Reducing the blood supply of tumors is one modality to combat cancer. Monoclonal antibodies are now established as a key therapeutic approach for a range of diseases. Owing to the ability of antibodies to selectively target endothelial cells within the tumor vasculature, vascular targeting programs have become a mainstay in oncology drug development. However, the antitumor activity of single agent administration of conventional anti-angiogenic compounds is limited and the improvements in patient survival are most prominent in combinations with chemotherapy. Furthermore, prolonged treatment with conventional anti-angiogenic drugs is associated with toxicity and drug resistance. These circumstances provide a strong rationale for novel approaches to enhance the efficacy of mAbs targeting tumor vasculature such as antibody-drug conjugates (ADCs). Here, we review trends in the development of ADCs targeting tumor vasculature with the aim of informing future research and development of this class of therapeutics.

Antibody Drug-Conjugates Targeting the Tumor Vasculature

Current and future developments

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Abbreviations: ADC, antibody-drug conjugate; AML, acute myeloid leukemia; FAAs, flavonoid; mAbs, monoclonal antibodies; ORR, objective response rate; PSMA, prostate specific stem cell antigen; VTA, vascular targeting agents; VT-ADC, vascular targeting ADC

Key words: tumor, vasculature, immunotherapy, antibody-drug conjugates, monoclonal antibody, cancer, angiogenesis

Therapeutic Antibodies and Antibody-Drug Conjugates for Cancer Therapy

Antibody-based therapeutics are of growing significance for cancer therapy as evidenced by twelve such drugs approved for oncologic indications since 1995, including nine in the USA. Among them, eight had more than US $1 billion global market revenues and the combined global revenues exceed US $50 billion. Currently, there are 121 oncology monoclonal antibodies (mAbs) in clinical development. Over the last six years, the success rates for approval of antibody therapeutics entering clinical development was 17%, which makes them an attractive class of therapeutics for oncology drug development.2,3 Despite the success of therapeutic mAbs in the clinic, naked antibodies targeting cell surface tumor antigens expressed on carcinomas are rarely curative by themselves, and most are administered in combination with chemotherapy.4 Similarly, antiangiogenic drugs when administered as single agents induced only limited therapeutic benefit in the clinic and were most successful when administered in combination with chemotherapy.5 These limitations spurred the development of a variety of technologies aimed at the enhancement of mAb therapeutics. Recent advances in antibody drug conjugate technology (ADC) allow for the combination of the selectivity of mAbs with the potency of cytotoxic drugs with the goal to reduce systemic toxicity and to increase efficacy and the therapeutic benefit to carcinoma patients.

ADCs consist of an antibody conjugated to a cytotoxic drug via a linker. The therapeutic concept of ADCs is to use an antibody as a vehicle to deliver a cytotoxic drug selectively to the tumor tissue by targeting an antigen expressed on the surface of a malignant cell. ADCs are prodrugs that require drug release for activation, which occurs commonly after ADC internalization into the target cell, providing greatly improved tumor-to-normal tissue ratios of ADC drugs compared to systemic chemotherapy. Numerous pre-clinical efficacy studies have shown that ADCs enhance the antitumor activity of "naked" antibodies and reduce the systemic toxicity of the cytotoxic drugs conjugated to the antibody.6,7

Important parameters for ADC development include the selection of the target antigen, the kinetics and efficacy of conjugate internalization by the tumor cells, the drug potency and the stability of the linker between drug and antibody (reviewed in ref. 8). Other parameters reported to be important include the drug/antibody ratios9-12 and the methods used for drug conjugation and their effects on the pharmacodynamic properties of the ADCs.13

During the development of ADCs, it became apparent that molecular mechanisms regulating drug linker cleavage, enabling the release of active drug, remained only partially understood.
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Certainly changes in the linker chemistry and drug conjugation methods resulted in alterations in the pharmacodynamic and safety characteristics of different drug linker types tested.9-12 The nature and magnitude of the biological consequences of these changes in the drug linker chemistry are difficult to predict based on the biological models currently available, and thus certain aspects of drug linker engineering remain rather empirical in nature.

Despite these changes in the development of ADC technology, clinical validation of the ADC concept has been provided earlier by gemtuzumab ozogamicin, a humanized anti-CD33 antibody conjugated to the tubulin binding agent, calicheamicin, which is approved in the USA for the treatment of acute myeloid leukemia (AML).14 Two recent cases of ADCs in the clinic were shown to induce unusually high objective response rates in early clinical studies: SGN-35 in Hodgkins lymphoma induced 54% objective response rates (ORR) when administered at doses of ≥1.2 mg/kg.15 Secondly, trastuzumab-DM1 induced 44% ORRs in breast carcinoma when administered at 3.6 mg/kg. These results from phase I and II clinical studies, respectively, provided clinical evidence for the extraordinary potential of these classes of compounds, with the corresponding naked antibody being only minimally active in the same indications.

Four Main Classes of Drug-Linker Compounds Currently Being Developed in the Clinic

Upon binding to cell surface antigens, many mAbs internalize through a process known as receptor mediated endocytosis into lysosomes. The lysosomal compartment is both acidic (pH 5) and rich in proteolytic enzymes (reviewed in ref. 16). The first and most advanced class of drug linker compounds employed for ADCs (doxorubicin, calicheamicin) consist of acid-labile hydrazide linkers, which are cleaved within the intracellular compartment of lysosomes as a consequence of the lower pH within this compartment compared to the systemic circulation (reviewed in ref. 4).9,11,17 The second class of drug linker compounds undergoing clinical testing are disulfide based. Disulfide linkers are selectively cleaved in the cytosol due the more reductive intracellular environment compared to the extra cellular milieu.9,11,18 A third class of "non-cleavable," thioether linkers was developed more recently. The release of free, active drug by this class of drug linker compounds is realized by catabolic degradation of internalized antibodies in the lysosomal compartment, releasing drug cleavage products that function as the active, cytotoxic drug component.18-20 Finally, a fourth class, peptide linkers, with the potential for selective cleavage within the lysosomal compartment by lysosomal proteases such as cathepsin-B, was developed.17,20,21 Peptide linkers are associated with increased serum stability of ADCs and improved anti-tumor effects compared to hydrazine linker compounds.

Biological Rationale to Develop Vascular Targeting ADCs (VT-ADCs)

The prerequisite for the success of the anti-angiogenic strategy is the identification of pathophysiological differences between endothelial cells within tumor and normal tissue vessels that can be harnessed therapeutically. A variety of biological and physiological differences between normal and tumor vasculature have been described, including the constant remodeling of tumor vessels, their reliance on a tubulin cytoskeletal network for functional integrity, a lack of associated pericyte cells and the increased vascular permeability of tumor vasculature.22-26

A growing body of experimental data also demonstrates significant differences in the expression of genes or splice forms between tumor and normal vasculature (reviewed in refs. 25 and 27), providing unique therapeutic targets for vascular targeting. More recently, gene expression profiling and proteomic mapping studies conducted with endothelial cells isolated from the vasculature of experimental models or patient derived tumors led to the identification of distinct molecular signatures within endothelial cells of the tumor vasculature. Several of the genes identified are currently being considered as putative targets for vascular targeting approaches (Table 1).28-35 In addition, the internalization and turnover rates of several membrane associated genes were found to be increased on endothelial cells located within tumors or when grown in culture simulating tumor like conditions.34 Importantly, significant differences in the proteolytic environment and changes in the composition of the extra-cellular matrix within the tumor vasculature were identified.34,35 Endothelial cells within the tumor vasculature proliferate at much higher rates compared to normal, quiescent vasculature.36,37 In support of this notion, small molecule compounds targeting the tubulin structures were shown to be efficacious vascular targeting agents. Among the most promising tubulin binding agents are derivatives of combretastatins, colchicines and dolastatin 10.38 In summary, the magnitude and the variety of the molecular and cellular changes between the endothelium in tumors versus normal tissues provide a wealth of opportunities for the development of vascular targeting agents (VTAs) that are designed to deliver a therapeutic agent selectively to the tumor vasculature, in particular, VT-ADCs that leverage these biological differences (reviewed in ref. 39).

Potential Advantages of VT-ADCs for Drug Development

The recent FDA approvals of therapeutic compounds interfering with VEGF induced angiogenesis for the treatment of solid tumors has validated the anti-angiogenic approach as a viable therapeutic strategy for the treatment of solid tumors.40 Given this clinical success, the focus of future clinical and preclinical oncology research is likely to include vascular targeting strategies. Importantly, VT-ADCs are not associated with some of the limitations of “naked” antibodies targeting antigens expressed on the tumor cells. For example, therapeutic antibodies and ADCs are known to permeate tumors inefficiently. Typically, only 0.001–0.01% of the injected antibody localizes to tumors in humans.41 Endothelial cells usually constitute the first cell layer encountered by therapeutics administered intravenously. Because the vascular endothelial cells are in direct contact with the circulating blood, the serum levels of vascular targeting agents represent the on-target drug exposure levels, and limited tumor perfusion does not affect target exposure.42,43 Such favorable exposure/efficacy relationship of vascular targeting agents may provide an important
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Development of VTAs

VTAs induce anti-tumor effects via rapid and selective destruction of blood vessels within established tumors, leading to a collapse in tumor blood flow and tumor cell death due to ischemia and extensive hemorrhagic necrosis. Low molecular weight VTAs are also known as vascular disrupting agents (VDAs). VTAs can be broadly divided into two classes, low-molecular-weight compounds and ligand-directed macromolecules such as monoclonal antibodies (reviewed in ref. 27). Small molecule VTAs exploit pathophysiological differences between endothelial cells present in tumor and normal tissues to achieve selective targeting of tumor vessels. The clinical data generated with different types of VTAs demonstrated their ability to effectively interfere with tumor angiogenesis. However, the dose levels required to achieve anti-angiogenic responses were frequently close to the maximal tolerated dose (MTD), suggesting a narrow therapeutic window (reviewed in refs. 27 and 50).

In support of this notion, several VTAs, when combined with other anti-neoplastic agents, induced improved therapeutic effects in preclinical models.48

Table 1 Promising, internalizing cell surface antigens overexpressed on human tumor vasculature for ADC development

| Target | Normal tissue expression | Non-endothelial expression | Immuno-conjugates tested | References |
|--------|--------------------------|-----------------------------|--------------------------|------------|
| Integrins αvβ3, α5β1, α6β4, αvβ5, αvβ6, αvβ8 | Vasculature, neutrophils, monocytes, lymphocytes. In most tissues during remodeling, inflammation | Melanoma, GBM, Ovarian, Breast, Prostate carcinoma | RGD-TF and RGD-DOX peptides/imaging targeting αvβ3 and αvβ5 nanoparticles | 59–61 |
| Annexin A1 | Lack of expression | Prostate carcinoma | 125-I-labeled MAb | 62 |
| Nucleolin | Lack of reports for humans tissues | Melanoma | Peptide F3 Imaging in MDA-MB-435 breast carcinoma model | 63, 64 |
| VCAM-1 (CD106) | Lung ECs, Macrophages | Prostate tumors | Tissue Factor fusion protein and peptide conjugates | 65–67 |
| PSMA | Normal prostate | Prostate tumors | Naked MAb ph 1, ADC: ph 1, 111I: ph 1, immunotoxins in preclinical studies | 57, 68, 69 |
| Endoglin (CD105) | Controversial reports regarding expression on normal vasculature | Heme-oncology Solid tumors | CD105 mAb used for imaging, ricin-A conjugates | 70–72 |
| ROBO4 | Controversial reports regarding expression on normal lung, kidney and liver parenchymal cells | TNF peptide fusions | | 73–75 |
| Amino-peptidase N (CD13) | Myeloid cells, renal proximal tubules, small intestine, prostatic epithelial cells, bile duct, mast cell, fibroblast and smooth muscle cells | | 76 |
| δ-like-4, DLL4 | Hematopoietic stem cells (HSCs) | | | 25, 77–79 |
| VEGFR-2 (CD309) | Pancreas, tonsil | Brest Lung Ovarian | VEGF-gelonin conjugates, VEGF-2-tissue factor fusions | 80, 81 |
| CXCR4 (CD184) | HSC, T- and B-cells, dendritic cells, macrophages | Heme-oncology Solid tumors | | 82–84 |
| Tie2 (CD202b) | Tonic expression in normal vasculature | | | 62, 85–88 |
| B7-H3 (CD276) | Lack of expression | Gastric Adenocarcinomas NSCL Breast Esophageal | | 32, 89, 90 |
Among the FAAs, LM985 and DMXAA were developed in early stage clinical trials (Table 2). Several tubulin binding agents such as colchicines, vincristine, vinblastine, auristatins and combretastatins were shown to destabilize the tubulin cytoskeleton and to induce disruption of the tumor vasculature. Among these tubulin binding agents, several were tested in clinical studies, including AVE8062A (Combretastatin A-4 prodrug, formerly AC-7700), Oxi4503 (Combretastatin A-1 disodium phosphate), ZD6126 (Phosphate prodrug of N-acetylcocoholin), ABT-751 (Sulfonamide, binding to β-tubulin), TZT-1027 (Synthetic derivative of dolastatin 10), LM985 (Flavonoid), DMXAA (Flavonoid). Despite the clinical successes of therapeutic antibodies in oncology and the remarkable potency of cytotoxic compounds targeting tumor vasculature, only limited efforts were directed in the past towards the investigation of the effects of ADCs targeting tumor vascular antigens. The reasons for the absence of such research activities are unclear. However, the most plausible explanation is that in most cases, hybridoma derived antibodies generated in mice immunized with human antigens failed to cross react with the murine orthologs. While these circumstances allowed for efficacy studies of antibodies targeting tumor antigens in mice implanted with human tumors, it precluded testing for vascular targeting compounds, and to study their ability to interfere with tumor angiogenesis and to block tumor growth.

Table 2  Cytotoxic agents with anti-angiogenic activities

| Name            | Class                      | Effects                                             | References |
|-----------------|----------------------------|-----------------------------------------------------|------------|
| AVE8062A (AC-7700) | Tubulin binder             | Decreased microvessel density, decreased proliferation and induction of apoptosis of tumor-associated endothelial cells | 91, 92     |
| Oxi4503         | Tubulin binder             | Selectively targets tumor vessels and causes immediate microvascular destruction. | 93, 94     |
| ZD6126          | Tubulin binder Phosphate prodrug of N-acetylcocoholin | Marked vessel destruction through loss of endothelial cells and thrombosis | 95, 96     |
| ABT-751         | Sulfonamide                | Marked reductions in tumor blood flow               | 97, 98     |
| TZT-1027        | Synthetic derivative of dolastatin 10 | Destruction of tumor microvessel network.            | 99, 100    |
| LM985           | Flavonoid                  | Discrepancies in activity between mouse and man seems to relate to differences in the ability of the immune system to respond to FAA | 52, 101    |
| DMXAA           | Flavonoid                  | Increased tumor vascular permeability both directly and through the induction of other vasoactive mediators, including TNF | 51, 102    |

Given these circumstances, it is not surprising that only a handful of immunotherapeutics conjugated to cytotoxic drugs (ADCs) or fused to cytokines (cytokine fusion chimeras) were developed in the clinic. The two most advanced clinical programs employ monoclonal antibodies targeting tumor specific splice forms of fibronectin and tenascin. L19 is a monoclonal antibody interacting specifically with an EDB-containing splice form of fibronectin, which is selectively expressed on the tumor vasculature within a variety of solid tumors. Several therapeutic derivatives of the L19 antibody were generated and shown to be efficacious when tested in preclinical models, and data from early clinical trials demonstrated tumor vasculature specific activities.

Tenascins comprise a family of four extracellular matrix glycoproteins that are widely expressed in different types of connective tissues. Detailed immunohistochemical analysis revealed that the C-terminal domain of tenasin-C is overexpressed in aggressive brain tumors and some lung tumors, with a prominent perivascular staining pattern. Two high affinity human antibodies, G11 and F16, were shown to bind to tumor vascular specific isoforms and to induce anti-tumor effects in preclinical models of human carcinomas.

Prostate specific stem cell antigen (PSMA) is a membrane glycoprotein that is predominantly expressed in the prostate, and serum concentrations are often increased in patients with prostate cancer. Several studies reported overexpression of PSMA in the neovasculature of different solid tumors in cancer patients. The anti-PSMA antibody huJ591 was conjugated to various toxins and radionucleotides and is currently being tested for therapeutic applications in prostate cancer and several solid tumor indications.

The recent technology improvements in antibody engineering, including the phage display technologies, will help to overcome the initial limitations of first generation, mouse hybridoma-derived antibodies which were selective for human antigens. The availability of novel platforms allowing the generation of cross species reactive targeting vehicles will enable the generation of novel, species cross-reactive VT-ADCs, representing an essential tool for the development of ADC with optimized drug-linker properties for vascular targeting purposes.
When combined with immunotherapeutics type and location agents such as auristatins. Response dependent on tumor Vascular endothelial cells are of hematopoietic origin and highly sensitive towards tubulin interfering antibodies in carcinomas. Limited anti-tumor activity of naked Have potential for improved safety profile and wider therapeutic index and/or subgroups of patients. Treatment restricted to tumor subtypes 100% of carcinomas are dependent on angiogenesis, broad target indications in oncology which leads to drug resistance. Vascular endothelial cells are of hematopoietic origin and highly sensitive towards tubulin interfering agents such as auristatins. Can either be developed in combination with or to replace chemotherapy.

**Future Perspectives**

VT-ADCs circumvent many of the limitations encountered when developing immunotherapeutics targeting antigens on neoplastic tumor cells (Table 3). In addition, major pathophysiological changes at the molecular and cellular level between the endothelium associated with tumors versus normal host tissues have been identified and provide an enormous opportunity to target tumor vasculature selectively. The development of VT-ADCs is particularly promising, as they harness several aspects of tumor vasculature, including the high proliferation rates of endothelial cells. However, the development of ADCs in oncology takes longer, is more complex and is less well defined than for naked antibodies. Two conceptually different approaches can be considered for the selection of novel drug linker compounds with utility for vascular targeting. The first strategy takes into account the empirical nature of successful drug linker design for ADCs in oncology and is based on the random screening of chemical libraries to identify drug linkers with optimal properties for vascular targeting purposes. An alternative strategy is based on the principles of rational drug design, taking advantage of the rapidly increasing knowledge of the pathophysiological changes between normal and tumor vasculature, such as provided by recent studies employing proteomic and genomic approaches. The rational drug-linker design approach may also include identification of novel peptide linkers that are cleaved selectively by proteases found to be upregulated predominantly on the tumor vasculature and/or on proliferating, activated endothelial cells. Alternatively, a novel approach towards the delivery of drugs to endothelial cells may be represented by the targeted delivery of drugs to the vicinity of blood vessels, including the subendothelial extracellular matrix (ECM), followed by hydrolytic release of the drug. The recent advances determining the changes in gene expression within tumor vasculature combined with the new technologies enabling cross species reactive targeting vehicles and the recent improvements in drug linker technology provide the basis for continuous development of novel VT-ADCs with improved activities in patients with solid tumors.

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