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An *in vitro* Model That Can Distinguish Between Effects on Angiogenesis and on Established Vasculature: Actions of TNP-470, Marimastat and the Tubulin-Binding Agent Ang-510

Jens van Wijngaarden [1] & Thomas J.A. Snoeks [1]
Ermond R. van Beek [1]
Henny Bloys [1]
Eric L. Kaijzel [1]
Victor W.M. van Hinsbergh [2]
Clemens W.G.M. Löwik [1]

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[1]Dept. of Endocrinology, LUMC, Leiden, The Netherlands
[2]Dept. for Physiology, Inst. of Cardiovasc. Res., VU-MC, Amsterdam, The Netherlands

Abstract

In anti-cancer therapy, current investigations explore the possibility of two different strategies to target tumor vasculature; one aims at interfering with angiogenesis, the process involving the outgrowth of new blood vessels from pre-existing vessels, while the other directs at affecting the already established tumor vasculature. However, the majority of *in vitro* model systems currently available examine the process of angiogenesis, while the current focus in anti-vascular therapies moves towards exploring the benefit of targeting established vasculature as well. This urges the need for *in vitro* systems that are able to differentiate between the effects of compounds on angiogenesis as well as on established vasculature. To achieve this, we developed an *in vitro* model in which effects of compounds on different vascular targets can be studied specifically. Using this model, we examined the actions of the fumagillin derivate TNP-470, the matrix metalloproteinase inhibitor marimastat and the recently developed tubulin-binding agent Ang-510. We show that TNP-470 and marimastat solely inhibited angiogenesis, whereas Ang-510 potently inhibited angiogenesis and caused massive disruption of newly established vasculature. We show that the use of this *in vitro* model allows for specific and efficient screening of the effects of compounds on different vascular targets, which may facilitate the identification of agents with potential clinical benefit. The indicated differences in the mode of action between marimastat, TNP-470 and Ang-510 to target vasculature are illustrative for this approach.

Introduction

A functioning and continuously expanding vascular network is essential for tumor development, growth, survival and metastasis. Given its pivotal role in these processes, tumor vasculature is a highly attractive target in anti-cancer therapy. Moreover, anti-vascular treatment may present with a low risk of developing drug resistance and promises to be effective against a broad spectrum of tumors.[1,2] Currently, two key approaches to target the tumor's blood vessel network have been developed.[3,4] One is directed at interfering with angiogenesis while the other aims to affect the already established tumor vasculature.

Angiogenesis is the process involving the outgrowth of new blood vessels from pre-existing vessels, and many compounds that affect tumor angiogenesis *in vitro* have been identified and are currently being investigated in clinical trials. Anti-angiogenic agents that have been tested interfere with different targets, such as angiogenic stimuli, receptor activity and endothelial cells.[4–6] The second approach aims at preferential targeting of the already established tumor vascular network and makes use of so-called vascular-disruptive agents (VDAs).[7–9] All VDAs currently examined draw on the differences between tumor and healthy vasculature to allow for highly selective targeting of tumor blood vessels.[10,11]

The VDAs can be divided into two categories: biologic and small-molecule agents (SMAs). Biologic agents include peptides and antibodies that deliver effectors to the tumor endothelium, where SMAs exploit the differences between healthy and tumor vasculature to induce selective vascular dysfunction.[12–14] Targeting angiogenesis and already established vasculature could both have their role in anti-cancer therapy.

Where tumor angiogenesis is well suited for treating micrometastatic disease and early-stage cancer, disrupting established tumor vasculature leads to rapid vascular collapse, vessel congestion and tumor necrosis and is therefore more efficacious against large, already established tumors. Both approaches have shown promising results in ongoing preclinical studies, but treatments either targeting tumor angiogenesis or established tumor vasculature alone are not fully effective.[10,15–19] For this reason, current research explores the benefit of combining these anti-vascular treatment strategies.[13,20–22]

When developing new anti-vascular compounds it would be of great benefit if one could determine if the overall anti-vascular action is mainly due to effects on inhibition of angiogenesis or to suppression of established vasculature or a combination of both. Therefore, in the present study, we developed an assay in which effects of substances on angiogenesis can easily be studied next to those on established vasculature in the same *in vitro* model. In order to validate this model system, we examined the actions of a number of different anti-vascular agents, among which a recently developed combretastatin like tubulin-binding agent Ang-510 (Figure 5.1).

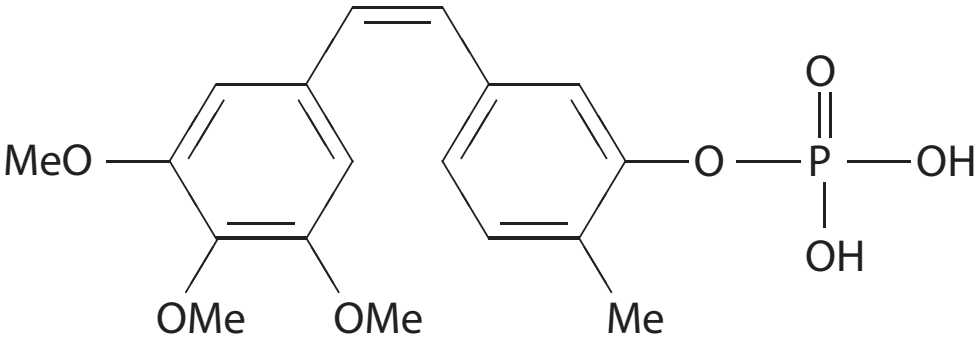

Figure 5.1: Chemical structure of the tubulin-binding agent Ang-510.

Materials & methods

Chemicals & reagents

Culture medium was α-MEM from Gibco BRL, Breda, The Netherlands, supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. rhVEGF-A was from Oncogene, Sanbio, Uden, The Netherlands. ER-MP12 directed against murine PECAM-1 (CD31) was kindly provided by Dr. P. Leenen, Erasmus University, Rotterdam, The Netherlands. The matrix metalloproteinase (MMP)-inhibitor marimastat was kindly provided by Chiroscience Inc. (Cambridge, United Kingdom). TNP-470, a kind gift from W. Landuyt, University Hospital, K.U. Leuven, Belgium. The newly developed tubulin-binding agent Ang-510 was a kind gift from Graeme J. Dougherty and Peter D. Davis, Angiogene Pharmaceuticals Ltd. (United Kingdom).

In vitro vascularization models

In vitro angiogenesis was measured as outgrowth of endothelial capillary structures from cultures of 17-day-old fetal mouse metatarsal bone explants, as described previously.[23] In short, isolated metatarsals were cultured for 48h in 24-well plates in 125µl α-MEM medium to allow for attachment to bottom of the culture plate. Subsequently, medium was replaced by 500µl fresh medium containing vascular endothelial growth factor (VEGF) (50ng/ml) and the test substances and the medium was replaced every 3-4 days. After a total of 10 days of culture, the explants were fixed and stained for PECAM-1.

In the pre-culture experiments, the explants were treated for 24h with the test substances, after attachment to the bottom of the plate, and were subsequently cultured for another 10 days in the presence of VEGF (50ng/ml).

The area of PECAM-1-positive tubular structures was determined by image anal-

ysis using Image Pro Plus 3.0 for Windows 95/NT (Media Cybernetics, Carlsbad, CA). Images were obtained using a digital camera with a fixed window of 768×576 pixels. Data are depicted as number of pixels per area.

In vitro effects on newly established vasculature were examined in fetal mouse bone explant cultures that were first cultured for 10 days in the presence of VEGF (50ng/ml) to stimulate capillary network formation. Subsequently, the medium was replaced with 500µl fresh medium containing the test substances and were cultured for another 24h after which they were fixed and stained for PECAM-1 and further analyzed as described above. After obtaining images for the quantification of PECAM-1 positive structures, the cultures were counterstained with Mayer's hematoxylin (H) for 30 seconds and eosin (1% in 96% ethanol) (E) for 90 seconds.

Statistics. Results are depicted as mean value standard error of the mean (SEM). Differences between groups were determined by one-way analysis of variance for multiple comparisons followed by Fisher's LSD test.

Results

Effects on angiogenesis

Figure 5.2a shows dose-inhibition curves of the effects of marimastat, TNP-470 and Ang-510 on VEGF (50ng/ml) stimulated PECAM-1 positive capillary outgrowth from 17-day-old fetal mouse metatarsal bone organ cultures. VEGF-stimulated outgrowth was significantly and dose-dependently suppressed by marimastat, TNP-470 and Ang-510 with IC_{50} values of approximately 0.6, 0.6 and 0.06µM, respectively. The effects of these agents on endothelial outgrowth are further illustrated in Figure 5.2b–d. Figure 5.2b shows a large PECAM-1 positive endothelial network that has been formed after 10 days stimulation with VEGF. Figure 5.2c and d show VEGF-stimulated cultures in the presence of 1µM TNP-470 and 1µM Ang-510, respectively. Both compounds inhibited the outgrowth of a capillary network, with Ang-510 being more potent than TNP-470. Explants cultured with 1µM marimastat showed inhibition of vascular outgrowth, similar to those treated with TNP-470 (not shown).

In order to study the effect of the three agents in our anti-angiogenic model in more detail, we examined the effect of pre-treatment with these agents on subsequent VEGF-stimulated vascular outgrowth. At time of explantation, PECAM-1 positive endothelial precursor cells are located in the perichondrium of the explants, as previously shown.[23] From these precursor cells the capillary structures sprout and form the vascular network. To target these precursor cells, directly after adhesion to the culture plate, the fetal bone explants were pre-treated for 24 h with the different anti-vascular compounds and were than subsequently cultured for 10 days in the presence of VEGF. As shown in Figure 5.3, pre-treatment with marimastat did not affect VEGF-stimulated capillary outgrowth, while both TNP-470 and Ang-510 significantly suppressed subsequent VEGF-stimulated outgrowth with IC_{50} values of approximately 0.7 and 0.08µM, respectively.

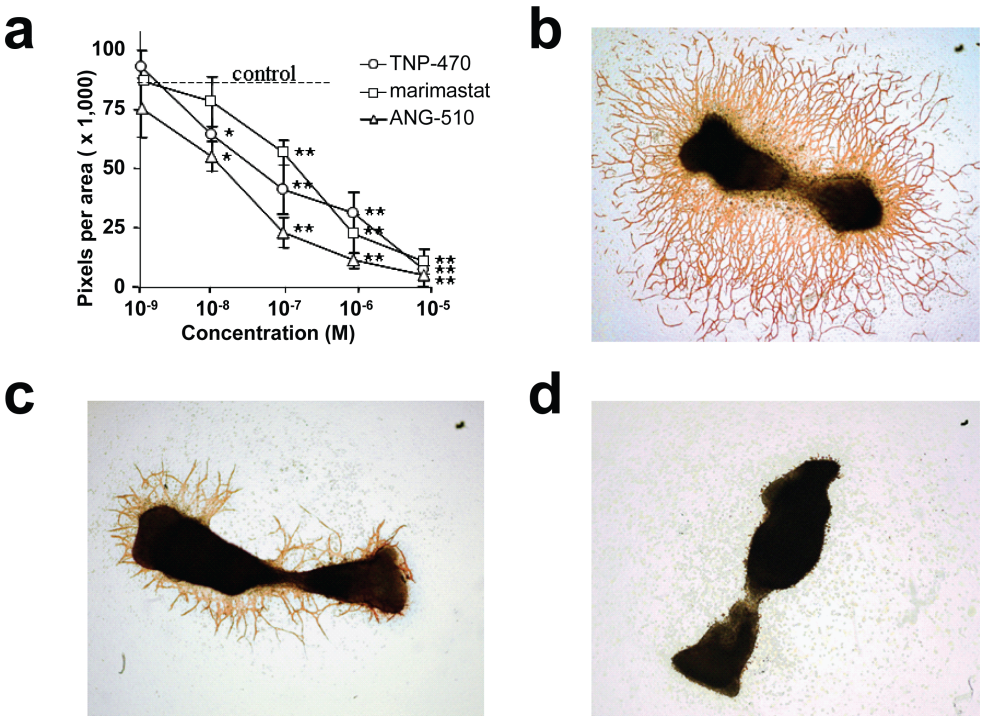

Figure 5.2: Effects on angiogenesis. (a) 17-day-old fetal mouse bone explants were stimulated for 10 days with VEGF (50ng/ml) in the absence or presence of different concentrations marimastat, TNP-470 or Ang-510 ($n = 6$). Quantification of the number of PECAM-1 positive pixels per area is given as mean ± SEM ($*p < 0.05$; $**p < 0.01$ compared to controls). (b–d) Endothelial outgrowth after stimulation with VEGF (b) and after simulation with VEGF together with TNP-470 (1μM) (c) and together with Ang-510 (1μM) (d).

Effects on newly established vasculature

To study the effects of the three compounds on newly established capillaries, first endothelial outgrowth was stimulated with VEGF (50ng/ml) for 10 days and subsequently the cultures were treated for 24h with the different anti-vascular agents. As shown in Figure 5.4a, marimastat and TNP-470 did not affect the VEGF-stimulated newly formed vasculature while Ang-510 showed a significant suppression of newly established vasculature with an IC_{50} of around 0.01μM.

Figure 5.4b shows control capillary outgrowth after 10 days stimulation with VEGF, stained for PECAM-1 and counter stained with HE. Figure 5.4c and d depicts newly formed vasculature after subsequent 24h treatment with TNP-470 (10μM) and Ang-510 (1μM). As shown, TNP-470 did not affect the established capillary network, similar results were obtained with marimastat (10μM) (not shown). In contrast, 24h treatment with Ang-510 caused a significant disintegration of the newly established capillary structures, with only fragments of the original network remaining. Histo-

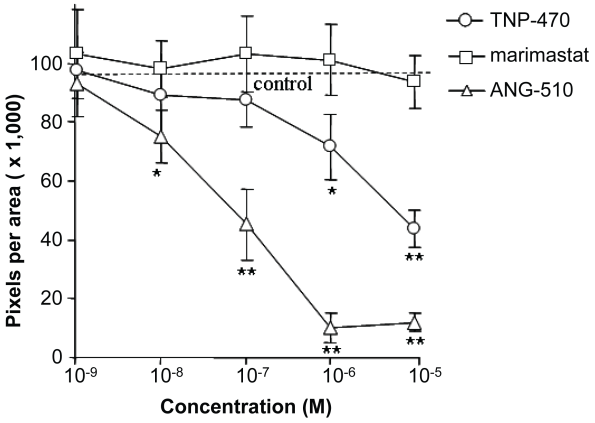

Figure 5.3: Effects of pre-treatment on vascular outgrowth. 17-day-old fetal mouse bone explants were, directly after adhesion to the culture plate, cultured for 24h with different concentrations of marimastat, TNP-470 or Ang-510 and subsequently stimulated for another 10 days with VEGF (50ng/ml) ($n = 6$). Quantification of the number of PECAM-1 positive pixels per area is given as mean ± SEM ($*p < 0.05$; $**p < 0.01$ compared to controls).

logical HE staining revealed that this degenerative effect was specific for the capillary network, as the layer of fibroblastic cells, originating from the periosteum on which the capillary network grows and expands,[23] remained morphologically fully intact.

Discussion

In this study, we developed an *in vitro* model that can distinguish between effects of compounds on angiogenesis and on newly established vasculature. We examined the effects of three anti-vascular agents, among which the recently developed tubulin-binding agent Ang-510. We showed that this compound effectively interfered with both angiogenesis as well as newly established vasculature, whereas the synthetic fumagillin derivate TNP-470 and the MMP-inhibitor marimastat selectively affected angiogenesis alone.

Angiogenesis is the process of generating new blood vessels from pre-existing vasculature, which is indispensable for solid tumor growth and metastasis. As such, targeting tumor angiogenesis, in anti-cancer therapy, is an intense field of interest. Current investigations towards the development of agents that inhibit tumor vascularization, however, not only focus on interference with the process of angiogenesis, but also on intervention with already established tumor vasculature.[3,4] Compounds that belong to this group are called VDAs; agents that selectively target tumor vasculature on basis of structural and functional abnormalities of these vessels.[7–9] In the development of new and more effective anti-vascular agents, it is of importance to have model systems available that can give accurate information about their mode

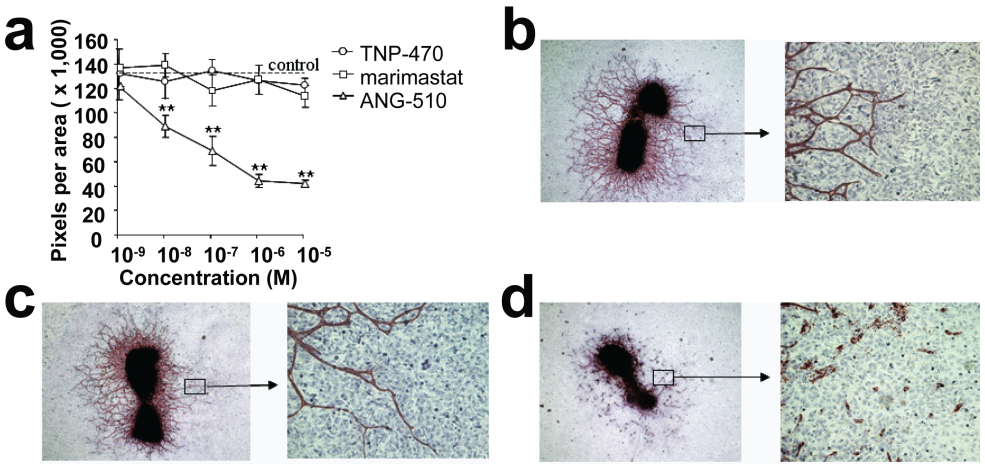

Figure 5.4: Effects on newly established vasculature. (a) 17-day-old fetal mouse bone explants were stimulated for 10 days with VEGF (50ng/ml) followed by 24h treatment with different concentrations marimastat, TNP-470 or Ang-510 ($n = 6$). Quantification of the number of PECAM-1 positive pixels per area is given as mean ± SEM ($*p < 0.05$; $**p < 0.01$ compared to controls). After culture, bone explant capillary outgrowth was visualized by staining for PECAM-1 in combination with HE. (b–d) The combined PECAM-1 and HE staining are shown for explants stimulated for 10 days with VEGF without subsequent treatment (control) (b), and for explants stimulated with VEGF with subsequent 24h treatment with TNP-470 (10μM) (c) or Ang-510 (1μM) (d).

of action and vascular targets involved and thus can differentiate between effects on angiogenesis or newly established vasculature. For this, we adapted our previously developed angiogenesis assay consisting of the outgrowth of capillaries from cultured fetal mouse metatarsals and suited it to study effects on established vasculature as well.[23] To validate this *in vitro* model, we examined the effects of three different compounds in several experimental settings.

We examined the anti-angiogenic actions of two well-known inhibitors of angiogenesis, marimastat and TNP-470, respectively, and that of the newly developed tubulin-binding agent Ang-510. In previous studies, it has been shown that both the synthetic MMP-inhibitor marimastat, as well as the synthetic fumagillin derivate TNP-470 possess strong anti-angiogenic properties in various *in vitro* models by interfering with endothelial cell invasion and proliferation.[24–28] In concordance with these observations, in our model, marimastat and TNP-470 potently and dose dependently inhibited angiogenesis, indicated by suppressed outgrowth of PECAM-1 positive capillaries. Moreover, the newly developed tubulin-binding agent Ang-510 also showed strong anti-angiogenic properties in our model system.

Previously, we have shown that PECAM-1 positive endothelial precursor cells are present in the perichondrium of the bone explants, before the outgrowth of vasculature.[23] In order to determine whether the observed anti-angiogenic effects might involve a direct action on these early-stage endothelial precursor cells from which

the capillaries are formed, we pre-incubated the bone explants with the different agents for 24h and subsequently cultured them for 10 days in the presence of VEGF. After pre-treatment of the bone explants with marimastat, at doses that actively suppressed angiogenesis, there was no effect on the subsequent outgrowth of vasculature. Recent studies have shown that MMP-inhibitors such as marimastat inhibit angiogenesis by blocking the invasion and migration of endothelial cells into the extracellular matrix,[24,29,30] which might explain why in our model, marimastat does not have a direct effect on endothelial precursors and their subsequent vascular outgrowth after pre-treatment, but strongly inhibits angiogenesis when it is continuously present. In line with this, it was previously shown that in a three-dimensional rat aortic model, marimastat potently inhibited angiogenesis, without affecting the proliferation of rat aortic endothelial cells in monolayer cultures.[24]

In contrast to marimastat, TNP-470, and even more potently Ang-510, inhibited vascular outgrowth after 24h pre-treatment of the bone explants. TNP-470 is a known angiogenesis inhibitor, which has been shown to induce a cell cycle arrest in the G1-phase, resulting in inhibition of endothelial cell proliferation and network formation, indicating that this compound acts via a cytostatic rather than a cytotoxic mode of action.[25–28] However, in our model, at higher concentrations, inhibition of outgrowth of vasculature by TNP-470 was not reversible after stimulation with VEGF, suggesting that, at these doses, the mode of action is cytotoxic and not cytostatic. Interestingly, a similar dual mode of action of TNP-470 has been described on the *in vitro* growth of human umbilical vein endothelial cells (HUVECs), showing cytostatic inhibition at lower doses and a cytotoxic suppression at higher doses.[31] Furthermore, we found that Ang-510 strongly inhibited the outgrowth of capillaries after pre-treatment of the bone explants, suggesting that this agent possess an irreversible cytotoxic mode of action on endothelial precursor cells. This observation is in line with findings of Ahmed *et al.* and Iyer *et al.* who showed that the combretastatin analog A4 phosphate (CA4P) was cytotoxic to proliferating HUVECs.[6,32]

Finally, we studied the effects of the three compounds on newly established vasculature. In contrast to their actions on angiogenesis, marimastat and TNP-470 did not affect newly established capillaries. *In vitro* studies exploring the effects on established vasculature are very rare, however, in one study, using cultures of rat aorta, marimastat showed to stabilize rather than to inhibit existing microvessels and to prevent their regression, resulting in the prolonged survival of microvascular networks.[33] To date, no studies on the effects of TNP-470 on established vasculature have been published. However, our observations, that this agent has no effect on established vasculature may be perceivable, since TNP-470 has been shown to act on endothelial cells via a cytostatic action through suppression of the cell cycle.[28] Furthermore, as expected, next to its strong inhibitory effects on angiogenesis and capillary outgrowth after pre-treatment of bone explants, Ang-510 showed to have a marked disintegrative effect on newly established vasculature. This damaging effect of Ang-510 was most likely specific for endothelial cells, as concomitant HE staining revealed that the underlying layer of fibroblastic cells remained unaffected. In line

with our findings, the VDA CA4P showed *in vitro* and *in vivo* rapid disruption of the tubulin cytoskeleton and changes in the three-dimensional shape of proliferating endothelial cells.[34–36]

In conclusion, the current search for more specific and more active VDAs is hampered by a lack of *in vitro* models that can accurately distinguish between effects on angiogenesis and on established vasculature, urging the need for models which can specifically differentiate between the two. The overlap in action of VDAs on angiogenesis and on newly established vasculature illustrates the usefulness of this *in vitro* model, which is able to differentially recognize effects on both vascular targets. This *in vitro* model provides an efficient and rapid way to screen for biological activity of anti-vascular compounds, which could prove of great benefit in the field of vascular research. Moreover, the ability to make a clear distinction between different vascular targets may facilitate the identification of pharmacological compounds with potential clinical benefit. The indicated differences between marimastat, TNP-470 and Ang-510 in targeting vascular networks are illustrative for this approach.

Acknowledgments

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