The history and future perspectives of anti-angiogenics

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Abstract

Angiogenesis is the process of formation of new blood vessels from existing ones and it is involved in both physiological and pathological conditions. In 1971, Dr. Folkman hypothesized that for tumour development, an angiogenic "switch" is activated, where the balance between pro-and anti-angiogenic regulators is tipped in favor of the first and induces neovascularization in the tumour. Tumour-associated angiogenesis is crucial for tumour growth, progression, and metastasis. For these reasons, the last decades there has been significant progress in the development of anti-angiogenic strategies. However, these strategies showcase severe limitations in both pre-clinical and clinical setting. Biological differences in anatomy and therapy response between the animal models and the human in clinical trials, low treatment efficiency, development of severe side effects, anti-angiogenic resistance and relapse post-treatment are but a few challenges that current therapies need to overcome. For that reason, development and application of novel approaches is required to improve anti-angiogenic therapy. Combination therapy constitutes the main way to overcome the limitations of conventional treatment. Application of chemotherapy or immunotherapy with administration of anti-angiogenic inhibitors demonstrated positive results with improved therapy efficiency. In this review, an overview of anti-angiogenic therapy is presented, analyzing the applications and limitations of anti-angiogenics and the developed novel strategies.

Layman's Summary

Angiogenesis is a process involved in the creation of new blood vessels. This process is important for the development and the sustainability of all the mammal organisms. In cancer, angiogenesis is often found deregulated. Tumour-associated angiogenesis is involved in its progression to malignancy and metastasis. For this reason, there is ongoing research for understanding the mechanisms of angiogenesis and developing new therapeutic strategies, termed anti-angiogenic therapy. These strategies have showed positive results in reducing the effect of tumour-associated angiogenesis. However, limitations, such as toxic side effects and cancer recurrence after treatment are obstructing effective treatment. Thus, it is necessary to develop novel, improved therapeutic approaches. Novel treatments combine the use of either chemotherapy or immunotherapy with the use of anti-angiogenic drugs. Combination of these therapies have demonstrated positive results in clinical trials and prolonged survivability of the patients. In this review, conventional and novel approaches of anti-angiogenic therapy and their limitations are discussed to provide a collective overview of past, present and future state of anti-angiogenic therapy.

Introduction

Angiogenesis is the formation process of blood vessels and capillaries and is critical for the development and sustainability of mammalian tissues. Mammalian cells cannot be metabolically active unless they are located less than a few hundred micrometres from a blood capillary. Diffusion of oxygen and nutrients from the capillaries results in survival and expansion of the cells. Angiogenesis is regulated by the balance of pro- and anti-angiogenic molecules. However, this process can be found deregulated in diseases and especially in cancer[1]. For cancer treatment, angiogenesis provides a double effect; on one hand tumours produce pro-angiogenic factors to grow and metastasize, thus inhibiting the production of these factors can reduce tumour growth and spread, on the other hand reducing the vessel creation in close proximity to the tumour results in an inefficient supply of anti-cancer drugs[2]. The above situation creates a dilemma on how we should apply anti-angiogenic therapy on cancer treatment. Based on research performed on the field of angiogenesis, understanding of the molecular regulatory mechanisms of angiogenesis has been improved and can be used to optimize cancer treatment. In this review, we will analyse the relation between cancer and angiogenesis, how this knowledge has been applied in clinical trials, the obstacles, novel anti-angiogenic strategies and the future perspectives of anti-angiogenic therapy.

Cancer and angiogenesis

Angiogenic switch

Tumours, as healthy tissue, require to be metabolically active to sustain their composition, to expand their growth and finally invade and metastasise. In order to achieve that angiogenesis is induced to create tumour-associate neovascularization. More importantly, Folkman hypothesized in 1971, that in order for tumours to transition from a quiescent to an invasive phenotype, angiogenic properties must be acquired. An early event in tumour development termed as an angiogenic "switch" is responsible for the acquisition of angiogenic properties [3], where the proportion of pro- and anti-angiogenic factors is imbalanced, with the first being the dominant[4] (Fig. 1).
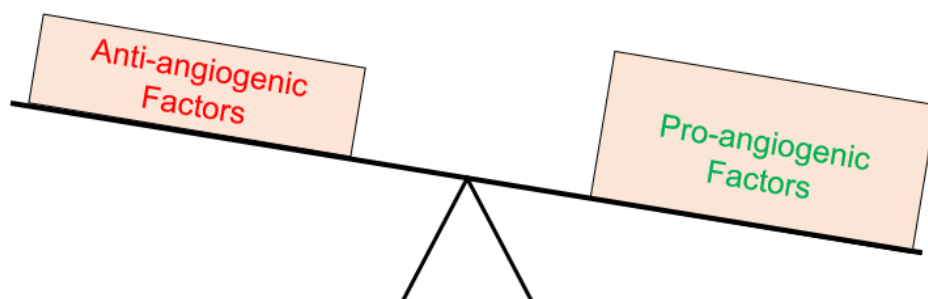
Figure 1: The balance hypothesis of the angiogenic switch. The quiescent vasculature can be set in motion by for the formation of new vessels by an angiogenic "switch" mechanism. The imbalance between the presence of pro- and anti-angiogenic factors regulates the "switch" activation. In tissues, where either there is an absence of pro-angiogenic factors, or the concentration of anti-angiogenic

factors is higher that the concentration of pro-angiogenic factors the angiogenic "switch" remains in an inactive state. In tumours, the angiogenic "switch" is activated by either an excess of pro-angiogenic factors or by loss of anti-angiogenic expression, leading to the growth of tumour-associated vasculature. Adapted from Douglas Hanahan and Judah Folkman, Cell Press, 1996[3].

Mechanisms of tumour angiogenesis

The angiogenic "switch" is an effect that remains active and is maintained throughout tumour development and induces tumour-associate neovascularization by three mechanisms: angiogenic sprouting, intussusceptive growth and angioblasts differentiation. In angiogenic sprouting the network of vessels is expanded by induction of endothelial sprouting. In intussusceptive growth, tumours induce the remodelling of the existing vascular network by inserting interstitial tissue columns. Lastly, tumours induce differentiation of endothelial precursors (angioblasts) from the bone marrow or the peripheral blood to create new vessels or embed in the lining of existing vessels[5]. By activating the above mechanisms the tumour can form vessels to support its growth.

Structure and function of tumour vessels

Tumour vessels compared to the normal vessels are characterized by abnormality both on their function and their structure. A unique characteristic of tumour vessels is their chaotic architecture. Their structure is twisted and there is excessive branching, while the vessels have increased dilation and uneven diameter, leading to an increased blood flow to the cancer cells[6]. Finally, due to their unique structure and composition, vessels walls have many openings resulting in high permeability[7,8], leading to hypoxic and acidic areas in the tumour[9]. These characteristics result in lower treatment efficiency[10] and enhance the survival of cancer cells with a more invasive phenotype, leading to larger number of metastatic sites[11]. The basis of this malformed architecture has been credited on the imbalance of various angiogenic regulators[12].

Regulation of angiogenic "switch"

For the induction of tumour-associated angiogenesis, the angiogenic "switch", the equilibrium between pro- and anti-angiogenic factors must be derailed. Various molecules are involved in the regulation of angiogenesis. Major activators of angiogenesis are: Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor (FGF), Angiopoietin-1 (Ang1), Platelet-Derived Growth Factor (PDGF) and Matrix Metalloproteinases (MMPs)[13] (Table 1). VEGF induces angiogenesis by regulating hematopoietic stem cell development, inducing remodelling of the extracellular matrix (ECM) by MMPs and regeneration of inflammation cytokines[14]. FGFs have a multidimensional effect on endothelial cell affecting their proliferation, their differentiation, their migration, their morphogenesis, their survival and stimulate ECM degradation by inducing the secretion of proteases[13]. Ang1 is responsible for endothelial cell survival and vascular branching, vascular stability and pericyte recruitment[15]. PDGF main function is the recruitment of pericytes and the enhancement of angiogenesis by inducing VEGF expression in tumour endothelial cells (TECs)[16]. Lastly, MMPs induce angiogenesis by releasing ECM-bound angiogenic growth factors, by aiding in the detachment of pericytes from vessels undergoing angiogenesis and by exposing pro-angiogenic integrin binding sites[17]. The expression of the pro-angiogenic factors in the tumour context can be induced by multiple factors such as a hypoxic tumour environment, activation of oncogenes and inhibition of tumour suppressors[18,19,20].

Opposing the function of the pro-angiogenic factors there are multiple anti-angiogenic factors that inhibit angiogenesis, and their function is disrupted in tumour-associated angiogenesis (Table 2). Examples of molecules that withstand vessel creation are: Angiostatin, Endostatin, Platelet Factor 4 (PF4) and Thrombospondin-1 (TSP-1)[13]. Angiostatin acts as a proliferation and migration inhibitor of endothelial cells, promotes of endothelial cell apoptosis, and induces the expression of other anti-angiogenic factors[21]. Endostatin main function is the inhibition of MMPs effect on the proteolytic degradation of the ECM[22]. PF4 is a strong angiogenesis inhibitor exhibiting anti-angiogenic effects by binding pro-angiogenic growth factors and blocking endothelial migration and proliferation[23]. Finally, TSP-1 has an inhibitory effect on proliferation, migration, and survival of endothelial cells[24].

Table 1: Angiogenesis activators

Pro-angiogenic Factors	Protein components	Function
VEGF Family members and VEGF receptor (VEGFR)[5,14]	VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGFR-2 , VEGFR-3	Stimulation of angiogenesis, increase permeability and leukocyte adhesion
FGF Family members and FGF receptor (FGFR)[25]	FGF-1, FGF-2 and FGFR-1	Stimulation of angiogenesis, endothelial cell proliferation
Angiopoietins[15]	Ang1	Endothelial cell survival, Stabilisation and branching of vessels
PDGF Family members and PDGF receptor (PDGFR)[16]	PDGF-A, PDGF-B, PDGF-C, PDGF-D and PDGFRα , PDGFβ	Pericyte recruitment
Hypoxia-Inducible Factor (HIF)[26]	HIF-1α , HIF-2α	Stimulation of angiogenesis, induction of pro-angiogenic gene expression
Neuropilin (NRP)[27,28]	NRP-1, NRP-2	Enhancement of cell migration
MMPs[17]	MMP-2, MMP-9, MMP-14	ECM remodelling, release and activation of growth factors
Integrins[5]	$\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$	Receptors for ECM molecules and MMPs function

Table 2: Inhibitors of angiogenesis

Anti-angiogenic Factors	Protein components	Function
Angiostatins[21]	Angiostatins	Suppression of angiogenesis
Endostatins[22]	Endostatins	Inhibition of MMPs effect
Thrombospondins[24]	TSP-1 , TSP-2	Inhibition of endothelial cell survival and migration
Angiopoietins[29]	Ang2	Counteracts the effects of Ang1
PF[23]	PF4	Pro-angiogenic factor binding and inhibition of endothelial cell migration and proliferation
MMP Inhibitors[30]	TIMP2	Inhibition of MMPs
Interferons (IFN)[31,32,33]	IFN-α, IFN-β,IFN-γ	Inhibition of endothelial cell migration

Unravelling the regulators of angiogenesis, it is clear that there is a set of angiogenic inducers and angiogenic inhibitors that affect directly or indirectly the endothelial

cells, which are the *de facto* mediators of neovascularisation. This realisation raises a crucial question; how all these factors are integrated by the signalling pathways in the endothelial cells that regulate the decision between quiescence and neo-angiogenesis, but most importantly by clarifying this regulatory mechanism of the angiogenic switch, how can we apply this knowledge to develop novel treatments in clinical oncology to restrict angiogenesis by manipulating the balance of angiogenic factors.

Challenges of clinical trials with anti-angiogenic drugs

Classification and strategies of developing angiogenic inhibitors

While the balance hypothesis for the angiogenic "switch", has been proposed by Folkman in 1971, it became widely acceptable by the middle of the decade, when plenty of laboratories decided the focus their research on developing angiogenic inhibitors. However, discovery of angiogenic inhibitors would be a challenge for scientists, due to the lack of valid bioassays for angiogenesis[34]. Finally, in 1980 the first angiogenic inhibitor was announced by Folkman's laboratory[35] This event paved the road for the discovery of additional angiogenic inhibitors in the decades to come.

The main strategies for the discovery and the development of endogenous angiogenic inhibitors were described early by Folkman and consist of these three research approaches: a) induction of downregulation of a specific molecules that induce the active state of angiogenic "switch" in tumorigenesis, b) transduction of the gene results in inhibition of angiogenesis and c) extraction and purification of the target molecule and administration to animal models lead to suppression of angiogenic activity[36]. These approaches are still in use in research, however with the development of computational approaches and improvement of genetic screening, computational models and analysers can deliver more results with higher efficiency[37].

The discovery of a plethora of angiogenic inhibitors revealed a distinct classification based on their function. Particularly, tumour-associated neovascularization in tumour niche can be inhibited either directly by targeting endothelial cells in the growing vasculature or indirectly by targeting either tumour cells or the other tumour-associated stromal cells. Therefore, angiogenesis inhibitors can be classified into direct and indirect inhibitors[38]. Characteristic examples of direct endogenous are the molecules of angiostatin and endostatin. These inhibitors prevent endothelial cell proliferation and migration in response to angiogenic promoters[39]. Indirect inhibitors of angiogenesis, such as two well-used drugs, Iressa an Epidermal Growth Factor receptor (EGFR) Tyrosine Kinase inhibitor (TKI) and the monoclonal antibody bevacizumab, which prevents the expression or the function of pro-angiogenic molecules[34].

The development of research approaches for the discovery of angiogenic inhibitors was a long-term process, especially before the development of modern equipment and computational models. However, it was a necessary step for the transition from the laboratory to the clinical studies.

Pre-clinical studies with angiogenic inhibitors

The evaluation of an anti-angiogenic factor for further use in clinical trials required the development of reliable bioassays. The absence of such assays constituted a serious issue in the early days, until 1980 where growth and long-term maintenance culture of vascular endothelial cell established by Folkman's laboratory[40]. Since then a plethora of semi-quantitive and quantitive in vitro assays have been established that involve sprouting angiogenesis, such as the cornea micropocket assay[41] and growth assay of blood vessels in 3D ECM-matrices such as collagen[42]. The creation of such systems has been the basis for the evaluation of the discovered angiogenic inhibitors in vitro.

However, the boost required for transitioning from the bench to the clinical trials was the use of animal models for tumour-associated angiogenesis therapy. Specifically, in 1993 Kim *et al.* demonstrated that the inhibition of the VEGF result in suppression of tumour growth in vivo[43]. In this landmark study for in vivo tumour-associated angiogenesis research, rhabdomyosarcoma, glioblastoma and leiomyosarcoma cancer cell lines were injected into nude mice and treatment of the mice with a monoclonal antibody specific for blocking VEGF resulted in inhibition of tumour growth in vivo. In another study by Auguste *et al.* proved that inhibition of FGF pathway resulted in inhibition of tumour growth and reduced number of blood vessels in immunodeficient mice with a depleted tyrosine kinase domain FGF receptor (FGFR) 1 and 2[44]. These results and other pre-clinical studies in animal models resulted in a better understanding on the mechanisms of anti-angiogenic drugs and constituted the foundation for the translation of the pre-clinical results to the clinical trials.

Challenges of pre-clinical studies

Success from *in vitro* and *in vivo* assays on the development and the effect of anti-angiogenics was the basis for the translation of pre-clinical data to the clinical setting. Nevertheless, these assays encountered some vital issues that justified caution in clinical trials. The most crucial issue involved the biological differences between the animal and the human setting. Organ anatomy between the human and the animal organism, mechanical properties, such as stiffness and finally the difference in gene expression are obstacles for the development of anti-angiogenics. Another issue is that animal tumours usually are induced in the subcutaneous layer, which is a rare site from human tumours, has a typical angiogenic properties with altered immune cell composition and thus this site of mouse tumour growth inappropriate and making difficult to predict the response in human tumours[5,45]. Furthermore, a fundamental issue of pre-clinical research in studies with angiogenic inhibitors is the use of tumour regression and not eradication as an end point. Often relapse can occur by a small number of surviving cancer cells, which are not considered in short term studies[5]. Additionally, in the majority of pre-clinical models a specialised targeting anti-vascular strategy is followed, which results in acute tumour regression. Use of such compounds that can cause such a dramatic reduction in tumour size might accompanied by major toxic side effects[12,46]. On the other hand, most anti-angiogenic drugs do not cause rapid tumour regression. Thus, administration of a specific compound in vivo might not yield rapid tumour regression over a short amount of time and potentially falsely estimated as non-effective[12]. Lastly but most importantly, the administration of anti-angiogenics in animal models occurs in newly developed tumours with immature vessels that are vulnerable to anti-angiogenics. In

contrast, in the human setting diagnosis occurs usually in a late stage where the tumour is well established, and vasculature is matured, thus being more resistant to the drug effects[12,47]. In conclusion, there were several obstacles that have hindered clinical translation of anti-angiogenics. However, some encouraging results have led to the administration of many compounds in cancer patients.

Clinical studies with angiogenic inhibitors

From the formulation of the angiogenic "switch" hypothesis by Folkman in 1971, until the approval of the first anti-angiogenic drug by the Food and Drug Administration (FDA) agency in 2004, many different anti-angiogenic factors were discovered, with Protamine discovered by Taylor and Folkman being the first. However, Protamine as other compounds would not reach the clinical trial level, due to cumulative toxicity in long-term administration and low ability to prevent angiogenesis[48].

It would take 32 years until the first approved anti-angiogenic drug; bevacizumab would be used in clinical trials against metastatic renal cancer carcinoma. The study proved that patients receiving a high dose of bevacizumab, demonstrated a significant prolongation in the progress of the disease in comparison to the placebo group[49]. The approval of bevacizumab by the FDA constituted a pioneering moment for the testing of anti-angiogenics in clinical trials.

Based on successful pre-clinical results, a plethora of other anti-angiogenic drugs are tested in clinical trials. These trials follow four strategies for the regression of tumour-associated angiogenesis: a) interfere with angiogenic ligands, their receptors or downstream components of the signalling cascade, b) deliver or upregulate endogenous angiogenesis inhibitors, c) inhibition of factors that promote cancer cell invasion through the blood vessels and d) obstruction of endothelial cell proliferation[50]. Clinically, other factors, besides the end result, are considered such as the whether the anti-angiogenic has a multitarget effect on the signalling pathways that promote angiogenesis, whether tumours can effectively develop resistance after long-term administration, whether the substance induces other anti-angiogenic pathways, its specificity and finally its impact on system toxicity and potential side effects[13].

Following these strategies, currently there are plenty of approved anti-angiogenic drugs (Table 3). The most applied in anti-angiogenic therapy amongst the currently available are: bevacizumab, sunitinib, pazopanib, and endostar[13,51].

Bevacizumab is an anti-vascular endothelial growth factor humanised monoclonal antibody targeting all isoforms of VEGF-A[49] and as a result it inhibits tumour-associated angiogenesis, restricts cancer cell growth, and prevents tumour spread[52]. It is effective against multiple cancer types, since it has been approved besides renal cell carcinoma, for treatment against colorectal cancer, lung cancer and glioblastoma[53]. Sunitinib is a receptor tyrosine kinase inhibitor (RTK), effecting multiple targets, specifically the VEGF receptor (VEGFR), PDGF receptor (PDGFR), colony-stimulating factor 1 (CSF1) and stem cell factor receptor (c-KIT)[13,51]. Its multifaceted inhibition results in reduction of tumour vascularization and apoptosis of cancer cells[51]. Since, its approval from FDA it has been used against renal cell carcinoma and gastrointestinal tumours[51]. Similar to sunitinib, pazopanib another tyrosine kinase inhibitor has passed through the phases of the clinical trials and has been used against renal cell carcinoma, epithelial ovarian cancer, and soft tissues

sarcoma[51]. Lastly, endostar constitutes of a human recombinant form of endostatin and it is used against the treatment of non-small-cell lung cancer[54].

All the above drugs have been approved for inhibiting tumour growth and tumour-associated angiogenesis. However, as it becomes apparent their efficacy is restricted into a small number of cancer types. Focusing on bevacizumab, a number of clinical trials that have used the drug demonstrated a modest response on anti-VEGF monotherapy. For example, while bevacizumab initially was approved for the treatment of metastatic breast cancer by FDA, it has been recalled due to a limited response of only 6.7% of cancer patients[55]. Furthermore, treatment with bevacizumab has been associated with severe side effects, including hypertension, proteinuria, and gastrointestinal perforations and bleeding. Moreover, post-treatment results reveal increased percentage of relapses due to invasion and resistance[56]. Thus, it becomes clear that application of monotherapy with anti-angiogenics has limitations in its application.

Table 3: Anti-angiogenic drugs currently used in clinics

Drug	Function	Approved indications	Year of approval
Bevacizumab[49]	VEGF antibody	Colorectal cancer and Non-small-cell lung carcinoma	2004
Lenalidomide[57]	Inhibitor of VEGF expression	Myelodysplastic syndrome	2004
Sorafenib[58]	RTK Inhibitor	Renal cell carcinoma	2005
Endostar[54]	VEGF Inhibitor	Non-small-cell lung carcinoma	2005
Sunitinib[51]	RTK Inhibitor	Gastrointestinal tract cancer, Renal cell carcinoma	2006
Thalidomide[59]	Tumour Necrosis Factor-α (TNF- α) and Interleukin-6 expression inhibitor	Multiple Myeloma	2006
Pazopanib[51]	RTK Inhibitor	Renal cell carcinoma	2009
Cabozantinib[60]	RTK Inhibitor	Renal cell carcinoma, Medullary thyroid cancer	2011
Ramucirumab[61]	VEGFR antibody	Non-small-cell lung carcinoma	2014

Challenges of clinical studies

The limitations of anti-angiogenic therapies become apparent through comparison of the number of anti-angiogenic substances experimented in clinical trials versus the number of anti-angiogenic approved by the responsible regulatory agencies. In particular, there are four main limitations that hinder the development and the

approval of new anti-angiogenic agents and therapy optimization: a) drug-associated toxicity, b) lack of biomarkers, c) drug resistance and d) tumour heterogeneity[12,62,63].

A major issue for the application of anti-angiogenic therapies is the toxicity accompanied by the chronic administration of anti-angiogenics. This issue usually goes undetected in the pre-clinical stage and the early clinical trials, but it surfaces after long-term use of newly developed drugs. The toxicity problem affects two aspects of the patient's health, the direct interaction between the angiogenic, the clotting and fibrinolytic pathways and the normal vascularization development. In the first aspect, the interrelationship of the pathways regulating coagulation, fibrinolysis and angiogenesis raise the possibility of bleeding or coagulation disorders or even the development and the recrudescence of cardiovascular defects in patients[64]. On the second aspect, administration of anti-angiogenics might impair or interfere with the normal human vasculature. Wound healing or the development of embryonic angiogenesis can be impaired by the use of anti-angiogenics[65]. This issue highlights the importance of optimizing the selection and development of drugs that are specialised in targeting only the tumour-associate vascularization.

The drug-associated toxicity is closely related to another fundamental issue in clinical studies, the lack of precise biomarkers for optimization of dosage of anti-angiogenic drugs. Usually, the classic approach for clinical studies is the achievement of highest dosage administration. While this approach might be effective in cytotoxic chemotherapies, in specialised therapy, such as anti-angiogenic therapy, the effect on the tumour can be accomplished before the development of toxic side effects[66]. Available anti-angiogenic agents main fashion of function is altering or reducing the tumour-associated vasculature and for this reason investigators are focused on developing suitable markers of the anti-angiogenic modulation of tumour vasculature. This task is challenging, due to variations in the tumour types, histology, size, and grade of differentiation[63,67,68].

Optimizing the dose through the development of biomarkers is beneficial, because it will reduce the occurrence of the most common issue of anti-angiogenic therapy, which is drug resistance. Although the clinically approved angiogenic drugs provide a satisfying effectiveness against tumour neovascularization, metastasis and mortality occur due to the development of an innate or adaptive resistance towards treatment[13]. The mechanisms of anti-angiogenic drug resistance include revascularization, protection of the tumour vessels, increased invasiveness of the cancer cells and augmented metastasis through different modes of vascularization[69]. The aetiology behind the acquisition of drug resistance involves amplification of pro-angiogenic genes, recruitment of pro-angiogenic factors and heterogeneity of tumour cells[13,70,71].

Tumours consists of a mass of cancer cells that in the later stages due to increased genetic instability demonstrate a non-uniform identity. As a result, increased clonal heterogeneity is present in the tumour leading to diverse histopathology and regions that differ in the context of proliferation, differentiation, vascularity, inflammation, and invasiveness[3]. For anti-angiogenic therapy, this is an obstacle to overcome for effective treatment and abolishment of drug resistance is heterogeneity of the TECs[71].

Heterogeneity of tumour endothelial cells and anti-angiogenic therapy

TECs are responsible for the inner lining of tumour blood vessels and consist the primary target of anti-angiogenic therapies. TECs are abnormal compared to the normal endothelial cells (NECs) and demonstrate intertumoral and intratumoral heterogeneity. Compared to the NECs, TECS have differences in their genetic profile, the protein receptor surface expression, their functional output, and their interaction with the tumour niche[72]. The basis behind TEC heterogeneity is the instability in the genome of TECs compared to NECs. TECs demonstrate an aneuploid karyotype with large chromosomal aberrations and abnormal centrosomes[73]. Thus, understanding heterogeneity in the context of TECs is important due to its impact on anti-angiogenic therapy.

TEC heterogeneity comprises a major obstacle for anti-angiogenic therapies. The heterogeneity of different types of TECs differs by grade of tumour malignancy, and their microenvironment. As a result, a universal anti-angiogenic therapy cannot be applied to all the patients. Furthermore, due to their heterogeneity different TECs can express a diverse set of angiogenesis receptors. A TEC receptor-specific phenotype makes different subpopulations of TECs being resistant to specific inhibitors, thus increasing the probability of drug resistance and subsequently reducing the effectiveness of anti-angiogenic therapy. Moreover, expression of multiple pro-angiogenic factor receptors indicates that inhibiting a specific pathway might not affect tumour neo-angiogenesis[71]. Lastly, a small subpopulation of TECs resistant to an anti-angiogenic agent after treatment, can prompt the creation of new vessels and relapse of the tumour[74,75]. Thus, in order to design and apply a more effective treatment, it becomes necessary to find ways to overcome TEC heterogeneity-related issues.

To counter the impact of tumour heterogeneity in anti-angiogenic therapy it is necessary to identify the different subpopulations of TECs. This can be achieved by developing biomarkers that reflect heterogeneity in tumour vasculature. TEC-specific markers comprise of cell surface proteins, secreted proteins, and cytoplasm proteins. For the effective targeting of TECs, the variety of different markers should be documented, subsequently allowing us to identify TECs and NECs, to recognise the resistances and the vulnerabilities of TECs in the context of a specific tumour type and to optimise drug delivery[71].

Another way to oppose the effect of TEC heterogeneity is the design and the development of novel anti-angiogenic strategies. Small interfering RNAs (siRNAs) have been proposed as a potential useful strategy, due to their ability of silencing genes that promote the diverse identities of different TEC subpopulations[71]. In fact, in 2014 Sakurai *et al.* developed an siRNA mediated VEGFR2 knockdown in renal cell carcinoma, which resulted in inhibition of tumour growth[76]. Considering the impact of TEC heterogeneity in anti-angiogenic therapy, identification of the composition of TEC population and optimization and development of novel strategies, could lead to overcoming the limitations of current treatment.

Novel anti-angiogenic strategies for cancer treatment

Chemotherapy and anti-angiogenic therapy

Challenges in the clinical trials and low efficacy of anti-angiogenic monotherapy were the major drivers for the design and the development of novel anti-angiogenic strategies and specifically the combination strategies. In 2000 Browder *et al.* established that applying chemotherapy on an "angiogenic" long-term and low-dose schedule on Lewis lung carcinoma and EMT-6 breast cancer drug resistant tumours in mice resulted in a more effective inhibition of tumour growth than the conventional high-dose schedule. Furthermore, this study demonstrated that the combination of the angiogenic schedule with the TNP-470 angiogenic inhibitor completely eradicated all the remained drug resistant tumours[77]. For this reason, this study was fundamental for the development a novel cancer therapy, termed metronomic chemotherapy.

The term metronomic chemotherapy describes the optimization of anti-angiogenic effects of chemotherapy by administrating cytotoxic chemotherapy agents in small doses below the maximum tolerated dose (MTD) on a frequent schedule in an uninterrupted manner, for extended periods[78]. Initially, application of solely frequently low-dosage chemotherapy in clinical trials yielded satisfying results by reducing the tumour size of patients that showed no response in treatment with MTD conventional therapy by having fewer side effects[79]. However, relapse occurred in the majority of the patients that had been benefited by metronomic therapy[80]. This was the main rationale on why the protocol of Browder *et al.* was altered and administration of an anti-angiogenic inhibitor in combination with metronomic chemotherapy was applied, thus improving the efficiency of this type of chemotherapy by minimizing the side effects.

The logic behind the combination of metronomic chemotherapy with anti-angiogenics was based on two main considerations. Firstly, there is extended evidence that the anti-proliferative or pro-apoptotic effect of cytotoxic chemotherapy agents on human endothelial cells in vitro is inhibited by VEGF[81,82]. Secondly, application of chemotherapy might be responsible for the upregulation of VEGF expression and the production of pro-angiogenic factors in tumour cells[83].

The effect of combination treatment has been confirmed both in the laboratory context as well as in the clinical context (Table 4). Experiments of combination therapy using xenografts models of neuroblastoma and other types of cancer proved that low-dose administration of vinblastine, a cytotoxic chemotherapy agent in combination with DC101, a monoclonal anti-VEGFR2 antibody caused a large regression of the tumour size, while the treatment could be maintained for several weeks without any side effects on immunodeficient mice[84]. Furthermore, standard chemotherapy agents could not completely eliminate drug resistance in tumours, however in the same murine tumours, combination with an angiogenic inhibitor, resulted in massive tumour regression and in some cases complete eradication of resistant tumours[77]. Moreover, in a randomized Phase-III clinical trial combination of standard chemotherapy with bevacizumab caused prolonged survival for patients with metastatic colorectal cancer compared to patients that were treated only with standard chemotherapy[85]. Lastly, in all studies where metronomic dosing was compared with conventional MTD, the conventional dosing was found to be inferior to metronomic in both the context of toxicity and survival[86,87]. Thus, the combination of metronomic chemotherapy with the administration of an anti-angiogenic substance

can provide both the cytotoxic and anti-angiogenic effects by improving the efficiency of anti-angiogenic therapy, while reducing the toxicity of side effects.

Table 4: Clinical studies of combination chemotherapy treatment with anti-angiogenics, modified from Jie Ma *et al.*[88]

Combination Therapy	Indications
Bevacizumab and Irinotecan/fluorouracil/leucovorin	Metastatic colorectal cancer ,Phase III
Bevacizumab and Paclitaxel	Metastatic breast cancer, Phase III
Bevacizumab and Paclitaxel/carboplatin	Recurrent Non-small-cell lung carcinoma , Phase III
Sorafenib and Paclitaxel/carboplatin	Non-small-cell lung carcinoma, Phase III
Pazopanib and Paclitaxel	Refractory advanced ovarian cancer, Phase II

However, there is a limited number of studies that have investigated the success of combination therapy and only a small number of chemotherapy agents have been tested. Moreover, while most studies showed that low dosage is sufficient to reduce the tumour size, other pre-clinical studies showed that, it would be beneficial to use in some cases MTD chemotherapy followed by metronomic chemotherapy, due to tumours being responsive only to a certain chemotherapy agents administered by the conventional function[84,89].

Immunotherapy and anti-angiogenic therapy

An established hallmark of tumour development is the ability to evade immune destruction. One parameter that confers to this hallmark is the recruitment of regulatory immunosuppressive T cells (Tregs) for the inhibition of cytotoxic lymphocytes function[3]. Blockage of immune-checkpoint proteins such as the cytotoxic T lymphocyte 4 (CTLA-4) and the T cell receptor programmed cell death protein 1 (PD-1) as well as the ligands of the PD-1 (PD-L1 and PD-L2) has demonstrated both in pre-clinical and clinical studies that increase cancer cell cytoxicity mediated by the immune cells[90,91,92]. Pro-angiogenic molecules have been associated with a variety of immunosuppressive effects, such as antigen presentation T cell priming, trafficking and infiltration[93]. Thus, such observations have promoted the development of anti-cancer therapy that combines immunotherapy and anti-angiogenic inhibitors.

To develop a combination therapy, it is important to understand the mechanism of immunomodulation by the angiogenic molecules. Pro-angiogenic factors can affect the interaction between the immune cells and the cancer cells by two ways: a) have a directly effect on immune cells and b) have an indirect effect on endothelial cells[93]. Molecules that effect the immune regulation directly are the VEGF and the PDGF. VEGF can inhibit the maturation of the antigen-presenting dendritic cells and differentiation of progenitor cells into CD8$^+$ or CD4$^+$ by respectively binding directly on the VEGFR2 of these cells[94,95]. Additionally, the PDGF family has been associated with the suppression of the immune system. A characteristic example is the heterodimer PDGFAB, which has been shown that inhibit dendritic cell maturation

and induces the expression of the immunosuppressive interleukin 10 (IL-10)[96]. Endothelial cells indirectly suppress immune cell cytotoxic function by the creation of a selective immune barrier and the expression of specific adhesion molecules and chemokines. In particular, VEGF interacts with prostaglandin E2 and IL-10 and induces the expression of the FAS antigen ligand (FASL), which reduces immune cell invasion by creating a selective barrier in tumour vasculature[97]. An additional factor, FGF2 confers to the indirect effect by inhibiting the adhesion natural killers (NK) cells in the vessels, by altering the expression of the molecules ICAM1 and VCAM1 of the endothelial cells. Thus, it is apparent that the pro-angiogenic factors can modulate the immune system in such way that tumour cells can evade and suppress immune-related cytotoxic effects.

The immunosuppressive effect of inducers of angiogenesis, is the main rationale for the development of a combination therapy with the administration of anti-angiogenics and immunotherapy. Currently, three types of immunotherapy combined with anti-angiogenics exist: a) immune-checkpoint inhibitors (ICI), b) tumour vaccines and c) adoptive immune-cell transfer[92].

The use of ICI is considered a valid option for combination therapy, due to positive results both in animal models, as well as in early clinical trials. In particular, use of axitinib, a VEGFR1-3 inhibitor in combination with anti-CTLA-4 antibodies resulted in an increased number of T cell effector cells, reduced immunosuppression by myeloid-derived suppressor cells (MDSCs) and extended survival in a melanoma mouse model[98]. Moreover, in a CRC mouse model use of anti-PD-1 antibodies in parallel with anti-VEGF antibodies resulted in reduction of tumour size, compared to mice that were administrated with each monotherapy alone[99]. In the clinical context, the treatment with ipilimumab and bevacizumab in a cohort of melanoma patients, demonstrated increased lymphocyte infiltration in the tumour, compared to patients which received monotherapy[100]. A similar effect was observed in another study, were the use of atezolizumab and bevacizumab showed increased T cell infiltration in patients with renal cancer carcinoma[101].

A different approach for of combination treatment, that yielded positive results is the use of tumour vaccines and anti-angiogenic drugs. Administration of a peptide-loaded dendrite cell vaccine against B16 melanoma cells in combination with sunitinib resulted in reduced number of T regulatory cells and increased cell infiltration in the tumours[102]. Additionally, in clinical trials use of a nanoparticle-based vaccine combined with the administration of low-dosage sunitinib in a cohort of melanoma patients, decreased the number of MDSCs and T regulatory cells, while significantly reducing tumour growth[103]. Lastly, treatment with an anti-VEGFR2 antibody before injection with an anti-NEU (known as HER2) vaccine demonstrated improved antitumour response, in comparison with using the vaccine alone[99].

Finally, the transfer of adoptive immune cells has provided valid results in use with anti-angiogenic substances. Tumour-antigen-specific CD8+ T cell transfer combined with sunitinib administration in hepatocellular carcinoma xenografts resulted in complete tumour regression[104]. In another melanoma murine cancer model adoptive transferred T cells administrated with an anti-VEGFR2 antibody resulted in increased T cell infiltration in the model[105].

Table 5: Clinical studies of combination immunotherapy treatment with anti-angiogenics, modified from Kabir A. Khan *et al.*[92]

Combination Therapy	Indications
Bevacizumab and Atezolizumab	Renal cancer carcinoma - Phase III. Metastatic colorectal cancer – Phase II
Bevacizumab and Durvalumab	Glioblastoma, Phase II
Bevacizumab and Pembrolizumab	Glioblastoma - Phase II, Non-small-cell-lung carcinoma – Phase II
Sorafenib and Nivolumab	Advance Hepatocellular carcinoma, Phase III
Ramucirumab and Durvalumab	Gastro-oesophageal adenocarcinoma, Phase I
Ramucirumab and Nivolumab	Gastric cancer, Phase I/II
Pazopanib and Pembrolizumab	Renal cancer carcinoma, Phase I/II

The above approaches have demonstrated positive results and provided much promise for treatment of cancer patients (Table 5). However, there are limitations to these strategies. In particular, in phase I clinical trials of renal cancer carcinoma patients with tremelimumab, an anti-CTLA-4 antibody, and sunitinib, severe toxic side effects were developed[106]. In addition, other studies showed that the combination treatment with sunitinib against the B16 marker for melanoma, lacked T cell responses in the lymph nodes, compared to use of the vaccine alone[107]. Lastly, it has been observed in mice models that tumours can develop resistance to VEGF inhibition, making the combination therapy inefficient[108]. Through these limitations in use of combination immunotherapy and anti-angiogenic therapy, it is clear that researchers have to evaluate which strategy is more beneficial and optimize the current strategies to achieve lower toxic effects and avoid tumour resistance.

Normalisation of tumour vascularisation through anti-angiogenic therapy

A fundamental obstacle for effective anti-angiogenic therapy is the abnormal structure and function of tumour vasculature. Abnormal structure results in impaired tumour blood flow and in combination with the mechanistic pressure generated by the proliferating cancer cells creates an anomalous tumour microenvironment characterised by increased hypertension, hypoxia, and acidosis. These conditions interfere and inhibit drug delivery to the tumour body. Hypoxia renders cancer cells resistant to the effect of chemotherapy and radiotherapy, increases their genetic instability and in combination with acidic pH, hinders the cytotoxic functions of immune cells[109]. This realisation lead Jain *et al.,* to hypothesise that normalising the structure and correcting the tumour microenvironment, would lead to a more efficient treatment[110].

In the past, many attempts for an effective anti-angiogenic therapy or chemotherapy have failed, due to the abnormality of the tumour vessels. These approaches used a high concentration of drugs and oxygen in order to treat the patient. However, the increased permeability and the discontinuous walls of the vessels[7] resulted in a flawed delivery system where there are still regions on the tumours that are

inaccessible for drugs delivery[111]. The solution for this problem is to "fix" the tumour vasculature and normalise it, thus more cancer cells will be affected by the drugs and the oxygen administration.

To achieve a normalised tumour vasculature, the driving force of the abnormality, angiogenic "switch" must be countered. The imbalance of pro- and anti-angiogenic factors is responsible for the derailed structure and function of the tumour vessels, thus Jain speculated that if the balance is restored then the tumour vessels can acquire a normal-like structure[109].

There is satisfying evidence that VEGF is a good target to achieve normalisation of tumour vasculature. Firstly, VEGF is responsible for the proliferation of endothelial cells, expression of adhesion molecules and regulates vessel permeability[5]. Furthermore VEGF is responsible for the initiation and the maturation of embryonic vasculature[112]. In the tumour context, downregulation of VEGF expression confers to shrinkage and elimination of immature vessels and actively remodels the tumour vasculature to resemble more a normal state with less permeability, leakiness, increased oxygenation, and improved drug delivery[7,109,113].

Another strategy to achieve tumour vessel normalisation is the recruitment of pericytes by targeting pericyte-regulating factors. Pericytes are responsible for vascular quiescence and integrity. Tumour vessels lack adequate pericyte coverage. By targeting factors that regulate the recruitment or the detachment of pericytes, a normalised state in the tumour vascularity can be achieved[114,115]. One of the major regulators of pericyte recruitment are angiopoietins[116]. Ang1 and Ang2 induce opposite effects on pericyte recruitment. Overexpression of Ang1 leads to increased mobilisation and recruitment of pericytes in the tumour vessels, while on the other hand Ang2 is involved in the destabilisation of endothelial cell-pericyte interaction[114,117]. Studies have demonstrated that dual inhibition of Ang2 and VEGF in orthotopic glioblastoma murine models results in increased survival, reduced growth, increased necrosis and promotes morphological vascularisation of the tumour vessels[118].

Lastly, an approach to promote tumour vessel normalisation is the enhancement of junction between the endothelial cells[114]. Endothelial cells are connected through adherent junctions, where their major component is vascular endothelial cadherin (VE-cadherin). Stabilisation of VE-cadherin through treatment with purified endothelial cells promotes the reorganisation of the cytoskeleton, stabilisation of the endothelial barrier and increased immune cell infiltration[119].

Strategies that involve normalisation of the tumour vasculature in anti-angiogenic therapy, are considered a promising approach for cancer treatment. Even so, there are three major problems that must be addressed. Firstly, it has been shown that there is a specific normalisation "window", where treatment with chemotherapy or radiotherapy can be effective. Indeed, researchers proved that after treatment with anti-VEGFR-2 antibodies, a period with increased oxygenation could yield the best therapeutic results on the tumour[120]. The second challenge is the reduction or elimination of toxic side effects. Patients treated with anti-VEGF therapies develop some serious side effects that include haemorrhage and thromboembolism. Furthermore, vascular regression has been associated with increased occurrence of metastasis. Moreover, post-treatment tumour relapse and development of resistance occurs in the majority of the patients[114]. Lastly, an important challenge to overcome is filling the gaps on understanding the molecular mechanisms that are involved in

tumour vessel normalisation process[114]. By tackling those challenges, we could optimise current anti-angiogenic treatments involving tumour vessel normalisation and improve their efficacy.

Future perspectives of anti-angiogenic therapy

Tumour-associated angiogenesis consists a crucial issue for anti-angiogenic treatment. Induction of the angiogenic "switch" early in tumour development, indicates the importance of angiogenesis in this process. Identifying and targeting the angiogenic factors that confer to the activation of angiogenic "switch" is important for the development of an effective anti-angiogenic treatment for cancer patients.

To develop a successful anti-angiogenic treatment, it is necessary to recognise the limitations of current anti-angiogenic therapies. Currently, a fundamental issue is the development of anti-angiogenic and the transition from pre-clinical to clinical setting. The differences in biology and the response between the animal models and the human is the major blockade for an effective transition. The dynamics and kinetics of a substance differ between the two organisms and side effects that cannot be detected in the animals are identified in the human context. In addition, a major flaw of studies with experimental models is the duration of treatment. Administration of a substance solely in a short-term period, can be deceiving, since relapse, severe administration-side effects or the effectiveness of a substance cannot be determined significantly. Moreover, besides the difficulties in pre-clinical level, in the clinical setting there are plenty of obstacles to overcome. Limitations of current anti-angiogenic therapies are low percentage of efficiency, severe side effects, increased possibility of resistance and high percentage of relapse post-treatment. All the above limitations demonstrate that it is necessary to design new approaches for anti-angiogenic therapy or optimise existing ones.

Optimisation of current treatments requires a multidimensional approach. Primarily, identifying the regulators and the mechanisms of angiogenesis, would lead to a better understanding of tumour-associated angiogenesis, aid to the creation of novel anti-angiogenic targets and would reveal possible resistance inducers that are activated during treatment, Secondly, development of sensitive and specific biomarkers for diagnosis and monitoring of anti-angiogenic therapy would be beneficial for identifying non-toxic angiogenic inhibitors and treating recurrent cancer before it poses a threat for patient health. Lastly, investing in the design and the application of novel approaches would be the key to eliminating tumour-associated angiogenesis impact. A study involving angiogenic gene therapy, especially gene transfer of VEGF showed positive biological results in pre-clinical level as well in phase I of clinical trials[121]. Another interesting approach is the use of nanotechnology for inhibition of angiogenesis within tumours. Nanoparticles with high affinity for endothelial cells could provide an efficient way to deliver anti-angiogenics to the tumour site, where delivery of conventional drugs would be impossible due to the abnormal tumour vasculature. Such an approach would provide increased treatment accuracy and efficiency and reduction of toxic side effects in the normal vasculature[122].

In 1971, when Folkman proposed the hypothesis of angiogenic "switch", it was impossible to realise its potential on cancer treatment. 50 years later and now we have achieved a better understanding of the process of angiogenesis, the effect on

tumour progression and its effects on cancer treatment. Development of novel agents and strategies of anti-angiogenic therapy have improved treatment of cancer patients. However, still more research is needed in order to completely block this process and succeed in improving treatment and subsequently the life of millions of cancer patients.

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