

Review

Molecular targeting of angiogenesis

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Abstract

The majority of pharmacological approaches for the treatment of solid tumors suffer from poor selectivity, thus limiting dose escalation (i.e., the doses of drug which are required to kill tumor cells cause unacceptable toxicities to normal tissues). The situation is made more dramatic by the fact that the majority of anticancer drugs accumulate preferentially in normal tissues rather than in neoplastic sites, due to the irregular vasculature and to the high interstitial pressure of solid tumors.

One avenue towards the development of more efficacious and better tolerated anti-cancer drugs relies on the targeted delivery of therapeutic agents to the tumor environment, thus sparing normal tissues. Molecular markers which are selectively expressed in the stroma and in neo-vascular sites of aggressive solid tumors appear to be particularly suited for ligand-based tumor targeting strategies. Tumor blood vessels are accessible to agents coming from the bloodstream, and their occlusion may result in an avalanche of tumor cell death. Furthermore, endothelial cells and stromal cells are genetically more stable than tumor cells and can produce abundant markers, which are ideally suited for tumor targeting strategies.

This review focuses on recent advances in the development of ligands for the selective targeting of tumor blood vessels and new blood vessels in other angiogenesis-related diseases.

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1. Introduction

Cancer chemotherapy relies on the expectation that anti-cancer drugs will preferentially kill rapidly dividing tumor cells, rather than normal cells. Since a large portion of the tumor cells has to be killed in order to obtain and maintain a complete remission, large doses of drugs are typically used, with significant toxicity towards proliferating nonmalignant cells [1]. The development of more selective anti-cancer drugs, with better discrimination between tumor cells and normal cells, is possibly the most important goal of modern anticancer research.

One avenue towards the development of more selective, better anti-cancer drugs consists in the targeted delivery of bioactive molecules (drugs, cytokines, procoagulant factors, photosensitizers, radionuclides, etc.) to the tumor environment by means of binding molecules (e.g., human antibodies) specific for tumor-associated markers.

Even though the concept of a selective delivery of therapeutics to the tumor environment was first envisioned by Paul Ehrlich at the end of the 19th century, several technologies had to be developed before this therapeutic strategy could become a reality. Indeed, the following considerations outline why discovery and validation of tumor-associated markers (as well as the corresponding ligands) remain an important challenge for the development of better, targeted anticancer agents for the treatment of disseminated solid tumors:

- (a) Most chemotherapeutic agents *do not* preferentially accumulate at the tumor site. Indeed, the dose of drug that reaches the tumor (normalized per gram of tissue) may be as little as 5–10% of the dose that accumulates in normal organs [2]! The high interstitial pressure and the irregular vasculature of the tumor account, in part, for the difficult uptake of drugs by tumor cells [3,4]. On top of that, the activity of multidrug resistance proteins may further decrease drug uptake [5].
- (b) Recent advances in protein engineering have made it possible to generate high-affinity human antibodies

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against virtually any biomolecular target [6]. Furthermore, technologies are becoming available for the generation of high-affinity binding peptides [7], aptamers [8] and synthetic organic molecules [9–11], which may be used as ligands for the development of targeted anti-cancer strategies.

- (c) Ligand-based tumor targeting approaches (e.g., those based on high-affinity monoclonal antibody fragments) allow to achieve excellent ligand localization in the tumor environment, with tumor/organ ratios of >10:1 already few hours after intravenous injection [12–19]. The search for novel targets and ligands is just as important as the development of strategies which convert a ligand (capable of selective localization in the tumor environment) into a therapeutic agent which retains the selectivity for the tumor.

Until now, most of the efforts in the development of tumor targeting agents have focused on the targeting of markers located on the membrane of tumor cells. However, strategies aimed at the direct killing of individual tumor cells are difficult, since distant cells may be hardly accessible to ligands. Furthermore, the intrinsic genetic instability of cancer cells often results in heterogeneous patterns of tumor marker expression. Consequently, markers which are selectively expressed around tumor blood vessels and in the tumor stroma may offer a number of potential advantages, such as better accessibility, stability and abundance.

This review provides an overview and presents the authors' view on recent advances in the ligand-based molecular targeting of tumor neo-vasculature.

1.1. *Angiogenesis and angiogenesis-related diseases*

Angiogenesis, defined as the development of new blood vessels from preexisting vessels, is one out of several mechanisms which build and maintain the blood supply of the body's tissues. As such, it can be distinguished from arteriogenesis and vasculogenesis. Arteriogenesis is a repair mechanism whereby bridging collateral arterioles are remodelled and grow to compensate for arterial occlusions in major vessels [20,21]. Vasculogenesis, on the other hand, is involved in the initial steps of the formation of the vascular system during embryogenesis. In this process, mesodermal cells differentiate into angioblasts which then give rise to the endothelial cells assembling into a first vascular network [22]. Because vasculogenesis only leads to an immature, poorly functional vasculature in the embryo, angiogenesis is essential for the subsequent development of the vascular network of arteries, veins, arterioles, venules and capillary blood vessels [23–25].

Angiogenesis is a physiological process in embryogenesis and during development. In the adult, angiogenesis is a prominent feature of the female reproductive cycle. Moreover, a high rate of endothelial cell turnover is observed in the testis [26,27]. Hair growth is also associated with

pronounced vascular endothelial growth factor (VEGF)-induced angiogenesis [28–31]. Otherwise, while angiogenesis is essentially a rare event in the adult (with the notable exceptions indicated above), it can occur in a number of relevant pathologies, such as cancer, blinding ocular disorders, rheumatoid arthritis, psoriasis [23,32].

The observation by Tannock [33] in 1968 that the vasculature is in rapid proliferation within the tumor was followed few years later by articles of Folkman [34], who postulated that the growth of new blood vessels is an essential requirement for tumors to grow beyond a certain size. As a consequence, inhibition of angiogenesis would represent an avenue for blocking tumor growth [35], possibly circumventing the multidrug resistance problem, since the endothelial cells which line tumor blood vessels are genetically stable, unlike the tumor cells [36]. The causal link between tumor hypoxia and induction of angiogenesis [37], as well as the molecular machinery for the sensing of and response to hypoxia, are by now well characterized [38–40].

Studies attempting to correlate tumor prognosis with vessel counts have often led to the misleading concept that tumor vascularity and tumor angiogenesis are synonymous [41]. Indeed, several examples are known of highly vascular lesions which are benign [42,43]. However, careful studies with double staining techniques (detecting proliferating endothelial cells, or blood vessels which stain with certain markers of angiogenesis) have shown that exuberant tumor angiogenesis remains the most important parameter associated with poor prognosis [41,43].

Recently, it has been suggested that tumor blood vessels may have a “mosaic” structure, with tumor cells lining the blood vessels wall instead of endothelial cells [44–46], but some authors question the relevance of these findings [47–49].

Furthermore, lymphangiogenesis (i.e., the proliferation of new lymphatic blood vessels) is emerging as a biological process which may facilitate tumor metastatic spread [50–53].

2. **Molecular targeting of angiogenesis: a definition**

A distinction between anti-angiogenesis therapeutic strategies (which aim at the inhibition of endothelial cell proliferation) and vascular targeting strategies (which aim at the selective destruction or occlusion of tumor neo-vasculature) is often used [54,55]. Generally, “vascular targeting” is used to indicate both compounds (such as combretastatins) which promote a change in shape of tumor endothelial cells, with consequent thrombosis of tumor blood vessels and tumor cell death [56] (a selective process which, however, does not necessarily implies a selective localization of the drug at the tumor site), and compounds capable of selective localization at the level of tumor neo-vasculature.

and in ocular disorders [76–79]. Immunohistochemistry studies have shown that a number of normal tissues stain positive for the antigen, though to a lower extent compared to tissues undergoing active angiogenesis [80]. A Phase I immunohistoscintigraphy clinical trial in 20 patients with cancer, using the radiolabeled humanized antibody Vitaxin, failed to image the tumor lesions in all but one patient [81].

RGD-containing peptides, capable of high affinity binding to $\alpha v\beta 3$ integrin, have been used successfully for the radio imaging of tumor in animal models [82,83]. While good tumor/blood ratios were observed at early time points (1–2 h), tumor/organ ratios were sometimes poor (particularly colon, kidney, liver, lung).

2.4. Prostate-specific membrane antigen (PSMA)

This enzyme has originally been used as a serum marker for prostate cancer, providing a clinical prognostic information complementary to the one of other markers [84–86]. A number of reports have indicated a strong expression of PSMA around blood vessels in a wide variety of carcinomas [87,88]. PSMA expression in blood vessels was also reported. An immunoscintigraphy clinical trial with radiolabeled de-Immunized mAb J591-DOTA-¹¹¹In is in progress at Cornell University.

2.5. Endoglin (CD105)

The initial excitement about the potential of endoglin as a marker of angiogenesis [89] has been slowed down by later reports of significant expression of the antigen in a number of normal organs [90,91]. While most of the quantitative biodistribution results obtained with radiolabeled anti-endoglin antibodies in tumor-bearing mice were rather poor [92], good imaging results in tumor-bearing dogs have also been reported [93].

2.6. VEGF and VEGF–receptor complex

VEGF (available in different forms) is one of the main mediators of the vascularization of solid tumors. In the tumor microenvironment, an up-regulation of both VEGF and its receptors occurs, leading to a high concentration of occupied receptors on tumor vascular endothelium. VEGF–receptor complexes were shown to be a specific target on tumor endothelium for antibodies in vivo. In a recent study, a monoclonal antibody (2C3) was shown to have anti-tumor activity against tumor xenografts in mice [94]. This antibody has also been shown to localize to tumor blood vessels by microscopic analysis, but quantitative biodistribution results have not yet been reported. Biodistribution studies in tumor-bearing mice with anti-VEGF antibodies or with VEGF itself as a ligand for its receptors have been disappointing [95,96]. Even though this review does not focus on inhibition of angiogenesis, it is nonetheless worth mentioning that a humanized neutralizing antibody to VEGF

(Avastin, Genentech) is currently in Phase III clinical trials [97].

2.7. CD44

CD44 is a cell adhesion receptor of great molecular heterogeneity due to alternative splicing and posttranslational modifications. In spite of its widespread pattern of expression in blood cells and tissues, a monoclonal antibody to a CD44 variant has been reported to display spectacular tumor targeting results in tumor-bearing mice, with prominent perivascular accumulation [98]. At this time point, it is not clear if the excellent targeting results (with % injected dose in tumor >75%, 1 h after injection in tumor-bearing mice) are due to a predominantly luminal pattern of expression of the antigen [99].

2.8. Phosphatidyl serine phospholipids

Phosphatidylserine phospholipids are normally located in the inner leaflet of the cell membrane and, therefore, not readily accessible to specific ligands. However, in cells undergoing apoptosis or under stress, they may become exposed in the outer cell membrane leaflet [100]. Recently, Thorpe and colleagues have postulated that phosphatidylserine may serve as marker of angiogenesis for ligand-based vascular targeting applications, on the basis of binding studies with Annexin V and monoclonal antibodies on endothelial cells undergoing oxidative stress. This hypothesis is supported by a fluorescence microscopic analysis of tumor targeting experiments with monoclonal antibodies injected in tumor bearing mice [101]. The real potential of phosphatidylserine for vascular targeting applications remains to be confirmed by quantitative biodistribution studies. A potential concern comes from the surface exposure of phosphatidylserine in activated platelets [102,103].

2.9. Large isoforms of tenascin C

Tenascin C is a component of the extracellular matrix, which exists in a “small” isoform (devoid of extra-domains) or in a more tissue-restricted “large isoform”, which contains additional domains inserted by alternative splicing (Fig. 1). Although expression of the large isoform of tenascin-C is detectable in certain normal tissues (e.g., at the interface between derma and epidermis), the protein is much more abundant in several aggressive tumors, with a prominent staining of the tumor stroma and of the tumor neo-vasculature [104]. Radiolabeled monoclonal antibodies specific for the large tenascin isoform have been investigated in the clinic for several years, both in diagnostic and radioimmunotherapeutic applications [105–108]. Recently, it has been discovered that the extra-domain C within the large isoform (Fig. 1) features an even more restricted pattern of expression, being undetectable in normal human tissues, but expressed in aggressive tumors such as high-

grade astrocytomas and lung cancers [109], with a predominantly vascular staining pattern.

2.10. Magic roundabout

Magic roundabout (MR or ROBO4) belongs to the roundabout family, which contains several closely related genes (three in man) that were previously thought to be only present in neuronal tissue and involved in axon guidance. Roundabouts have five IgG and three fibronectin-like extracellular domains. They are large transmembrane receptors for ligands known as slits. The discovery of an endothelial specific roundabout has been demonstrated by a combination of Northern blotting, in situ hybridisation and immunohistochemistry, confirming a highly restricted pattern of expression [110]. MR is highly expressed during embryonal development, but is absent from adult tissues except at sites of active angiogenesis, including tumors. A similar pattern of expression has also been found for delta4, an endothelial specific member of the delta family [111].

3. Vascular targeting antibodies

Putative markers of angiogenesis have been discussed in the previous section, together with some of the properties of the corresponding antibodies. Until now, very few publica-

tions describe vascular targeting antibodies, which have moved to advanced preclinical experimentation to clinical studies.

In collaboration with the group of Luciano Zardi (Genova, Italy), our group has extensively characterized the human antibody L19, specific to the EDB domain of fibronectin, a marker of angiogenesis. In addition to biodistribution studies in animal models [16,17,19,72,73,96,112], the radiolabeled antibody fragment (in noncovalently associated scFv homodimeric format) has been characterized in an immunoscintigraphy clinical trial in patients with cancer [74] (Fig. 2).

A number of derivatives of scFv(L19), with a therapeutic potential, have been studied in animal models. The antibody, coupled to a photosensitizer, was shown to be able to mediate a complete and selective photo-occlusion of new blood vessels in a rabbit model of ocular angiogenesis. Indeed, the EDB domain of fibronectin is strongly expressed in angiogenesis-related ocular disorders [113]. A fusion of scFv(L19) with truncated tissue factor (a pro-coagulant protein) [114] mediated the rapid intraluminal blood coagulation in tumor blood vessels, but not in normal blood vessels. As a result, a dramatic collapse of the tumor mass could be observed, particularly for large tumor masses. This work follows previous demonstrations of the principle with artificially-induced markers of angiogenesis (e.g., MHC-II) [115].

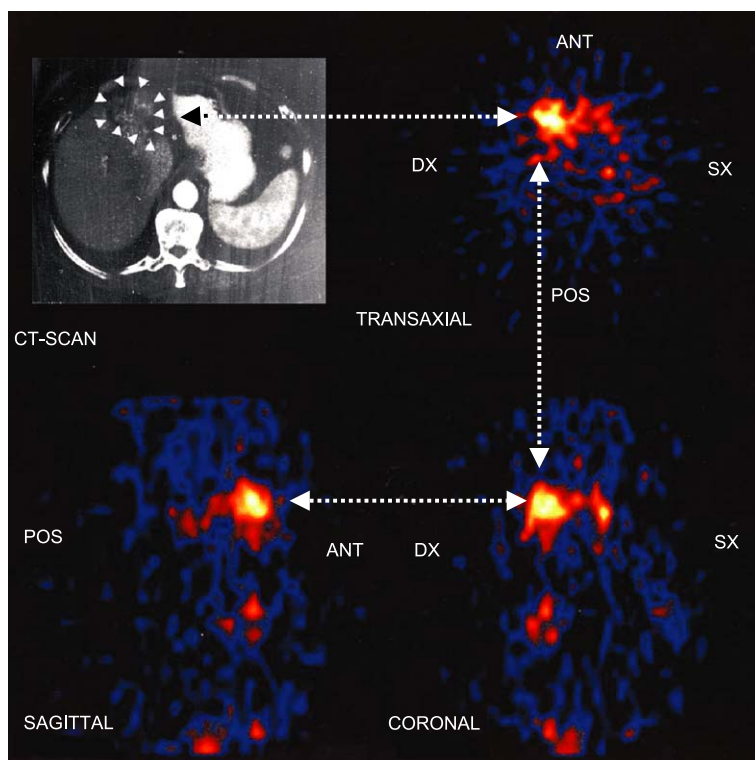


Fig. 2. Localization of ^{123}I -L19(scFv) $_2$ in bulky liver metastases from patients with colorectal cancer. CT scan and SPECT images, obtained 21 h after antibody injection, showing intersecting transaxial, sagittal, and coronal projection of the abdomen. The liver metastasis is indicated with arrowheads in the CT scan (reproduced from Ref. [74] with permission).

Systemic administration of cytokines, such as interleukin-2 (IL-2), tumor necrosis factor (TNF), GM-CSF or IL-12, can render some non-immunogenic tumors immunogenic, activating a protective immunity. However, systemic administration of cytokines is often associated with severe toxic side effects, preventing the administration of a curative dose. A possible way of increasing the therapeutic index of cytokines consists of fusing them to antibodies, which mediate a preferential accumulation of the cytokine at the tumor site. Indeed, in the past decade, several groups have reported different antibody-cytokine fusions for different tumor-associated antigens. ScFv(L19) has been expressed as fusion protein with potent immunostimulatory cytokines (such as IL-2, IL-12, TNF). These novel proteins were shown to retain both antibody and cytokine functions and to show superior anti-cancer activities, as compared to equivalent amounts of free cytokine (and antibody) [18, 116, 117].

Small antibody fragments are rapidly cleared from circulation through the kidneys. For scFv and Fab fragments, more than 80% of the injected antibody dose typically disappears from serum within 1 h from injection. Considering the physical barriers to antibody extravasation (endothelial cells, extracellular matrix components, irregular microcirculation, high hydrostatic pressure), it has been suggested that the majority of antibody fragments directed against tumor-associated markers located on the membrane of tumor cells are indeed “vascular targeting” antibodies. In fact, if these antibodies are injected at low doses and have a high-affinity for the antigen, they should be trapped by the first layer of tumor cells, in the immediate surrounding of tumor blood vessels [118, 119]. Experimental microscopic studies with antibody fragments of different affinity for the antigen appear to support this intuitive picture [120].

How fast can antibody-mediated vascular targeting be? And how efficient? Some of the answers to these questions are coming from biodistribution studies performed with vascular markers located in the luminal or abluminal aspects of new blood vessels. Schnitzer and co-workers have developed methods for the selective recovery of endothelial cell membrane proteins and caveolar structures following terminal perfusion experiments, which can be used for proteomic investigations [58, 121, 122]. As a result, a marker was identified, which is preferentially expressed on the lung endothelium. Biodistribution experiments with a specific monoclonal antibody exhibited a very rapid and efficient lung uptake of the antibody already 1 h after intravenous injection, suggesting that (at least for some abundant antigens and for some antibodies) vascular targeting can be an extremely efficient process [58]. Dose/response biodistribution studies will be necessary to elucidate whether the efficiency of ligand-based luminal vascular targeting process can be predicted on the basis of a single compartment pharmacokinetic system and of chemical binding kinetics. In a recent publication, we have described equations which

indicate that luminal vascular targeting can be an inefficient process, if the target is present in low amount and/or if the binding kinetic properties of the ligand are insufficiently good [96].

For antigens predominantly located on the abluminal aspect of tumor blood vessels (e.g., EDB, tenascin-C), we have shown that targeting efficiency is independent of the amount of antibody injected [16]. These data are compatible with a two-compartment pharmacokinetic model, and with a local concentration of antigen in the perivascular space being in large excess to the concentration of antibody (“antigen binding to the antibody, and not vice versa”). Furthermore, a number of barriers may exist, which prevent an antibody derivative from reaching a marker of angiogenesis, located outside of the tumor vessel. For example, we have recently described how anti-EDB antibody-based fusion proteins, characterized by extremely high or low pI values [123] or large molecular weight [117], display a completely abrogated tumor targeting ability, in spite of being fully immunoreactive and well behaved in gel electrophoresis and in gel filtration assays.

Even though ligand-based applications of magnetic resonance imaging (MRI) and of ultrasound imaging have been proposed [79, 124], the main imaging applications of vascular targeting antibodies are expected in the field of optical imaging and of nuclear medicine. The transmittance of light at 850 nm through 1 cm of tissue is approximately 10% [125]. Consequently, antibodies labeled with infrared fluorophores may open imaging applications for superficial lesions, for endoscopically accessible lesions and for certain parts of the body, like brain, breast, and prostate [112, 126–130]. On the Nuclear Medicine side, even though SPECT imaging of angiogenesis has been shown to be possible [74], we expect most of the future exciting developments to originate in the field of positron emission tomography (PET), ideally in a PET/CT combination. PET imaging may couple high sensitivity with the possibility of obtaining very good spatial resolution and quantitative pharmacokinetic results. Recent advances in animal micro-PET will facilitate developments in this field [131, 132].

For therapeutic applications, it is not clear which antibody format and/or derivative may be the most effective, but it is likely that both disease type and intended application will strongly influence the choice of a particular molecular format. In addition to the antibody derivatization strategies mentioned above (photosensitizers, cytokines, pro-coagulant factors), other possibilities include the use of naked antibodies, glycosylation-engineered antibodies [133], drug conjugates, radiolabeled antibodies, immunotoxins, etc. (for a review, see Ref. [134]).

4. Vascular targeting peptides

Following the introduction of *in vivo* biopanning of peptide phage-display libraries [59], a number of peptides

have been postulated as organ-specific and tumor-specific vascular targeting agents [135]. To our knowledge, this attractive possibility remains to be experimentally confirmed by quantitative biodistribution experiments with labeled purified peptides. In our hands, the site-specifically Cy7-labeled RGD-4C peptide [136] did not target tumors. This, however, could be due to the labeling procedure. The potent antitumor activity of certain peptide-doxorubicin conjugates is sometimes used as confirmation of the vascular targeting ability of the corresponding peptides [136]. However, the chemical procedures presented in the article suffer from several flaws (e.g., absent characterization and purification of the conjugates, coupling chemistry which gives rise to polymeric doxorubicin derivatives), making a proper evaluation of the results difficult. Peptides isolated by *in vivo* panning procedures, which contain a NGR sequence, have been shown to potentiate the anti-tumor effect of TNF, when fused to this cytokine [137]. However, the peptide–TNF fusion protein did not preferentially accumulate in the tumor after intravenous injection in murine models, when studied by quantitative biodistribution analysis.

Peptides have also been used to target pathological features which are sometimes associated with angiogenesis. For example, Tepe et al. [138] described radiolabeled endothelin derivatives, which preferentially accumulate on atherosclerotic plaques (e.g., in Watanabe rabbits) after intravenous administration.

As a result of a systematic analysis of the tumor endothelial extracellular matrix, and of the corresponding proteolytic peptides, Maeshima et al. [139] identified tumstatin, a collagen IV-derived peptide. Furthermore, tumstatin was shown to selectively localize to tumor blood vessels (but not to other types of angiogenic blood vessels!) by microscopic analysis [140]. These findings, if confirmed by quantitative biodistribution analysis, could be very important, as they would indicate the existence of vascular targets which are differentially expressed in the tumor endothelium and in other types of neo-vasculature (e.g., in the endometrium during the proliferative phase).

5. Discussion and outlook

The identification of markers which are overexpressed in the tumor endothelium, together with the generation of specific binding molecules, opens the way to diagnostic and therapeutic strategies, based on the targeted delivery of molecules to the tumor environment. Because of its accessibility, the tumor endothelium appears to be a particularly attractive target for anti-cancer strategies. As an illustrative example, it is worth considering the lessons learned from isolated limb perfusion procedures, featuring the high-dose administration of TNF, interferon- γ and melphalan to limbs carrying in transit melanoma metastases or sarcomas [141,142]. First, the spectacular anti-tumor results obtained

by the groups of Lejeune and Eggermont show the therapeutic potential of TNF, a cytokine which mainly displays its anti-tumor effect at the level of the tumor neo-vasculature, causing intraluminal blood coagulation and (as a consequence) tumor cell death. Second, thanks to its vasoactive properties, TNF potently enhances the tumor uptake of cytotoxic drugs in the tumor [4]. Third, knowledge of the effective concentration of TNF necessary to mediate a macroscopic effect provides direct information about how much the therapeutic ratio of TNF has to be improved (e.g., by means of a fusion with a vascular targeting antibody) in order to allow a successful treatment of patients with systemic administration of the fusion protein.

At this moment, it is not clear whether some of the vascular targeting therapeutic strategies presented in this review (which have shown promising results in animal models of cancer) will be successful in the clinic. For some strategies (e.g., immunocytokines), issues such as immunogenicity and blockade by endogenous receptors will be resolved only when these new prototypes move to clinical trials. Therapeutic approaches based on chemical derivatives of antibodies (e.g., antibody-drug conjugates, antibody-photosensitizer conjugates) appear more straightforward for pharmaceutical development, provided that very active drugs are used, thus minimizing the stoichiometric problem of attaching a sufficient amount of low molecular weight drug to a macromolecular carrier (antibody). It is possible that, in a near future, non-peptidic binding molecules specific for markers of angiogenesis may become more and more important for vascular targeting applications. Such small organic compounds should not be immunogenic, and may display an improved tissue penetration, compared to antibodies. Novel technologies based on bidentate ligands may allow the isolation of binding molecules with sufficient affinity, to be used in targeted anticancer applications [9–11].

Anti-EDB antibody fragments have demonstrated that the ligand-based molecular imaging of angiogenesis is possible. Future advances in this field are likely to depend not only on the quality of the target molecule and of the ligand used (e.g., high affinity human antibody fragment) but also on the imaging modality used (PET vs. SPECT for radiolabeled antibodies; conventional near-infrared imaging vs. near-infrared diffuse optical tomography [143]). Ultimately, much of what we will learn about vascular targeting in the next few years will depend on how rapidly clinical-grade reagents will become available, and on the determination of both industrial and academic groups to perform translational clinical research with this novel class of compounds. Many of the most promising anti-cancer vascular targeting compounds available today have been tested only in rodent animal models; mechanistic clinical trials will elucidate whether the animal data reflect a true pharmaceutical potential, or only an abnormally high dependence of the pathology on angiogenesis.

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