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Potential role of vascular targeted therapy to combat against tumor

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Tumors, like other tissues, have a fundamental requirement for access to the nutrients, oxygen and waste removal functions of the circulatory system. Vascular targeted therapy exploits this basic need, along with molecular heterogeneity observed between normal and tumor blood vessels, to develop efficient and selective chemotherapies that essentially starve tumors by destroying their vasculature. As the basic principle on which this therapy is based differs from agents that directly target cancerous cells, combining it with traditional therapies such as radiation, surgery and existing chemotherapies has the potential to create powerful new anticancer strategies. As the requirement for vascularization is universal to solid tumors, vascular targeted therapies have the potential for broad applicability. Vascular targeted therapies include both angiogenesis inhibitors, which inhibit neovascularization, and vascular disrupting agents, which destroy existing vasculature. Applications of this model include finding peptides that bind specifically to cell surface markers on tumor vessel endothelial cells and might deliver chemotherapeutic agents. Expression profiling with microarrays, serial analysis of gene expression, and *in vitro* and *in vivo* screening of phage display libraries have identified candidate peptides for targeted delivery to the tumor endothelium.

Keywords: angiogenesis inhibitor, combination therapy, phage displayed peptide, vascular disturbing agent, vascular targeted therapy

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1. Introduction

The principal topic of this review is vascular targeted therapy, focusing on its potential for the treatment of gastric cancer. Antiangiogenic and vascular disrupting therapies will be briefly compared. Progress in the discovery of targets for vascular disrupting agents will be presented, and possibilities for the development of new agents for treating solid tumors, particularly those of gastrointestinal cancer. The focus in this latter section is on using phage display to find targeting peptides that bind specifically to vascular endothelial cells of gastric cancer.

In 1971, Folkman laid the groundwork for research on therapies that target tumor vascularization by proposing that angiogenesis is necessary for tumor growth and invasion, and this system could be a legitimate target for anticancer agents [1]. Subsequent research showed that growth and metastasis of solid tumors indeed requires angiogenesis and a functioning vasculature [2,3], and targeting this system can be an effective anticancer therapy [4,5]. Vascular targeted therapy may be categorized as either antiangiogenic, preventing the production of new vessels, or vascular disrupting, destroying the tumor's existing vascular supply [6,7]. The mechanism of these therapies is entirely different from cytotoxic therapies, which target the cancer cells directly, providing the potential for a multi-pronged therapeutic approach. In fact, the combination of cytotoxic and antivascular agents may constitute a powerful complementary therapy program [8-12].

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2. Process and characteristics of tumor vascularization

The complexity of tumor neovascularization provides several targets for vasculardirected anticancer agents [6,7]. The current model for angiogenesis is that a balance exists between antiangiogenic and proangiogenic factors, with no angiogenesis occurring until an 'angiogenic switch' is triggered by external factors such as anoxia, mechanical stress, or immune system signals. Mutations in genes that control angiogenesis may also contribute [13,14]. Activation of the angiogenic switch leads to expression of proangiogenic factors that signal for endothelial cell proliferation, degradation of the basement membrane by matrix metalloproteases, and finally formation of new vessels. Angiogenesis inhibitors (AIs) target these processes. Once tumor vessels are established, vascular disrupting agents (VDAs) may potentially damage existing vessels by the induction of a rapid and selective vascular shutdown in tumors and extensive secondary neoplastic cell death due to ischemia, although abnormalities in tumor vessels and heterogeneity among the vascularization of different tumor types may influence the effectiveness of vascular targeted therapies [15-17].

3. Advantages of vascular targeted therapies

Vascular targeted therapy has several advantages compared with conventional antitumor therapies and non-targeted antitumor therapies. Vascular targeted therapy is said to have easy accessibility, because tumor endothelial cells are directly available to systemically delivered, circulating drugs. The fundamental requirement for vasculature means vascular targeted therapies can be used against a wide variety of different tumor types. As the entire tumor is dependent on the vascular system, effective AIs and VDAs should affect the entire tumor [6,17]. In addition, vascular targeted therapies may be less susceptible to drug resistance, because the targeted cells are genetically normal and stable [8,9,18]. This hypothesis must be tested clinically, however, with treatment regimes optimized to minimize resistance [19].

Balanced antiangiogenic therapy may increase the efficiency of conventional chemotherapy by contributing to the normalization of tumor vasculature, allowing more efficient action of cytotoxic agents [20,21]. Interestingly, in preclinical studies of combination therapies, more promising data seem to come from those involving antiangiogenic agents and radiotherapy [22], which seems to be contradictory to the well-known hypothesis that oxygenation has radiosensitizing effects on radiotherapy. Several explanations are provided to account for the synergistic effects, including the so-called 'normalization' of the tumor vasculature following antiangiogenic treatments [20], and the deduction that the use of antiangiogenic compounds might sensitize endothelial cells to the toxic effects of ionizing radiations and also prevent radiotherapy-stimulated tumor vessel regrowth [6,23]. Further investigations are still continuing.

4. Angiogenesis inhibitor therapies

Vascular endothelial growth factors (VEGFs) were identified as diffusible factors that induced angiogenesis *in vivo* [24-26]. Their specificity for vascular endothelial cells makes them a particularly attractive target for antiangiogenic agents. VEGF inhibitors are especially promising for gastrointestinal cancer, as VEGF is overexpressed in most gastrointestinal (GI) cancers, and overexpression is associated with metastasis and poor prognosis [27-29], it was shown that VEGF production is required for *in vivo* tumorigenicity and angiogenesis of two human colorectal cancer cell lines. Gastrointestinal cancers are an especially promising candidate for antiangiogenic agents because colon carcinomas are highly angiogenic [15]. The recombinant anti-VEGF monoclonal antibody bevacizumab (Avastin®, Genentech, South San Francisco, CA, USA) is an AI that is at present approved for antiangiogenic therapy [30]. Early evidence is promising for the use of bevacizumab against cancers of the GI. Phase II trials combining bevacizumab with chemotherapy to target the tumor cells showed significant improvements in survival for patients with untreated, metastatic colorectal cancer (CRC) [10,11]. At present, bevacizumab is in active development for the following indications: brain cancer, breast cancer, colorectal cancer, diffuse large B-cell lymphoma, fallopian tube cancer, gastric cancer, gastrointestinal stromal tumors, glioblastoma, head and neck cancer, liver cancer, malignant melanoma, multiple myeloma, non-Hodgkin's lymphoma, non-small cell lung cancer (NSCLC), small cell lung cancer, ovarian cancer, pancreatic cancer, peritoneal cancer, prostate cancer and renal cancer (**Table 1**).

Other promising candidates that are not yet clinically available include endostatin, a ligand of a5ß1 integrin. Chen *et al.* [31] used adenoviral vector-mediated expression of endostatin to show antiangiogenic effects *in vivo*, in mice. Antibodies and peptides that are antagonistic against the integrin family have been studied in treatment for colon cancer, breast cancer, lung cancer and prostatic cancer [32]. Interferon (IFN), which acts through inhibition of VEGF and basic fibroblast growth factor (bFGF), has been used in treatment for leukemia, uterine cervix cancer and lung cancer, among others [6]. Fathallah-Shaykh *et al.* [33] increased survival times in a mouse metastatic brain tumor model with IFN-γ gene therapy. The results suggested the mechanism was through antiangiogenesis rather than through an immune-mediated response or direct cytotoxic effects. Carboxyamidotriazole (CAI), an inhibitor of calcium channels and their related signal transduction pathways, is antiangiogenic, in addition to being antiproliferative and antimetastatic [34] *in vivo*. Its mechanism of action against neovascularization appears to involve downregulation of matrix metalloproteases and VEGF [8]. In a Phase II clinical trial, CAI showed promise as a cytostatic stabilizing agent in ovarian cancer treatment [35], whereas in some Phase II/III clinical trials it failed to provide clinical benefit or improvement in quality of life over placebo

Table 1. Several vascular targeted therapeutic agents in clinical trials.

Drug	Target	Cancer type	Clinical trial	Ref.
Bevacizumab	VEGF	GI cancers, NSCLC, breast cancer, glioblastoma, etc.	Approved for clinical use; Phase II/III	[10,30]
CAI	Calcium channel	NSCLC, ovarian cancer, renal cell carcinoma, etc.	Phase II/III	[34,35]
CA4P	β-tubulin	Thyroid cancer, breast cancer, etc.	Phase I/II	[39,40]
AVE8062	Tubulin	Various solid tumors	Phase I	[44,45]
ZD6126	Tubulin	Various solid tumors	Phase I	[46,48]
ABT-751	Tubulin	Various solid tumors	Phase I/II	[49]
TZT-1027	Tubulin	Advanced refractory cancer	Phase I	[42,43]
DMXAA	Not clear	NSCLC, recurrent ovarian cancer, prostate cancer	Phase III	[51,52]
RGD	Integrin	Breast cancer	Phase I	[72]
NGR	Aminopeptidase N	Various solid tumors	Phase I	[78]

CAI: Carboxyamidotriazole; CA4P: Combretastatin-A4 phosphate; DMXAA: 5,6-Dimethylxanthenone-4-acetic acid; GI cancer: Gastrointestinal cancer; NGR: Asn-Gly-Arg; RGD: Arg-Gly-Asp; VEGF: Vascular endothelial growth factor.

in advanced NSCLC, glioblastoma multiforme or advanced renal cell carcinoma [35,36].

5. Vascular disrupting agent therapies

An advantage of VDAs over AIs is they tend to be cytotoxic rather than cytostatic. VDAs can be classified as tubulin-binding agents, flavonoids and ligand-directed cytotoxic agents such as TNF-α inducers [6,17,37]. The most well-studied tubulin-binding VDA is combretastatin-A4 phosphate (CA4P), a stilbenoid phenol that binds the cholchicine binding site of β-tubulin in endothelial cells. The resulting microtubule and cytoskeletal disruptions cause changes in cell shape, which leads to vascular disruption by increasing vessel permeability and changing interstitial fluid pressure, ultimately leading to vessel damage and vascular failure [38,39]. Existing abnormalities in tumor vascular systems, including already heightened permeability, make the pathological vasculature more susceptible than the physiological vasculature to CA4P [17,28]. Several Phase I/II trials with CA4P have been conducted (**Table 1**) [40,41]. In these studies, a maximum tolerated dose in the range 60 – 68 mg/m^2 was established, and significant changes to tumor perfusion were achieved across a wide range of doses. The dose-limiting toxicities include tumor pain, ataxia and cardiovascular changes. The maximum tolerated dose was independent of schedule, indicating the absence of cumulative toxicity. Although unexpected from preclinical studies, some evidence of clinical response was seen using CA4P as a single modality, and in addition there was also evidence to indicate that CA4P can be combined effectively with chemotherapy or radiotherapy. Also, the potential of combining VDAs with antiangiogenic therapies has shown considerable promise in preclinical models and such combinations are being evaluated in patients [7].

Other tubulin-binding agents include AVE8062, ZD6126, ABT-751, MN-029, TZT-1027 [42,43], and others. AVE8062 is a water-soluble analogue of CA4P. In preclinical studies, it showed markedly enhanced antitumor effects, with rapid and irreversible vascular shutdown in various tumor models. In some Phase I clinical trials [44,45], decreased tumor blood flow induced by AVE8062 administration (15.5 mg/m^2) was observed by dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI), and clinical studies exploring the possible synergistic activity of AVE8062 with oxaliplatin and docetaxel are also continuing. ZD6126 is also involved in several Phase I trials [46], and in all these studies it was well tolerated, with some mild side effects, such as anemia, nausea, vomiting and

constipation [47]. So far no obvious tumor responses have been detected; further studies are being carried out [48]. ABT-751 is a sulfonamide molecule that can be given orally. In a Phase I study involving 39 patients with solid tumors, 1 minor response and 4 patients with stable disease lasting for 6 months were observed, without any specific side effects. Phase I/II studies are continuing, evaluating the safety and efficacy of this molecule in combination with pemetrexed or docetaxel in NSCLC patients [49]. The flavonoid 5,6-dimethylxanthenone-4-acetic acid (DMXAA) has apoptotic effects on endothelial cells, possibly through upregulation of tumor necrotic factor-α (TNF-α) [50]. Phase II trials of DMXAA are underway [51,52]. Ligand-directed VDAs are cytotoxic agents targeted specifically to the tumor vascular endothelium. This strategy has several advantages over traditional chemotherapies. For example, one of the most promising results with the inflammatory cytokine TNF-α has been gene therapy with the *TNF*-α gene delivered specifically to melanoma xenografts [53] or to lymphomas and melanomas in mice [54]. However, gene therapy is not at present a common clinical practice. The toxicity of TNF-α means that it cannot be delivered systemically. An alternative to systemic therapy with TNF-α or gene therapy with the *TNF*-α gene is delivery of the toxic agent itself to vascular endothelial cells by targeting a cell surface molecule specific to these cells.

Undoubtedly, vascular targeted therapies possess attractive advantages over conventional antitumor treatments and are becoming increasingly important in the treatment of different cancers, alone or in combination with classic cytotoxic agents. However, a range of predicted and sometimes unpredictable side effects are observed in clinical use of these drugs [55]. The paradigm of this phenomenon is the side effects observed with bevacizumab, such as venous thromboembolim, hypertension, proteinuria, epistaxis, wound healing, gastrointestinal perforation and bleeding [55,56]. In clinical trials of other vascular targeted drugs (as mentioned above), several dose-limiting toxicities are observed, including acneiform rash, bowel ischemia, transient myocardial ischemia, myocardial infarction, tumor pain and reversible urinary incontinence. These side effects may be related to the specific molecular target in normal tissues inhibited or modulated by certain drugs. To solve these problems, of critical importance should be the assessment of biological activity at doses that can be administered safely; also, some so-called 'magic bullets' that could target specifically and accurately at tumor vasculature without disturbing normal vessels are being investigated and may become an important part of the anticancer armamentarium.

6. Targeting drugs using vascular zip codes

On the basis of the above idea, the discovery and investigation of vascular heterogeneity, specifically the molecular differences between normal and tumor blood vessels, and variation among different kinds of tumor, have been a major focus of research [56,57]. The goal of this research has been to find 'vascular zip codes', or molecular identifiers that could be used to deliver anticancer agents specifically to the tumor vascular system. Expression profiling by microarray analysis and serial analysis of gene expression (SAGE) have been used to find identifiers that distinguish between physiologically expressed markers and pathologically expressed markers that are associated only with vascular endothelial cells of tumors. This approach has identified genes that show differential expression in the blood vessel endothelial cells of normal and colorectal cancer cells, and in normal or tumor-bearing liver, providing potential targets for vascular targeted therapy [58-61].

Phage display libraries have also been used successfully to find peptides that specifically bind vascular endothelial cells of tumors [62-64]. These methods have the advantage of direct identification of peptides that, in their native conformation, interact with targeted cell types. It was demonstrated that specific immunogenic epitopes could be identified even without the recognition of natural antigens or the receptors [65]. *In vitro* methods with phage display libraries use cultured tumor cells to identify peptides expressed from the library that bind to specific tumor types [66]. Another successful *in vitro* technique using phage display has been biopanning and rapid analysis of selective interacting ligands (BRASIL). In this process, suspended cells that bind to components of a phage display library are selected by a quick differential centrifugation step, which eliminates the need for repeated washing and resuspension that can result in sample loss [67]. *In vivo* phage display techniques have also been used to identify candidates for targeting endothelial cells [56,64,68]. In this method, phage peptide libraries are injected into a model organism, typically mouse, or into human patients [63,69]. When tumors are removed or biopsied, bound phage are collected and the peptides they express are identified from the DNA sequence of the library fragments they carry.

These techniques have yielded promising candidates for targeted drug delivery, including peptides that appear to be tumor-specific (**Table 1**) [54,57,70,71]. Potential drug-delivering peptides can be classified into three groups. Some are capable of homing only, or delivering a drug to a specific cell. Others are capable of both homing and uptake. These allow both delivery and internalization of a drug. Finally, the most efficient molecules would be those that can also act as the drug, so that homing, uptake and cell destruction are performed by the same molecule. The tumor vessel homing peptide RGD (Arg-Gly-Asp) interacts with cell surface integrins, and is an example of a peptide that potentially can allow both delivery and internalization [72,73]. The peptide alone directs the cellular uptake of phage that are displaying RGD into mammalian cells [74]. The peptide NGR (Asn-Gly-Arg) is a homing peptide isolated by *in vivo* phage display methods that binds to aminopeptidase N (CD13), which is upregulated in tumor endothelial cells [56,75-77]. Clinical first application of low dosages of NGR conjugated with truncated tissue factor (tTF-NGR) in man revealed good tolerability

and decrease of tumor perfusion as measured by MRI. Targeted thrombosis in the tumor vasculature induced by tTF-NGR may be a promising strategy for the treatment of cancer [78]. F3 peptide, which binds cell surface-expressed nucleolin, has the potential not only to deliver and internalize cytotoxic agents, but also to direct their entry into the nucleus [79]. This could be an advantage for DNA-damaging chemotherapeutic agents. Finally, the peptide GEBP11 (CTKNSYLMC) and peptide GX1 (CGNSNPKSC), both targeting gastric cancer vascular endothelial cells, have been identified recently [63,66]. GEBP11 is isolated by *in vitro* screening of phage-displayed peptide library in a co-cultured cell line, called Co-HUVEC (human umbilical vein endothelial cells co-cultured with human gastric adenocarcinoma cells). Immunofluorescence microscopy showed that GEBP11 located at the membrane and perinuclear cytoplasm of Co-HUVECs. GX1 is identified by *in vivo* screening with an immunosuppressed mouse model bearing human gastric adenocarcinoma xenograft. $^{99}Tc^{m}O_4^-$-labeled GX1 shows selective homing in gastric cancer tissues [80], which, together with other reports, indicates that certain homing peptides can be conjugated with molecules used for imaging, to improve diagnosis and monitoring techniques [70]. *In vivo* studies are continuing for clarification of the possible activities of these newly identified peptides and the underlying mechanisms, and preliminary results suggest that targeting peptides can be used as delivery systems for vascular directed agents [75]. In addition, using an immunoprecipitation technique, screening of microvascular cDNA library, and other methods, some candidates for receptors of these homing peptides are isolated, although further work for identification is necessary [81].

7. Conclusion

In summary, progress in AI and VDA drug development has generated many candidates and lead compound candidates for vascular targeted therapy. In addition, microarray expression profiling, SAGE and phage display methods have helped to identify specific markers of tumor endothelial cells, as well as peptides that interact with molecules on their cell surface. The combination of advances in AI and VDA compounds and new techniques for targeted drug delivery holds promise for more specific and effective anticancer therapies.

8. Expert opinion

Conventional antitumor therapies, including surgery, chemotherapy and radiotherapy, have long been known to have limitations in fighting human tumors. Since the first proposal that tumors cannot grow beyond 1 – 2 mm^3 without stimulating the sprouting of new blood vessels [1], vascular targeted therapies have attracted the increasing interest and attention of many researchers. Vascular targeted strategies disturb the process of tumor angiogenesis or impair an existing tumor blood vessel network, and therefore have distinct advantages over conventional antitumor therapies, including easy accessibility to circulating agents, extensive effects on a wide variety of tumor types and a decreased tendency to develop drug resistance. Among a myriad of investigations, an exciting report has come from the first successful Phase III clinical trial of an antiangiogenic drug, bevacizumab. This neutralizing antibody against VEGF was subsequently approved as a first-line treatment in combination with standard chemotherapy drugs for patients with metastatic colorectal cancer [82]. This development has strengthened the medical community's confidence in this therapeutic approach, and supports the feasibility of transforming preclinical achievements in vascular targeted therapy into clinical application.

Based on recent data from preclinical and clinical studies, new progress in antitumor therapy is most likely to come from combination therapy, and from interdisciplinary cooperation. For example, several clinical trials on tumor vascular homing peptides have found that when used alone, these molecules may not be able to control completely the targeted tumor, or at least are not as powerful as predicted from preclinical studies [83]. This may partly due to the fact that nearly all clinical trials for vascular targeted therapeutic agents have been carried out in patients with advanced tumors, which might limit the therapeutic efficacy and lead to underestimation of a drug's role in adjuvant antitumor treatment. Alternatively, the results may be caused by the sophisticated regulatory networks of tumors. If this is true, a combination of vascular homing peptides and other antitumor treatments may yield synergistic effects. An effective regimen could be combining peptides possessing internalization properties (e.g., RGD, HN-1) with cytotoxic drugs, to overcome the present limitation that most anticancer drugs appear to be unable to access and penetrate solid tumors efficiently. Another reasonable approach is to conjugate antiangiogenic treatments with drugs that target tumor cells or mesenchymal cells (e.g., smooth muscle cells). Tumor cells and mesenchymal cells have been shown to secrete pro-survival factors such as VEGF to protect vascular endothelial cells from destruction under the stress induced by antiangiogenic agents. Therefore, inhibition of these non-endothelial cells may sensitize the endothelial cells and indirectly facilitate tumor vascular targeted treatment.

Attempts at interdisciplinary cooperation have shown promising prospects for antitumor therapy. For example, quantum dots coupled to vascular homing agents have been shown to maintain the ability to home to the targeted tumor sites [70], and interestingly, the complex stays in the vasculature without tissue penetration. Such properties have provoked interest in vascular homing agents as potential intravascular probes for imaging and drug delivery. Another promising strategy is the combination of tumor vascular targeted agents with various so-called 'smart' promoters. Such promoters are designed to be activated by specific factors that

exist only in tumors, not in adjacent normal tissues. An example is cathepsin B, which is often overexpressed in human colonic neoplasms but not in normal tissues [84]. Placing the cytotoxic agent under the control of a tissue-specific promoter, or placing a tissue-specific activating motif upstream of an inactive prodrug that will be activated only in the proper location, could minimize the unwanted cytotoxic effects on normal tissues; in addition, this technique may be applied to tumor imaging.

Research in this field is still in its infancy, however, and far from sophisticated. Along with progress in the development of relevant techniques, more targeting candidates need to be identified, and a better understanding of their mechanisms of function will help to generate more potent agents, as well as identify their possible receptors. Finally, despite all the promising data from preclinical studies, there is still a long way to go before vascular targeted therapy is translated into clinical work.

Declaration of interest

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