

Review Article

Angiogenesis and melanoma - from basic science to clinical trials

Maxine Sylvia Emmett^{1*}, Daemon Dewing^{1*}, Rowan Oliver Pritchard-Jones^{1,2}

¹Mersey Academic Plastic Surgery Group, Liverpool Cancer Research UK Centre, Department of Molecular and Clinical Cancer Medicine, The Duncan Building, Daulby Street, Liverpool, L69 3GA, UK ²Department of Plastic Surgery, Whiston Hospital, Warrington Road, Prescott, Merseyside, L35 5DR, UK. *joint first author

Received July 7, 2011; accepted July 27, 2011; Epub August 8, 2011; Published August 30, 2011

Abstract: The effective management of malignant melanoma has remained centred around the surgeon. The arrival of anti-angiogenic agents as the 'fourth' cancer treatment joining the ranks of surgery, chemotherapy and radiotherapy has been a source of renewed hope. This article provides an up-to-date review of the focus, state and rationale of clinical trials of anti-angiogenic therapies in metastatic malignant melanoma. Vascular Endothelial Growth Factor (VEGF) is by no means the only target, although perhaps the most extensively studied following the successful introduction of the anti-VEGF Antibody bevacizumab. This has been combined with other established therapies to try and improve outcomes in metastatic disease, and is being trialled in the UK to prevent metastasis in high-risk patients. We describe the encouraging preclinical work that lead to great enthusiasm for these agents, assess the key trials and their outcomes, discuss why these therapies have not revolutionised melanoma care and explore how they might be better targeted in the future.

Keywords: Cancer, angiogenesis, metastatic melanoma, melanoma, anti-angiogenic therapy, vascular endothelial growth factor, VEGF, receptor tyrosine kinase inhibitors, signal transduction inhibitors, bevacizumab

Introduction

Despite being the least common of the skin cancers, malignant melanoma is by far the most lethal. Worldwide it has the highest increasing incidence rates of any common cancer, doubling approximately every 10-20 years in white populations [1]. In common with many other cancers, incidence increases with age, yet the fastest rising melanoma population appears to be young women.

Surgery remains the cornerstone of melanoma treatment and is effective in early stage disease. However, thicker melanomas, even when completely excised, are associated with only a 32% survival at 10 years [2] and even 15% of patients diagnosed with a thin melanoma (less than 1mm thick) will die within 10 years [3]. The challenge has been to manage those tumour cells that have effectively broken away from the primary site. Regional spread is most commonly first seen via the lymphatics, and distant spread

via the vasculature. Both conventional chemotherapies and radiotherapy have been largely ineffective in patients presenting with metastatic disease. A typical response rate to any heralded drug has been in the order of 10%. As a result there has been a desperate need for novel targets in the treatment of melanoma.

Angiogenesis

Angiogenesis is defined as the growth of new blood vessels from a pre-existing vasculature and is as an absolute requirement for tumour survival and progression beyond a few hundred microns in diameter. In order for a cell to survive it must be assured of having a constant supply of both oxygen and nutrients and as a result cannot be more than a few hundred micrometers from the nearest blood vessel [4]. For a tumour to proliferate beyond this size, it must therefore produce adequate levels of pro-angiogenic growth factors to induce local angiogenesis. It is this pivotal role that defines angio-

genesis as one of the hallmarks of cancer [5], proving vital not only for initial development of the primary lesion but also during the establishment of distant metastases.

Although angiogenesis is essential during embryogenesis, once in adulthood it has only a relatively limited role in normal physiology and is almost exclusively linked to pathological processes. Furthermore, when angiogenesis is induced as part of normal physiological processes, such as wound healing and the female menstrual cycle, it tends to be only a transient process. This is in stark contrast to tumourigenesis, which involves the almost constant activation of angiogenesis to keep pace with the constantly growing, metabolically demanding tumour [5, 6]. This makes for an attractive therapeutic target, as not only is angiogenesis a central element of tumour growth and survival, but the relatively quiescent nature of much of the adult vasculature suggests that targeting active vessel proliferation ought not result in any significant or insurmountable side effects if systemically blocked. Furthermore, unlike the tumour cells themselves, the vasculature is hypothesised to remain relatively genetically stable and thus less likely to develop drug resistance and cease to respond to therapy [4]. In fact, there have been notable successes seen with anti-angiogenic therapies in the treatment of metastatic disease in several cancer types including breast cancer, colorectal cancer, small cell lung cancer and renal cell carcinoma to name but a few. So much so that in 2004, after the FDA approval of the anti-angiogenic therapy bevacizumab for treatment of advanced colorectal cancer, the FDA Commissioner at the time heralded anti-angiogenic therapy as “the fourth modality of cancer treatment” after surgery, chemotherapy and radiotherapy, establishing it as a distinct and valid weapon in the fight against cancer [7].

Angiogenesis and melanoma

Malignant melanoma has been well documented as an angiogenic tumour type, clearly demonstrating new vessel formation as an important step in disease progression from atypical melanocytes, through radial growth to the aggressive vertical growth phase [8]. However, the precise role of angiogenesis in the clinical setting has remained unclear and shrouded in debate. Several studies have sought to estab-

lish prognostic links with angiogenesis, correlating microvessel density (MVD) in and around the tumour with disease progression, metastasis and survival, yet have yielded mixed and uncertain results. In 1997 Erhard *et al.*, described increasing angiogenesis with disease transition from the horizontal growth phase, that has a low metastatic potential, to the more aggressive vertical growth phase [9], suggesting a link between angiogenic status and metastatic potential. This is supported by several other studies that demonstrated a positive correlation between angiogenesis, vascularity and poor outcome [10-14], including a large prospective study of over 400 patients which was undertaken in 2002 and identified vascularity as the most important determinant of overall survival as determined by multivariate analysis [15]. However, several studies have equally identified either no correlation or a direct correlation, with increasing angiogenesis predicting paradoxical, and somewhat counter-intuitive, *improved* outcome and survival [16-18].

Despite this contradictory data, it is the fundamental role of angiogenesis in tumourigenesis that maintains it as an attractive target for novel therapies. Thus, given the successes seen with anti-angiogenic therapies in other solid cancer types, and the relatively limited therapeutic options currently available for melanoma patients presenting with metastatic disease, there has resulted an explosion of research into the potential role of anti-angiogenic therapy in advanced malignant melanoma.

Targetable controllers of angiogenesis

Like many physiological processes, angiogenesis is not a simple response to one solitary factor, but rather a complex, multi-step progression controlled by a fine balance of pro- and anti-angiogenic signals. During tumour growth an “angiogenic switch” is activated, disrupting this balance such that pro-angiogenic growth factor expression vastly outweighs that of anti-angiogenic signals and results in a corresponding growth of new vasculature [6]. Thus, all the emerging targets for therapy are aimed at redressing this balance to once again favour anti-angiogenic signals and so achieve a quiescent vasculature once more. However, the diversity of the factors involved in this process highlights the complexity of angiogenic control that must exist. This raises distinct challenges when tar-

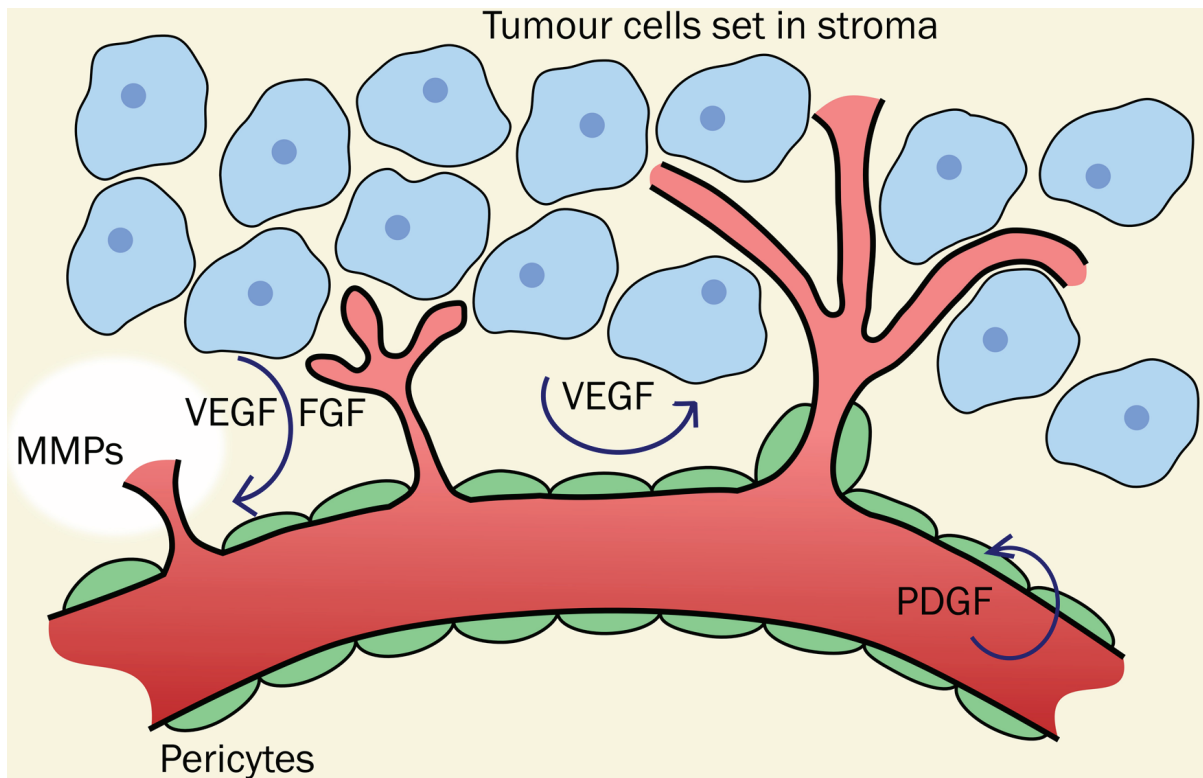


Figure 1. Schematic of tumour angiogenesis. Several different growth factors are secreted by the tumour to induce local angiogenesis through sprouting from nearby vasculature. The predominant factor is VEGF that acts on both endothelial cells and in an autocrine manner on the tumour itself. Matrix Metalloproteinases (MMPs) drive the corresponding re-modelling of the extracellular matrix, and are also able to release any bound growth factors simultaneously, thereby optimising the pro-angiogenic environment. Immature vessels, which lack pericyte coverage, are susceptible to anti-VEGF therapy, whereas those covered with pericytes are protected by the secretion of paracrine growth factors.

getting therapy by manipulation of any one of these factors because it is likely that the *interplay* of these molecules holds the key to success [19, 20], and single agent/ligand approaches are at the mercy of resistance, be it inherent or acquired.

VEGF

Vascular Endothelial Growth Factor (VEGF) exists in mammals as several related families of glycoproteins including VEGF-A, B, C, D, and E [21], which mediate their effects through activation of the class III tyrosine kinase receptors VEGFR-1 (Flt-1), VEGFR-2 (KDR), and VEGFR-3 (Flt-4) [22-24]. VEGF-A, which is often simply referred to as VEGF, is considered to be one of the most important mediators of angiogenesis, both in the physiological and pathological setting [23, 25-27], and has been shown to be

overexpressed in all known solid tumours [28]. The angiogenic response to VEGF is predominantly mediated through VEGFR-2 [22], with activation resulting in endothelial cell proliferation and migration, along with lumen formation and increased vessel dilatation and permeability [22, 29-31] (for schematic **Figure 1**).

Through alternate splicing within exons 6 and 7, VEGF exists as a series of isoforms of differing amino acid length, after which they are named VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅ (the dominant isoform), VEGF₁₈₉ and VEGF₂₀₆ [21, 32, 33]. These differing isoforms are all highly angiogenic and only differ in their solubility as the variably expressed exons encode heparin-binding domains, which is important in controlling the bioavailability of these isoforms and thus in establishing chemotactic gradients to guide the newly developing vasculature [21, 34, 35]. In-

deed, this has important implications for melanoma as the precise isoforms expressed by the developing tumour have a significant impact on tumour and vasculature growth. When overexpressed in an otherwise non-tumorigenic, non-VEGF expressing melanoma cell line, VEGF₁₂₁ and VEGF₁₆₅ resulted in an aggressive tumour with a highly extensive supporting vasculature, whereas VEGF₁₈₉ expression resulted in a relatively dormant tumour with limited vascular supply as the expressed VEGF isoform remains almost entirely bound to the expressing cells [36].

The potential role of anti-angiogenic therapies which target this VEGF pathway are made an even more attractive target by the fact that VEGF receptors are known to be expressed by melanoma cells, but not melanocytes [37, 38]. This suggests a potential autocrine role for VEGF expression by the developing tumour, with increasing VEGF expression not only improving local vascular supply, but perhaps also acting as a direct stimulator of tumour growth. Therefore any therapy that directly targets this pathway, may not only starve the tumour of vital oxygen and nutrients through blockade of its vascular supply, but also directly inhibit tumour cell survival and proliferation, perhaps even rendering them more susceptible to more conventional treatment with chemo- or radiotherapy [39].

This dogma of the pro-tumorigenic role of VEGF isoforms has however, recently been challenged by the description of an alternative family of VEGF splice variants termed VEGF_{xxx}b where xxx denotes amino acid number [40]. This family of almost identical sister isoforms results from selection of a differential splice site in a region previously believed to be the 3'-untranslated region (3'UTR). As a result, the terminal exon of VEGF, exon 8, actually exists as two subexons, exons 8a and 8b. Conventional VEGF results from the selection of the proximal splice site and includes exon 8a encoding six amino acids. This novel family of sister isoforms however, is generated by selection of the distal splice site and includes exon 8b, which encodes a different set of six amino acids [40]. Although this alternative splicing event only alters the terminal six amino acids, it has profound effects on both the structure and resulting function of the translated protein, with the VEGF_{xxx}b family demonstrating highly anti-angiogenic effects [40-42]. These VEGF_{xxx}b isoforms are highly expressed as part of normal physiology [42] and unlike conventional VEGF, have been seen to be

downregulated in many solid tumours [40, 41, 43]. Indeed, when overexpressed in the A375 melanoma cell line, VEGF_{165b} was shown to inhibit tumour growth *in vivo* [41] and furthermore, expression of this inhibitory VEGF_{xxx}b in archival primary melanoma samples was associated with reduced metastatic spread irrespective of tumour thickness [44].

Neuropilin

Neuropilin-1 and 2 (Nrp-1, Nrp-2) are known to act as co-receptors for the VEGFRs and enhance VEGF signalling upon binding [23, 45], and there is evidence to suggest that VEGF may be able to signal independently through Nrp in the absence of VEGFR-1 or 2 [46-48]. In addition, rather like VEGFRs, Nrp receptors have been shown to be expressed not only by tumour associated endothelial cells but also by the tumour cells themselves, suggesting it may play a crucial role in tumourigenesis, although such a role is yet to be fully understood [47, 48]. Intriguingly, there is growing evidence to suggest that the combination of neuropilin blockade and anti-VEGF therapy is synergistic in its effects. In an animal model of melanoma growth, blocking Nrp-2 using a mutated soluble neuropilin-2 B domain in combination with the anti-VEGF antibody bevacizumab (Avastin) resulted in a greater inhibition of overall tumour growth, than either treatment achieved alone [49]. Similarly, the blockade of Nrp-1 in combination with the same anti-VEGF treatment as part of an animal model utilising a lung cancer cell line, demonstrated an additive effect on inhibiting tumour growth. In their paper, Pan *et al.* propose a possible mechanism for this observation, describing an enhanced susceptibility of the tumour associated vasculature to the anti-VEGF therapy in the presence of the Nrp-1 inhibition as a result of a disrupted relationship between the newly developing vessels and their usually protective pericytes [50].

Pericyte coverage

Pericytes are well known to play a vital role in supporting normal vasculature and although it was previously believed that these cells were largely absent from the aberrant neovasculature of the tumour, there is growing evidence to suggest that they are present in most, if not all tumours [51-53]. These pericytes are known to cover the surface of the developing vessels and have been shown to demonstrate a paracrine

relationship in which they secrete survival signals to support the underlying endothelial cells [5, 53, 54]. These pericyte covered vessels are considered far more stabilised and are often referred to as 'mature' vessels as opposed to the 'immature', leaky, unstable vessels that lack pericyte association. When treated with anti-VEGF therapy, it is possible that these supporting pericytes protect the underlying endothelial cells such that it is only the immature vessels that appear to be susceptible to treatment [50], thus rendering anti-VEGF therapy potentially less effective in established advanced disease, where of course, novel therapies are first evaluated. It appears that these supporting pericytes are driven by the tumour and endothelial cell production of Platelet Derived Growth Factor β (PDGF- β) [54] and indeed upon blockade of PDGF- β and VEGF in combination, results in a more effective treatment regimen [55], though it has been suggested that this combination therapy may only be able to target a certain subset of pericytes [56]. Further complicating the seductive idea that anti-VEGF and PDGF therapy is the key to destabilising and disrupting neovasculature, is the fact that anti-VEGF therapy can also induce an increase in Angiopoietin-1 (Ang-1) that can increase pericyte coverage and thus counteract the effects of the anti-VEGF therapy [57]. These many compensatory pathways only highlight the importance of considering the role of pericytes in contributing to the development of resistance to anti-angiogenic therapy.

Angiopoietin

Angiopoietin-1 and 2 (Ang-1 and Ang-2) have both been shown to play an important role during angiogenesis and exert their effects by binding to the Tie receptor tyrosine kinase receptors (Tie-1 and Tie-2) [58, 59]. As with many angiogenic factors, this role is a complex one that can be very context dependent, with Ang-1 stimulating pericyte coverage of vessels, thereby stabilising the developing vasculature and Ang-2 (acting as an Ang-1 antagonist) stimulating the pericytes to become dissociated from the endothelial cells so that they may respond to VEGF and proliferate and migrate as part of a pro-angiogenic response [58]. Furthermore, it has been demonstrated that VEGF can also directly activate the Tie-2 receptor [60], suggesting important crosstalk between the VEGF and angiopoietin/Tie receptor pathways. This is further

supported in that blockade of both VEGF and angiopoietins appears to have an additive effect on reducing tumour neovascularisation [61, 62]. In melanoma, high levels of Ang-2 in the blood stream have been identified as a poor prognostic marker associated with disease progression, metastasis and reduced overall survival, indicating that this pathway may be an important target [63].

FGF

It is all too tempting to focus on the importance of VEGF in angiogenesis and we neglect, at our patients' peril, other key angiogenic pathways. The fibroblast growth factor (FGF) ligands are highly angiogenic and in particular, basic FGF (bFGF) expression has been demonstrated in melanoma cells but not melanocytes [64, 65]. Expression of bFGF has been shown to have both paracrine effects, through the stimulation of local angiogenesis, but also autocrine effects, directly increasing tumour cell proliferation through activation of FGF receptors expressed on the melanoma cells themselves [8, 64]. This dual role of FGF suggests it to be a very appealing target and indeed FGF expression was one of the first noted mechanisms mediating resistance to an anti-VEGF therapy [66]. This was identified as part of an animal model of pancreatic cancer, where initial treatment with a VEGFR-2 monoclonal antibody was able to reduce tumour vascularity, however this response was only short lived with a rebound in growth and neovascularisation noted just four weeks later. This was associated with an increase in FGF expression and thus, when an FGF-trap was introduced in combination with the VEGFR-2 antibody treatment, there was a reduction in resistance development [66, 67]. This serves to highlight the importance of abeyant pathways of angiogenic signalling that provide diversity in normal physiology, and can be activated to facilitate drug resistance in pathological angiogenesis. Furthermore this has been clinically evidenced in glioblastoma patients receiving anti-VEGF therapy, where progressive disease was associated with increasing levels of circulating bFGF [66, 68].

Extracellular matrix re-modelling

In order for new blood vessels to form there must be corresponding remodeling of the surrounding extracellular matrix (ECM), a process

that is usually under the tight regulation of Matrix Metalloproteinases (MMPs) and Tissue Inhibitor Metalloproteinases (TIMPs). Expression of MMPs has been clearly shown to support tumour angiogenesis and indeed increasing expression is well correlated with metastasis and aggressive disease in melanoma [69-71]. The gelatinases, MMP-2 and MMP-9 have been highly linked with angiogenesis as they are both able to digest basement membrane components and are the most studied MMPs with regards to melanoma [72]. MMP-9 has further been identified as having the ability to release heparin bound VEGF from the extracellular matrix, effectively increasing the angiogenicity of the local microenvironment [73]. There is however, growing evidence to suggest that it is in fact the cell surface associated membrane-type matrix metalloproteinase (MT1-MMP) that is most relevant during melanoma neovascularisation. It not only demonstrates direct effects on vessel formation [74-76], but also has been clearly shown to be upregulated in melanoma [77].

While it is clear the MMPs have an important role to play during melanoma progression and neovascularisation, the role of the naturally occurring inhibitors, TIMPs, is far less clear. Some evidence suggests that increased expression of TIMPs can reduce neovascularisation and tumour development [78], however TIMP expression in pancreatic cancer cells has also been linked to an increase in tumour cell proliferation [79]. Indeed, an increase in TIMP expression has even been correlated to the level of tumour invasion, though it is hypothesized that this may reflect a host response in an attempt to control the corresponding increase in MMP expression and protect the ECM integrity [80].

Other factors

Although these are some of the major players that are currently under investigation for their role in melanoma angiogenesis, this is by no means an exhaustive list. For example, Placental Growth Factor (PlGF) has been implicated as yet another alternative pro-angiogenic signal through which anti-VEGF therapy resistance may develop, with increasing circulating levels of PlGF noted as part of both preclinical and clinical trials following VEGF signalling blockade [66, 68, 81]. Equally, there are several naturally occurring inhibitors of the angiogenic process,

such as thrombospondin, angiostatin and endostatin, which have all been identified as being potentially able to reduce tumour growth if over-expressed [82]. There is also growing interest in the role of integrins during angiogenesis as another potential therapeutic target, though as yet this work has not reached clinical trials [83]. Efforts to control the angiogenic nature of the tumour are further complicated by the role of other cell types within the tumour microenvironment. Both cancer associated fibroblasts and immune inflammatory cells recruited as part of the tumour associated inflammatory response, have been shown to contribute to the angiogenicity of the tumour by directly producing pro-angiogenic growth factors [5, 84], adding yet another layer of control that needs to be considered. Indeed, despite the many successes that have been achieved with developing anti-angiogenic therapies, none have yet proved to be the reliable panacea many had anticipated. It is possible that their true potential will only be fully realised when we properly understand the role and interplay of each factor.

Current therapy

VEGF targets

Dacarbazine, is currently the generally accepted standard of care for patients with advanced melanoma, and with response rates in phase III trials of 9.8%–12% [85], reflects the disappointing results seen with chemotherapy overall. VEGF is the principle ligand targeted by anti-angiogenic therapies of which bevacizumab (Avastin (ROCHE)), a humanized monoclonal IgG antibody against VEGF, is shown to have anti-angiogenic effects in numerous tumours [86], recognizing all isoforms of VEGF but not other peptide growth factors. It was the first anti-angiogenic agent to be granted FDA approved in 2004 for use in colorectal cancer based upon the publication of results of a pivotal combination therapy trial in advanced colorectal cancer (Study AVF2107g) [87]. The UK license was granted the following year in 2005. To date, there are 24 US trials either recruiting or in analysis assessing bevacizumab, alone or in different combinations with chemotherapeutic agents for cutaneous melanoma (a further 4 trials are targeting ocular variants) (www.clinicaltrials.gov). The majority of these trials are investigating bevacizumab in advanced metastatic disease.

Table 1 Melanoma trials: published data

Published Trials	Therapy	Phase	Patient (n)	PR%	medPFS (months)	TTP (months)	mOS (months)	Year	Ref
Varker <i>et al.</i>	Bevacizumab ± interferon- α2b	(II)	32	6 0	3.0 3.0		10 8.5	2007	[89]
Von Moos <i>et al.</i>	Temozolomide, bevacizumab	(II)	62	15	4.2		9.6	2011	[96]
Grignol <i>et al.</i>	Bevacizumab, high dose interferon α-2b	(II)	25	25	4.8		17	2011	[90]
Egberts <i>et al.</i>	Pegylated interferon- α2b, sorafenib	(II)	55	3.6	2.5		9.6	2011	[123]
Carvajal <i>et al.</i>	Imatininib	(II)	28	21		3.0	11	2011	[114]
Hainsworth <i>et al.</i>	Bevacizumab, everolimus	(II)	57	12	4.0		8.6	2010	[124]
Del Vecchio <i>et al.</i>	Fotemustine, bevacizumab	(II)	20	15		8.3	20.5	2010	[94]
Vihinen <i>et al.</i>	Bevacizumab, dacarbazine, IFN-α2a	(II)	26	15	2.3, 8.1 responders		11.5	2010	[91]
Ott <i>et al.</i>	Sorafenib	(II)	36	3		2.0		2010	[102]
Hodi, O'Day <i>et al.</i>	Ipilimumab ± gp100 vaccine	(III)	676	9.5 5.5		2.86 2.76	10.0 6.4	2010	[125]

PR - Partial Response, medPFS – median Progression Free Survival, TTP - Time to Progression, mOS – median Overall Survival

Table 2 Melanoma trials: selected conference abstracts

Conference abstracts	Therapy	Phase	Patient (n)	PR%	medPFS (months)	mOS (months)	Year	Ref
BEAM Trial	Carboplatin, paclitaxel ± bevacizumab	(II)	214		5.6 4.2	12.3 8.6	2009	[92]
Hodi <i>et al.</i>	Ipilimumab, bevacizumab	(I)	22	36			2011	[126]
BRIM 3	Vemurafinib vs dacarbazine	(III)	675	48 vs 5.5	5.3 vs 6.1	84% vs 64% at 1 year	2011	[127]

PR - Partial Response, medPFS – median Progression Free Survival, TTP - Time to Progression, mOS – median Overall Survival

Initial evaluations of bevacizumab in cutaneous melanoma were conducted in 2007 in a Phase II trial as either a monotherapy or combined with low dose interferon, IFN-α2b (inhibiting FGF from tumour cells [88]). 25% of patients showed increased disease stabilisation ranging 24-146 weeks (but there was no significant improvement in Overall Survival (OS) between mono and combined therapy (8.5 vs 10 months [89])), though it would appear that in combination with either a higher dose of IFN-α2b [90], or dacarbazine, these response rates were further improved [91] (**Table 1**). The BEAM trial (NCT00434252), a randomised multi-centre Phase II trial for melanoma treatment with around 200 patients, evaluated carboplatin and paclitaxel with or without bevacizumab as first line therapy for patients presenting with metastatic disease. This trial demonstrated an im-

provement in Progression-Free Survival (PFS) of 22% and OS of 21% by the addition of bevacizumab to the standard chemotherapy. Whilst the results were not statistically significant (95% CI: 0.55-1.13; p=0.19), bevacizumab still appeared beneficial [92] (**Table 2**). This was further supported in 2009, with a Phase II trial combining twice-weekly bevacizumab at 10mg/kg with a regime of paclitaxel/carboplatin in 53 patients with stage IV unresectable melanoma reporting disease stabilisation in 57% of patients for 8 or more weeks, with median progression-free survival of 6 months and overall survival of 12 months [93]. This is in contrast to the more usual OS of 6 months for this advanced stage of disease.

In 2010 Del Vecchio *et al.* published a multi-centre Phase II single arm trial using bevacizu-

mab (15mg/kg every 3 weeks), and the nitrosourea alkylating agent fotemustine (100 mg/m² by intravenous administration on days 1, 8, and 15, repeated after 4 weeks). The authors report mean Time To Progression (TTP) of disease to be 8 months and OS 20.5 months in 20 chemo-naïve advanced melanoma patients. Serum VEGF-A, VEGF-C, VEGFR-1 and VEGFR-2 and overall all of the sixteen measured pro-angiogenic serum markers were significantly reduced post treatment [94]. This compares favourably with combination treatment using CVD combination (cisplatin, vinblastine or vindesine, and dacarbazine) plus interferon (IFN)- α and interleukin (IL)-2, in 190 melanoma patients achieving mean TTP of 4.9 months in a phase III trial [95].

Results published this year combining temozolomide (150mg/m²) and bevacizumab (10mg/kg per 2 weeks) in a Phase II trial [96], demonstrated OS of 12 months vs. 9.2 months (mOS 9.6), in patients with BRAF mutated melanomas vs. wild type. Temozolomide is of particular interest because it crosses the blood brain barrier and hence may improve the outcome of patients with cerebral metastases (a key driver of mortality in stage IV disease). Overall temozolomide combined with bevacizumab improved the quality of life in end-stage of disease.

Whilst bevacizumab is a generally well-tolerated drug, concerns have been raised in Ranpura's 2011 meta-analysis that has reported a 1.46 fold increase in fatal adverse events in cancer patients treated with bevacizumab compared to chemotherapy alone (incidence 2.5% vs. 1.7%; $p=0.01$) [97]. This appears to contradict the original hypotheses that the adult vasculature is relatively quiescent and as such targeting angiogenesis ought not result in any significant side effects, suggesting that we still do not fully understand the actions of these therapies.

In all of these prior investigations, the patients recruited have all had advanced disease and it has been suggested that it is possible that anti-angiogenic therapy may provide more successes if targeting an earlier stage when active angiogenesis is likely to be more pivotal. As a result, the UK Adjuvant Avastin Trial in High-Risk Melanoma (AVAST-M) study has focused on a preventative strategy targeting angiogenesis to disrupt early metastasis following surgical resection of primary disease (and corresponding

microscopic metastasis detected at Sentinel Lymph Node Biopsy). This is an actively recruiting Phase III trial offering adjuvant therapy to 1320 patients following resection of AJCC stage IIB (T3bNOMO and T4aNOMO), IIC (T4bNOMO) and III (TxN1-3MO) cutaneous melanoma randomised to either bevacizumab 7.5mg/kg three weekly for 1 year, or observation. The primary endpoint of the study is OS, with secondary endpoints relating to Disease Free Interval (DFI), Distant Metastasis Free Interval (DMFI) and Toxicity. Thus far toxicity profiling has been encouraging and there is a significant translational component to the trial to evaluate biomarker proteins and candidate genes as predictors of response or progression in the cohort. This is a unique trial that will go a long way to establishing the role of bevacizumab in preventing disease recurrence by controlling any microscopic disease that has escaped beyond the reach of the surgeon.

Whilst bevacizumab, a monoclonal antibody that binds all known isoforms of VEGF-A, is the most well known of the anti-angiogenic therapies, there are alternative approaches. Other VEGF targeted agents use soluble receptor constructs preventing binding to, and activation of VEGFR-1, VEGFR-2, NRP-1 and NRP-2. There is also the rise of broad Tyrosine Kinase Inhibitors (TKIs) or Small-molecule Signal Transduction Inhibitor (STI) that can, in addition to anti-VEGF signalling functions, inhibit the activity of PDGFR activity [98].

Broad blockade

Sorafenib (BAY 43-9006) was first developed as a BRAF inhibitor but later found to have anti-angiogenic properties inhibiting VEGFR in several xenograft models [99]. Significant clinical responses have been seen in both leukaemia and Gastro Intestinal Tumours (GIST) [100, 101]. There was understandable enthusiasm for this approach in melanoma, but as a monotherapy, sorafenib has proved disappointing with no significant responses reported [102-104]. When used in series against a variety of advanced solid tumours (including malignant melanoma), sorafenib followed by bevacizumab was anticipated to demonstrate additive effects by combining a specific anti-ligand antibody with the more promiscuous STI. It was also anticipated that lower doses of each could be used because these agents would offer reduced

Table 3. Melanoma trials: ongoing trials

Ongoing Trials	Therapy	Phase	Patient (n)	Ref
NCT00387751	Sorafenib, bevacizumab	(II)	45	[107]
NCT00538005	Bevacizumab, oxaliplatin sorafenib	(I/II)	56	[108]
NCT01321437	Axitinib	(III)	60	[112]
NCT00667953	Temozolamide, imatinib mesylate	(I/II)	56	[128]
NCT01174238	Axitinib + carboplatin/paclitaxel	(II)	36	[111]
NCT00936221	AZD6244 +/- dacarbazine	(III)	91	[129]

VEGF production courtesy of disrupted VEGFR-2 signalling, and inhibition of vascular proliferation and survival pathways via feed-forward and feed-back loop disruption [105]. The authors found however, that the only additive effects were seen in relation to significantly increased toxicity that prevented the investigators escalating doses of either drug to the standard therapeutic doses. Furthermore, the only group of patients to see any meaningful clinical activity were those with ovarian cancer [106].

Currently sorafenib and bevacizumab are being assessed (NCT00387751) in a multicentre Phase II trial with 45 patients [107], with another ongoing study (NCT00538005) combining a Phase I (bevacizumab) /Phase II dose escalation study (oxaliplatin/sorafenib) [108]. Temozolamide which has recently shown improved disease stabilization combined with bevacizumab in a Phase III trial [96], also showed benefits in combination therapy with sorafenib in a Phase II trial treating patients with advanced melanoma [109] (**Table 3**).

Axitinib (AG013736) is a small molecule inhibitor of VEGFR-1, VEGFR-2 and VEGFR-3. A Phase II trial with 32 melanoma patients demonstrated a median OS of 6.8 months and OR rate of 15.6% [110]. Currently, a trial of axitinib and carboplatin/paclitaxel in melanoma (NCT01174238) is measuring primary outcomes of Objective Response Rate (ORR), and seeking to evaluate the optimal time between drug dosing [111]. A 60 patient trial (NCT01321437) is projected to start recruiting shortly, investigating axitinib monotherapy in stage III melanoma [112] (**Table 3**).

Some 28% of melanomas arising from acral, mucosal, and chronically sun-damaged sites harbor activating mutations and amplification of the type III transmembrane receptor tyrosine

kinase KIT [113]. The TKI imatinib targets c-KIT and PDGFR, described earlier as playing a role in pericyte recruitment and modulation of autocrine tumour growth. The 2005 phase II study of imatinib was disappointing with no ORs reported and median OS of only 3.9 months. The recent trial from Memorial Sloane Kettering has reported OR rate of 16% and OS of 11.5 months using imatinib in c-KIT mutated melanoma [114]. A new study is investigating temozolamide with imatinib (NCT00667953) with no published data yet available.

Back in the 1990s there was an enormous enthusiasm for targeting MMPs and a number of small molecule MMP inhibitors such as marimastat (British Biotech) were developed and used both alone and in combination with other conventional chemotherapies following promising pre-clinical trials. Sadly, the considerable musculoskeletal toxicity and limited anti-tumour activity in most melanoma patients [115] has seen the decline of this target and there are currently no active trials.

A new hope?

Perhaps the most encouraging new targets for melanoma are the BRAF inhibitors such as vemurafenib (Roche/Plexxikon) with startling early results in metastatic melanoma. Phase I data presented in Milan in 2010 described reduction in brain metastases in 9 out of 10 patients treated. The reduction in size was 20- 100%. The drug has been shown to be most active in patients whose melanoma harbours the BRAF V600E mutation (seen in some 50% of melanoma patients), but wild-type tumours also show considerable response. BRAF is a controller in the important MAPK signalling pathway responsible for controlling cell survival, growth, proliferation and angiogenesis. The main concern, however, is the emergence of resistance

at 8-12 months. This is thought to be mediated via upstream receptor tyrosine kinases such as N-RAS signalling via alternative compensatory pathways such as AKT and mTOR. It is reasonable to anticipate therefore that targeting these pathways with inhibitors may prolong the effectiveness of BRAF inhibitors [116].

Why doesn't it work?

Despite early hopes that anti-angiogenic therapies would offer a turning point in oncology, progress in the treatment of melanoma has been limited. Some patients simply show no response, while others show partial response, quickly followed by further progression. There are many possible issues that may underlie this, and it is possible that for each patient the precise mechanisms are specific, further complicating the challenge of circumventing these problems.

Drug resistance

Lee Ellis elegantly describes the idea of *inherent* and *acquired* resistance to anti-angiogenic therapy [66]. He highlights the need to consider whether a particular tumour type best responds to anti-VEGF monotherapy, suggesting resistance is modulated via the VEGF pathway alone, or (as is more likely the case in melanoma) clinical benefit is most commonly seen in poly therapy – and thus a more complex interplay of resistant pathways and mutations will exist. Ellis clearly dissects the many diverse mechanisms through which this resistance may develop from compensatory signalling pathways and interplay with the surrounding microenvironment, to recruitment of myeloid cells and resistance mediated via the combined chemotherapy rather than the anti-VEGF drug. As frequently evidenced in pre-clinical models, success may lie in the combination of therapies, whether that be in the form of targeting multiple angiogenic pathways simultaneously, sequencing different anti-angiogenic therapies as resistance develops, or combining alternative chemo or bio-therapies with anti-angiogenics.

There remain a number of pathways in angiogenesis that are yet to be fully understood. For example, the identification of novel inhibitory isoforms of VEGF in both the kidney and renal cell carcinoma [40, 41]. This has led to the description of inhibitory isoforms of VEGF in

melanoma that follow a non-metastatic course, in contrast to a loss of this expression in aggressive tumours that have metastasised, in many cases despite being apparently early stage, thin primary lesions [44]. This underscores the complexities of angiogenic control, and until these pathways, and the often surprising route they take to a final destination are better understood, it is possible that we will not fully comprehend the many possible resistance mechanisms.

Advanced drug targeting

It would be naïve of us to consider all the different available therapies as being simply interchangeable; some combinations cause unexpected increases in toxicity such as seen with sorafenib followed by bevacizumab [106], whereas others may be truly synergistic. Clinicians also have a duty to look beyond the PR rate, the SD rate and OS rate and remain aware of changes in performance status, co-morbidity and quality of life for the patients receiving these treatments.

As we increasingly appreciate the heterogeneous nature of melanoma, the issue of biomarkers to predict an individual patient's most efficacious treatment regimen, remains relevant. Not least because in the UK, bevacizumab has been denied funding by the regulatory body NICE (in spite of encouraging outcomes in trial) because it is simply not deemed effective enough across the entire trial population. Were we able to reliably identify sub-populations of patients to receive high cost therapies that in fact deliver impressive outcomes, the story may be very different.

Other problems

There is now growing evidence to support the occurrence of 'vasculogenic mimicry' in cutaneous melanoma, in which aggressive tumour cells are able to begin to form vascular channels that are devoid of endothelial cells [117, 118]. Although there is still some debate as to the exact nature of these channels, it does raise the concern that many anti-angiogenic therapies may fail to target these channels and thus tumour perfusion would continue unfettered. This suggests the need for an anti-angiogenic agent that will target the pathways that drive both classical neovascularisation and vascu-

logenic mimicry, such as the use of MMP inhibitors, with several MMPs including MT1-MMP having been implicated in channel formation [117, 119, 120]. However as yet clinical trials utilising MMP inhibitors have been relatively unsuccessful with the reporting of significant toxicity and limited effectiveness [70, 72, 115]. Alternatively, it may necessitate the use of a combination of therapies in which an anti-angiogenic is combined with conventional chemotherapy, such that any regions of tumour supported by vascular channels that fail to respond to the anti-angiogenic treatment itself, are still targeted with an alternative therapeutic strategy [117]. This is further supported by the demonstration of the highly heterogeneous nature of melanomas, with some melanoma cells having been shown to successfully withstand both hypoxia and metabolic stress, suggesting they may not be susceptible to anti-angiogenic therapy alone [121, 122]. This appears to be well reflected in patients, with increasing successes reported when anti-angiogenic treatments have been combined with conventional chemotherapy.

Future directions

It is conceivable that clinicians and scientists caring for patients with melanoma, and seeking new strategies to treat this unpredictable and aggressive disease, stand at the dawn of a new era in melanoma therapy with the emergence of some of the first ever credible adjuvant therapies. It is anticipated that anti-angiogenics will continue to develop as part of the cocktail of drugs needed to reduce the burden of disease, and maintain metastases in quiescence. We wait to see if the AVAST-M Trial will describe a new and credible role for bevacizumab in early disease, and constantly strive to understand the subtle complexities of angiogenesis that we may control this phenomenon for benefit of our current, and future patients.

Declaration of conflicts of interest

We declare that we have no conflicts of interest relating to this article.

Address correspondence to: Mr Rowan Pritchard-Jones, Mersey Academic Plastic Surgery Group, Liverpool Cancer Research UK Centre, Department of Molecular and Clinical Cancer Medicine, The Duncan Building, Daulby Street, Liverpool, L69 3GA, UK, E-mail: rowanpj@liv.ac.uk

References

- [1] CRUK. Cancer Research UK Malignant Melanoma - UK CancerStats Report. 2008.
- [2] Retsas S, Henry K, Mohammed MQ and MacRae K. Prognostic factors of cutaneous melanoma and a new staging system proposed by the American Joint Committee on Cancer (AJCC): validation in a cohort of 1284 patients. *Eur J Cancer* 2002; 38: 511-516.
- [3] White RR, Stanley WE, Johnson JL, Tyler DS and Seigler HF. Long-term survival in 2,505 patients with melanoma with regional lymph node metastasis. *Ann Surg* 2002; 235: 879-887.
- [4] Carmeliet P and Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000; 407: 249-257.
- [5] Hanahan D and Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; 144: 646-674.
- [6] Hanahan D and Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996; 86: 353-364.
- [7] Folkman J. Endogenous angiogenesis inhibitors. *Apmis* 2004; 112: 496-507.
- [8] Streit M and Detmar M. Angiogenesis, lymphangiogenesis, and melanoma metastasis. *Oncogene* 2003; 22: 3172-3179.
- [9] Erhard H, Rietveld FJ, van Altena MC, Brocker EB, Ruiter DJ and de Waal RM. Transition of horizontal to vertical growth phase melanoma is accompanied by induction of vascular endothelial growth factor expression and angiogenesis. *Melanoma Res* 1997; 7 Suppl 2: S19-26.
- [10] Srivastava A, Laidler P, Davies RP, Horgan K and Hughes LE. The prognostic significance of tumor vascularity in intermediate-thickness (0.76-4.0 mm thick) skin melanoma. A quantitative histologic study. *Am J Pathol* 1988; 133: 419-423.
- [11] Marcoval J, Moreno A, Graells J, Vidal A, Escriba JM, Garcia-Ramirez M and Fabra A. Angiogenesis and malignant melanoma. Angiogenesis is related to the development of vertical (tumorigenic) growth phase. *J Cutan Pathol* 1997; 24: 212-218.
- [12] Straume O, Salvesen HB and Akslen LA. Angiogenesis is prognostically important in vertical growth phase melanomas. *Int J Oncol* 1999; 15: 595-599.
- [13] Graham CH, Rivers J, Kerbel RS, Stankiewicz KS and White WL. Extent of vascularization as a prognostic indicator in thin (< 0.76 mm) malignant melanomas. *Am J Pathol* 1994; 145: 510-514.
- [14] Vlaykova T, Laurila P, Muhonen T, Hahka-Kemppinen M, Jekunen A, Alitalo K and Pyrhonen S. Prognostic value of tumour vascularity

- in metastatic melanoma and association of blood vessel density with vascular endothelial growth factor expression. *Melanoma Res* 1999; 9: 59-68.
- [15] Kashani-Sabet M, Sagebiel RW, Ferreira CM, Nosrati M and Miller JR, 3rd. Tumor vascularity in the prognostic assessment of primary cutaneous melanoma. *J Clin Oncol* 2002; 20: 1826-1831.
 - [16] Dadras SS, Paul T, Bertoncini J, Brown LF, Muzikansky A, Jackson DG, Ellwanger U, Garbe C, Mihm MC and Detmar M. Tumor lymphangiogenesis: a novel prognostic indicator for cutaneous melanoma metastasis and survival. *Am J Pathol* 2003; 162: 1951-1960.
 - [17] Ilmonen S, Kariniemi AL, Vlaykova T, Muhonen T, Pyrhonen S and Asko-Seljavaara S. Prognostic value of tumour vascularity in primary melanoma. *Melanoma Res* 1999; 9: 273-278.
 - [18] Busam KJ, Berwick M, Blessing K, Fandrey K, Kang S, Karaoli T, Fine J, Cochran AJ, White WL, Rivers J and et al. Tumor vascularity is not a prognostic factor for malignant melanoma of the skin. *Am J Pathol* 1995; 147: 1049-1056.
 - [19] Gourley M and Williamson JS. Angiogenesis: new targets for the development of anticancer chemotherapies. *Curr Pharm Des* 2000; 6: 417-439.
 - [20] Shojaei F and Ferrara N. Refractoriness to antivasculature endothelial growth factor treatment: role of myeloid cells. *Cancer Res* 2008; 68: 5501-5504.
 - [21] Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* 2004; 25: 581-611.
 - [22] Ferrara N. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *Am J Physiol Cell Physiol* 2001; 280: C1358-1366.
 - [23] Neufeld G, Cohen T, Gengrinovitch S and Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. *Faseb J* 1999; 13: 9-22.
 - [24] Karkkainen MJ and Petrova TV. Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis. *Oncogene* 2000; 19: 5598-5605.
 - [25] Dvorak HF, Brown LF, Detmar M and Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 1995; 146: 1029-1039.
 - [26] Detmar M. Molecular regulation of angiogenesis in the skin. *J Invest Dermatol* 1996; 106: 207-208.
 - [27] Ferrara N. Vascular endothelial growth factor: molecular and biological aspects. *Curr Top Microbiol Immunol* 1999; 237: 1-30.
 - [28] Dvorak HF. Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. *J Clin Oncol* 2002; 20: 4368-4380.
 - [29] Ferrara N. Role of vascular endothelial growth factor in physiologic and pathologic angiogenesis: therapeutic implications. *Semin Oncol* 2002; 29: 10-14.
 - [30] Bates DO and Harper SJ. Regulation of vascular permeability by vascular endothelial growth factors. *Vascul Pharmacol* 2002; 39: 225-237.
 - [31] Bates DO, Hillman NJ, Williams B, Neal CR and Pocock TM. Regulation of microvascular permeability by vascular endothelial growth factors. *J Anat* 2002; 200: 581-597.
 - [32] Ferrara N. Molecular and biological properties of vascular endothelial growth factor. *J Mol Med* 1999; 77: 527-543.
 - [33] Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC and Abraham JA. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem* 1991; 266: 11947-11954.
 - [34] Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D and Betsholtz C. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol* 2003; 161: 1163-1177.
 - [35] Ruhrberg C, Gerhardt H, Golding M, Watson R, Ioannidou S, Fujisawa H, Betsholtz C and Shima DT. Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. *Genes Dev* 2002; 16: 2684-2698.
 - [36] Yu JL, Rak JW, Klement G and Kerbel RS. Vascular endothelial growth factor isoform expression as a determinant of blood vessel patterning in human melanoma xenografts. *Cancer Res* 2002; 62: 1838-1846.
 - [37] Gitay-Goren H, Halaban R and Neufeld G. Human melanoma cells but not normal melanocytes express vascular endothelial growth factor receptors. *Biochem Biophys Res Commun* 1993; 190: 702-708.
 - [38] Mehnert JM, McCarthy MM, Jilaveanu L, Flaherty KT, Aziz S, Camp RL, Rimm DL and Kluger HM. Quantitative expression of VEGF, VEGF-R1, VEGF-R2, and VEGF-R3 in melanoma tissue microarrays. *Hum Pathol* 2009; 41: 375-384.
 - [39] Harmey JH and Bouchier-Hayes D. Vascular endothelial growth factor (VEGF), a survival factor for tumour cells: implications for anti-angiogenic therapy. *Bioessays* 2002; 24: 280-283.
 - [40] Bates DO, Cui TG, Doughty JM, Winkler M, Sugiono M, Shields JD, Peat D, Gillatt D and Harper SJ. VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. *Cancer Res* 2002; 62: 4123-4131.
 - [41] Woolard J, Wang WY, Bevan HS, Qiu Y, Mor-

- bidelli L, Pritchard-Jones RO, Cui TG, Sugiono M, Waive E, Perrin R, Foster R, Digby-Bell J, Shields JD, Whittles CE, Mushens RE, Gillatt DA, Ziche M, Harper SJ and Bates DO. VEGF165b, an inhibitory vascular endothelial growth factor splice variant: mechanism of action, in vivo effect on angiogenesis and endogenous protein expression. *Cancer Res* 2004; 64: 7822-7835.
- [42] Woolard J, Bevan HS, Harper SJ and Bates DO. Molecular Diversity of VEGF-A as a Regulator of Its Biological Activity. *Microcirculation* 2009; 10: 1-21.
- [43] Varey AH, Rennel ES, Qiu Y, Bevan HS, Perrin RM, Raffy S, Dixon AR, Paraskeva C, Zaccheo O, Hassan AB, Harper SJ and Bates DO. VEGF 165 b, an antiangiogenic VEGF-A isoform, binds and inhibits bevacizumab treatment in experimental colorectal carcinoma: balance of pro- and antiangiogenic VEGF-A isoforms has implications for therapy. *Br J Cancer* 2008; 98: 1366-1379.
- [44] Pritchard-Jones RO, Dunn DB, Qiu Y, Varey AH, Orlando A, Rigby H, Harper SJ and Bates DO. Expression of VEGF(xxx)b, the inhibitory isoforms of VEGF, in malignant melanoma. *Br J Cancer* 2007; 97: 223-230.
- [45] Grunewald FS, Prota AE, Giese A and Ballmer-Hofer K. Structure-function analysis of VEGF receptor activation and the role of coreceptors in angiogenic signaling. *Biochim Biophys Acta* 1804; 1804: 567-580.
- [46] Murga M, Fernandez-Capetillo O and Tosato G. Neuropilin-1 regulates attachment in human endothelial cells independently of vascular endothelial growth factor receptor-2. *Blood* 2005; 105: 1992-1999.
- [47] Soker S, Takashima S, Miao HQ, Neufeld G and Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* 1998; 92: 735-745.
- [48] Ellis LM. The role of neuropilins in cancer. *Mol Cancer Ther* 2006; 5: 1099-1107.
- [49] Geretti E, van Meeteren LA, Shimizu A, Dudley AC, Claesson-Welsh L and Klagsbrun M. A mutated soluble neuropilin-2 B domain antagonizes vascular endothelial growth factor bioactivity and inhibits tumor progression. *Mol Cancer Res* 2010; 8: 1063-1073.
- [50] Pan Q, Chantry Y, Liang WC, Stawicki S, Mak J, Rathore N, Tong RK, Kowalski J, Yee SF, Pacheco G, Ross S, Cheng Z, Le Couter J, Plowman G, Peale F, Koch AW, Wu Y, Bagri A, Tessier-Lavigne M and Watts RJ. Blocking neuropilin-1 function has an additive effect with anti-VEGF to inhibit tumor growth. *Cancer Cell* 2007; 11: 53-67.
- [51] Raza A, Franklin MJ and Dudek AZ. Pericytes and vessel maturation during tumor angiogenesis and metastasis. *Am J Hematol* 2010; 85: 593-598.
- [52] Bergers G and Song S. The role of pericytes in blood-vessel formation and maintenance. *Neuro Oncol* 2005; 7: 452-464.
- [53] Liu W, Ahmad SA, Reinmuth N, Shaheen RM, Jung YD, Fan F and Ellis LM. Endothelial cell survival and apoptosis in the tumor vasculature. *Apoptosis* 2000; 5: 323-328.
- [54] Reinmuth N, Liu W, Jung YD, Ahmad SA, Shaheen RM, Fan F, Bucana CD, McMahon G, Gallick GE and Ellis LM. Induction of VEGF in perivascular cells defines a potential paracrine mechanism for endothelial cell survival. *Faseb J* 2001; 15: 1239-1241.
- [55] Shaheen RM, Tseng WW, Davis DW, Liu W, Reinmuth N, Vellagas R, Wiczorek AA, Ogura Y, McConkey DJ, Drazan KE, Bucana CD, McMahon G and Ellis LM. Tyrosine kinase inhibition of multiple angiogenic growth factor receptors improves survival in mice bearing colon cancer liver metastases by inhibition of endothelial cell survival mechanisms. *Cancer Res* 2001; 61: 1464-1468.
- [56] Hasumi Y, Klosowska-Wardega A, Furuhashi M, Ostman A, Heldin CH and Hellberg C. Identification of a subset of pericytes that respond to combination therapy targeting PDGF and VEGF signaling. *Int J Cancer* 2007; 121: 2606-2614.
- [57] Winkler F, Kozin SV, Tong RT, Chae SS, Booth MF, Garkavtsev I, Xu L, Hicklin DJ, Fukumura D, di Tomaso E, Munn LL and Jain RK. Kinetics of vascular normalization by VEGFR2 blockade governs brain tumor response to radiation: role of oxygenation, angiopoietin-1, and matrix metalloproteinases. *Cancer Cell* 2004; 6: 553-563.
- [58] Huang H, Bhat A, Woodnutt G and Lappe R. Targeting the ANGPT-TIE2 pathway in malignancy. *Nat Rev Cancer* 2010; 10: 575-585.
- [59] Loughna S and Sato TN. Angiopoietin and Tie signaling pathways in vascular development. *Matrix Biol* 2001; 20: 319-325.
- [60] Singh H, Milner CS, Aguilar Hernandez MM, Patel N and Brindle NP. Vascular endothelial growth factor activates the Tie family of receptor tyrosine kinases. *Cell Signal* 2009; 21: 1346-1350.
- [61] Hashizume H, Falcon BL, Kuroda T, Baluk P, Coxon A, Yu D, Bready JV, Oliner JD and McDonald DM. Complementary actions of inhibitors of angiopoietin-2 and VEGF on tumor angiogenesis and growth. *Cancer Res* 2010; 70: 2213-2223.
- [62] Brown JL, Cao ZA, Pinzon-Ortiz M, Kendrew J, Reimer C, Wen S, Zhou JQ, Tabrizi M, Emery S, McDermott B, Pablo L, McCoon P, Bedian V and Blakey DC. A human monoclonal anti-ANG2 antibody leads to broad antitumor activity in combination with VEGF inhibitors and chemotherapy agents in preclinical models. *Mol Cancer Ther* 2010; 9: 145-156.
- [63] Helfrich I, Edler L, Sucker A, Thomas M, Chris-

- tian S, Schadendorf D and Augustin HG. Angiopoietin-2 levels are associated with disease progression in metastatic malignant melanoma. *Clin Cancer Res* 2009; 15: 1384-1392.
- [64] Lazar-Molnar E, Hegyesi H, Toth S and Falus A. Autocrine and paracrine regulation by cytokines and growth factors in melanoma. *Cytokine* 2000; 12: 547-554.
- [65] Friesel RE and Maciag T. Molecular mechanisms of angiogenesis: fibroblast growth factor signal transduction. *Faseb J* 1995; 9: 919-925.
- [66] Ellis LM and Hicklin DJ. Pathways mediating resistance to vascular endothelial growth factor-targeted therapy. *Clin Cancer Res* 2008; 14: 6371-6375.
- [67] Casanovas O, Hicklin DJ, Bergers G and Hanahan D. Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. *Cancer Cell* 2005; 8: 299-309.
- [68] Batchelor TT, Sorensen AG, di Tomaso E, Zhang WT, Duda DG, Cohen KS, Kozak KR, Cahill DP, Chen PJ, Zhu M, Ancukiewicz M, Mrugala MM, Plotkin S, Drappatz J, Louis DN, Ivy P, Scadden DT, Benner T, Loeffler JS, Wen PY and Jain RK. AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients. *Cancer Cell* 2007; 11: 83-95.
- [69] Nikkola J, Vihinen P, Vuoristo MS, Kellokumpu-Lehtinen P, Kahari VM and Pyrhonen S. High serum levels of matrix metalloproteinase-9 and matrix metalloproteinase-1 are associated with rapid progression in patients with metastatic melanoma. *Clin Cancer Res* 2005; 11: 5158-5166.
- [70] Overall CM and Lopez-Otin C. Strategies for MMP inhibition in cancer: innovations for the post-trial era. *Nat Rev Cancer* 2002; 2: 657-672.
- [71] Hofmann UB, Westphal JR, Van Muijen GN and Ruiter DJ. Matrix metalloproteinases in human melanoma. *J Invest Dermatol* 2000; 115: 337-344.
- [72] Egeblad M and Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* 2002; 2: 161-174.
- [73] Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z and Hanahan D. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol* 2000; 2: 737-744.
- [74] Arroyo AG, Genis L, Gonzalo P, Matias-Roman S, Pollan A and Galvez BG. Matrix metalloproteinases: new routes to the use of MT1-MMP as a therapeutic target in angiogenesis-related disease. *Curr Pharm Des* 2007; 13: 1787-1802.
- [75] Sounni NE, Paye A, Host L and Noel A. MT-MMPs as Regulators of Vessel Stability Associated with Angiogenesis. *Front Pharmacol* 2011; 2: 111.
- [76] Chun TH, Sabeh F, Ota I, Murphy H, McDonagh KT, Holmbeck K, Birkedal-Hansen H, Allen ED and Weiss SJ. MT1-MMP-dependent neovessel formation within the confines of the three-dimensional extracellular matrix. *J Cell Biol* 2004; 167: 757-767.
- [77] Iida J, Wilhelmson KL, Price MA, Wilson CM, Pei D, Furcht LT and McCarthy JB. Membrane type-1 matrix metalloproteinase promotes human melanoma invasion and growth. *J Invest Dermatol* 2004; 122: 167-176.
- [78] Anand-Apte B, Bao L, Smith R, Iwata K, Olsen BR, Zetter B and Apte SS. A review of tissue inhibitor of metalloproteinases-3 (TIMP-3) and experimental analysis of its effect on primary tumor growth. *Biochem Cell Biol* 1996; 74: 853-862.
- [79] Bloomston M, Shafii A, Zervos E and Rosemurgy AS. TIMP-1 antisense gene transfection attenuates the invasive potential of pancreatic cancer cells in vitro and inhibits tumor growth in vivo. *Am J Surg* 2005; 189: 675-679.
- [80] Airola K, Karonen T, Vaalamo M, Lehti K, Lohi J, Kariniemi AL, Keski-Oja J and Saarialho-Kere UK. Expression of collagenases-1 and -3 and their inhibitors TIMP-1 and -3 correlates with the level of invasion in malignant melanomas. *Br J Cancer* 1999; 80: 733-743.
- [81] Ebos JM, Lee CR, Christensen JG, Mutsaers AJ and Kerbel RS. Multiple circulating proangiogenic factors induced by sunitinib malate are tumor-independent and correlate with antitumor efficacy. *Proc Natl Acad Sci U S A* 2007; 104: 17069-17074.
- [82] Ribatti D. Endogenous inhibitors of angiogenesis: a historical review. *Leuk Res* 2009; 33: 638-644.
- [83] Basu B, Biswas S, Wrigley J, Sirohi B and Corrie P. Angiogenesis in cutaneous malignant melanoma and potential therapeutic strategies. *Expert Rev Anticancer Ther* 2009; 9: 1583-1598.
- [84] Pietras K, Pahler J, Bergers G and Hanahan D. Functions of paracrine PDGF signaling in the proangiogenic tumor stroma revealed by pharmacological targeting. *PLoS Med* 2008; 5: e19.
- [85] Middleton MR, Grob JJ, Aaronson N, Fierlbeck G, Tilgen W, Seiter S, Gore M, Aamdal S, Cebon J, Coates A, Dreno B, Henz M, Schadendorf D, Kapp A, Weiss J, Fraass U, Statkevich P, Muller M and Thatcher N. Randomized phase III study of temozolomide versus dacarbazine in the treatment of patients with advanced metastatic malignant melanoma. *J Clin Oncol* 2000; 18: 158-166.
- [86] Willett CG, Boucher Y, di Tomaso E, Duda DG, Munn LL, Tong RT, Chung DC, Sahani DV, Kalva SP, Kozin SV, Mino M, Cohen KS, Scad-

- den DT, Hartford AC, Fischman AJ, Clark JW, Ryan DP, Zhu AX, Blaszkowsky LS, Chen HX, Shellito PC, Lauwers GY and Jain RK. Direct evidence that the VEGF-specific antibody bevacizumab has antivasculature effects in human rectal cancer. *Nat Med* 2004; 10: 145-147.
- [87] Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R and Kabbinavar F. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 2004; 350: 2335-2342.
- [88] Dinney CP, Bielenberg DR, Perrotte P, Reich R, Eve BY, Bucana CD and Fidler IJ. Inhibition of basic fibroblast growth factor expression, angiogenesis, and growth of human bladder carcinoma in mice by systemic interferon-alpha administration. *Cancer Res* 1998; 58: 808-814.
- [89] Varker KA, Biber JE, Kefauver C, Jensen R, Lehman A, Young D, Wu H, Lesinski GB, Kendra K, Chen HX, Walker MJ and Carson WE, 3rd. A randomized phase 2 trial of bevacizumab with or without daily low-dose interferon alfa-2b in metastatic malignant melanoma. *Ann Surg Oncol* 2007; 14: 2367-2376.
- [90] Grignol VP, Olencki T, Relekar K, Taylor C, Kibler A, Kefauver C, Wei L, Walker MJ, Chen HX, Kendra K and Carson WE, 3rd. A Phase 2 Trial of Bevacizumab and High-dose Interferon Alpha 2B in Metastatic Melanoma. *J Immunother* 2011; 34: 509-515.
- [91] Vihinen PP, Hernberg M, Vuoristo MS, Tynnelä K, Laukka M, Lundin J, Ivaska J and Pyrhonen S. A phase II trial of bevacizumab with dacarbazine and daily low-dose interferon-alpha2a as first line treatment in metastatic melanoma. *Melanoma Res* 2010; 20: 318-325.
- [92] O'Day SJ, Atkins MB, Boasberg P, Wang HJ, Thompson JA, Anderson CM, Gonzalez R, Lutzky J, Amatruda T, Hersh EM and Weber JS. Phase II multicenter trial of maintenance bi-therapy after induction concurrent Biochemotherapy for patients with metastatic melanoma. *J Clin Oncol* 2009; 27: 6207-6212.
- [93] Perez DG, Suman VJ, Fitch TR, Amatruda T, 3rd, Morton RF, Jilani SZ, Constantinou CL, Egner JR, Kottschade LA and Markovic SN. Phase 2 trial of carboplatin, weekly paclitaxel, and biweekly bevacizumab in patients with unresectable stage IV melanoma: a North Central Cancer Treatment Group study, N047A. *Cancer* 2009; 115: 119-127.
- [94] Del Vecchio M, Mortarini R, Canova S, Di Guardo L, Pimpinelli N, Sertoli MR, Bedognetti D, Queirolo P, Morosini P, Perrone T, Bajetta E and Anichini A. Bevacizumab plus fotemustine as first-line treatment in metastatic melanoma patients: clinical activity and modulation of angiogenesis and lymphangiogenesis factors. *Clin Cancer Res* 2010; 16: 5862-5872.
- [95] Eton O, Legha SS, Bedikian AY, Lee JJ, Buzaid AC, Hodges C, Ring SE, Papadopoulos NE, Plager C, East MJ, Zhan F and Benjamin RS. Sequential biochemotherapy versus chemotherapy for metastatic melanoma: results from a phase III randomized trial. *J Clin Oncol* 2002; 20: 2045-2052.
- [96] von Moos R, Seifert B, Simcock M, Goldinger SM, Gillessen S, Ochsenbein A, Michielin O, Cathomas R, Schlappi M, Moch H, Schraml PH, Mjhić-Probst D, Mamot C, Schonewolf N and Dummer R. First-line temozolomide combined with bevacizumab in metastatic melanoma: a multicentre phase II trial (SAKK 50/07). *Ann Oncol* 2011; 28: 28.
- [97] Ranpura V, Hapani S and Wu S. Treatment-related mortality with bevacizumab in cancer patients: a meta-analysis. *Jama* 305: 487-494.
- [98] Karaman MW, Herrgard S, Treiber DK, Gallant P, Atteridge CE, Campbell BT, Chan KW, Ciceri P, Davis MI, Edeen PT, Faraoni R, Floyd M, Hunt JP, Lockhart DJ, Milanov ZV, Morrison MJ, Pallares G, Patel HK, Pritchard S, Wodicka LM and Zarrinkar PP. A quantitative analysis of kinase inhibitor selectivity. *Nat Biotechnol* 2008; 26: 127-132.
- [99] Wilhelm SM, Carter C, Tang L, Wilkie D, McNabola A, Rong H, Chen C, Zhang X, Vincent P, McHugh M, Cao Y, Shujath J, Gawlak S, Eveleigh D, Rowley B, Liu L, Adnane L, Lynch M, Auclair D, Taylor I, Gedrich R, Voznesensky A, Riedl B, Post LE, Bollag G and Trail PA. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res* 2004; 64: 7099-7109.
- [100] Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, Lydon NB, Kantarjian H, Capdeville R, Ohno-Jones S and Sawyers CL. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med* 2001; 344: 1031-1037.
- [101] Demetri GD, von Mehren M, Blanke CD, Van den Abbeele AD, Eisenberg B, Roberts PJ, Heinrich MC, Tuveson DA, Singer S, Janicek M, Fletcher JA, Silverman SG, Silberman SL, Capdeville R, Kiese B, Peng B, Dimitrijevic S, Druker BJ, Corless C, Fletcher CD and Joensuu H. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. *N Engl J Med* 2002; 347: 472-480.
- [102] Ott PA, Hamilton A, Min C, Safarzadeh-Amiri S, Goldberg L, Yoon J, Yee H, Buckley M, Christos PJ, Wright JJ, Polsky D, Osman I, Liebes L and Pavlick AC. A phase II trial of sorafenib in metastatic melanoma with tissue correlates. *PLoS One* 2010; 5: e15588.
- [103] Eisen T, Ahmad T, Flaherty KT, Gore M, Kaye

- S, Marais R, Gibbens I, Hackett S, James M, Schuchter LM, Nathanson KL, Xia C, Simantov R, Schwartz B, Poulin-Costello M, O'Dwyer PJ and Ratain MJ. Sorafenib in advanced melanoma: a Phase II randomised discontinuation trial analysis. *Br J Cancer* 2006; 95: 581-586.
- [104] Hauschild A, Agarwala SS, Trefzer U, Hogg D, Robert C, Hersey P, Eggermont A, Grabbe S, Gonzalez R, Gille J, Peschel C, Schadendorf D, Garbe C, O'Day S, Daud A, White JM, Xia C, Patel K, Kirkwood JM and Keilholz U. Results of a phase III, randomized, placebo-controlled study of sorafenib in combination with carboplatin and paclitaxel as second-line treatment in patients with unresectable stage III or stage IV melanoma. *J Clin Oncol* 2009; 27: 2823-2830.
- [105] Akula SM, Ford PW, Whitman AG, Hamden KE, Bryan BA, Cook PP and McCubrey JA. B-Raf-dependent expression of vascular endothelial growth factor-A in Kaposi sarcoma-associated herpesvirus-infected human B cells. *Blood* 2005; 105: 4516-4522.
- [106] Azad NS, Posadas EM, Kwitkowski VE, Steinberg SM, Jain L, Annunziata CM, Minasian L, Sarosy G, Kotz HL, Premkumar A, Cao L, McNally D, Chow C, Chen HX, Wright JJ, Figg WD and Kohn EC. Combination targeted therapy with sorafenib and bevacizumab results in enhanced toxicity and antitumor activity. *J Clin Oncol* 2008; 26: 3709-3714.
- [107] Anderson MD. Bevacizumab and Sorafenib in Treating Patients With Unresectable Stage III or Stage IV Malignant Melanoma. <http://www.clinicaltrials.gov/ct2/show/NCT00387751?term=NCT00387751&rank=1>
- [108] Clay EF. Sorafenib, Bevacizumab, and Oxaliplatin in Treating Patients With Metastatic Malignant Melanoma. <http://www.clinicaltrials.gov/ct2/show/NCT00538005?term=NCT00538005&rank=1>
- [109] Amaravadi R SL, McDermott D et al. Updated results of a randomised Phase II study comparing two schedules of temozolamide in combination with sorafenib in patients with advanced melanoma. *J.Clin. Oncol.* 25(18s) 2007; Abstract 8527:
- [110] Fruehauf J, Lutzky J and McDermott D. Axitinib (AG-013736) in patients with metastatic melanoma: a phase II study. *J. Clin. Oncol.* 26, (2008) 2008; Abstrat 9006.
- [111] Daud A. A Two Arm Trial of Axitinib and Carboplatin/Paclitaxel in Melanoma (CC# 10852). <http://www.clinicaltrials.gov/ct2/show/NCT01174238?term=NCT01174238&rank=1>
- [112] Fruehauf JP. Study of the Anti-Angiogenesis Agent Axitinib in Patients With Stage III Malignant Melanoma. <http://www.clinicaltrials.gov/ct2/show/NCT01321437?term=NCT01321437&rank=1>
- [113] Becker JC BE, Schadendorf D, Ugurel S. Imatinib in melanoma: a selective treatment option based on KIT mutation status? . *J. Clin. Oncol.* 25(7) 2007; Abstract e9:
- [114] Carvajal RD, Antonescu CR, Wolchok JD, Chapman PB, Roman RA, Teitcher J, Panageas KS, Busam KJ, Chmielowski B, Lutzky J, Pavlick AC, Fusco A, Cane L, Takebe N, Vemula S, Bouvier N, Bastian BC and Schwartz GK. KIT as a therapeutic target in metastatic melanoma. *Jama* 2011; 305: 2327-2334.
- [115] Quirt I, Bodurth A, Lohmann R, Rusthoven J, Belanger K, Young V, Wainman N, Stewar W and Eisenhauer E. Phase II study of marimastat (BB-2516) in malignant melanoma: a clinical and tumor biopsy study of the National Cancer Institute of Canada Clinical Trials Group. *Invest New Drugs* 2002; 20: 431-437.
- [116] Hong S and Han SB. Overcoming metastatic melanoma with BRAF inhibitors. *Arch Pharm Res* 2011; 34: 699-701.
- [117] Zhang S, Zhang D and Sun B. Vasculogenic mimicry: current status and future prospects. *Cancer Lett* 2007; 254: 157-164.
- [118] Maniotis AJ, Folberg R, Hess A, Seftor EA, Gardner LM, Pe'er J, Trent JM, Meltzer PS and Hendrix MJ. Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry. *Am J Pathol* 1999; 155: 739-752.
- [119] Seftor RE, Seftor EA, Koshikawa N, Meltzer PS, Gardner LM, Bilban M, Stetler-Stevenson WG, Quaranta V and Hendrix MJ. Cooperative interactions of laminin 5 gamma2 chain, matrix metalloproteinase-2, and membrane type-1-matrix/metalloproteinase are required for mimicry of embryonic vasculogenesis by aggressive melanoma. *Cancer Res* 2001; 61: 6322-6327.
- [120] Hendrix MJ, Seftor EA, Hess AR and Seftor RE. Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma. *Nat Rev Cancer* 2003; 3: 411-421.
- [121] Rak J, Yu JL, Kerbel RS and Coomber BL. What do oncogenic mutations have to do with angiogenesis/vascular dependence of tumors? *Cancer Res* 2002; 62: 1931-1934.
- [122] Yu JL, Rak JW, Carmeliet P, Nagy A, Kerbel RS and Coomber BL. Heterogeneous vascular dependence of tumor cell populations. *Am J Pathol* 2001; 158: 1325-1334.
- [123] Egberts F, Gutzmer R, Ugurel S, Becker JC, Trefzer U, Degen A, Schenck F, Frey L, Wilhelm T, Hassel JC, Schadendorf D, Livingstone E, Mauch C, Garbe C, Berking C, Rass K, Mohr P, Kaehler KC, Weichenthal M and Hauschild A. Sorafenib and pegylated interferon-(alpha)2b in advanced metastatic melanoma: a multicenter phase II DeCOG trial. *Ann Oncol* 2011; 10: 10.
- [124] Hainsworth JD, Infante JR, Spigel DR, Peyton JD, Thompson DS, Lane CM, Clark BL, Rubin MS, Trent DF and Burris HA, 3rd. Bevacizumab

- and everolimus in the treatment of patients with metastatic melanoma: a phase 2 trial of the Sarah Cannon Oncology Research Consortium. *Cancer* 2010; 116: 4122-4129.
- [125] Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbe C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A and Urba WJ. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010; 363: 711-723.
- [126] Hodi FS, Friedlander PA, Atkins MB, McDermott DF, Lawrence DP, Ibrahim N, Wu X, Zhou J, Giobbie-Hurder A, Murphy G, Hollman T, Velazquez E, Russell S, Dipro P, Yap JT and Van Den Abbeele AD. A phase I trial of ipilimumab plus bevacizumab in patients with unresectable stage III or stage IV melanoma. *J Clin Oncol* 29: (suppl; abstr 8511) 2011;
- [127] Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, Dummer R, Garbe C, Testori A, Maio M, Hogg D, Lorigan P, Lebbe C, Jouary T, Schadendorf D, Ribas A, O'Day SJ, Sosman JA, Kirkwood JM, Eggermont AM, Dreno B, Nolop K, Li J, Nelson B, Hou J, Lee RJ, Flaherty KT and McArthur AG. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* 2011; 364: 2507-2516.
- [128] Novartis. Study of Temzolomide and Gleevec in Advanced Melanoma. <http://www.clinicaltrials.gov/ct2/show/NCT00667953?term=NCT00667953&rank=1>
- [129] Middleton MR and Robert C. Comparison of AZD6244 in Combination With Dacarbazine Versus (vs) Dacarbazine Alone in BRAF Mutation Positive Melanoma Patients <http://www.clinicaltrials.gov/ct2/show/NCT00936221?term=NCT00936221&rank=1>