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Review

Angiogenesis as a target in neuroblastoma

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ABSTRACT

Several research investigations on neuroblastoma (NB) have shown the important dependency of this embryonic tumour on angiogenesis, especially in the advanced and aggressive stages. However, the first pre-clinical data on anti-angiogenic drugs in NB have not been published until recently and clinical trials with anti-angiogenic agents in NB treatment protocols are still missing.

Here, we summarise current knowledge on the important role and mechanisms of angiogenesis in NB, and report available pre-clinical results of anti-angiogenic agents used to treat NB. This review clearly shows that angiogenesis is a target in NB and that clinical trials are urgently needed to bring forward promising anti-angiogenesis treatment strategies into NB therapy.

1. Angiogenesis in neuroblastoma

Angiogenesis represents an attractive new target for tumour therapy and studies on tumour vessel formation have been performed in nearly all types of solid tumours. Whilst the vast majority of this research has focused on adult malignancies, neuroblastoma (NB) has been the focal point of angiogenesis research in paediatric oncology.[1] Although the biological mechanisms that underlie the clinical heterogeneity observed in NB is not completely understood, several recent studies implicate angiogenesis as an essential mechanism regulating NB growth. Therefore, from this point of view, studies on angiogenesis and anti-angiogenic therapies are extremely interesting and necessary in NB. This review summarises recent data on angiogenesis as well as anti-angiogenesis strategies for NB found in the current literature.[2–4]

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1.1. *Angiogenesis*

The first study on tumour vessel formation was reported by Folkman et al. in 1962[5] in which Folkman described the sprouting of new tumour blood vessels from pre-existing ones. Since, the formation of new blood vessels has been shown to be a multi-step process consisting of endothelial cell proliferation, migration and tubule formation. It is now widely accepted that solid tumours require neo-vascularisation for growth beyond a very small size (2–3 mm^3) and for metastatic spread. Within the tumour, this process has been coined 'tumour angiogenesis'. However, as the initial observation was restricted to the formation of blood vessels, a more precise denomination would have been 'tumour blood-angiogenesis'. Dissemination of tumour cells into lymph nodes via the lymphatic vascular system is also a well known problem. Recent research has unravelled another phenomenon named 'tumour lymph-angiogenesis' in which tumour cells not only stimulate the formation of blood vessels, but also the formation of lymphatic vessels as well.[6] Today, understanding the tumour vasculature as a global scheme must take into account both blood and the newly described lymph-angiogenesis processes.

The origin of neo-vessels within the expanding tumour tissue is the result of different mechanisms. Accumulating evidence indicates that in addition to the sprouting of neighbouring pre-existing vessels, tumour angiogenesis is supported by the mobilisation and functional incorporation of endothelial progenitor cells (EPC) that originate from the bone marrow.[7] EPC are highly proliferative cells that can be mobilised to circulate in the peripheral blood and arrest at the site of the developing tumour. Recent reports have described the functional incorporation and contribution of EPC to tumour neovessels, suggesting that the process of 'vasculogenesis' – the progenitor-cell-driven *de novo* generation of new vessels[8] – is not restricted to embryonic development. EPC appear to home to sites of angiogenesis and participate in the generation of new blood vessels in adults as demonstrated by animal models of ischaemia. Other studies have shown that blood vessel formation in tumours may occur primarily through endothelialisation of adjacent and pre-existing blood vasculature, a process called 'co-option'.[9] Exactly how tumours generate new blood vessels is still a matter of debate. Most authors agree that the various mechanisms involved (sprouting, vasculogenesis, or co-option) may not only depend on the nature of the tumour, but may also coexist as a complex and multi-faceted process. To make matters even more confusing, it has been shown that tumour cells may mimic and transform themselves into vascular cells. The term 'vasculogenic mimicry' was recently introduced to reflect this embryonic-like ability of tumour cells to form a blood vessel network.[10] The role and significance of these different angiogenesis mechanisms, recently reviewed by Dome et al.,[11] must be further clarified.

1.2. *Neuroblastoma*

Derived from cells of the primitive neural crest,[12] neuroblastoma (NB) is the most common extra-cranial solid tumour of childhood and the most frequent tumour in children less than one year of age. NB has a wide range of clinical virulence reflective of its underlying biological heterogeneity. Tumour stage, as defined by the International NB Staging System (INSS), and patient age at diagnosis are important clinical prognostic factors that strongly correlate with survival.[13] Genetic abnormalities play a role in determining tumour phenotype and predicting outcome and include amplification of the *MYCN* oncogene, deletion of chromosome 1p, DNA polyploidy, grade of neuronal differentiation and messenger RNA expression level of the neurotrophin receptor Trk-B. Some of these factors have been used to develop a risk-based classification scheme and categorise patients as having low, intermediate or high-risk of tumour relapse in order to help guide treatment protocols.[14] Whilst the majority of children with low-risk NB may be cured by surgery alone, less than 30% of children with high-risk NB become long-term survivors despite aggressive therapy associating surgery, high-dose chemotherapy with autologous stem cell transplantation, radiation and administration of differentiating agents.[15] These children are in desperate need of novel therapeutic strategies. A recent seminar on NB specifically focused on current advances in understanding NB tumour biology as well as potential new approaches to treat this complex paediatric tumour.[16]

2. Mechanisms of angiogenesis in neuroblastoma

Different angiogenesis mechanisms have been described in the formation of NB tumour vasculature. Sprouting of new blood microvessels from pre-existing capillaries under the influence of pro-angiogenic growth factor expression, such as vascular endothelial growth factor-A (VEGF-A), bFGF and angiopoetien (Ang), has been reported.[17–19] A recent study analysing the origin of endothelial cells in NB tumours described the discovery of microvessels harbouring *MYCN* amplification and therefore originating from NB cells. This report provided proof for the existence of 'vasculogenic mimicry' as another mechanism of angiogenesis in NB.[20] Also, we have observed the presence of circulating EPC in patients with NB, demonstrating for the first time that vasculogenesis is also involved in NB tumour vessel formation.[21] Thus, all known mechanisms of angiogenesis described to date are also present in NB. A summary of mechanisms of angiogenesis in NB is given in Table 1.

2.1. *Microvessel density in neuroblastoma*

Microvessel density can be evaluated by counting the number of immunohistochemically identified blood vessels in vascular hot spots of tumour samples. Several other techniques also are available such as Chalkley counting, vascular grading and the use of image analysis systems.[22] Meitar et al. were the first to demonstrate in NB that tumour angiogenesis was associated with a clinical phenotype.[23] They calculated a vascular index, defined as the number of vessels/mm^2 of tissue, and found that a high vascular index correlated strongly with widely disseminated disease, amplification of *MYCN* and unfavourable histology and was associated with poor survival. Multivariate analysis confirmed that tumour vascularity was an important and independent prognostic variable. Another study used a computerised system to determine vas-

Table 1 – Mechanisms of angiogenesis in neuroblastoma

Neuroblastoma	Angiogenesis	Reference
Low risk		
– Favourable histology with presence of Schwann cells	– Highly expressed activin A	Schramm et al.[30]
– Trk-A expression	– Down-regulated angiogenesis factor expression	Eggert et al.[98]
High risk		
– Unfavourable histology with stroma poor in Schwann cells	– Higher vascular index	Meitar et al.[23]
– MYCN amplification	– Higher expression levels of VEGF-A, VEGF-B, bFGF, PDGF-A, TGF-alpha	Eggert et al.[18]
	– Down regulation of endothelial cell growth inhibitors	Fotsis et al. [17]
– TrK-B expression	– Increased levels of MMP-2 and MMP-9	Ribatti et al.[27]
	– More highly expressed levels of integrins alpha (v) beta 3 and alpha (v) beta 5	Erdreich-Epstein et al.[28]

cular parameters in hot spots of NB tissue.[24] In contrast to the previous report, the authors did not find that disseminated disease or local relapse was influenced by the angiogenic characteristics of the tumours, which therefore did not demonstrate predictive value for survival. However, it is widely accepted that localised tumours with favourable biological features are often less vascular and have a rich stromal component largely composed of nonmalignant Schwann cells, which have recently been shown to produce potent anti-angiogenic molecules.[25] Further data supporting the importance of the number and the function of microvessels in NB is presented in a study with an orthotopic NB nude mice model in which tumour vessel morphology and vascular perfusion were analysed *in vivo* by ultrasound (Fig. 1).[26] Unlike orthotopic NB tumours, subcutaneous tumours lacked the expression of the highly angiogenic integrin alpha(v)beta3. The authors suggested that the differences observed were due to the impact of the regional microenvironment on tumour biology. Although not fully understood, NB vascularity seems to play a major role in clinical phenotype and be intrinsically related to the underlying tumour biology.

2.2. *Angiogenesis growth factors in neuroblastoma*

Several pro-angiogenic growth factors have been shown to be differentially expressed in NB in a pattern suggesting the promotion of a pro-angiogenic phenotype in high-risk tumours. Significantly higher expression levels of VEGF-A, VEGF-B, bFGF, Ang-2, transforming growth factor alpha (TGF-alpha) and platelet-derived growth factor A (PDGF-A) were found in advanced-stage tumours (stages 3 and 4) compared to low-stage tumours (stages 1, 2, and 4s).[18] In this study, PDGF-A was significantly associated with survival. In a smaller series, VEGF-A was the only factor found to correlate with advanced tumour stage. Matrix metalloproteinases (MMP) and their inhibitors (MMPI) play a key role in maintaining the balance between extra-cellular matrix deposition and degradation especially important for the migration of endothelial cells during angiogenesis.[27] Whilst MMPI has been shown to suppress tumour invasion and angiogenesis, overexpression of MMP-2 and MMP-9 is associated with tumour invasion and metastasis in many types of cancer. In NB, an association between increased levels of MMP-2 and -9 and advanced tumour stage has also been observed. Furthermore, several studies have shown that high levels of MMP-2 expression correlate with poor outcome. Finally, integrins alpha(v)beta3 and al-

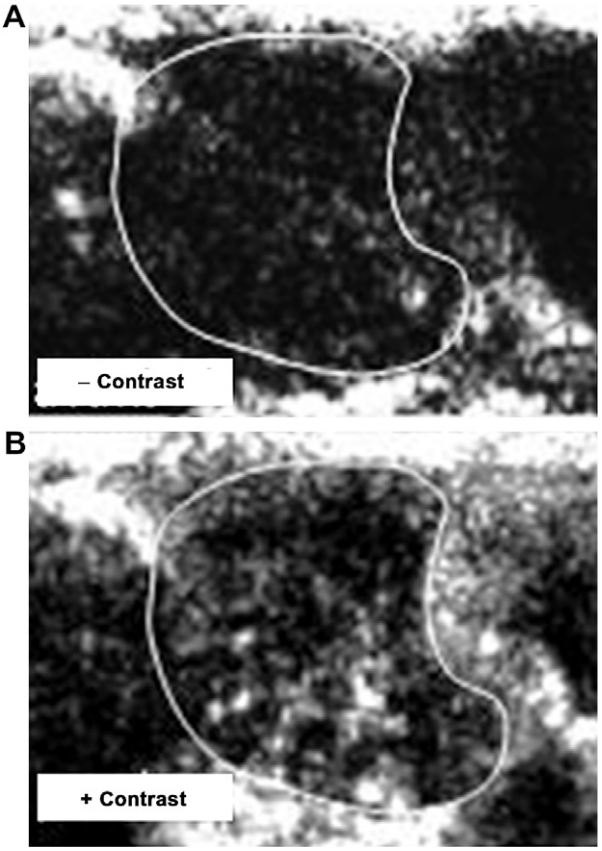

Fig. 1 – Contrast ultrasound to visualise vascularisation of a neuroblastoma (NB) orthotopic tumour. (A) NB cells injected into the right adrenal gland develop into a measurable tumour which can be visualised by ultrasound. (B) After intraocular injection of a contrast agent, vascular perfusion is visible and measurable.

pha(v)beta5 – markers of angiogenic endothelium – were also found to be more highly expressed in blood vessels of high-risk versus low-risk NB tumours.[28]

3. Regulation of angiogenesis in neuroblastoma

Most malignant cells are potently angiogenic as a result of an increased secretion of angiogenic stimulators and a decreased production of inhibitors. However, several other mechanisms appear to play a role in the regulation of angiogenesis in NB, as described below.

3.1. *Oncogene MYCN*

The *MYCN* oncogene is frequently amplified in high-risk NB and is associated with an increased vascular index and poor prognosis.[23] Consequently, it has been hypothesised that *MYCN* might regulate some aspects of NB angiogenesis either by up-regulating angiogenic stimulators or by down-regulating inhibitors. Most investigators have not found a correlation between *MYCN* amplification and increased expression of pro-angiogenic factors such as VEGF, bFGF and MMP. However, recent data suggest that the aggressive biology of *MYCN*-amplified NB is due to suppressed expression of three angiogenesis inhibitors.[17] One of these inhibitors, recently identified as activin A, represses NB growth, endothelial cell proliferation and angiogenesis both *in vitro* and *in vivo*.[29] Activin A is highly expressed in differentiated NB, and increased expression is strongly correlated with favourable outcome.[30] *In vitro* studies demonstrated that *MYCN* inhibited activin A by suppressing its promoter activity.[31] Also, *MYCN* appears to regulate two other inhibitors, interleukin (IL-6) and leukaemia inhibitory factor (LIF),[17,32] which are currently under investigation for their potential as therapeutic targets in NB.

3.2. *Schwann cells*

In contrast to *MYCN*-amplified tumours, low-risk NB is characterised by favourable histology in which a rich stromal component made up of quiescent Schwann cells is frequently observed.[33] Schwann cells produce anti-proliferative and differentiation-inducing factors and a 'cross-talk' between Schwann cells and neuroblasts has been proposed to describe the biology and clinical behaviour of NB tumours.[34] In support of this hypothesis, laboratory studies have shown that Schwann cells also produce several inhibitors of angiogenesis including tissue inhibitor of metalloproteinases (TIMP2), pigment epithelial-derived growth factor (PEDGF) and the secreted protein acidic and rich in cysteine (SPARC).[25,35,36] Therefore, the presence of Schwann cells most likely contributes to the relatively avascular nature of most low-risk NB, further highlighting that differential expression of angiogenesis-related genes contributes to specific phenotype in NB.

3.3. *Hypoxia*

Situations of cellular stress such as hypoxia lead to rapid response mechanisms that ensure adaptive strategies for cellular survival. Genes are rapidly transcribed via the transcription factor hypoxia-inducible factor-1 (HIF) in order to allow for this response[37] and target genes include the pro-angiogenic growth factor VEGF-A. Subsequently, angiogenesis is also regulated by hypoxia.[38] Within the tumour, the initial vasculature is insufficient to cope with the high-index proliferation of tumour cells leading to areas of low oxygen supply. This can be visualised by radiological examinations after minor perfusion of contrast medium into embryonic tumours.[39] We have also demonstrated that hypoxia leads to up-regulated VEGF-A secretion and subsequent stimulation of endothelial cell proliferation in NB cell lines.[19] Furthermore, erythropoietin (EPO) – the prototype of hypoxia-regulated expression via the transcription factor HIF – has a role in erythropoiesis as a stimulator of endothelial proliferation[40] and is also up-regulated in NB cells of neuronal phenotype exclusively.[41,42] However, EPO did not modify NB cell proliferation *in vitro*. More interestingly, in a tissue microarray study, the NB tumour specimens with the highest expression of the EPO receptor had a significantly better overall survival.[43]

4. Lymph-angiogenesis in neuroblastoma

Despite the important role of lymphangiogenesis in tumour growth and metastasis, most studies have focused on evaluating this process in adult cancers, notably in melanoma,[44] and not in children for which very little data are available. Studies concerning embryonic tumours and the relationship between the number of lymph vessels, tumour stage and prognosis are lacking. One study showed significantly higher expression levels of the lymphangiogenesis growth factor VEGF-C in NB of stages 3 and 4 compared to low-stage tumours.[18] In another study using immunohistochemistry, we demonstrated that lymph vessels of different morphology and localization are present in NB tumours.[45] With the help of the antibodies D2_40, specific for lymphatic endothelial cells, and CD34, a surface marker which identifies blood endothelial cells, we could discriminate between both types of tumour vessels in NB tumours (Fig. 2). Lymphatic vessel density was determined by a computer-aided method and ranged from complete absence to as much as 80 lymph vessels per hot spot in available NB specimens. Further studies on lymphangiogenesis are needed as they would help understand its underlying biology and verify its prognostic relevance in NB.

5. Anti-angiogenesis inhibitors in neuroblastoma

Targeting tumour vasculature represents a promising new tool for cancer therapy. The first angiogenic inhibitor, interferon-alpha, was described by Folkman in 1971.[46] Successful treatment of haemangioma, the most frequent vascular tumour in very young infants, was reported with interferon.[47] TNP-470 is the first selective inhibitor of endothelial proliferation and is a synthetic analogue of an antibiotic naturally secreted by *Aspergillus fumigatus* and isolated from an endothelial cell culture contaminated by the fungus.[48] More

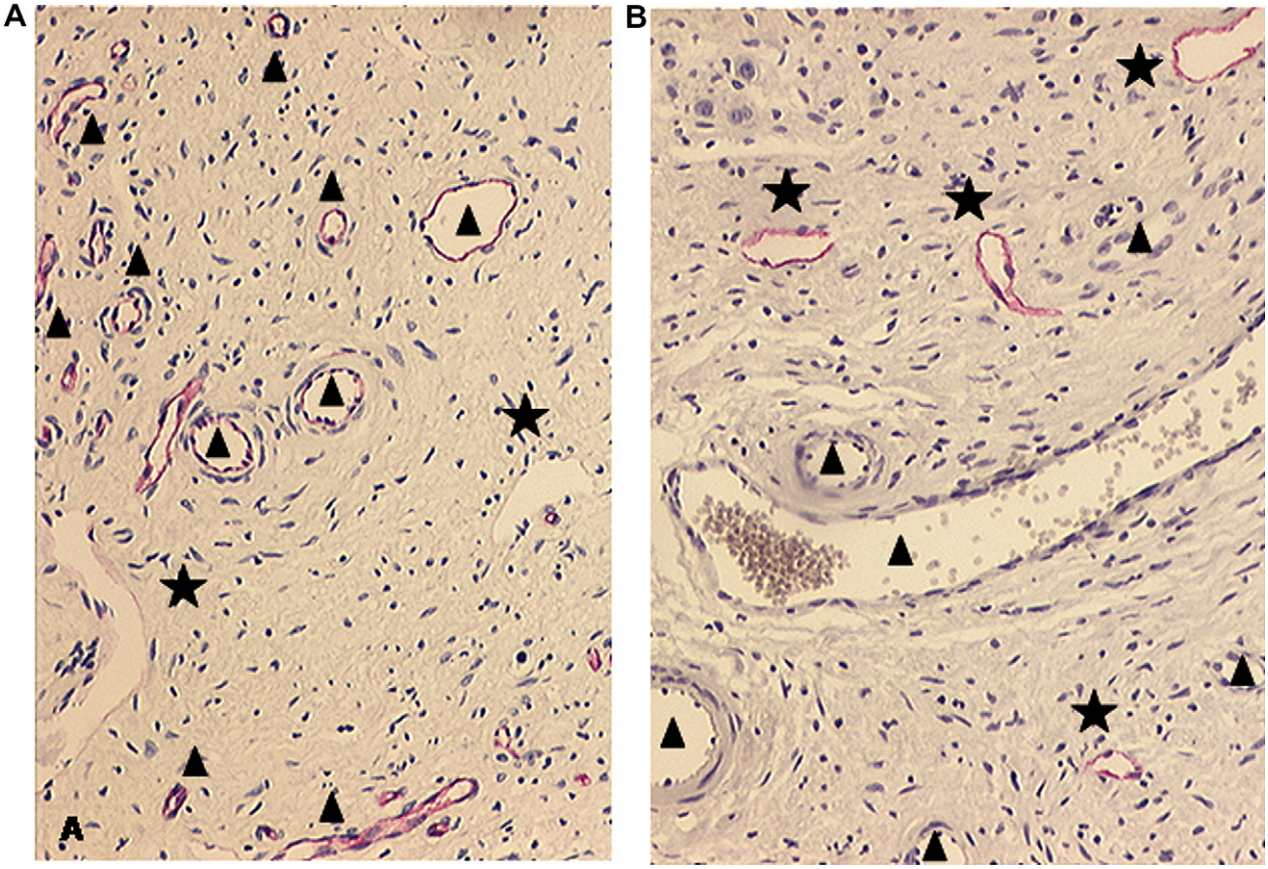

Fig. 2 – Tumor blood and lymphatic vessels identified by immunohistochemistry. (A) The CD34 antibody directed against a cell surface marker of blood endothelial cells shows positively stained tumour blood cells (▲) and negatively stained tumour lymph vessels (★). (B) Immunohistochemistry with the D2_40 antibody marked tumour lymph endothelial cells (★) but negatively stained tumour blood vessels (▲).

than 70 anti-angiogenic molecules are described today. Most of these molecules are being tested in clinical trials, mainly for adult cancer and 18 are currently in phase III studies.[49]

Three classes of angiogenic inhibitors can be distinguished: direct, indirect and mixed inhibitors. Direct angiogenesis inhibitors, such as angiostatin, endostatin or thrombospondin, target microvascular endothelial cells involved in proliferation, migration and formation of new blood vessels. Indirect inhibitors block the production or activity of pro-angiogenic molecules produced by the tumour itself such as VEGF, or the receptors of VEGF or PDGF. The so-called mixed angiogenic inhibitors, such as multipotent tyrosine kinase inhibitors (TKI) or interferon-alpha, affect both tumour endothelial cells and malignant cells.[50]

Recently, a major step in anti-angiogenesis therapy was the approval of bevacizumab (Avastin®) by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) for the treatment of metastatic colorectal cancer with fluoropyrimidine combined regimes.[88] Bevacizumab is a recombinant monoclonal antibody that binds VEGF-A and subsequently blocks the activation of its receptors. Sunitinib (Sutent®, SU11248) was registered by the FDA and the EMEA for the treatment of advanced or metastatic renal cell carcinoma after failure of Interferon alfa or Interleukin 2 therapy, as well as imatinib-resistant gastro-intestinal stromal tumour (GIST). Sunitinib is a multi-targeted TKI showing both anti-proliferative and anti-angiogenic effects as a result of its inhibition of VEGFR, PDGFR-beta and c-Kit.[51] A third anti-angiogenic drug, sorafenib (Nexavar®, BAY 43-9006), has officially been licensed for use in adult oncology after receiving FDA approval for the treatment of advanced renal cell carcinoma. Initially, this bi-aryl urea was developed as a specific inhibitor of the intracellular kinase raf but subsequent studies have shown that this compound inhibits several other TK involved in tumour progression including VEGFR.[52]

Compared to conventional cytotoxic cancer treatment, anti-angiogenic strategies offer several advantages: (1) the damage caused to a single tumour blood vessel impacts a high number of tumour cells dependant on its blood supply; (2) anti-angiogenic therapy appears to normalise tumour vessels, resulting in improved delivery and efficacy of chemotherapy agents; (3) targeting endothelial cells may help control minimum residual disease due to quiescent cells in the $G_{0/1}$ phase which are not accessible to standard chemotherapy; (4) anti-angiogenic drug resistance is rare and contrasts with tumour resistance frequently developed against standard chemotherapy drugs.

Direct angiogenic inhibitors are not currently used in NB therapy protocols. Interestingly, retinoic acid, which is now administered in stage 4 patients after autologous stem cell

transplantation as a maintenance treatment,[53] has not only been chosen for its differentiating effect on immature NB cells, but also for its anti-angiogenic potency as demonstrated in several pre-clinical studies. A summary of pre-clinical studies evaluating anti-angiogenic agents in NB is presented in Table 2.

5.1. *Retinoids*

Retinoids exert their effects by inducing differentiation of NB cells. Furthermore, retinoids and notably fenretinide, a newly developed synthetic retinoid, have demonstrated an anti-angiogenic effect in different experimental models. These effects include prevention of tumour-associated angiogenesis *in vivo*, inhibition of vessel sprouting in chicken chorion allantois membrane (CAM), and tumour cell transformation from an angiogenic to an anti-angiogenic phenotype *in vitro*.[54–56] All these effects are mediated by the inhibition of endothelial migration along with the production of stimulating or inhibiting factors for vessel growth.[54] Furthermore, retinoic acid induced expression of thrombospondin, an important physiological angiogenesis inhibitor, in NB cell lines.[57] Therefore, retrospectively, the use of retinoids in NB is the first anti-angiogenesis strategy to have been used in treating embryonic tumours.

5.2. *TNP-470*

The synthetic derivative of fumagillin TNP-470 (or AGM-1470) inhibits an important enzyme for endothelial cell proliferation and migration known as methionine aminopeptidase-2 (MetAP2).[3] There are several ongoing clinical phase I and II trials with TNP-470 for adult patients with cervical, pancreatic or renal cell carcinoma.[58] When given in combination with other common cytotoxic drugs like cisplatin, paclitaxel or cyclophosphamide, treatment with TNP-470 synergistically potentiates the anti-tumour effects of these molecules.[3] Another MetAP2 inhibitor, A-357300, demonstrated tumour growth suppression in pre-clinical models without the toxicities observed with TNP-470.[59] Several pre-clinical studies report the use of TNP-470 in NB models (Table 2). Altogether, current data suggest that TNP-470 may be useful as adjuvant therapy for high-risk NB patients when administered either between cycles of induction therapy or at the end of cytotoxic chemotherapy, and perhaps more particularly when used in the setting of minimal disease status.

5.3. *Thalidomide*

Thalidomide was first introduced in the 1950s as a nontoxic sedative but withdrawn because of its marked teratogenicity. Recent studies have demonstrated that the aetiology of the limb defects caused by foetal exposure to thalidomide was most likely due to inhibition of blood vessel growth in developing limb buds.[60] Therefore, thalidomide is an anti-angiogenic molecule with potential to treat cancer. In adult oncology, a phase I study showed safety, tolerance and potential efficacy of thalidomide in treatment of recurrent epithelial ovarian cancer.[61] However, in a phase II study for gynaecological sarcoma, thalidomide showed no activity and caused major adverse effects such as constipation, fatigue, drowsiness and worsening of performance status.[62] Consequently, derivatives of thalidomide are currently under development.[63] One study tested thalidomide in a xenotransplant NB mouse model and reported reduced angiogenesis although tumour growth was not altered significantly.[64] The first paediatric clinical trial using thalidomide in combination with radiation in children with brain stem glioma and glioblastoma did not demonstrate efficacy and displayed higher toxicity as shown by increased use of corticosteroids.[65]

5.4. *Matrix metalloproteinase inhibitors*

In normal tissues, a balance between MMP and MMPI exists. However, in tumour tissue, there is an imbalance/switch in favour of the MMP. Consequently, another attempt to control angiogenesis is to use MMPI in order to prevent degradation of the basal membrane and the surrounding connective tissue. Marimastat® (BB-2516) is a synthetic inhibitor for MMP-1, -2, -3, -7 and -9[58] and represents the first orally bioavailable MMPI to be tested in humans.[50,58] Results of phase I, II and III clinical trials using Marimastat® alone or in combination with other chemotherapeutic regimes showed dose-limiting toxicity in musculoskeletal disorders. BMS-275291, another broad-spectrum MMPI, was tested in combination with chemotherapy but showed increased toxicity without improving survival in advanced NSCLC.[66] Therefore, the use of MMPI is considered disadvantageous in this setting. As the expression of MMP-2 and MMP-9 correlates with poor prognosis in ovary, lung, breast and colon cancer, BAY 12-9566 (Tanomastat®) was developed as a selective, non-peptidic and orally available inhibitor of these two MMP. Tanomastat® demonstrated activity in humans without musculoskeletal toxicities in phase I studies,[58] but failed to show efficacy as maintenance therapy in patients with advanced ovarian cancer.[67] On the other hand, RO 28-2653, another MMPI for MMP-2 and MMP-9, showed promising results in a pre-clinical model for pancreatic cancer.[68] In NB, both MMP-2 and MMP-9 seem to play a major role in tumour angiogenesis and studies evaluating MMPI against these two MMP should be performed in pre-clinical models.

5.5. *Endostatin*

Endostatin, a 20 kDa C-terminal fragment of collagen XVIII, inhibits endothelial proliferation *in vitro* and tumour growth *in vivo* when given systemically.[3] Its anti-angiogenic activity is mediated through zinc-binding of endostatin. Results of phase I studies have shown that human endostatin is well tolerated and does not show drug-related toxicity when administered as daily bolus injections. The effects were lower VEGF-A and bFGF urinary levels, reduced tumour blood flow (measured by dynamic magnetic resonance imaging), reduced EPC levels and up-regulation of apoptosis in endothelial cells. However, recent data have questioned the actual efficacy of endostatin in tumour therapy as the reported results have not been reproducible in other laboratories.[50] Differences in terms of storage, handling and purification techniques may be responsible for differential endostatin behaviour.[58] Two studies have tested recombinant endostatin as a possible

Table 2 – Pre-clinical studies on anti-angiogenesis in NB

Drug	Study result	Reference
Retinoids	Pre-treatment of LAN-5 and GI-LI-N cells with 10^{-5} and 3×10^{-5} M RA for 24 h reduced the ability of conditioned medium to stimulate endothelial cell proliferation	Ribatti et al.[56]
	Treatment of SMH-KCNR cells with RA increases both cell associated and soluble forms of thrombospondin within 24 h	Castle et al.[57]
	13-*cis*, 9-*cis* RA and Ro 13-6307 decreased tumour growth in SH-SY5Y xenografts (subcutaneous (s.c.) in nude rats) significantly	Ponthan et al.[99]
TNP-470	Reduction of tumour growth rate and decreased microvascular density in SH-SY5Y xenografts (s.c. in nude rats)	Wassberg et al.[100]
	Induction of metabolic stress, resulting in chromaffin differentiation and apoptosis in SH-SY5Y xenografts (s.c. in nude mice)	Wassberg et al.[101]
	Reduction of primary tumour volume, size of axillary lymph nodes and liver metastases. Improvement of survival in TBJ and C1300 grafts (s.c. murine neuroblastoma (NB) cell lines)	Nagabuchi et al.[102]
	Inhibition of liver metastasis and increased survival of C1300 cells (murine NB cell line) injected in the spleen	Yoshizawa et al.[103]
	Decreased mean rate of tumour growth in NBL-W-N grafts (s.c. in nude mice) with small tumours but not in animals with large tumours. Treatment inversely correlated with tumour burden	Katzenstein et al.[104]
	Inhibition of tumour growth rate of CHP-134 cells inoculation by tail vein injection in mice, increased apoptotic index in treated tumours	Shusterman et al.[105]
Thalidomide	No significant alteration in tumour growth of NGP xenografts (s.c. in athymic mice). Suppression of angiogenesis (as measured by fluorescein angiography, immunohistochemical staining) and induced apoptosis of endothelial cells	Kaicker et al.[64]
Endostatin	Angiogenesis inhibition or immunomodulation alone resulted in only a modest delay in tumour growth of NB cell grafts transfected with endostatin and the immunogene GFP prior to inoculation (in syngenic immunocompetent mice). In combination, prevention of the formation of appreciable tumours	Davidoff et al.[70]
	Tumor growth slowed down only in endostatin-treated SK-N-AS mice (s.c. in mice). No statistically significant difference in endostatin serum levels	Jouanneau et al.[69]
	Continuous administration of recombinant endostatin resulted in more significant tumour regression than intermittent administration in TNB9 xenograft (s.c. in nude mice)	Kuroiwa et al.[106]
Angiostatin	Treatment with AdK3-HSA (a recombinant adenovirus encoding the human angiostatin kringle 1–3 directly fused to human serum albumin HSA) showed no delay in tumour growth of IGR-N835 xenografts (s.c. nude mice)	Jospeh et al.[72]
Bevacizumab (Avastin®)	Alterations in tumour vessel physiology allowing improved delivery and efficacy of chemotherapy in NB-1691 xenografts (orthotopic in SCID mice)	Dickson et al.[79]
	Reduction of SK-N-AS, IMR-32 and SH-SY5Y xenografts (s.c. in mice) without toxicity due to the reduction of angiogenesis. Two–Six-fold increase in serum concentrations of VEGF-A during therapy without faster tumour growth	Segerstrom et al.[78]
DC-101	Inhibition of tumour growth of unfavourable WAC2 xenografts (s.c. in nude mice) potentiated by simultaneous irradiation	Gong et al.[83]
	Significant but transient regression of SK-N-MC, SK-N-AS xenografts, diminished tumour vascularity and inhibited angiogenesis. In combination with low-dose vinblastine: full and sustained regression of large established tumours without an increase in host toxicity or any signs of acquired drug resistance	Klement et al.[82]
SU5416	Reduction in the growth of SH-SY5Y xenografts (s.c. nude mice) without apparent toxicity.	Beckmann et al.[107];
	Suppression of tumour angiogenesis, despite an increase in plasma VEGF-A levels per ml tumour volume during therapy. Combination with chemotherapy increased efficacy	Svensson et al.[108]
	Inhibition of tumour growth in WAC2 xenografts (s.c. in nude mice) by simultaneous irradiation	Gong et al.[83]

anti-angiogenic drug for NB (Table 2). In one report, only a 'deceleration' in tumour growth was reported in endostatin-treated mice when compared to control mice.[69] However, drug efficacy was increased when endostatin was administered as a continuous infusion or combined with an immunomodulating approach.[70,71]

5.6. *Angiostatin*

Angiostatin is a 38 kDa circulating endogenous protein that mediates its anti-angiogenic activity through binding ATP synthetase on the surface of human endothelial cells. By this, angiostatin causes apoptosis of tumour cells and inhibition of endothelial cell migration and tubule formation.[58] Pre-clinical testing of angiostatin in NB has been evaluated in a gene therapy approach using a recombinant adenovirus encoding the human angiostatin kringle 1–3 directly fused to human serum albumin HSA (AdK3-HSA).[72] Intravenous injection of this vector into the human NB IGR-N835 tumour model showed no delay in tumour growth when compared to tumours treated with the empty virus AdCO1. In early-stage tumours, kinetics of tumour occurrence and tumour growth were similar in

both AdK3-HSA and AdCO1-treated animals, respectively. Because of higher VEGF levels measured in the NB IGR-N835 tumours, the VEGF/VGEFR system has been proposed as a more promising target to inhibit NB angiogenesis.

5.7. *Thrombospondin*

Thrombospondin-1 (TSP-1) is a glycoprotein secreted in extra- and pericellular matrixes capable of inhibiting endothelial cell proliferation and migration. TSP-1 knockout mice display an up-regulation of tumour vascularisation with a subsequent increase in tumour growth.[73] TSP-1 has been shown to be silenced in a subset of undifferentiated, advanced-stage NB tumours and cell lines by promoter methylation.[74] In contrast, expression of this angiogenesis inhibitor was present in most localised NB tumours. ABT-510, a peptide derivative of TSP-1, significantly suppressed the growth of NB xenografts established from two different MYCN-amplified cell lines.[75] In combination with the histone deacetylase inhibitor valproic acid, ABT-510 inhibited even more effectively the growth of small NB xenografts compared to single-agent treatment. In animals with large NB xenografts, total cessation of tumour growth was achieved with this treatment approach. Further evaluation of ABT-510 in NB should be performed in early clinical trials.

5.8. *Monoclonal VEGF-A antibody*

Early studies using a monoclonal antibody against VEGF or using VEGF-TRAP (a composite decoy receptor based on VEGF receptor-1 and -2 fused to an Fc segment of immunoglobulin G1 (IgG1)) in a murine model of human NB were performed by Kim et al.[76,77] By combining the chemotherapy agent topotecan with these anti-VEGF strategies, the authors observed partial suppression of tumour growth and significant inhibition of rebound tumour growth. The first pre-clinical study to evaluate bevacizumab in NB demonstrated a significant reduction in tumour growth *in vivo* without toxicity due to the reduction of NB angiogenesis.[78] Recently, another study showed that bevacizumab-mediated VEGF blockade caused alterations in tumour vessel physiology that, in turn, allowed improved delivery and efficacy of chemotherapy.[79] One can imagine a similar impact in a clinical setting where 'normalisation' of the tumour vasculature and improved tumour perfusion could improve the delivery of systemic chemotherapy. This further emphasises that VEGF blockade in NB is an extremely interesting strategy and should be evaluated as it may help to optimise anti-tumour activity when given with correct drug scheduling.

5.9. *Monoclonal antibodies against VEGF receptors*

Several antibodies against VEGFR-2 (KDR) have been developed. Among them, a high affinity fully human anti-KDR antibody fragment[80] provided proof-of-principle that an anti-VEGFR-2 antibody could strongly inhibit tumour growth.[81] DC-101, a monoclonal rat anti-mouse VEGFR-2 antibody, was shown to inhibit the growth of NB cells without *MYCN* amplification when administered alone or in combination with low-dose vinblastine.[82] The rationale was to combine the anti-vascular effects of the low-dose chemotherapy which would selectively enhance VEGF survival signals in endothelial cells of neo-vessels that would in turn be simultaneously blocked by the anti-VEGFR-2 antibody. In fact, a full and sustained regression of large established tumours was achieved by this combination. Activity of DC-101 was also shown in a NB cell line bearing MYCN over-expression,[83] in which tumour growth delay was increased by simultaneous irradiation. Finally, another chimeric monoclonal antibody specific for VEGFR-2, IMC-IC11, is currently being evaluated for anti-angiogenesis efficacy.[84]

5.10. *Small molecules as inhibitors of the tyrosine kinase of VEGF receptors*

Sugen 5416 (SU5416, Semoxinal®) is a specific VEGFR-1 (Flt-1) and -2 (KDR) antagonist capable of blocking VEGF-stimulated Flk-1 phosphorylation.[3,58] SU5416 was evaluated in clinical trials for Kaposi's sarcoma, non-small cell lung (NSCLC), ano-rectal, renal cell, adenoid cystic and basal cell carcinomas in which stable disease was observed after six months of treatment.[58] It was reported to be able to induce apoptosis in gastric cancer by inhibiting tumour angiogenesis. SU5416 also exhibits strong inhibitory effects on primitive tumour growth and liver metastasis of gastric cancer in pre-clinical mouse models. Furthermore, SU5416 has shown efficacy as an angiogenesis inhibitor in NB *in vivo* models (Table 2). Efficacy was increased when SU5416 was given in combination with irradiation or chemotherapy. The current status of TKI of VEGFR in oncology has been reviewed.[85] Recently, we have also given an overview of TKI with special focus on their potential in paediatric solid tumours.[86] Although these small molecules are of special interest for their potential use in NB, data concerning their use in NB have not been published to date.

6. Anti-angiogenesis treatment strategies in neuroblastoma

Today, anti-angiogenic treatment is mostly used in combination with standard chemotherapy [87]. In treatment for colorectal cancer, bevacizumab is administered in parallel with standard chemotherapy.[88] However, recent studies have suggested that administration of conventional or high-dose chemotherapy might actually cause vascular rebound with endothelial cell recovery during rest periods, a process that could counteract the anti-cancer effect of chemotherapy.[89] Other schedules have been proposed for anti-angiogenic molecule administration in combination with classical chemotherapy: (i) administration before chemotherapy could increase the efficacy of chemotherapy via normalisation of the tumour vasculature and (ii) administration afterwards could have an effect on CEP that are mobilised during bone marrow regeneration and might contribute to tumour angiogenesis and vascular repair. In the case of NB, addition of an anti-angiogenic drug to efficient combination chemotherapy warrants evaluation in a clinical trial.

Another strategy to target the tumour endothelium is through administration of protracted low doses of chemo-

therapeutic agents in a combination schedule, a concept called metronomic chemotherapy.[90] Several metronomic chemotherapy protocols are available for paediatric solid tumours. In the study by Sterba et al., 22 patients with relapsed solid tumours were treated with the combined oral maintenance biodifferentiating and anti-angiogenic therapy (COMBAT) protocol using celecoxib, 13-*cis*-retinoic acid and cycles of metronomic temozolomide and low-dose etoposide.[91] Four NB patients were successfully treated with this protocol (2 VGPR, 1 PR and 1 SD). In another study by Stempak et al. using celecoxib in combination with vinblastine or cyclophosphamide, 32 patients with recurrent paediatric solid tumours were treated including three NB patients. Four patients (13%) had durable stable disease (28–78 weeks) although no complete or partial responses were observed.[92] Therefore, metronomic chemotherapy may play a role as a maintenance treatment in NB to consolidate remission and fight minimal residual disease.

A strategy taking into account the formation of lymph vessels and the prevention of tumour cell dissemination via lymph vessels should be taken into account in future anti-angiogenesis strategies. Anti-lymphangiogenesis has been established in animal experiments using antibodies against VEGF-C and VEGF-D[93,94] and as a major result, lymphogenic metastisation could be omitted. Another way to inhibit lymphangiogenesis is via blocking of VEGFR-3 by antibodies or tyrosine kinase inhibitors.[95] However, the efficacy of anti-lymphangiogenesis is controversial as the destruction of lymph vessels could result in a higher interstitial pressure within the tumour leading to increased haematogenous metastisation.[96] Because of limited data, lymphangiogenesis in NB should be further studied in pre-clinical models before evaluating its role in the clinical setting.

Finally, potential toxicity of anti-angiogenic drugs needs to be addressed in the paediatric population. Studies with bevacizumab in adult patients have reported many side-effects including a greater risk of grade 3 hypertension and grade 1 or 2 proteinuria, a slight increase (<2 percentage points) in grade 3 or 4 bleeding and impaired surgical wound healing in patients who undergo surgery during treatment with bevacizumab.[97] Potentially life-threatening events (arterial thrombotic events and gastro-intestinal perforation) have occurred in a small number of patients. In young children and adults free of cardiovascular risk factors, these side-effects may be rare. However, the impact of hitherto unrecognised damage to angiogenesis dependent processes such as skeletal or CNS maturation must be carefully considered when proposing anti-angiogenic therapies in young children.

7. Conclusions and perspectives for anti-angiogenesis in neuroblastoma

Relevant studies on angiogenesis and its regulation in NB is crucial as they highlight the importance of its role in NB tumour biology. The vast majority of pre-clinical study results indicate that specific targeting of a single angiogenic molecule or pathway is most likely to be insufficient and unsuccessful. The most promising anti-angiogenic strategies in NB will most certainly include either drugs that can affect more broadly the different steps of angiogenesis (endothelial cell proliferation, migration and tubule formation), or combinations of agents targeting several mechanisms of tumour angiogenesis (sprouting, co-option, vasculogenic mimicry and vasculogenesis). However, many questions on current concepts as well as on how anti-angiogenic therapies work still have to be addressed in order to optimise their use in the clinical setting.

Only a translational research approach evaluating anti-angiogenesis agents and strategies in regard to standard therapy through pre-clinical models will be able to bring forward the development of anti-angiogenic therapy protocols for NB. Clinical phase I and II trials will answer the question on dosage, toxicity and efficacy of anti-angiogenic drugs in NB.

Conflict of interest statement

None declared.

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