New ways in RGD-peptide mediated drug targeting to angiogenic endothelium

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Summary and Concluding Discussion

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Summary

The endothelium is a prime target for intervention in cancer. Tumor growth starts with the outgrowth of a cluster of a few malignant cells, whose growth is subsequently inhibited by a limited diffusion of oxygen and nutrients from the surrounding tissue. The lack of oxygen induces transcription of the hypoxia inducible factor 1α, which in turn initiates the transcription and shedding of proangiogenic factors like VEGF [1]. These factors stimulate angiogenesis, the growth of new blood vessels towards the tumor. Angiogenesis is one of a few ways how a tumor can ensure continuous supply of oxygen and nutrients, besides for example vascular mimicry or vessel cooption [2, 3]. Thereby, the tumor can grow beyond a diameter of 2 mm and spread to other places in the body (metastasis). Due to the importance of angiogenesis for development and progression of cancer, vasculature-directed therapies are widely investigated [4].

The involvement of endothelial cells is also crucial in chronic inflammatory diseases. Proinflammatory cytokines like TNFα and IL-1β activate ECs via p38MAP kinase or NFκB [5]. The activated EC excrete cytokines and chemokines, which in concert with cellular adhesion molecules promote leukocyte recruitment and infiltration, and mediate vascular remodeling [6, 7]. Blockade of these processes has obtained immense attention in recent years and many new signal transduction inhibitors against p38MAP kinase or NFκB were developed [8, 9].

In this thesis, I developed novel drug targeting conjugates aiming at the inhibition of proinflammatory or proangiogenic stimuli in the endothelium. The first part of the thesis concerns the specific delivery of kinase inhibitors into these cells. In the second part of the presented work, the drug targeting concept was applied to antimitotic agents, in order to selectively kill angiogenic endothelium and thereby deprive a tumor from oxygen and nutrients.

I applied a cyclic arginine-glycine-aspartic acid tripeptide as targeting ligand. This so called RGD-peptide selectively binds to αvβ3-integrin, a receptor that is upregulated on activated and angiogenic EC but hardly expressed on dormant endothelium. Chapter 2 summarizes the structural requirements for RGD-peptides and RGD-mimetics to be a high affinity and high specificity ligand for αvβ3-integrin. The importance of multivalent RGD-constructs for high avidity binding and internalization is highlighted.

Chapter 3 is dedicated to the cell-specific delivery of the p38MAP kinase inhibitor SB202190 into activated endothelial cells. For that purpose, SB202190 needed to be coupled to a RGD-peptide-modified albumin carrier. I succeeded with the coupling of this drug by application of the Universal Linkage System (ULS), a platinum-based drug linker which formed a coordination bond with the pyridyl group of SB202190. The stability of the drug-ULS linkage in serum was high. Drug release was accelerated by coincubation with sulfur containing ligands like glutathione (GSH) or dithiothreitol. Thus, high intracellular concentrations of GSH or other sulfur donors will facilitate drug release at the site of action. The cyclic RGD-peptide was introduced using a conventional bifunctional polyethylene glycol (PEG) linker. The peptides clearly facilitated binding and uptake of the drug targeting conjugates, ultimately leading to intracellular drug release and inhibition of TNFα induced proinflammatory gene and protein expression.

Chapter 4 describes the development of a novel series of RGD-peptide-modified albumin carriers. RGD-peptides, monovalent PEG, or bivalent PEG were combined to achieve optimal binding characteristics together with the best solubility. The VEGFR kinase inhibitor PTK787 was
coupled via ULS to the albumin based carriers after the modifications with RGD and PEG. The coupling of PTK787 to RGD-HSA was only limited by solubility of the final product, due to the hydrophobic nature of PTK787. The solubility was significantly improved by incorporation of PEG-moieties or PEG-linkers for the RGD-peptide. Introduction of the drug did not influence the excellent binding affinities of these carriers. I demonstrated inhibition of VEGF-induced proangiogenic gene expression by targeted PTK787, revealing the value of our targeting approach.

Two auristatin derivatives were specifically delivered to \(\alpha_v\beta_3\)-integrin expressing cells in the studies described in chapters 5 and 6. A valine-citrulline linker was applied for coupling of auristatin E and F (MMAE and MMAF) to the albumin drug carrier. I created drug targeting conjugates that bound with high affinity and were taken up by \(\alpha_v\beta_3\)-integrin expressing cells. Primary endothelial cells and C26 colon carcinoma cells were killed by these conjugates at low nanomolar concentrations. Identical conjugates with only a slight modification in the homing ligand (RAD instead of RGD) demonstrated absence of effect at 50-fold higher concentrations. A pilot in vivo experiment indicated accumulation of drug targeting conjugate in the tumor tissue 24 h after injection. Liver, spleen and kidney were free of drug targeting conjugate at this time point. The distribution pattern of RGD-albumin coupled with auristatin was widespread in the tumor and not restricted to the tumor endothelium, since the tumor cells in the used in vivo model also expressed \(\alpha_v\beta_3\)-integrin.

In chapter 7 I further evaluated targeted PTK787 and MMAF for their tumor growth inhibitory effect in s.c. C26 carcinoma bearing mice. The in vivo evaluation of RGD-albumin drug carriers revealed high immunogenicity of e(RGDfK)-albumin conjugates. Multiple injections of drug targeting conjugates with a RGD to albumin ratio of 12:1 induced antibody formation and a fatal anaphylactic shock. Strikingly, effect on tumor growth was noticed for all conjugates that provoked the anaphylactic shock, regardless of any drug modifications. Thus RGD-albumin inhibited tumor growth similar to RGD-albumin-drug conjugates. Most likely the effect of RGD-albumin on the tumor was due to a non-specific arousal of the immune system. In addition, I modified RGD-HSA with a label for positron emission tomography (PET), to study the distribution of RGD-HSA in tumor bearing mice. Contrary to our expectations, the labeled RGD-HSA was eliminated rapidly from the bloodstream and therefore did not allow sufficient time for accumulation in the tumor tissue. Accordingly, PET images visualized liver and spleen as the organs responsible for the fast elimination and the subcutaneous tumor was not visible. The fast elimination, absence of tumor accumulation, and immunogenicity problems led me to conclude that the present RGD-albumin constructs are not suitable yet for in vivo targeted drug delivery.

In chapter 8, I briefly reviewed current antiangiogenic and antivascular therapies, mainly aiming at creating awareness of how tumors progressively recruit nonmalignant cells. By highlighting the role of endothelial progenitor cells in tumor recurrence as indicated by a recent publication of Shaked and coworkers [10], I reviewed how nonmalignant cells can turn out to be suitable target cells for future cancer therapy.
Discussion

Drug targeting devices are complex macromolecular constructs consisting of many moieties each designed to fulfill a specific task. Here, I highlight the single moieties and evaluate their performance within the drug targeting approach and their benefits and limitations for targeted drug delivery in general.

**Drugs:** Drug targeting approaches are developed for drugs that either exert severe side effects or have a detrimental distribution which limits their efficacy. Targeting of a drug to a specific tissue or even cell type can diminish side effects in non-target tissue and render a drug more effective. However, targeted drugs have to be very potent. The importance of this rather trivial statement becomes apparent when taking a closer look at the route of cell entry. I aimed at receptor mediated uptake of the drug targeting conjugate, which is naturally limited by the number of receptors per cell and the rate of receptor recycling. The drugs used in this thesis are potent as they have pharmacological IC$_{50}$s for their targets in the nanomolar range. But intracellular concentration and effect depend also on the efflux rate of the delivered drug. This rate is influenced by size, charge, and lipophilicity of the drug and the concentration gradient. Here we touch upon a general problem in drug targeting: Most small molecule drugs like e.g. PTK787 and SB202190 are developed to rapidly cross cell membranes. Thus they are small (<500 g/mol), not charged, and lipophilic. Upon coupling to a carrier molecule, the lipophilic nature of the drug can give rise to solubility problems of the final product. This was demonstrated in chapter 4 in which not more than 6 drugs could be coupled to RGD-HSA. Furthermore, rapid efflux of the released drug from the target cell is driven by a concentration gradient. This gradient inevitably forms, since in the circulation all drug is carrier-bound (thus low free drug concentration in the circulation) but in the cell the drug is released in the restricted cell volume (thus high intracellular drug concentration). A typical small, non-charged, and lipophilic drug can quickly redistribute in such a situation, as a consequence of which the efficacy of such a targeting approach may be limited. Size, charge, and lipophilicity requirements are completely opposite for targeted drugs compared to non-target drugs. In the same situation as described above, a charged and hydrophilic drug will be retained in the cell for a prolonged period of time, since it will not readily cross the cell membrane. This will eventually lead to a higher efficacy. An example of a good drug candidate is MMAF, which is relatively large (1250 g/mol) and charged at physiological pH, ensuring a longer retention in the target cell after release from its carrier. I demonstrated that drug targeting conjugates containing the charged MMAF showed a lower EC$_{50}$ in cell killing than drug targeting conjugates modified with the non-charged MMAE [11]. However, I also demonstrated that even targeting of small and lipophilic kinase inhibitors is promising in malignant or inflammatory disease, as was also further demonstrated for fibrotic disease in other theses from our group (Prakash et al., Gonzalo et al.) [12]. In order to further improve therapeutic efficacy, kinase inhibitors with an improved redistribution profile should be selected for future intracellular targeting studies. Optimal, for example, would be a compound that is charged at pH 7 but largely not charged at pH 5, which will allow the compound to cross the lysosomal membrane after release from the carrier but will hamper further redistribution.

**Drug Linkers:** Drug linkers are designed to firmly hold on to the drug during storage and in the circulation, but to release the drug at the target site. In the study presented here, I applied two different reversible drug linkers. The valine-citrulline linker is a cathepsin-cleavable self-immolative drug linker, used for the conjugation of auristatins to albumins [11, 13] and antibodies [14].
stability was demonstrated in the circulation but rapid enzymatic release occurred upon internalization of drug targeting conjugates [15, 16]. Thus high intracellular drug concentrations can be achieved due to the rapid drug release. However, the intracellular concentration of free drug also depends on the rate of internalization and redistribution as discussed above. The intracellular concentration profile of auristatins was excellent in order to induce apoptosis. Consequently this resulted in low nanomolar EC<sub>50</sub>s for the killing of endothelial and tumor cells. In contrast, intracellular concentrations of signal transduction inhibitors ideally need to be maintained above a certain threshold over a prolonged period of time to achieve an extended blockade of disease associated signaling pathways. We therefore applied the Universal Linkage System, which is novel in the context of targeted drugs, for coupling of kinase inhibitors to carrier proteins. The release from these carriers was generally slow in serum but could be accelerated by high intracellular concentrations of glutathione [17-19]. Thus, the linker proved to be stable in the circulation, but demonstrated a sustained release of the drug upon internalization. In a renal drug targeting approach, Prakash et al., showed that this linker can be employed to achieve an effective concentration of the p38MAP kinase inhibitor SB202190 in the kidneys and maintain this concentration over 72 h [19].

An additional advantage of ULS is that it extends the possibilities for the preparation of drug targeting conjugates. Drugs like PTK787 cannot be conjugated via any other linker to a carrier as they lack common reactive groups needed for the coupling reaction (e.g. amino, carboxyl, or sulfhydryl-groups). We demonstrated the versatility of the ULS linker by coupling a range of different drug molecules to different carrier molecules (scheme 1). From pharmacological point of view, the drugs can be classified into kinase inhibitors [12, 17, 19, 20], an anti-inflammatory drug [18] and an angiotensin-II receptor blocker. The common denominator of the compounds is that they all can bind to ULS via an aromatic nitrogen that can donate an electron pair to the platinum(II) atom in the linker. Similarly, the drug-ULS intermediates were conjugated to the carrier proteins by coordination linkage, most likely at methionine residues. The simplicity of the coupling reaction was remarkable and is unmatched in the field of reversible drug linkers. The drug molecules were conjugated to the ULS in a single reaction step and in a second step these drug-ULS constructs were readily coupled to albumin with yields of 60-90 %. In comparison, multiple reaction steps are needed for the coupling of maleimidyl-val-cit-linker to auristatin and further steps for the conjugation of maleimidyl-val-cit-auristatin to albumin.

I also addressed concerns that were raised about toxicity due to ULS being a cisplatin related compound. Cisplatin toxicity involves cross-linking of DNA [21] with the availability of a reactive ligand site at the platinum atom being essential for the toxicity of platinum compounds. ULS can therefore only react with DNA after release of the drug or carrier. In view of the sustained intracellular drug release, we expected only slow formation of reactive platinum species in the target cell, that will be readily detoxified, as was described for cisplatin [22, 23]. The absence of acute toxicity of ULS-containing drug targeting conjugates in vitro on human endothelial cells [17], renal tubular cell [19], and hepatic stellate cells [18], provided evidence that indeed the released platinum is detoxified. Accordingly, no signs of acute platinum related toxicities were observed in vivo in different rat models of disease [18, 19]. However, the fate of the platinum in the body has not been fully elucidated yet. Further in vivo studies will have to show if and how platinum is excreted or if it resides in the body for a prolonged period of time like cisplatin does possibly provoking chronic toxicities. In conclusion, the ULS is an excellent reversible drug linker for targeted drug delivery with
a sustained release profile. Therefore, we will apply this linker in new project aiming at cell specific delivery of kinase inhibitors for the treatment of liver and kidney fibrosis.

Scheme 1: Several drugs have been coupled by the Universal Linkage System to a number of different carriers demonstrating the versatility of this linker. The drugs at the left side of the scheme from top to bottom are losartan (angiotensin II receptor blocker), AG1295 (PDGFR kinase inhibitor), PTK787 (VEGFR kinase inhibitor), RWJ67657 (p38MAP kinase inhibitor), an ALK5 inhibitor, SB202190 (p38MAP kinase inhibitor), Y27632 (ROCK inhibitor), a PDGFR kinase inhibitor, and pentoxifylline (antiinflammatory drug). As drug carriers we used (right; from top to bottom) HSA, several dendrimers, lysozyme, and a number of antibodies.

**Drug Carrier:** The task of a carrier is to carry the drug, prevent its excretion, and to present the targeting ligand. For this purpose, liposomes, natural and synthetic polymers, antibodies (ligand and carrier), nanoparticles and even erythrocytes have been used. I chose human serum albumin due to its high number of primary amino-groups, its long circulation property, and its biodegradability. Using conventional and novel linkers, we modified it with up to twelve RGD-peptides and nine drug molecules, leading to a receptor binding drug carrier with a high avidity. For some of these products like RGD-PTK-HSA problems with solubility were observed and this only allowed modification with six PTK787 per albumin. Using different approaches for PEGylation like the coupling of monovalent
PEG or the use of a bivalent PEG-linker for the RGD-peptide coupling. I resolved these issues. More drugs have been coupled to pegylated albumin without precipitation of the final products. However, the combination of RGD-peptides and albumin appeared to be immunogenic in our study and the long circulation property of parental HSA was lost. It should be stressed that RGD-HSA conjugates were immunogenic but modified HSA in general was not, as can be appreciated by the absence of an immunogenic response against PTK-HSA. HSA has also proven useful as a carrier for methotrexate (MTX), exploiting passive retention in the tumor via EPR. The MTX-HSA conjugate displayed anti-tumor activity in a variety of human xenograft tumors in nude mice [24] and was well tolerated in a clinical phase I trial [25]. HSA is also a suitable drug carrier in combination with other targeting ligands like mannose-6-phosphate [26] or cyclic, PDGF-mimicking peptides [27], which both have been administered multiple times to immune-competent animals without raising an immune response [28] (Theses of T. Gonzalo, W.I. Hagens, and R. Greupink). Therefore, such drug carriers have been and still are under investigation in our lab.

Targeting ligand: The purpose of a targeting ligand is to provide target cell selectivity and, in this targeting approach, to trigger internalization of the drug targeting conjugate. Apart from the RGD-peptide many ligands are used for the delivery of mostly toxic payloads to malignant tissues. These ligands can be divided mainly into two groups aiming either at specific receptors on the tumor cell surface or at receptors on tumor endothelium. Among others, folate, somatostatin analogs, and several monoclonal antibodies have been applied successfully for tumor cell targeting in animal models [29-31]. It was shown that macromolecular carriers (> 10 kDa) accumulate in tumors by EPR, allowing the carriers to reach tumor cells in close proximity to the vasculature. However, deeper penetration into the tumor tissue was hampered [32]. Human tumors are believed to be even less accessible than animal tumors due to the heterogeneous blood supply, elevated interstitial pressure, and large transport distances in the interstitium [33]. This hampers the penetration of drug targeting conjugates deep into the tumor mass and, as a consequence, their ability to reach the targeted receptors. Drug delivery targets on tumor endothelial cells are in that respect much more accessible than tumor-cell expressed targets. Popular ligands for drug delivery strategies are RGD-peptides, NGR-peptides, and monoclonal antibodies against E-selectin or phosphatidylserine [34-36]. But the target receptors of these ligands are not always expressed throughout the whole tumor endothelium. This endothelium consists of dormant vessels, angiogenic vessels, mosaic vessels, and even vessel lacking endothelial cells [3]. Finding a target receptor covering all tumor blood vessels seems highly unlikely. A combination of two or more targeting ligands might overcome this limitation [37].

Existing drug carriers should be combined instead of the modification of a single carrier with multiple ligands, which would needlessly increase the complexity of a construct. The combination of different drug targeting conjugates harnessed with the same drug can deliver a cytotoxic or antiangiogenic compound to most of the tumor endothelium. Drug carriers modified with multiple c(RGDfK)-peptides can play an important role in such a combined approach. This thesis reports how multivalency greatly improved the affinity of the drug targeting conjugates in comparison to the single peptide by coupling of multiple peptides per carrier. The multivalency also facilitated internalization in contrast to monovalent drug targeting approaches which demonstrated poor internalization [13, 17, 38, 39]. The drug targeting conjugates developed in this thesis showed high binding avidity, proven internalization and adequate drug release properties, since the uptake eventually led to effect of the released drug. Prevailing that the immunogenic properties of the RGD-albumin carriers can be removed and the target-cell specific homing in vivo can be restored by
modification of the carrier, such multivalent RGD-equipped carriers are interesting candidates for a combined targeting approach directed to the angiogenic endothelium.

**Perspectives for RGD-mediated drug targeting:** In this thesis, I have shown that multivalent RGD-albumin constructs with 12 RGD-peptides per carrier are immunogenic while carriers with less RGD-peptide demonstrated only little immunogenicity. This finding can have a large impact on the future of RGD-mediated drug targeting since multivalent carriers are widely studied [34]. Therefore, it has to be studied whether this effect is specific for RGD-albumin conjugates or if multimeric presentation of RGD-peptides in general induces antibody formation. In that respect, it would be interesting to administer multiple injections of the RGD-peptide modified HPMA copolymer of Mitra et al [40, 41] which closely resembles our RGD-HSA conjugates. The absence of an immunogenic response would indicate that only the combination of HSA and RGD induced antibody formation. Possibly, the good biodegradability of HSA allows for HSA-RGD fragments to be presented on MHC complexes. This might not occur for RGD-polymer constructs or RGD-modified liposomes. If the immune system also recognizes RGD-HPMA conjugates, it might be the multimeric representation of the c(RGDfK)-peptide in general triggering the immune system. Although this would be a setback, there are also a number of other RGD-peptides like RGD4C or RGD10, suitable for drug targeting purposes, which might not be immunogenic and could replace c(RGDfK)-peptides. However, the immunogenic profile of these peptides is not known yet. In chapter 2 I also reviewed RGD-mediated targeting approaches aiming at delivery to the cell surface of tumor endothelium, like RGD-tTF or radiolabeled RGD-peptides [37, 42]. These drug targeting conjugates do not represent multimeric forms of RGD but only a single high affinity peptide, since uptake is not required or would even hamper their mode of action. Based on our results we do not expect an immunogenic response to such a construct, regardless of the outcome of a possible study of RGD-HPMA copolymer. Such single peptide targeting approaches have been especially successful in delivery of radioactive compounds for imaging or radiotherapy [42, 43] as exemplified by $^{18}$F-Galacto-RGD, which has already been tested successfully in patients [44].

Although the features of the single moieties have been discussed, a drug targeting approach has to be evaluated as a whole. A drug targeting conjugate has to be scrutinized for e.g. binding affinity and selectivity, uptake, drug release, effect, immunogenicity, half-life, but also overall yield of synthesis, complexity and scalability of synthesis, and formulation issues of complex constructs. Our studies demonstrated that the demands of many of these listed parameters were met but the negative outcome for immunogenicity did terminate further work and led us conclude that c(RGDfK)-albumin constructs are not suited for targeted drug delivery. Nevertheless, our drug targeting approach must be transferred to new drug targeting conjugates like albumins modified with multiple NGR-peptides or artificial polymers (e.g. dendrimers) modified with RGD-peptides. Thereby, we can exploit our experience on multivalent peptide-carrier conjugates that demonstrated valuable properties as shown in this thesis. The novel linking technology will facilitate straightforward drug conjugation and will allow us to develop new classes of drug targeting conjugates.

**Reference List**


Summary & Discussion
Chapter 9


