 5. L1-10 treatment induces in vivo necrosis in GBM xenografts, but does not significantly alter hypoxia levels. On H&E stained mouse brain sections necrosis area was quantified in relation to total tumor area (A, scale bar = 200 µm). At T1 necrotic areas were only observed in the xenografts of mice that were treated with L1-10, which was deemed a significant increase in necrosis (B). At T2 no differences in necrosis were observed, but due to presentation with neurological symptoms none of the mice treated with L1-10 only survived until this time point. The level of necrosis was unrelated to the total tumor volume (C). Hypoxia levels were assessed after staining for FITC and hypoxic areas were quantified in relation to total tumor area (D, scale bar = 200 µm). At T1 L1-10 treatment seemed to increase hypoxia levels, but this trend was insignificant (E). The comparison of hypoxia levels between tumors with or without necrosis returned an insignificant trend for increased hypoxia in tumors with necrosis (F). Horizontal lines represent median values; *: P<0.05.
The quantification of these areas at the different termination time points revealed that particularly the mice treated with L1-10 presented with extensive necrotic areas at T₁ (Figure 5B, P<0.05). No differences in necrosis were observed at T₂, at which time it was no longer possible to include L1-10-treated mice (as commented on before).

Hypoxic areas can often be detected surrounding necrotic tissue areas due to tumor cell migration from the necrotic areas and a consequential increase of cell density in these areas. The staining for pimonidazole allowed us to identify nuclei of hypoxic cells (Figure 5D), and also here a trend for increased hypoxia in L1-10-treated tumors could be observed at T₁ (Figure 5E). The difference in the percentage hypoxia was determined between tumors with and without necrosis, and a non-significant increase in average hypoxia percentage was observed in tumors with necrotic areas (Figure 5F). These results suggest that necrosis and hypoxia levels could be induced through the administration of an anti-Ang-2 treatment.

Discussion

In the current study we have evaluated the benefit of Ang-2 inhibition with L1-10 alone and in combination to the VEGFA inhibitor Bevacizumab in a mouse GBM xenograft model. To allow for the analysis of effects while mice were on treatment and after discontinuation of treatment, tissue was collected for examination right after the dosing schedule was completed and 10 days after treatment discontinuation. In this xenograft model tumor growth was unaffected by the applied treatments, although the physiological effects of the inhibitors could be detected through morphological assessments.

The manuscript commenced with the exploration of more physiological effects of the inhibitors on the developing CAM, which confirmed that both drugs reduced capillary formation and exerted anti-angiogenic effects [30]. Similarly, in the GBM xenograft model we noticed that the established physiological effects of both inhibitors could be detected. We reach this conclusion through the reduced CD34 positivity in Bevaziumab-treated xenografts, while the increased α-SMA staining in L1-10-treated xenografts could be explained by reduced Ang-2-mediated pericyte detachment. These effects explicitly focus on vascular responses, which could potentially signify substantial cross-reactivity between the humanized drugs that were applied in this study with host tissue components. The situation however remains complicated and relatively uncontrolled for, and these findings do not substantiate the cross-reactivity of alternate induced messengers or signaling processes.

Another challenging aspect is the comparison and relation of the in vivo collected results to reports from clinical experiences with Bevacizumab especially in regard to the vascular leakiness data. Following Bevacizumab treatment, the contrast enhancement on T1-weighted MRI reduces in patients [31], but in our in vivo model vascular integrity was unaffected by both Bevacizumab and L1-10 treatment. It should be noted that the variation in the groups is substantial and the group sizes were small, but a trend could not be appreciated either. This discordance is highly undesirable and it calls the translational value of the in vivo xenograft model into question. To the
contrary, the fact that L1-10 treatment resulted in abundant necrosis did introduce a clinically important GBM characteristic that is infrequently observed in in vivo GBM models.

Invasion has been a topic of interest with respect to Bevacizumab treatment since several in vitro and clinical reports as well have described increased invasiveness following treatment [25-28,32]. In this regard, particularly the loss of the L1-10 group before termination point \( T_2 \) is disappointing, since Ang-2 has been suggested as the mediator of the induced invasive behavior with Bevacizumab treatment [27,28]. Our data however do seem to support the suggestion that Bevacizumab discontinuation can enhance the invasive behavior of GBM cells, and the addition of L1-10 as a treatment to Bevacizumab seems to reduce the invasive behavior after treatment has been discontinued. Due to data variation and group sizes, it is currently not possible to draw stronger conclusions from this study.

Looking more closely at angiogenesis, extensive similarities can be appreciated with endothelial cell activation. In response to endothelial cell activation vascular integrity decreases and cytokine production is increased, and importantly endothelial cells are able to participate in the inflammatory response through upregulation of adhesion molecules [33]. In this study we have focused on the vascular effects of Ang-2 and VEGFA inhibition, but recent evidence signifies that angiogenic factors can have important contributions to inflammatory responses as well [34]. Ang-2 is required for the generation of an inflammatory response [35] and acts as a chemoattractant for immune cells in gliomas [20,36,37]. Increased infiltration of immune cells has been associated with glioma grade [38] and immune cells have been reported to mediate anti-angiogenic therapy refractoriness [39]. It would therefore be very interesting to evaluate the effects of the current therapies and those after therapy discontinuation in an immunocompetent animal model.

To conclude, we have shown that in vivo inhibition of Ang-2 and VEGFA modulates isolated vascular parameters, but tumor progression was unaffected. Since the survival prolonging effects of the combinatorial inhibition of Ang-2 and VEGFA in vivo have been associated with a M2-to-M1 phenotype switch of macrophages [23] and the benefit from combination therapy was impaired when immune cell recruitment was prevented [22], it is to be expected that the interactions with the immune compartment are critical to the ultimate success of anti-angiogenic therapies.
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