Antiangiogenic Therapy of Cerebral Melanoma Metastases Results in Sustained Tumor Progression via Vessel Co-Option

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ABSTRACT
Purpose: In the brain, tumors may grow without inducing angiogenesis, via co-option of the dense pre-existent capillary bed. The purpose of this study was to investigate how this phenomenon influences the outcome of antiangiogenic therapy.

Experimental Design: Mice carrying brain metastases of the human, highly angiogenic melanoma cell line Mel57-VEGF-A were either or not treated with different dosages of ZD6474, a vascular endothelial growth factor (VEGF) receptor 2 tyrosine kinase inhibitor with additional activity against epidermal growth factor receptor. Effect of treatment was evaluated using contrast-enhanced magnetic resonance imaging (CE-MRI) and (immuno)morphologic analysis.

Results: Placebo-treated Mel57-VEGF-A brain metastases evoked an angiogenic response and were highlighted in CE-MRI. After treatment with ZD6474 (100 mg/kg), CE-MRI failed to detect tumors in either prevention or therapeutic treatment regimens. However, (immuno)histologic analysis revealed the presence of numerous, small, nonangiogenic lesions. Treatment with 25 mg/kg ZD6474 also resulted in efficient blockade of vessel formation, but it did not fully inhibit vascular leakage, thereby still allowing visualization in CE-MRI scans.

Conclusions: Our data show that, although angiogenesis can be effectively blocked by ZD6474, in vessel-dense organs this may result in sustained tumor progression via co-option, rather than in tumor dormancy. Importantly, blocking VEGF-A may result in undetectability of tumors in CE-MRI scans, leading to erroneous conclusions about therapeutic efficacy during magnetic resonance imaging follow-up. The maintenance of VEGF-A–induced vessel leakage in the absence of neovascularization at lower ZD6474 doses may be exploited to improve delivery of chemotherapeutic agents in combined treatment regimens of antiangiogenic and chemotherapeutic compounds.

INTRODUCTION
The continuously increasing demand for oxygen and nutrients of growing solid tumors can be met by induction of a neovascular bed. This is accomplished by a hypoxia-driven mechanism, which results in expression of a number of effector molecules, among which is vascular endothelial growth factor (VEGF)-A, the most potent angiogenic factor known to date (1). The notion that abrogation of the blood supply to a tumor will prevent further outgrowth and induce dormancy has led to the development of numerous compounds that target one of the events in the multistep process of angiogenesis. These include naturally occurring angiogenesis inhibitors such as tumstatin, endostatin, and angiostatin (proteolytic fragments of collagen IV and XVIII and plasminogen; respectively; refs. 2–9); protease inhibitors that prevent degradation of the basal lamina of blood vessels (10); and compounds that interfere with the adherence of endothelial cells to matrix proteins (e.g., via blockade of αvβ3 integrin; refs. 11 and 12). However, because VEGF-A by itself has the potency to initiate most, if not all, necessary steps in the angiogenic process, most research has focused on targeting VEGF-A or its receptors, VEGF receptor (VEGFR)-1 (Flt-1) and VEGFR-2 (KDR/Flik-1). This has resulted in the development of VEGF antagonists (13, 14), humanized antibodies against VEGF-A (15, 16) or VEGFR-2 (17, 18), and soluble chimeric VEGF receptor ectodomains (19), compounds with tumor growth-inhibitory activities in a number of animal models (19, 20). One novel class of compounds targeting VEGF comprises small molecule agents that can be administered orally against the tyrosine kinase moieties of VEGFRs (21–23). Included in this class is ZD6474, which selectively inhibits VEGF-2 kinase activity and has additional inhibitory activity against epidermal growth factor receptor (EGFR) tyrosine kinase. It has previously been shown that this compound has potent antitumor activity against a broad spectrum of histologically diverse subcutaneous tumor xenografts in mice (21).

These promising preclinical data have led to high expectations for antiangiogenic approaches in the clinic. However, it seems unlikely that inhibition of VEGF-dependent angiogenesis will demonstrate the same pan-tumor activity in the clinic that has been seen in preclinical models. There are several reasons to expect discrepancies in results between preclinical models and clinical trials in man. First, slowly growing, large human tumors may differ in their sensitivity toward angiogenesis inhibitors from the rapidly growing...
tumors that are often used in mouse models of cancer, and human tumors may have regions with relatively mature and stable tumor vessels that are less susceptible to anti-VEGF therapy. Indeed, in Rip-Tag mice (a transgenic mouse model of spontaneous pancreatic islet carcinoma formation), it was shown that targeting VEGFRs resulted in inhibition of tumor angiogenesis and growth only in early stages of tumor development. Affecting vessel integrity by targeting platelet-derived growth factor receptors leading to pericyte loss resulted in regression of vessels in more established tumors. In this model, simultaneous targeting of VEGFRs and platelet-derived growth factor receptors was more efficient than targeting of either receptor alone (24). Secondly, candidate patients for phase I trials with antiangiogenic therapy are predominantly patients with disseminated cancer, for which other therapeutic options are no longer available, and one may ask whether disseminated tumors have the same angiogenesis dependency as the originating tumor: because tumor metastasis occurs through lymph and blood vessels, outgrowth of metastases will occur mostly in vessel-dense organs such as the lung, liver, and brain. In these organs, tumors (both primary and metastatic) may grow independently of angiogenesis via a process of co-option of pre-existent vessels (25–28). This implies that, whereas compounds may be efficient inhibitors of angiogenesis and tumor growth in angiogenesis-dependent tumors (such as subcutaneous tumor xenografts), their effects may be limited in tumors growing in tissues with an intrinsic vascular density that allows for co-option by infiltrative tumors.

Here we investigated this hypothesis by treating mice carrying brain tumors of the human melanoma cell line Mel57 with the antiangiogenic agent ZD6474. Whereas parental Mel57 xenografts grow in brain parenchyma of immunocompromised mice exclusively via co-option of brain capillaries, Mel57 tumors stably expressing VEGF-A165 evoke a fulminating angiogenic response (29). In contrast to parental tumors, those expressing VEGF-A are readily detected in contrast-enhanced magnetic resonance imaging (CE-MRI) due to VEGF-induced vascular leakage (30). Here we show that ZD6474 is a potent inhibitor of angiogenesis in this tumor model. However, this inhibition does not lead to tumor regression or increased survival. Tumor growth is able to continue via co-option of pre-existent vessels, a growth pattern that is not detected by Gd-diethylenetriaminepentaacetic acid (DTPA)-enhanced magnetic resonance imaging (MRI). Intermediate doses of ZD6474 inhibited new vessel formation but not VEGF-A–induced vessel leakage, still allowing MRI detection of treated tumors.

These data indicate that in clinical settings where switching to vessel co-option is possible, the interpretation of CE-MRI data to monitor antitumor effects should be used with caution. Decreases in CE-MRI signal can effectively determine whether an agent reduces tumor vessel permeability but may be a poor indicator of antitumor response, particularly if the tumor can adopt a vessel co-option phenotype.

**MATERIALS AND METHODS**

**Cell Lines and Transfections.** Culture of the Mel57 human melanoma cell line and generation of stably transfected Mel57 cell lines expressing enhanced green fluorescent protein (EGFP) or VEGF-A165 have been described previously (27, 29). EGFP and VEGF-A165 cDNAs were under control of the cytomegalovirus promoter, and expression of the proteins of interest was linked to that of the neomycin resistance gene product via an internal ribosome entry site, enabling effective selection for EGFP- or VEGF-A-expressing cells.

**Animal Experiments.** Specific pathogen-free male BALB/c nu/nu mice were purchased from the university central animal facility breeding program. Experiments were carried out in accordance with national animal protection laws, and approval was obtained from the Animal Experimental Committee. To establish brain metastases, 7-week-old mice were anesthetized (1.3% isoflurane/O2/N2O), and tumor cells (1 × 10^5 Mel57-EGFP or Mel57-VEGF-A165 cells in 100 μL of PBS) were injected in the right internal carotid artery as described previously (31). Three sets of experiments were performed. In one experiment, mice carrying Mel57-VEGF-A165 brain lesions were treated with 100 mg/kg ZD6474 (n = 9) or placebo (n = 10) starting at day 2 or 10 after tumor cell injection. In a second experiment, tumor-bearing mice (n = 10) were treated with 50 mg/kg ZD6474, starting at day 1, 5, 9, 13, or 17 after injection (n = 2 for each time point). Finally, an experiment was performed in which a 1:1 mixture of Mel57-VEGF-A165 and Mel57-EGFP cells was injected to compare the effects of ZD6474 on angiogenic and co-opting lesions within one animal (n = 10). In this case, mice were treated with 25 mg/kg ZD6474 starting at day 2 after injection. ZD6474 (as a suspension in 1% polysorbate-80) or placebo (1% polysorbate-80) was administered orally once daily in a volume of 100 μL. After 16 to 20 days, when clinical symptoms due to tumor growth were apparent (weight loss and neurologic defects), CE-MRI was performed according to the protocol described below.

**Contrast-Enhanced Magnetic Resonance Imaging.** CE-MRI was performed as described previously (30). Briefly, mice were anesthetized [1.3% isoflurane; 1:1 (v/v) N2O/O2 mixture], catherized in the tail vein, and placed in a MR spectrometer (S.M.I.S. console equipped with a Magnex Scientific 7T/200 mm horizontal bore magnet and a 150 mT/m gradient set). Body temperature was maintained by placing the mice on a 37°C circulating warm water bed. A 12-mm–diameter surface coil was positioned over the skull. After initial monitoring of the brain with fast gradient-echo scout images, 16 contiguous images were acquired with a T1-weighted multislice gradient-echo sequence (T1 = 8 ms; T2 = 100 ms; flip angle = 90°; number of averages = 1; field of view = 25 × 25 mm; matrix size = 256 × 256; slice thickness = 1 mm) before and 1, 2, 10, and 20 minutes after bolus injection of Gd-DTPA (Magnevist; Schering, Berlin, Germany) at a dose of 0.2 mmol/kg.

**Histologic and Immunohistochemical Analysis.** After MRI, mice were sacrificed by injecting an overdose of barbiturate and brains were removed and fixed in buffered formalin. Brains were cut into coronal slices and embedded in paraffin. Sections of 4 μm were processed for conventional hematoxylin and eosin staining or immunostaining for mouse IgG (to examine the presence of extravasated proteins), endothelial marker CD34 (Hycult, Uden, the Netherlands), GLUT-1 (as a blood-brain barrier marker and a hypoxia marker; DAKO, Glostrup, Denmark), MIB-1 (antineuman Ki67; DAKO), and antimouse Ki67 (Dianova, Hamburg, Germany). MRI images were...
matched to histology as closely as possible. To confirm stable VEGF-A expression, tumors were also subjected to VEGF in situ hybridization (ISH) using a digoxigenin-labeled VEGF-A antisense RNA probe. The corresponding sense probe was used as a control.

RESULTS

We have recently described that Mel57 tumors grow in mouse brain via co-option of pre-existent vessels without inducing an angiogenic switch, whereas the same tumors, engineered to express VEGF<sub>165</sub>, evoked a fulminant angiogenic response (25, 29). Parental Mel57 lesions were invisible in Gd-DTPA-enhanced MRI scans, whereas VEGF-A<sub>165</sub>-induced vessel leakage led to clear MRI visibility (30). To investigate the effects of the anti-VEGF therapy on MRI representation and tumor phenotype, we treated mice carrying highly angiogenic Mel57-VEGF-A<sub>165</sub> brain tumors with ZD6474. Treatment with 100 mg/kg ZD6474, starting early during tumor development (2 days after injection of tumor cells), led to complete inhibition of VEGF-A effects. In treated animals, tumors grew via co-option of pre-existent vessels, as was evident from a number of observations. First, vessel dilation as observed in placebo-treated controls was absent in the treated tumors (Fig. 1, A and F). Secondly, the up-regulation of a number of endothelial markers such as CD34, which was evident in placebo-treated Mel57-VEGF-A<sub>165</sub> tumors (Fig. 1B), was absent in ZD6474-treated tumors (Fig. 1G). Furthermore, in contrast to tumor vessels in placebo-treated animals, tumor vessels in ZD6474-treated animals expressed GLUT-1, a characteristic property of the highly specialized blood-brain barrier, suggesting that these vessels were pre-existent rather than newly formed (compare Fig. 1, C and H). Vessels were not leaky, as evidenced by the absence of extravasated mouse immunoglobulins (data not shown). Also, VEGF-A-induced proliferation of mouse endothelial cells, which occurred abundantly in the control animals, failed to appear in the ZD6474-treated animals (see mouse-specific Ki67 stainings in Fig. 1, D and I). Importantly, in both the control and the ZD6474-treated animals, tumor cells had a high proliferation index (see Mib-1 immunostaining in Fig. 1, E and J) indicating that blood supply from co-opted pre-existent brain capillaries was sufficient for tumor progression. This was also evident from the fact that hypoxia and necrosis were never observed in these tumors. Importantly, using the intracarotid injection technique, multiple lesions develop in brain parenchyma. Typically, 20 to 50 lesions are found in each brain. All lesions responded to therapy in a similar manner.

Mel57 cells express VEGFR-2 but do not respond to VEGF-A by increased proliferation. The high proliferation index in ZD6474-treated tumors (Fig. 1J) confirms that the effects of ZD6474 are indeed the result of inhibition of VEGFR-2 on endothelial cells rather than a direct effect on the tumor cells.

The absence of vessel leakage in ZD6474-treated tumors was reflected in MRI: treated tumors were not visible (Fig. 2B), whereas lesions in control-treated animals were clearly enhanced after injection of Gd-DTPA (Fig. 2A). To exclude the possibility that the co-opting phenotype of ZD6474-treated tumors was due to incidental loss of VEGF-A expression, we performed ISH with an antisense VEGF-A probe, which demonstrated that the co-opting phenotype was caused by ZD6474 treatment and not by incidental loss of VEGF-A expression (Fig. 1K).

In this tumor model, 12 days after tumor cell injection, small lesions were present that already exerted marked effects on the vasculature such as vasodilatation and vessel leakage, as demonstrated by IgG leakage around the lesions (data not shown), and were detectable by Gd-DTPA in MRI as small hyperintense spots (Fig. 2D, arrows). To investigate the effects of ZD6474 on tumors in which VEGF-A effects are already distinct, mice received injection with Mel57-VEGF-A cells, and treatment with 100 mg/kg ZD6474 was initiated 10 days later. After another 5 days, mice were subjected to Gd-DTPA–enhanced brain MRI and sacrificed. Again, tumor lesions grew by vascular co-option, with some lesions showing remnants of dilated vasculature (data not shown). However, vessels were not leaky, as illustrated by the absence of Gd-DTPA extravasation (Fig. 2C). This was confirmed once more by the presence of only very little extravasated mouse immunoglobulin (data not shown).

At a dose of 100 mg/kg, ZD6474 very efficiently prohibited the development of an angiogenic tumor phenotype, forcing tumor cells to grow by co-option of pre-existent vessels, much like parental Mel57 tumors. To better understand the switch to a vessel co-option phenotype, we tested the effects of lower doses of ZD6474 (25 and 50 mg/kg) in this model.

Mice that were treated with 50 mg/kg ZD6474 starting at day 2 after tumor cell injection showed a strikingly different tumor phenotype compared with the 100 mg/kg treatment group. Now, tumors had an expansive appearance and were poorly vascularized. In approximately 50% to 70% of the lesions, central hypoxia (evidenced by GLUT-1 staining of tumor cells; arrow in Fig. 3C) and necrosis (Fig. 3A) were observed. At the tumor rims, cells co-opted pre-existent, GLUT-1–positive vessels (Fig. 3C). Surprisingly, a similar tumor phenotype was observed when 50 mg/kg treatment was initiated 1, 5, 9, and 13 days after tumor cell injection (Fig. 4; data not shown). Only when treatment was given for 2 days, starting at day 17 after tumor cell injection, was a tortuous, dilated, and leaky tumor vasculature without apparent signs of hypoxia or necrosis observed (Fig. 3, E–G; Fig. 4D). Whereas in these tumors, cells proliferated abundantly, as demonstrated by anti-pan–Ki67 stainings, proliferating mouse endothelial cells in the dilated vasculature were only sparsely detected (data not shown). This suggests that ZD6474 treatment for as little as 2 days led to marked inhibition of endothelial cell proliferation.

Although ZD6474 at 50 mg/kg effectively inhibited angiogenesis, it did not completely prevent vascular leakage, as evidenced by the presence of extravasated mouse immunoglobulins around the lesions (Fig. 3D) and the fact that tumors were readily detected in Gd-DTPA–enhanced MRI as ring-enhancing lesions (Fig. 4). The ring enhancement observed in Fig. 4, A–C corroborated the histologic finding that within these tumor lesions, the vessel density was low, leading to central

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4 W.P.J. Leenders and B. Küsters, unpublished results.
Fig. 1 Effects of 100 mg/kg ZD6474 on Mel57-VEGF-A\textsubscript{165} brain tumor morphology. Mice received injection in the internal carotid artery with Mel57-VEGF-A\textsubscript{165} cells, and oral treatment with placebo (A–E) or 100 mg/kg ZD6474 (F–J) was initiated 2 days after injection. Sixteen days after injection of tumor cells, mice were sacrificed, and brains removed and fixed in formalin. Four-micrometer sections were stained with conventional hematoxylin and eosin (A and F) and with antibodies against the antiendothelial marker CD34 (B and G), brain endothelium marker GLUT-1 (C and H), Ki67 (proliferation antigen, mouse specific; D and I) and with MIB-1 (anti-pan-Ki67; E and J). In treated tumors, GLUT-1 was expressed on tumor vessels (arrow in H), whereas it was absent on placebo-treated tumors (arrow in C points at a tumor vessel). Note the absence of proliferating mouse endothelial cells in the ZD6474-treated tumors (I), whereas numerous proliferating (endothelial) cells are present in placebo-treated tumors. By contrast, tumor cell proliferation was not inhibited by ZD6474 (compare MIB staining in E and J), and ZD6474-treated tumors progressed by vascular co-option. Treated tumors were highly invasive but still expressed VEGF-A as demonstrated by ISH using an antisense VEGF probe (K). ISH using a sense control probe was negative (data not shown).
(nonenhancing) necrosis. The bulky enhancement of the lesions in Fig. 4D (arrows) is in agreement with the absence of necrosis and the high vessel density in these tumors (Fig. 3, E–G).

Lowering the dose even further to 25 mg/kg yielded similar results: vessel leakiness was not inhibited in contrast to angiogenesis, again resulting in central necrosis in approximately 50% of the lesions (data not shown).

In our brain tumor model, lesions arise by clonal expansion. This enabled us to compare the effects of ZD6474 on angiogenic Mel57-VEGF \(_{165}\) tumors and co-opting Mel57-EGFP tumors within one animal by making use of EGFP expression to discriminate between the different Mel57 transfectants. Similar experiments have recently been published in which clonality of metastases was determined using this approach (32). In a time-matched experiment, a 1:1 mixture of Mel57-EGFP and Mel57-VEGF \(_{165}\) was injected in carotid arteries, and treatment with 25 mg/kg ZD6474 or placebo was initiated 2 days later (\(n = 5\) for each group). Again, tumors were analyzed at day 20 after injection.

EGFP staining of brain tissue revealed that necrosis only occurred in Mel57-VEGF-A tumors, and never in Mel57-EGFP tumors (arrow in Fig. 4E), indicating that co-opting Mel57-EGFP lesions were not notably affected by ZD6474 treatment. As in the 50 mg/kg experiment, ZD6474 treatment led to a ring-shaped appearance of tumors in Gd-DTPA–enhanced MRI, presumably because of the presence of central, nonenhancing necrosis (data not shown).

**DISCUSSION**

ZD6474 has been shown to be a promising antiangiogenic agent that inhibits VEGFR-2 tyrosine kinase activity and has additional activity against EGFR tyrosine kinase (21). It prevents tumor growth in a wide range of histologically diverse tumor models and is currently being evaluated in phase II clinical trials. It is generally accepted that tumor growth is heavily dependent on angiogenesis, but a number of reports have been published that now suggest that angiogenesis is not always a prerequisite for tumor growth because tumor cells may exploit pre-existent vasculature, a process known as vascular co-option (25–28, 33). In this report, we investigated the effects of ZD6474 on tumor behavior in a mouse model of brain colonization by the human melanoma cell line Mel57. This cell line homes to brain parenchyma (31), where it grows rapidly to relatively large, vital infiltrative tumors by co-option of pre-existent vessels. Blood supply from these brain vessels was adequate for tumor growth because no hypoxia developed in the lesions (27). Consequently, the trigger for up-regulation of VEGF-A expression and the angiogenic switch did not occur. Furthermore, co-opted blood vessels were not notably affected
by the tumor because the blood-brain barrier remained intact (30). In contrast, stably transfected Mel57 cells expressing human VEGF165 evoked a fulminant angiogenic response in which the conceptual hallmarks of angiogenesis were present: tumor vessels were dilated and tortuous and contained numerous proliferating endothelial cells. Furthermore, the blood-brain barrier was disrupted, enabling Gd-DTPA–enhanced detection in MRI. Importantly, the fact that these tumors induce angiogenesis does not preclude co-option: in fact, at the tumor rim, co-option of vessels can be observed.

When treatment with 100 mg/kg ZD6474 was initiated 2 days after carotid injection, effects of VEGF-A were completely abrogated, whereupon the tumor adopted the pre-existing vessel co-opting phenotype in which the blood-brain barrier remained intact. Accordingly, treated tumors were invisible in Gd-DTPA–enhanced MRI. Importantly, these co-opting tumors still expressed high levels of VEGF-A, excluding the possibility that the development of this phenotype was due to loss of VEGF-A expression.

These results were anticipated because abrogation of the effects of VEGF-A in Mel57-VEGF-A cells would indeed be expected to result in a parental Mel57 tumor-like phenotype. However, Mel57-VEGF-A tumors were also invisible in Gd-DTPA–enhanced MRI when mice were treated from day 10 to 17 after injection of tumor cells. This illustrates that ZD6474 not only prevents but probably also reverts VEGF-A–induced vascular changes. This is in accordance with previous observations that ZD6474 led to regression of certain established subcutaneous tumors (21). We did not perform Kaplan-Meier analysis; therefore, we cannot make definite conclusions about the effects of treatment on survival. It appeared, however, that ZD6474 treatment, despite significant inhibition of tumor angiogenesis
Effects of Antiangiogenic Therapy on Brain Tumors were still visible by Gd-DTPA age. Consequently, tumors treated with 25 or 50 mg/kg ZD6474 – VEGF-A induced effects: increased vascular permeability and formation but did not prevent VEGF-A – symptoms. mg/kg ZD6474 treatment did not delay the onset of neurologic and placebo groups. This may explain our finding that 100 the overall number of lesions is not different between treatment colonization model are smaller in the ZD6474-treated groups, travasated. Therefore, although individual tumors in our brain can directly take profit from the vessels from which they ex- grow via vessel co-option because, on extravasation, tumor cells develop, whereas stereotactic tumor cell implantation will give rise to one tumor. In the latter case, the effects of anti-VEGF therapy are predicted to result in slower growth of a single lesion because only the tumor rim has the possibility of outgrowth via infiltration/co-option. In our model, all lesions may grow via vessel co-option because, on extravasation, tumor cells can directly take profit from the vessels from which they extravasated. Therefore, although individual tumors in our brain colonization model are smaller in the Z6474-treated groups, the overall number of lesions is not different between treatment and placebo groups. This may explain our finding that 100 mg/kg ZD6474 treatment did not delay the onset of neurologic symptoms.

At lower doses, ZD6474 potently inhibited new vessel formation but did not prevent VEGF-A–induced vascular leakage. Consequently, tumors treated with 25 or 50 mg/kg ZD6474 were still visible by Gd-DTPA–enhanced MRI. Thus, at lower doses, ZD6474 appears to have differential effects on two VEGF-A–induced effects: increased vascular permeability and endothelial proliferation, both activities that are mediated by VEGFR-2 (13). This suggests that endothelial cell proliferation and vascular permeability are the result of a divergence in cell signaling pathways downstream of VEGFR-2. Such a diver- gence has indeed been described recently (35). Permeability depends on a Rac-dependent pathway, whereas proliferation is mediated by extracellular signal-regulated kinase 1/2. Our data suggest that the extracellular signal-regulated kinase 1/2 pathway requires a stronger signal from VEGFR-2 than the Rac pathway.

An intriguing observation from this study was that ZD6474 treatment at 100 mg/kg resulted in tumor progression via co-option of pre-existent brain vessels, a process that appears efficient because no hypoxia or necrosis develops in the tumor. In contrast, tumors treated with lower doses of ZD6474 often showed central necrosis, whereas in the same animals, necrosis in co-opting Mel57-EGFP tumors was never observed. These data support the following interpretation: ZD6474 at 25 or 50 mg/kg inhibits new vessel formation but does not prevent vascular leakage. Increased vessel permeability presents a potential benefit to the tumor because it leads to an increased supply of nutrients to the tumor cells and, consequently, to increased and expansive tumor growth. However, because compensating angiogenesis does not occur, due to a lack of endothelial cell proliferation, central hypoxia and, eventually, necrosis develop.

Interestingly, we found that on the morphologic and MRI level, there was little or no difference between brain tumors in the different groups of mice in which treatment was started from day 1 or day 13 onward. It is possible that this is due to both the potent antivascular and antiangiogenic activity of ZD6474.

Differential effects on angiogenesis and vascular permeability have the potential to be exploited in brain tumor therapy because it creates the possibility of manipulating the blood-brain barrier for therapeutic benefit. For instance, by creating a situation in which VEGF-dependent angiogenesis, but not vascular leakage, is inhibited, combination treatment with chemotherapeutic agents may be more effective. Where both processes are

Fig. 4 Brain MRI of tumor-bearing mice treated with 50 mg/kg ZD6474 from day 1, 5, 9, or 17 after inoculation of tumor cells, as indicated. T1-weighted Gd-DTPA–enhanced images were acquired at day 20, before (A–D, top panels) and directly after (A–D, bottom panels) intravenous injection of the contrast agent. Clearly, at this dose of ZD6474, VEGF-induced vessel leakage is not inhibited. Note the ring enhancement of the tumors when treatment is started at earlier time points, whereas bulky enhancement (arrows in D) was observed in the group treated from day 17 to 19. The enhancement patterns correlate with the histology of the lesions (see Fig. 3), where tumor centers are very poorly vascularized and often necrotic, leading to poor enhancement. E. EGFP immunostaining on the brain of a mouse carrying both EGFP-expressing and VEGF-A–expressing Mel57 tumors and treated with 25 mg/kg ZD6474. Note that co-opting Mel57-EGFP tumors were not affected by ZD6474 treatment (arrow), whereas the VEGF-expressing tumors display central necrosis (n).
inhibited, leading to restoration of the blood-brain barrier, reduced accessibility of chemotherapy for the tumor cells may occur. This subject is currently under investigation in our laboratory.

ZD6474 has potent activity against VEGF-R2 signaling and has additional activity against EGFR. EGFR is up-regulated on endothelium and involved in angiogenesis (36). Because the particular vascular phenotype in Mel57-VEGF-A brain tumors has an unambiguous VEGF-A effect (29), the effects of ZD6474 are likely due to VEGF-R2 antagonism rather than EGFR antagonism.

In animals treated with 100 mg/kg ZD6474, tumor cells still had a high proliferation rate (>50% of the cells were positive for the proliferation antigen Ki67), whereas endothelial cell proliferation was completely abolished. This provides strong evidence that the effects of ZD6474 on the tumor phenotype might be entirely attributed to effects on the vasculature and not on the tumor cells. This is in agreement with the fact that Mel57 cells in vitro do not respond to VEGF-A, although they express VEGFR-2, similar to other melanoma cell lines (data not shown).

At present, in clinical practice, the best noninvasive way to diagnose brain tumors is by CE-MRI. Our model system indicates that tumors that adopt a vessel co-opting phenotype would be invisible by Gd-DTPA–enhanced MRI. If a switch to this phenotype occurred while VEGFR-2 was fully antagonized by a treatment regimen, it might falsely suggest that tumor regression occurred while VEGFR-2 was fully antagonized by a

VEGF-A variant inhibits VEGF-stimulated endothelial cell proliferation and not migration or invasiveness (31).

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