Doctoral Thesis

Molecular targeting of carbonic anhydrase IX using human monoclonal antibodies and small organic ligands

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MOLECULAR TARGETING OF CARBONIC ANHYDRASE IX
USING HUMAN MONOCLONAL ANTIBODIES AND
SMALL ORGANIC LIGANDS

A dissertation submitted to
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presented by
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1. SUMMARY

A major feature of solid tumors is hypoxia, the decreased availability of oxygen, which increases patient treatment resistance and favors tumor progression. Hypoxic conditions trigger a molecular response via the hypoxia-inducible factor. Among the target genes of this key transcription factor are carbonic anhydrases IX and XII, two transmembrane proteins frequently upregulated in solid tumors. Due to their role in pH regulation, they confer a survival advantage to tumor cells within an acidic and hypoxic tumor environment.

This thesis describes the generation of high-affinity human monoclonal antibodies specific to CA IX. These antibodies were able to stain CA IX \textit{ex vivo} and to target the cognate antigen \textit{in vivo}. After intravenous administration of the antibodies their staining pattern was compared to the one of a chemical marker of hypoxia (pimonidazole) by multi-fluorescence analysis. While there was a perfect overlap for both agents in the colorectal adenocarcinoma model LS174T, a complementary pattern of targeted tumor regions could be observed in the second model SW1222. Moreover, not all CA IX positive areas were reachable in this tumor model.

The results support the hypothesis of a dynamic blood flow within tumors that may vary over time, thereby controlling the accessibility of certain tumor regions.

The new human anti-CA IX antibodies are expected to be non-immunogenic and may serve as broadly applicable reagents for the non-invasive imaging of hypoxia and for pharmacodelivery applications.

The second part of the thesis describes the synthesis and characterization of two acetazolamide derivatives, which contain either a charged fluorophore or an albumin-binding moiety. The fluorescent acetazolamide was shown to preferentially target tumors \textit{in vivo}. The rationale for the synthesis of the albumin-binding acetazolamide was to prevent cellular internalization through the engagement of serum albumin thereby restricting the scope of the inhibitor to membrane-associated carbonic anhydrases. The ability of the bifunctional compound to cause tumor growth retardation was assessed in
two xenograft models of cancer (LS174T and SK-RC-52) alone and in combination with chemotherapeutic drugs (5-fluorouracil and sunitinib).
2. ZUSAMMENFASSUNG


Der durch Sauerstoffmangel aktiv gewordene Transkriptionsfaktor “hypoxia-inducible factor” induziert die Transkription von Genen, welche die Tumorzellen an die veränderte Umgebung anpassen. Dazu zählen auch die Gene der carbonischen Anhydrasen (CA) IX und XII. Ihre Überexpression in zahlreichen Krebsarten ist bekannt. Aufgrund ihrer pH-regulierenden Rolle erhalten die Tumorzellen einen Selektionsvorteil in dem sauren und hypoxischen Tumormilieu.

Es wird erwartet, dass die neuen Antikörper nicht immunogen sind und dass sie eine breite Anwendung im nicht-invasiven „imaging“ von Hypoxie finden werden oder als Vehikel für Pharmazeutika in der gezielten Krebstherapie dienen werden.

3. INTRODUCTION

3.1. Ligand-based tumor targeting

The vast majority of pharmacological approaches for the treatment of solid tumors is limited by the lack of specificity. Chemotherapeutic agents exhibit poor accumulation in the tumor mass owing to poor blood perfusion, irregular vasculature and high interstitial pressure in the tumor environment (Bosslet et al, 1998). Moreover, multidrug resistance proteins may decrease drug uptake. As a consequence, the development of therapeutic agents, which preferentially accumulate in solid tumors represents a main focus 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 specific for tumor-associated markers.

Ever since Paul Ehrlich first envisioned the possibility of selective delivery of antibodies to the tumor environment at the end of the nineteenth century, the concept of “magic bullets” capable of tumor targeting has been extended to other molecules like peptides, aptamers, small globular proteins and small organic molecules.

Tumor-associated markers (antigens) can either be located on the surface of tumor cells, in the extracellular matrix of the tumor (stroma) or around the tumor-neovasculature.

3.1.1. Targeting the tumor cells

Many efficient and currently successful antibodies target surface receptors. Besides mediating antibody dependent cytotoxicity (ADCC) and complement-mediated cell lysis, they often interfere with signaling. Table 3.1 summarizes approved monoclonal antibodies, with the majority being directed to cell surface markers.

A major drawback of targeting antigens on the cells of solid tumors, is their poor accessibility. In the case of CA IX, a tumor marker that becomes upregulated under
hypoxic conditions, it was unclear at the beginning of this study whether in vivo targeting would be possible, considering that hypoxic areas are typically located at a 100-200 µm distance from the nearest tumour blood vessel (Kerbel & Folkman, 2002) and may be thus more difficult to reach. Indeed, while the antibody-based targeting of markers of tumour neo-vasculature is a rapid and efficient process [e.g., L19 antibody specific to the alternatively-spliced EDB domain of fibronectin; (Berndorff et al, 2005; Borsi et al, 2002; Borsi et al, 2003; Tarli et al, 1999; Tijink et al, 2006; Viti et al, 1999)], the tissue penetration of monoclonal antibodies to certain abluminal structures is often impaired by several factors, including molecular size (Adams et al, 1998; Low et al, 2008; Yokota et al, 1992), antigen barrier (Dennis et al, 2007) and tumour interstitial pressure (Jain, 1987).

| Table 3.1 | Approved monoclonal antibodies. Adapted from (Reichert et al, 2005). |
3.1.2. Targeting the tumor neo-vasculature and the stroma

The basis for vascular tumor targeting is provided by the existing anatomical and physiological differences in the endothelium and surrounding stroma of tumors compared to normal tissues. The tumor vasculature is strikingly disorganized and tortuous (Konerding et al., 2001). Indeed, it is often difficult to distinguish arterioles and venules, and the occurrence of vascular shunts (in which blood passes directly from an arteriole to a venule) is common. The flow of blood through the tumor capillaries is frequently sluggish, and at times might be stationary or even experience a reversal in the direction of flow (Tozer et al., 2005). The haemoglobin in the erythrocytes is depleted of oxygen and the microenvironment - which includes both the blood and the endothelium lining the vessels - is profoundly hypoxic (Helmlinger et al., 1997). In this environment, the endothelial cell is strikingly different from that in a normal tissue, in which the endothelium is remarkably quiescent. The endothelium in tumors proliferates rapidly and contributes to active angiogenesis (Denekamp & Hobson, 1982). The tumor is also nutrient (for example, glucose) starved, acidic (owing to lactate production from anaerobic glycolysis and carbonic acid production by carbonic anhydrases (CA) IX and XII) and under oxidative stress (Brown & Bicknell, 2001). The endothelial cell has been shown to respond transcriptionally to all these stimuli, and the existence of hypoxically induced endothelial specific genes indicates that the endothelial cell is no stranger to a hypoxic environment.

Since vascular tumor targeting aims at the rapid and selective shutdown or damage of the established tumor vasculature, it is superior to strategies attacking the tumor cells directly, because occluding one blood vessel will cut the blood, oxygen and nutrient supply of a large number of tumor cells. Moreover, a wide range of solid tumors should in principle be treatable with one single vascular targeting agent. In addition, the problem of multidrug resistance is overcome due to the given genetical stability of endothelial cells.
3.2. Ligands for tumor targeting applications

3.2.1. Antibodies

In pharmaceutical biotechnology, antibodies are indisputably the best-established class of binding molecules for tumor diagnosis and therapy. At present, monoclonal antibodies and possibly aptamers represent the only classes of binding molecules that can be rapidly isolated and that have strong affinity for virtually any antigen.

Monoclonal antibodies of rodent origin were first obtained in 1975 by means of hybridoma technology (Kohler & Milstein, 1975). In 1986, the group of Greg Winter pioneered the generation of humanized antibodies by grafting the complementarity determining regions (CDRs) of murine antibodies onto a human antibody framework (Jones et al, 1986), thereby minimizing immunogenicity.

Antibody humanization was later complemented by the generation of human antibodies by immunization of transgenic animals carrying human immunoglobulin genes (Zou et al, 1993) and by antibody phage technology (Winter et al, 1994).

The display of antibody fragments (scFv or Fab) on the surface of filamentous phage allows the easy construction of large libraries of human antibodies (>10^9), from which monoclonal antibodies can be isolated by panning the phage library against the antigen of choice. Affinity maturation of selected antibody fragments using combinatorial mutagenesis of the antibody gene may be applied in order to achieve dissociation constants in the nanomolar to picomolar range (Pini et al, 1998). In 1999, ribosome display has been proposed as a fully in vitro avenue for the isolation and affinity maturation of human antibodies (Schaffitzel et al, 1999).

Different antibody formats show different pharmacokinetics and different tumor-targeting properties (Borsi et al, 2002). Full IgGs exhibit a slow elimination from the blood and readily accumulate in the liver. For imaging applications in nuclear medicine, rapidly clearing antibody fragments are typically preferred [Figure 3.1].
Figure 3.1 Antibody formats

However, intact immunoglobulins remain the antibody format of choice, whenever activation of signaling pathways, antibody-dependent cellular cytotoxicity (ADCC) or the complement system is required for the observation of a therapeutic benefit. Antibody fragments isolated e.g. by phage display may therefore easily be converted into full IgGs by subcloning the genes encoding the variable antibody domains into a suitable expression vector.

A major drawback of these blockbuster drugs is the expensive production in mammalian cells required for proper disulfide bond formation and glycosylation. Furthermore, expression yields are usually low and antibodies tend to aggregate, especially when fused to additional domains or proteins like cytokines.

3.2.2. Peptides

Several internalizing peptides, specific to receptors, which are overexpressed on tumor cells, have been used for the imaging of tumors and for the selective delivery of therapeutic radionuclides to neoplastic lesions. The somatostatin analogue Sandostatin LAR (octreotide, Novartis) has been approved in Europe and the USA for the imaging of tumors (Kowalski et al., 2003). Several other agents are in development (Behr et al., 2001; Ginj & Maecke, 2004), such as integrin-binding peptides comprising an arginine-glycine-aspartate (RGD) tripeptide-motif (Chen et al., 2004) or bombesin peptide analogs (Chen et al., 2004; Okarvi & al-Jammaz, 2003).
Peptide phage libraries have been used for *in vivo* panning by the groups of Ruoslahti and Pasqualini (Pasqualini & Ruoslahti, 1996; Rajotte *et al.*, 1998), but there is need to confirm the imaging and therapeutic potential of these phage-derived peptides in advanced animal models and in the clinic.

In general, peptide phage display has lower success rates than antibody phage technology and yields binders of affinities, which rarely exceed the micromolar range. This limitation can be overcome by multimerization resulting in an improvement of avidity (Terskikh *et al.*, 1997).

Natural occurring peptides, e.g., peptides resulting from the degradation of extracellular matrix components, or peptides derived from physiological angiogenesis inhibitory proteins have been shown to selectively target the tumor vasculature by microscopic techniques (Maeshima *et al.*, 2002). Analogy to the CDR loops of binding antibodies has also been exploited for peptide design in order to construct tumor-targeting molecules (Tan *et al.*, 2006).

Nonetheless, the generation of peptidic binders of sufficient affinity is not possible for every antigen. In addition, the *in vivo* stability of peptidic drugs remains a major cause of concern.

### 3.2.3. Aptamers

Besides antibodies, aptamers (single-stranded nucleic acids capable of adopting a complex three-dimensional structure) are possibly the only other class of molecules from which specific binding molecules against a variety of target proteins can be isolated (Nimjee *et al.*, 2005). Aptamer technology enables the generation of large libraries (>10^{12} members) of single-stranded DNA or RNA molecules, which can be panned for target antigen binding. The nucleic acids selected during this procedure are then amplified using PCR-based techniques in order to generate the input material for a further selection cycle. At 8-15 kDa, aptamers are intermediate in size between antibodies (150 kDa) and small peptides (1-5 kDa) and are slightly smaller than scFvs (25 kDa). The small size and polyanionic nature of aptamers may minimize the residence in organs of excretion, providing some potentially useful features for imaging and radiotherapy. Dissociation
constants of aptamers usually lie in the micromolar to subnanomolar range. Spiegelmer technology has led to advances concerning the in vivo stability of aptamers (Vater & Klussmann, 2003). Pegaptanib (Macugen™, Pfizer and Eyetech) for the treatment of age-related macula degeneration is the first aptamer to be approved by the FDA (Doggrell, 2005). After intravitreal injection Macugen™ binds to VEGF-165, thereby inhibiting ocular angiogenesis. Promising imaging studies of rodent models of cancer with radiolabeled aptamer TTA1 specific to tenascin C have been described (Hicke et al, 2006). The potential of aptamers for tumor targeting applications is currently being investigated in clinical trials.

3.2.4. Small organic molecules

Small organic molecules constitute the vastest class of drugs on the market. They have several advantages over biopharmaceuticals, namely oral bioavailability, easy manufacture, lack of immunogenicity and favorable tissue distribution properties. The isolation of high-affinity small organic binders to flat protein surfaces remains a big challenge (Arkin & Wells, 2004), whereas typical dissociation constants can be nanomolar for antigens with cavities (e.g., enzymes). There is experimental evidence which suggests that affinity and specificity can be increased by linking two or more organic compounds which recognize adjacent epitopes on the target antigen, thus exploiting the chelate effect (Melkko et al, 2006). Methods for the identification of such bidentate ligands include “SAR by NMR” (Shuker et al, 1996), dynamic combinatorial chemistry (Ramstrom & Lehn, 2002) and tethering approaches (Erlanson et al, 2003). This list has been expanded by DNA-encoded chemical libraries, in which individual compounds are covalently attached to a DNA fragment serving as amplifiable identification bar code. DNA encoded chemical libraries can be constructed in single (Mannocci et al, 2008) or dual pharmacophore format, also termed termed Encoded Self-Assembling Chemical (ESAC) libraries (Melkko et al, 2004). We have described the isolation of ESAC-derived bidentate molecules with nanomolar affinity to carbonic anhydrase (Melkko et al, 2004). Isoenzymes of carbonic anhydrase have been identified
as interesting disease targets. In renal cell carcinoma (RCC) carbonic anhydrase IX may be found upregulated under hypoxic conditions (Grabmaier et al, 2004).

More in general, it appears that combinatorial chemistry methodologies such as Speed Screen (Muckenschnabel et al, 2004) and DNA-encoded chemical library technology (Gartner et al, 2004) may allow the screening of chemical libraries of unprecedented size. It remains to be seen whether these approaches are efficient enough to compete with recombinant antibody technology for the generation of specific binders to protein targets of interest.

3.3. Classes of targets

Several protein antigens expressed either in the tumor blood vessel or in the adjacent matrix have been the subject of in vivo targeting studies using radiolabeled targeting agents or compounds delivering a payload to the tumor vasculature.

3.3.1. Extra-Domain B (EDB) and A (EDA) of Fibronectin

Fibronectin is a large glycoprotein present in large amounts in plasma and tissues. Under tissue remodeling conditions a 91-amino acid type III homology domain, the extra-domain B (EDB), becomes inserted into the fibronectin molecule by a mechanism of alternative splicing at the level of the primary transcript (Zardi et al, 1987). In healthy adult individuals EDB is essentially undetectable. However, in many aggressive solid tumors EDB-containing fibronectin is abundant and displays either predominant vascular or stromal expression patterns, depending on the tumor type (Carnemolla et al, 1989). The EDB sequence is identical in mouse, rat, rabbit, dog, monkey and man. This feature prevented the generation of anti-EDB antibodies using hybridoma technology in the past, probably due to tolerance. Nowadays the conservation of the EDB domain facilitates animal experiments in immunocompetent syngeneic settings using human monoclonal phage derived antibodies (Carnemolla et al, 1996; Neri et al, 1997; Pini et al, 1998). These include the high-affinity human antibody L19, which has been shown to efficiently
localize to the subendothelial layer of tumor blood vessels in animal models (Borsi et al., 2002; Demartis et al., 2001; Tarli et al., 1999; Viti et al., 1999) and patients with cancer following intravenous injection (Santimaria et al., 2003). The three-dimensional structure of EDB has been solved in solution using nuclear magnetic resonance (NMR) methods (Fattorusso et al., 1999). A large number of therapeutic derivatives of the L19 antibody have been produced [Figure 3.2]. Importantly, the anti-EDB antibody L19, in homodimeric scFv format and labeled with iodine-123, has been studied in over 40 patients with cancer. The results obtained in the first 20 patients have recently been described (Santimaria et al., 2003). The derivatives L19-\(^{131}\)I, L19-IL2 and L19-TNF are currently being studied in phase II clinical trials.

![Figure 3.2](image)

**Figure 3.2** Schematic representation of antibody derivatives, which can be used either for tumor imaging or for therapeutic applications.

Fibronectin contains a second alternatively spliced domain [extra-domain A (EDA)] (Rybak et al., 2007a; Villa et al., 2008), which has been shown to display a restricted pattern of expression in plasma and normal human tissues, while being over-expressed in
the neo-vasculature and/or stroma structures of some aggressive solid tumors (Borsi et al, 1998). Recently, Villa et al. reported the isolation of the high-affinity human monoclonal antibody F8 specific to both human and mouse EDA. ScFv(F8), when used in homodimeric format, was shown to efficiently target the tumor neo-vasculature in immunocompetent 129SvEv mice, bearing subcutaneous syngeneic F9 murine tumors (Villa et al, 2008). The fusion protein F8-IL10 is about to enter phase I clinical trials in arthritis.

3.3.2. Large tenascin-C isoforms

Tenascins are family of four glycoproteins, which contribute to extracellular matrix (ECM) structure and influence the behaviour of cells that are in contact with the extracellular matrix. Tenascins are all built from a common set of structural motifs. Several isoforms of tenascin-C can be generated as a result of different patterns of alternative splicing in the region between fibronectin type III domains A1 and D. Certain extra-domain containing isoforms have been identified as tumor-associated antigens showing a more restricted expression pattern in normal tissues compared to the small tenascin isoforms (Borsi et al, 1992). The extra-domain showing the most restricted expression pattern is the C-domain of Tenascin-C. Using a phage-derived antibody it was undetectable in immunohistochemistry and northern-blot analysis in normal adult tissues, but showed a prominent perivascular staining pattern in aggressive brain tumors and some lung tumors [Figure 3.3] (Carnemolla et al, 1999).

![Figure 3.3](image)

**Figure 3.3** Immunohistochemical analysis on U87 human glioblastoma tumor model. The sections were stained with (A) SIP(G11) for domain C of tenascin-C and (B) scFv(D11) for domain D of tenascin-C.
Radiolabeled derivatives of monoclonal antibodies to domains A1 and D of tenascin-C have been used for over a decade for imaging and radioimmunotherapy in patients with cancer (Paganelli et al., 1999; Riva et al., 1999). The staining pattern of these antibodies varies between different tumors, the two extremes being a predominantly vascular and a diffuse stromal staining. Encouraged by promising preclinical studies (Marlind et al., 2008) F16-\(^{131}\)I and F16-IL2 are now being studied in clinical studies in cancer patients.

### 3.3.3. Integrins

Integrins are a family of transmembrane heterodimeric receptors that engage cells with their environment. The extracellular domains bind to components of the ECM usually through recognition of an RGD tripeptide, thereby mediating cell invasion and proliferation (Ruoslahti & Pierschbacher, 1986). \(\alpha_\beta_3\) was the first integrin shown to be associated with tumor angiogenesis. \(\alpha_\beta_3\) is highly expressed in angiogenic endothelial cells in wound granulation tissue and in malignant tumors but not, or to a much lower extent, in quiescent endothelial cells. A high-affinity humanized antibody (Vitaxin, MedImmune) is in clinical development as an anti-angiogenic therapeutics (Gutheil et al., 2000). However its tumor-targeting performance for cancer imaging has been unsatisfactory so far (Posey et al., 2001). Anti-\(\alpha_\beta_3\) antibodies have been shown to preferentially localize to tumor blood vessels using \textit{ex vivo} fluorescence microscopy detection (Baluk et al., 2003). Moreover, a paramagnetic contrast agent targeted to the LM609 monoclonal antibody, which is specific to \(\alpha_\beta_3\) has been described for the \textit{in vivo} imaging of angiogenesis using magnetic resonance (Sipkins et al., 1998).

### 3.3.4. Annexin A1

Annexins are cytosolic proteins that associate with cell membranes in a calcium dependent manner. Some annexins may translocate the lipid bilayer to the external cell surface.
Schnitzer and co-workers recently discovered annexin A1 as a tumor endothelial target by the terminal perfusion of tumor-bearing rats with silica beads, as described later. A monoclonal antibody to this antigen has been used for the radioimmunoscintigraphic detection of solid tumor lesions in a rat model. Furthermore, relatively low radioactive doses of the same antibody labeled with iodine-125 showed therapeutic benefit in rats (Oh et al, 2004).

3.3.5. Vascular endothelial growth factors and their receptors

The discovery of vascular endothelial growth factors (VEGFs), and especially VEGF-A, as prime mediators of angiogenesis has stimulated interest in the use of VEGFs, VEGF receptors and their complexes as antigens for the targeted delivery of antibody derivatives to the tumor neovasculature (Brekken et al, 2000). Only recently, the approval of bevacuzimab (Avastin; Genentech) for first-line cancer treatment has highlighted the contribution of VEGF-A to cancer progression (Ferrara). In addition, the selective localization of monoclonal antibodies to VEGF receptor 2 and the VEGF-A/VEGF receptor 2 complex has been studied (Prewett et al, 1999). In general, the absolute amounts of antibody that have been delivered to the tumor were modest, which possibly reflects kinetic limitations in the targeting of low or medium abundance antigens, even when they are accessible to binding agents injected into the bloodstream (Halin et al, 2002).

3.3.6. Prostate-specific membrane antigen

Prostate-specific membrane antigen (PSMA) is a membrane glycoprotein with proteolytic activity. Whereas it is usually predominantly expressed in the prostate, serum concentrations in patients with prostate cancer are often found to be elevated (Bostwick et al, 2000). There have been several studies on the overexpression of PSMA in the neovasculature of various different tumors (Liu et al, 1997). In normal vasculature the expression is restricted to some vessels of the breast, duodenum, kidney and prostate. Imaging of patients after receipt of radiolabeled humanized monoclonal antibody J591
demonstrated excellent tumor targeting (Bander et al, 2003). Anti-proliferative properties in vivo have been observed in rodent models of cancer using targeted alpha therapy with the J591 antibody. Alpha-particles might be uniquely suited as selective depositors of toxic radiation to tumor vascular structures, because of their short tissue penetration (a few cell layers) and high-energy (Kennel et al, 2002).

In a clinical phase I trial the tolerability and preliminary efficacy when conjugated to $^{90}$Yttrium was investigated (Milowsky et al, 2004).

### 3.3.7. Endoglin

Endoglin, also termed CD105, is a homodimeric transmembrane glycoprotein acting as a transforming growth factor-β (TGF-β) co-receptor. It was initially characterized as a marker of leukemia, although it is mainly being overexpressed by the endothelial cells of solid tumors. Despite the occurrence of endoglin in normal tissues as shown by immunohistochemistry (Matsubara et al, 2000), biodistribution and imaging studies have been carried out in rodents and dogs (Bredow et al, 2000).

### 3.3.8. Roundabout-4

Roundabout-4 (Robo4), also known as Magic Roundabout, is the most recently discovered member of the Roundabout family and was identified through data mining for endothelial-specific genes (Huminiecki & Bicknell, 2000; Huminiecki et al, 2002). Robo4 comprises an extracellular domain than smaller than the one of the neuronal roundabout receptors, but the intracellular domain shows significant roundabout homology.

Analysis of the promoter sequence upstream of Robo4 has identified a 3kb sequence that codes for endothelial specific expression (Okada et al, 2007). Expression of Robo4 is abundant on tumor vessels of brain, colon, breast, kidney, and bladder, but undetectable in normal tissues in vivo. However, when cultured in vitro, Robo4 was found to be expressed on the endothelium of such normal tissues (Huminiecki et al, 2002; Seth et al, 2005). Jones et al. reported that the binding of Slit2 to Robo4 regulates vascular integrity.
by counteracting the effects of VEGF (Jones et al., 2008). Due to its specific expression on tumor endothelial cells, Robo4 constitutes an attractive target for cancer therapy (Neri & Bicknell, 2005).

3.3.9. Nucleolin

Nucleolin is the major nucleolar protein of growing eukaryotic cells. It is found associated with intranucleolar chromatin and pre-ribosomal particles. It induces chromatin decondensation by binding to histone H1. Nucleolin is thought to play a role in pre-rRNA transcription and ribosome assembly. Schnitzer and co-workers identified nucleolin together with annexin A1 as a tumor endothelial target by the terminal perfusion of tumor-bearing rats with silica beads, as described later (Oh et al., 2004). Christian et al. showed that cell surface nucleolin is a specific marker of angiogenic endothelial cells and describe furthermore a tumor-homing peptide specific to this marker (Christian et al., 2003). The expression of nucleolin in normal human tissues remains unclear.

3.3.10. Aminopeptidase N

Aminopeptidase N (CD13) is a widely expressed ectoenzyme with functions that do not always depend on its enzymatic activity. As an extracellular peptidase, Aminopeptidase N plays a role in angiogenesis by degrading matrix proteins, by generating peptides with pro- or anti-angiogenic properties or by modulating the signaling of angiogenic factors (Bauvois, 2004). In vivo peptide phage panning led to the discovery of the asparagine-glycine-arginine (NGR) tumor-homing peptide (Pasqualini et al., 1995), which was later shown to recognize Aminopeptidase N (Pasqualini et al., 2000). This peptide, when coupled to TNF-α, melphalan, doxorubicin, interferon-α and endothelial-monocyte-activating polypeptide II, has been demonstrated to improve the therapeutic index of the corresponding bioactive moiety in vivo (Crippa et al., 2008; Sacchi et al., 2006). Curnis et al. reported that different CD13 forms are expressed in myeloid cells, epithelia and tumor-associated blood vessels and claim that a TNF-NGR peptide fusion binds
selectively to the tumor vasculature. However, the group did not publish the amount of tumor uptake in a biodistribution experiment comparing radioiodinated TNF-NGR with unmodified TNF (Curnis et al, 2002).

3.3.11. Delta-like 4

Delta-like 4 (DLL4), a member of the Notch/Delta family of signaling molecules, has been shown to be expressed at sites of vascular development, as well as in physiologic and pathologic angiogenesis (Claxton & Fruttiger, 2004; Gale et al, 2004; Mailhos et al, 2001). The protein is located in the plasma membrane. Mailhos et al. described the upregulation of DLL4 in ovarian vessels surrounding growing follicles and within tumor vasculature when compared with adjacent normal tissues. Xenograft studies revealed high expression of mouse DLL4, but not of human derived DLL4, in the tumor vasculature (Mailhos et al, 2001). Human renal clear cell carcinoma vasculature displayed an almost 9-fold higher DLL4 expression than the vasculature of normal human kidney (Patel et al, 2005). DLL4 is induced both by VEGF (Liu et al, 2003) and hypoxia (Mailhos et al, 2001).

3.3.12. Chemokine receptor 4

Chemokine receptor 4 (CXCR4) has been identified as a tumor marker by our group in a transcriptomic and proteomic study of human umbilical vein endothelial cells (HUVECs) exposed to hypoxic conditions (Scheurer et al, 2004). Malignant cells from at least 23 different types of cancer have been shown express CXCR4 and to respond to its ligand CXCL12. However, there is wide expression of this chemokine receptor and its ligand in normal tissues (Balkwill, 2004). There is evidence that CXCR4 and CXCL12 play a fundamental role in foetal development, mobilization of haemopoietic stem cells and trafficking of naïve lymphocytes (Rossi & Zlotnik, 2000). CXCR4 also plays a role in AIDS, where it functions as the coreceptor necessary for the CD4-mediated infection of T-cells by the HIV virus. Single chain Fv antibodies raised against CXCR4 inhibited
prostate cancer cell migration and invasion in vitro, but their in vivo targeting performance remains to be investigated (Vaday et al, 2004).

3.4. Methodologies for the discovery of novel tumor targets

3.4.1. Differential expression analysis of tumor endothelial cells

Early approaches for the identification of tumor vascular targets relied on the direct comparison of endothelial cells cultured in vitro under conditions thought to mimic those in normal and tumor tissues. The culture conditions included those favoring proliferation or quiescence; hypoxia or normoxia; as well as exposure to tumor-cell-conditioned media. Protein electrophoresis clearly revealed differences in gene expression (Clarke & West, 1991), however, it proved difficult to identify the differentially expressed proteins at the molecular level. Others groups have tried to raise antibodies either by immunization (Borsi et al, 1987) or by antibody phage technology to different endothelial cell cultures (Mutuberria et al, 2004). These efforts were successful in terms of the discovery of endothelial markers, but failed to identify differentially expressed genes, possibly because such proteins represent a minority of all proteins present on the cell surface.

However, real advances have come with the advent of techniques that allow full genome analysis. By means of a serial analysis of gene expression (SAGE) study several tumor endothelial markers (TEMs) upregulated in tumor endothelial cells could be identified (St Croix et al, 2000). For this purpose normal and colon cancer-derived endothelial cells were isolated in order to construct SAGE libraries and compare them directly for differential expression. Since target accessibility from the blood stream is fundamental in tumor vascular targeting, the work was pursued on proteins with predicted transmembrane domains (Carson-Walter et al, 2001; Nanda & St Croix, 2004; St Croix et al, 2000). TEM1, TEM7, and TEM8 all show a single-pass transmembrane domain, whereas TEM5 is an orphan seven-pass transmembrane G-protein coupled receptor (GPCR) with a long extracellular amino-terminal domain belonging to the adhesion
family of GPCRs. An investigation carried out on the expression of mouse orthologues in normal and tumor tissue, revealed the exclusive expression of TEM1, TEM5 and TEM8 in tumor endothelium (Carson-Walter et al, 2001). TEM1 has been shown to be identical to endosialin (Christian et al, 2001), suggesting a contamination of the endothelial cells from which the SAGE-libraries were derived with pericytes and fibroblasts, which are the main source of this marker in typical conditions (MacFadyen et al, 2005).

The anti-tumor activity of the anthrax toxin may be explained by the fact that TEM8 is identical to the anthrax-toxin receptor, thus enabling the toxin to bind to the tumor endothelium (Duesbery et al, 2001).

Bicknell and co-workers have developed bioinformatics strategies, which utilize the wealth of expression data now available in the public domain. The application of a subtractive algorithm to the expressed sequence tag expression data with a subsequent screening by in situ hybridization led to the discovery of roundabout-4 (Huminiecki et al, 2002), described in a previous section, and an endothelial-specific protein disulphide isomerase (EndoPDI) (Sullivan et al, 2003) as tumor endothelial markers.

Other groups used chip analysis of endothelial cells undergoing morphogenetic changes into tube-like structures in three in vitro models. Out of over 1000 upregulated genes, all putative transmembrane and secreted proteins were overlapped with genes that are upregulated in colon tumors compared to normal colon tissue. This resulted in the identification of stanniocalcin (STC1) as a putative endothelial marker (Gerritsen et al, 2002). The striking overexpression in the neovasculature of colorectal cancer compared to normal colon mucosa was confirmed by in situ hybridization. However, it is also expressed at high levels in various adult organs (Chang et al, 2003).

3.4.2. Perfusion with silica beads

A promising avenue for the discovery of novel markers of angiogenesis consists in the in vivo labeling of vascular structures, followed by recovery and comparative proteomic
analysis. Schnitzer and co-workers described the use of colloidal silica for the in vivo coating of the vasculature (Jacobson et al, 1992). The physical modification enabled the enrichment of silica-coated structures such as luminal cell plasma membranes and their caveolae. The isolated plasma membranes were then analyzed by 2D-gel electrophoresis and/or multiple multidimensional mass spectrometry techniques to produce high-resolution protein maps. Differential spot analysis followed by mass spectrometry of tryptic peptides, database searching and immunoblotting allowed the characterization of differentially expressed proteins. Recently, the analysis of plasma membranes from rat lungs bearing breast adenocarcinoma and from normal rat lungs led to the identification of a number of tumor-specific vascular proteins (Oh et al, 2004). Two of these markers, annexin A1 and nucleolin, have been described in a previous section.

3.4.3. In vivo and ex vivo biotinylation

Our group has developed a methodology for the in vivo and ex vivo chemical labeling and identification of vascular proteins. In the first approach, tumor-bearing mice were perfused with derivatives of biotin. The active ester moiety of the biotinylation reagent subsequently reacts with primary amino groups of proteins, which are readily accessible from the blood stream (Rybak et al, 2005). After homogenization in the presence of SDS, biotinylated proteins were captured onto a streptavidin column subjected to a tryptic digest and identified using LC-MS/MS methodologies (Rybak et al, 2004). The biotinylation of mice with F9 subcutaneous tumors or orthotopic kidney tumors revealed both quantitative and qualitative differences in the recovery of biotinylated proteins compared to normal tissues (Rybak et al, 2005). More recently, this approach has been extended to the discovery of vascular markers of metastasis and to the ex vivo biotinylation of surgically resected human organs with cancer (Castronovo et al, 2006).
3.5. Generation of antibodies \textit{in vitro}

3.5.1. Phage display

Phage display is a powerful methodology that allows the selection of a particular phenotype (e.g. a ligand specific to a desired antigen) from repertoires of polypeptides displayed on phage. The methodology was originally described in 1985 by Smith (Smith, 1985), who reported the use of the non-lytic filamentous bacteriophage fd for the display of specific binding peptides on the phage coat. The power of phage display was further enhanced by the groups of Wells (Lowman \textit{et al}, 1991) and Winter (McCafferty \textit{et al}, 1990) who demonstrated the display of functional folded hormones and antibody fragments, respectively, on the phage surface. By inserting the gene coding for a polypeptide into the phage genome, the polypeptide can be displayed on the phage surface, where it can perform a certain function, typically the specific binding to an antigen of interest. Phage display hence relies on the coupling of genotype and phenotype \[\text{Figure 3.4}\].

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{phage_display.png}
\caption{Phage displaying a binding protein (in this case a scFv fragment) as fusion protein of a minor coat protein pIII.}
\end{figure}

It is possible to create repertoires of phage (phage display libraries) in which the proteins displayed on each phage represent a population of different molecules with different properties. If a phage particle is isolated by virtue of its phenotype displayed on the surface (e.g. the binding specificity), the genetic information coding for the protein is co-
isolated. As an example, one can consider the selection of a binding specificity from a repertoire of binders [Figure 3.5]. The phage library is panned against an antigen of interest; unbound phages are washed away whereas specifically binding phages are enriched and subsequently amplified in bacteria. In the case of antibody phage libraries, usually 2-4 rounds of selection are performed. As a consequence, within 7 days even very rare phenotypes present in large repertoires can be selected and amplified from a background of phage displaying undesired phenotypes.

**Figure 3.5** Selection of binders from a phage display library. A library of proteins displayed on the phage surface is used as input for the selection. Phage, which displays a binding protein (here in red) is captured on immobilized target antigen, and after unbound phages are washed off, bound phages can be eluted. The eluted phage population is then used to infect *E.coli* cells and can be propagated in bacterial cultures. The resulting phage population is enriched with target-specific binders and can be used for subsequent rounds of panning.

Phage particles remain infectious when treated with acids, bases, denaturants and even proteases, hence allowing a variety of selective elution protocols. The stability properties have furthermore been exploited for applications other than selection for binding, such as the selection of proteins with altered thermal stability (Bothmann & Pluckthun, 1998;

Filamentous phages infect strains of *E.coli* that harbor the F conjugative episome by attaching to the tip of the F pilus and translocating the phage genome (a circular single-stranded DNA molecule) into the bacterial cytoplasm. The genome is replicated involving both phage- and host-derived proteins, and packaged into elongated (“filamentous”) viral particles of approximately 6 nm in diameter and 900 nm in length [Figure 3.6].

**Figure 3.6** Life cycles of filamentous phage fl (M13/fd). Sequential binding of pIII to the tip of the F-pilus and then to the host Tol protein complex results in depolymerization of the phage coat proteins, their deposition in the cytoplasmic membrane (where they are available for reutilization), and entry of the ssDNA into the cytoplasm. The ssDNA is converted by host enzymes to a double-stranded reading frame (RF), the template for phage gene expression. Progeny ssDNA, coated by pV dimers (except for the packaging sequence hairpin (PS) that protrudes from one end), is the precursor of the virion. A multimeric complex that spans both membranes mediates conversion of the pV-ssDNA complex to virions and secretion of virions from the cell. This process involves removal of pV dimers and their replacement by the five coat proteins that transiently reside in the cytoplasmic membrane. Adapted from (Clackson & Lowman, 2004).
Several thousand copies of the major coat protein (pVIII) cover the surface of filamentous phage particles, whereas only few copies of the minor coat proteins pIII and pVI are displayed on one extremity of the phage particle and pVII and pIX are present at the other extremity. The minor coat protein pIII, the product of gene III, is displayed in 3-5 copies and mediates the adsorption of the phage to the bacterial pilus.

Peptides and/or proteins have been displayed on phage as fusions with the coat proteins pIII (Smith, 1985), pVIII (Greenwood et al, 1991), pVII and pIX (Gao et al, 2002; Gao et al, 1999). Display of proteins encoded by a cDNA library as carboxy-terminal fusion with the minor coat protein pVI has also been reported (Jespers et al, 1995).

The first peptides and proteins were displayed on phage using phage vectors (essentially the phage genome with suitable cloning sites for pIII or pVIII or fusions and an antibiotic resistance gene). Phage vectors carry all the genetic information required for the phage life cycle. Using phage vectors, most peptides and folded proteins can be displayed as pIII fusions, while only short peptides of 6-7 residues without cysteine give rise to functional phage when displayed as pVIII fusions (Iannolo et al, 1995).

A more popular vector for display are phagemids, plasmid vectors that carry the gene III with appropriate cloning sites and a packaging signal as the only phage-derived sequences [Figure 3.7] (Hoogenboom et al, 1991). In order to produce functional phage particles, phagemid-containing bacteria have to be superinfected with helper phage particles containing the missing information to complete the phage genome. The resulting phage particles may incorporate either pIII derived from the helper phage or the polypeptide-pIII fusion, encoded by the phagemid. However, phagemid vectors encoding the polypeptide-pIII fusion are preferentially packaged into the phage particles, because helper phages used for superinfection (such as M13K07 or VCS-M13) have a defective origin of replication that at the same time serves as packaging signal. Depending on the phagemid type, growth conditions and the nature of the polypeptide fused to pIII, ratios of polypeptide-pIII-fusions:pIII ranging between 1:9 and 1:10000 have been reported (Demartis et al, 1999; Kristensen & Winter, 1998; Silacci et al, 2005). In addition, proteolytic cleavage of protein-pIII fusions contributing to further decreased levels of displayed protein on the phage has been reported (McCafferty et al, 1990).
Display rate influences the outcome of selection experiments. Whereas phage particles obtained by using phage vectors are polyvalent (i.e. 3 to 5 identical polypeptides displayed on one phage particle), the use of phagemids often delivers monovalent phage, which is instrumental for the isolation of high-affinity binders, and thus for affinity maturation. In contrary, polyvalent phage enable the selection of lower affinity binders due to avidity effects. By performing superinfections with hyperphage, a phage lacking gene III in its genome, polyvalent phage can also be generated with phagemids (Rondot et al, 2001).

![Figure 3.7 General scheme for phage display using phage or phagemid vectors. The difference between phage and phagemid vectors is illustrated for pIII display. Sequences for display are inserted between a secretion signal sequence (Sig.) and gene III. Both phage and phagemid vectors carry an Ff origin of replication to permit production of ssDNA and hence virions. Phagemid vectors also have a plasmid origin (here pBR322) and an antibiotic resistance marker to allow propagation as plasmids in E.coli. Phage vectors are also often modified with antibiotic resistance markers for convenience, as illustrated here. In many phagemid vectors, an amber stop codon (tag) is interposed between the displayed sequence and gene III, to allow soluble protein expression by transferring the vector into a non-supE suppressor strain. Adapted from (Clackson & Lowman, 2004).]
Phage display was the first methodology available for the generation of antibodies fully in vitro (Hoogenboom & Winter, 1992; Winter et al, 1994). It is still widely used for the selection of human recombinant antibodies from antibody libraries. Antibody fragments displayable on phage include scFv fragments (Huston et al, 1988) and Fab fragments (Better et al, 1988; Cabilly, 1989) [see also Figure 3.1]. ScFv fragments have a molecular weight of about 30 kDa and are non-glycosylated. The order of the variable domains in scFv fragments may vary, with the VH-domain at the amino- or at the carboxy terminus (Bird et al, 1988; Huston et al, 1988). The linker length has to be adjusted for optimal spatial arrangement of the two variable domains (Huston & Haber, 1996).

The most frequently used format used for the construction of various antibody libraries is VH-(Gly$_4$Ser)$_3$-VL (Clackson et al, 1991; Marks et al, 1991).

### 3.5.2. Alternative methodologies

#### 3.5.2.1. Yeast surface display

Yeast display of antibody fragments represents an efficient methodology for the directed evolution of scFv antibodies with increased affinity and thermal stability. More recently, yeast display has also been applied to the display and screening of nonimmune scFv and immune Fab libraries. Its compatibility with FACS enables the screening of mid-sized libraries and thus replaces the need of selections (Boder & Wittrup, 1997). Since *Saccharomyces cerevisiae* is of eukaryotic nature posttranslational modifications and processing of proteins resembles that of mammalian cells and alleviates expression biases present in bacterially propagated libraries.

Antibodies are fused to the Aga2 protein, which engages in a disulfide bridged complex with the Aga1 protein at the cell surface of *S. cerevisiae* (Boder & Wittrup, 1997). By labeling surface displayed antibodies with fluorescent antigen, yeast cells can be screened by FACS for desired binding properties. Since FACS allows the facile screening of to $10^7$–$10^8$ cells, screening of entire libraries replaces selections.
A peptide tag appended to the scFv [hemagglutinin and myc tag in Figure 3.8] allows detecting the amount of displayed antibodies using a second fluorescent probe. This enables to discriminate between good expression and bad expression clones, and eventually to rank clones according to their affinity during screening [Figure 3.9].

**Figure 3.8** The scFv Aga2 fusion protein surface expression system. Aga1 is bound to a cell wall glucan and connected by a disulfide bond to Aga2. The protein to be displayed is cloned in frame with Aga2 protein. The N-terminal hemagglutinin (HA) tag and C-terminal c-myc tag allow the monitoring of fusion protein display by suitable anti-peptide tag antibodies. By addition of labeled antigen, yeast cells displaying antibody fragments that bind to the antigen can be isolated by FACS (e.g. fluorescently labeled antigen) or affinity purification (e.g. biotinylated antigen).

Enrichment of antigen-binding clones can be achieved by multiple subsequent rounds of enrichment (typically four in the case of antibody libraries) on a cell sorter. Sort gates are drawn such that the best 0.05 -1% clones are collected, depending on the stage of enrichment. High affinity clones are sorted regarding both antigen binding (first fluorophore) and surface display (second fluorophore).

Yeast surface display in combination with FACS is carried out in solution and does not require immobilisation on a solid support, which often leads to avidity effects. Moreover, performing screening in solution allows controlling the precise antigen concentration and consequently to establish conditions to distinguish between high-affinity and low-affinity antibody clones (Boder & Wittrup, 1998) along with subtle affinity improvements (VanAntwerp & Wittrup, 2000).
Yeast display permits the determination of the dissociation constant ($K_d$), the kinetic dissociation rate ($k_{off}$) and the stability without need for subcloning or purifying the antibodies. Affinities are determined using equilibrium-based kinetic analysis by measuring the degree of binding over a wide range of antigen concentrations by FACS. The kinetic dissociation rate $k_{off}$ is determined by competition assays using an excess of unlabeled antigen as competitor (Colby et al, 2004). Stability can be determined by measuring the ability to bind antigen after exposure of the scFv displayed on the cell surface to different temperatures (Orr et al, 2003).

**Figure 3.9** Flow cytometric analysis of yeast-displayed antibody fragments in a population greatly enriched for antigen binding. Yeast cells were labeled with biotinylated antigen/streptavidin-phycoerythrin (detection of bound antigen), and anti-c myc/ anti-mouse-FITC (monitoring of scFv display level). The subpopulation of yeast cells in the blue circle does not display scFv fragments. Despite good display of scFv, the subpopulation in the yellow circle does not bind to the antigen. The subpopulations in the red and in the green circles represent clones with high levels of scFv display (meaning good expression) and antigen binding. However, the subpopulation in the green circle represents binders with higher affinities than the red population. Adapted from (Feldhaus et al, 2003).

Using four cycles of affinity mutagenesis and screening, Boder et al. decreased the dissociation rate of scFv(4-4-20) recognizing fluorescein > 1000-fold (Boder et al, 2000). Graff et al. reported the directed evolution of an anti-carcinoembryonic antigen (CEA) scFv with a 4-day monovalent dissociation half-life at 37°C. Two rounds of mutagenesis and screening led to a 1000-fold improvement of $k_{off}$ over the parental scFv. The greatest
improvement corresponded to a half-life of binding to CEA of 4-7 days at 37°C versus 10 min for the parental antibody hMFE-23. This is the slowest reported dissociation rate constant engineered for an antibody against a protein antigen.

In the same study, the authors also engineered antibody expression by yeast surface display and achieved an increase of the expression level from 80 µg/l yeast culture to 8 mg/l (Graff et al., 2004).

Yeast display is limited by FACS throughput and therefore restricts library size to <10^7. Big naïve yeast surface display antibody libraries have been used for subsequent rounds of panning using first magnetic bead capturing in order to reduce the complexity of clones, followed by FACS screening (Feldhaus et al., 2003). However, as soon as the antigen is not monovalent, strong avidity effects may come into play due to dense scFv display on the yeast cell wall, making yeast display inappropriate for the isolation of high-affinity antibodies. Moreover, in comparison to filamentous phage yeast cells are less resistant to harsh experimental conditions.

3.5.2.2. Ribosome display

Both phage display and yeast surface display require the transformation of either bacteria or yeast. This step is inefficient and limits the size of libraries, which can be achieved to approximately 10^10 clones.

Ribosome display constitutes a fully in vitro display methodology, thus allowing repertoires of >10^12 different clones. The time required to generate diverse libraries is also shortened significantly, thereby allowing to repeatedly introducing new mutations after each round of selection.

Ribosome display was first described by Mattheakis and colleagues (Mattheakis et al., 1994), who displayed short peptides on polysomes. Considerable improvements were needed to allow the display and selection of folded proteins (Hanes & Pluckthun, 1997), such as scFv antibody fragments with dissociation constants as low as 10^{-11} M (Hanes et al., 1998). Proper folding in vitro is supported by introducing an unstructured spacer region to the C-terminal end of proteins. This peptide spacer fills the ribosomal tunnel and provides some extra flexibility, thus allowing the protein of interest to fold as an
independent unit and bind to the target. Additionally, chaperones and protein disulfide isomerases (for proteins depending on disulfide bonds) are added to the translation reaction (Ryabova et al, 1997).

In ribosome display [Figure 3.10], a DNA library encoding a repertoire of proteins is transcribed in vitro, the mRNA is purified and finally used for in vitro translation. Since the mRNA lacks a stop codon, the ribosome stalls at the end of the mRNA, giving rise to a ternary complex of mRNA, ribosome and functional protein. Thus, the ribosome links the phenotype and genotype. The ribosomal complexes, which are stabilized by high concentrations of magnesium ions and low temperature (Jermutus et al, 2001), are directly used for selections on either surface-immobilized or in solution ligand. The mRNA incorporated in the bound ribosomal complexes is eluted by addition of EDTA, purified, reverse-transcribed and amplified by PCR. During the PCR step, the T7 promotor and the Shine–Dalgarno sequence are reintroduced by appropriate primers enabling the direct use of the PCR product for further selection cycles.

Hanes and colleagues have used a large synthetic antibody library, HUCAL-1, of 2 x 10⁹ independent members (Knappik et al, 2000) for the selection of antibodies specific to bovine insulin (Hanes et al. 2000) performing six rounds of selection. After each round, further diversity was introduced into the selected genes by error prone PCR, a procedure that mimics the process of somatic hypermutation of antibodies in vivo. Ribosome display has been shown to work especially well for affinity maturation of scFv fragments. So far, two studies have been reported in which a given antibody was evolved to higher affinity. In both cases, stringent off-rate selections combined with error-prone PCR were used.

An antibody fragment specific to fluorescein with a dissociation constants of about 100 pM was generated by Jermutus and colleagues (Jermutus et al, 2001). Interestingly, the majority of the 4 to 11 mutated residues which were present in selected antibodies were found to be unlikely to contact the antigen.
In a second study, the dissociation constant of a scFv fragment specific to a peptide deriving from the transcription factor GCN4 was improved from 40 to 5 pM (Zahnd et al., 2004). Libraries of antibody mutants were generated with error-prone PCR and DNA shuffling, and selected for decreased off-rates. Crystallographic analysis of the scFv in its antigen bound and free states showed that only a few mutations, which do not make direct contact to the antigen, lead to the affinity improvement.

Figure 3.10 Schematic representation of a selection cycle of ribosome display. Linear DNA fragments coding for a protein library (here scFv variants) are transcribed in vitro and purified before subsequent translation in vitro. After having reached the end of the mRNA during translation, the ribosome is unable to dissociate from the mRNA because the stop codon is absent. The resulting ternary complex comprising the ribosome, mRNA and the nascent polypeptide can be stabilised by high concentrations of magnesium ions and low temperature, thereby creating a stable linkage between the mRNA (genotype) and the encoded polypeptide (phenotype). Ribosomes displaying a binding protein can be isolated by affinity selection on immobilised antigen, the selected mRNA molecules can be eluted, and the genetic information is amplified by reverse transcription and PCR, which allows to introduce further variability by error prone PCR.

These results might suggest that the affinity optimization of very high affinity binders is achieved by modulating existing interactions via subtle changes in the framework rather than introducing new contacts.
ScFv antibody fragments have in addition been evolved for stability by using ribosome display (Jermutus et al, 2001).

3.5.3. The ETH-2-Gold library

The ETH-2-Gold library is a synthetic human phage display library which consist of 3 x $10^9$ different antibody clones (Silacci et al, 2005). The library is cloned in scFv format (VH-linker-VL) in the phagemid vector pHEN1 (Hoogenboom et al, 1991), which appends the short peptidic myc-tag to the C-terminus of the recombinant antibody followed by pIII.

The ETH-2-Gold library is based on one single VH segment (DP47) and one single Vk (DPK22) or Vλ (DPL16) segment, respectively, linked by the flexible polypeptide Gly4SerGly4SerGly4 (Huston et al, 1988). These variable segments dominate the functional repertoire in humans and are well represented in binders isolated from synthetic phage libraries (Griffiths et al, 1994; Kirkham et al, 1992). The use of the DP47 VH germline segment offers a number of advantages, ranging from a higher thermodynamic stability (Ewert et al, 2003) to the possibility of using Protein A for antibody purification and detection (Hoogenboom & Winter, 1992). Variability was confined to the VL CDR3 (positions 91, 92, 93, 94, and 96 for Vk, 92, 93, 94,95, 95a and 95b for Vλ) and to four to six residues in the VH CDR3 (positions 95–100) [Figure 3.11], in accordance with their role as common antigen contacts and with the high variability of naturally occurring antibodies in these regions (Padlan, 1994).

It was shown before that high affinity antibodies can be obtained with short CDR3 in the heavy chain (Lavoie et al, 1992). The choice of short CDR3 also limited the potential diversity of the library and reduced clone to clone variability. Furthermore, short CDR3 in VH and VL immunoglobulin domains are generally associated with better antibody stability to proteolysis, improved binding and reduced immunogenicity.

With this modular design, the ETH-2-Gold phage display library allows the facile affinity maturation of antibodies of interest by introducing diversity in CDR1 and CDR2 using standard primers (Pini et al, 1998).
The ETH-2-Gold library was shown to be highly functional. 90% of the clones exhibit good expression of soluble antibody, and antibodies have been isolated against more than 20 different antigens (Silacci et al, 2005).

**Figure 3.11** The design of the ETH-2-Gold library. (A) ScFv antibody fragment structures. Upper panel: the DPK22 (V\(\kappa\)) backbone is represented in blue and the DP47 (VH) in green (PDB file 1IGM). Residues subject to random mutation (spacefill representation) are DPK22 CDR3 positions 91, 92, 93, 94, and 96 (light blue) and DP47 CDR3 positions 95, 96, 97, 98, 99, and 100 (yellow). The residues are numbered according to (Tomlinson et al. 1995). Lower panel: DPL16 (V\(\lambda\)) backbone is represented in red and the DP47 (VH) in green (PDB file 8FAB). Residues subject to random mutation (spacefill representation) are DPL16 CDR3 positions 92, 93, 94, 95, 95a and 95b (orange) and DP47 CDR3 positions 95, 96, 97, 98, 99, and 100 (yellow). (B) The sequence of relevant residues of the variable heavy and light chains, together with the human antibody germline segments from which they are derived. Adapted from (Silacci et al, 2005).

### 3.5.4. In vitro affinity maturation

*In vitro* affinity maturation may become necessary whenever the affinity of selected antibodies is suboptimal and does not meet the requirements of therapeutic applications, such as tumor targeting or virus neutralization. It is generally accepted that improving the
affinity of an antibody will improve its targeting performance or virus neutralization potency (Adams et al., 1998; Kuan et al., 2000; Viti et al., 1999; Wu et al., 2005), even though there is certain evidence that ultra-high affinity hinders efficient tumor targeting (Graff & Wittrup, 2003; Saga et al., 1995; Weinstein et al., 1987).

In vivo, antibodies are affinity matured stepwise by the gradual incorporation of mutations causing small improvements in affinity. The in vitro affinity maturation process involves the introduction of diversity in the antibody V-genes of the parental binder to create an affinity maturation library followed by selection or screening to isolate clones with improved affinity. This procedure can be repeated until antibodies with desired affinity are obtained. All selection/screening methodologies discussed previously are well suited for affinity maturation.

Strategies to introduce mutations for the generation of affinity maturation libraries include chain shuffling, where one chain of the parental antibody, for instance the light chain, is replaced by a repertoire of VL chains isolated from lymphocytes. Alternatively, only the V segments (comprising CDR1 and CDR2 but not CDR3) can be shuffled (Marks et al., 1992; Schier et al., 1996a). The isolation of genes from lymphocytes requires a lot of time and effort, unless the VL repertoire is already available, for instance from a naïve antibody library.

Diversity can also be introduced as point mutations randomly distributed over the variable gene by error-prone PCR or by using E.coli mutator strains (Coia et al., 2001; Low et al., 1996).

Affinity maturation by ribosome display often involves error prone PCR [see also Figure 3.10]. At first glance, it might seem that the strategy of introducing random point mutations, followed by selection of the best clones, mimics somatic hypermutation of the immune system. However, somatic hypermutation is rather a targeted than a randomly occurring process (Jolly et al., 1996; Wagner et al., 1995). Targeted somatic hypermutation has been mimicked for affinity maturation of antibodies by mutating a subset of residues which are naturally prone to hypermutations (Chowdhury & Pastan,
Clones with a 15- to 55-fold increase in affinity could be isolated from libraries of $10^3 - 10^4$ different clones.

A third strategy, which preserves the backbone sequence of an antibody, is the mutation of CDR loops by oligonucleotide directed mutagenesis (Pini et al., 1998; Schier et al., 1996b) [Figure 3.12]. In vivo, the diversity introduced by somatic hypermutation in the maturation process is mainly located at the periphery of the antigen-binding site in the CDR1 and CDR2. This suggests that the CDR1 and CDR2 regions should be targeted for in vitro affinity maturation. This strategy for affinity maturation is particularly well suited for libraries based on single scaffolds, such as the ETH-2-Gold library. The modular design allows introducing mutations in the peripheral CDR1 and CDR2 with standard primers identical for all clones.

**Figure 3.12** Schematic representation of an example for in vitro affinity maturation by mutation of CDR loops of an antibody isolated from the ETH-2-Gold library. An antibody selected from the ETH-2-Gold library (primary library with CDR3 of VH and VL in red spacefill) is used as template for the construction of a secondary library with mutated residues in CDR1 of VH and VL (in yellow spacefill). The best performing clone selected from the secondary library is used as template for the construction of a third library with mutations in CDR2 of VH and VL (in green spacefill). This procedure can be extended to more residues and repeated until the desired affinity is obtained.
3.6. Carbonic anhydrase IX and carbonic anhydrase XII as anti-cancer targets

3.6.1. Carbonic anhydrases: physiological function

Sixteen α-carbonic anhydrase (CA) isozymes have been identified so far in higher vertebrates. The isozymes differ in their subcellular localization, catalytic activity and susceptibility to different classes of inhibitors [see also Figure 4.11] (Supuran, 2008). Some of the active isozymes are cytosolic (CA I, CA II, CA III, CA VII and CA XIII), others are membrane bound (CA IV, CA IX, CA XII and CA XIV), two are mitochondrial (CA VA and CA VB), and one is secreted in saliva (CAVI). The three acatalytic forms are called CA-related proteins (CARPs): CARP VIII, CARP X and CARP XI (Supuran, 2008). The recently reported CA XV is not expressed in humans or in other primates, but is abundant in rodents and other higher vertebrates (Hilvo et al, 2005). CAs catalyze the reversible hydration of carbon dioxide to bicarbonate and a proton (\( \text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{HCO}_3^- + \text{H}^+ \)) and are thus involved in physiological processes like respiration, electrolyte secretion, acid-base regulation, calcification, bone resorption and biosynthetic processes requiring bicarbonate as a substrate. In humans, CAs are expressed throughout various tissues, among them the gastrointestinal tract, the nervous system, kidneys, lungs, skin and eyes (Supuran, 2008).

Recently, two CA isozymes, CA IX and CA XII have been shown to be overexpressed in multiple epithelial tumor types (Supuran, 2008; Wykoff et al, 2000), where they promote cancer progression and response to therapy (Tureci et al, 1998; Zavada et al, 1993).

3.6.2. Carbonic anhydrases: role in hypoxia

A major feature of solid tumors is hypoxia, the decreased availability of oxygen. Massive tumor cell proliferation distances cells from the vasculature, leading to a deficiency in the local environment of blood carrying oxygen and nutrients. Tumor hypoxia usually occurs at a distance of 100-200 µm from blood vessels (Hockel & Vaupel, 2001; Wykoff et al,
Tumors often display a central core of necrotic cells, which has been suggested to result from a drop in the oxygen availability and glucose deprivation leading to cell death [Figure 3.13]. Hypoxic tumors are known to be resistant to radiotherapy, due to the decrease in oxygen-free radicals required for DNA-damage (Moeller et al., 2007). Furthermore, hypoxic tumors are considered to be chemotherapy-resistant as a consequence the lowered extracellular pH (pH_e), which might impair the uptake of weakly basic anticancer drugs. (Raghunand et al., 1999). By activating proteases and thereby the degradation of the extracellular matrix, the lowered pH_e has also been associated with tumor progression and invasion (Stubbs et al., 2000).

Figure 3.13 The characteristics of a hypoxic tumor mass. Blood capillaries carry oxygen to tissues, but since oxygen has a diffusion limit, its concentration decreases with distance from the capillaries. Necrotic cells can be found in the central region, whereas expanding tumor cells reside in proximity to capillaries. This gradient of cell viability in dependence of oxygen availability is accompanied by an increase in HIF-1α, a decrease of the extracellular pH (pH_e) and an increase in the resistance to radio- and chemotherapy in direction of the central region. Adapted from (Brahimi-Horn et al., 2007).

Hypoxia regulates the expression of various genes, via the activation of the heterodimeric hypoxia-inducible factor (HIF-1). The HIF-1α subunit is unstable under normoxic conditions, as a consequence of proline-hydroxylation. Hydroxylated HIF-1α is recognized by the von Hippel-Lindau tumor suppressor protein (pVHL), which promotes its polyubiquitinylation, followed by proteasomal degradation (Hockel & Vaupel, 2001).
However, hypoxia attenuates proline-hydroxylation, owing to the inactivity of prolyl-4-hydroxylases (PHDs) in the absence of oxygen. This results in the non-recognition of HIF-1α by pVHL and thus the stabilization of HIF-1α. After translocation to the nucleus and pairing to HIF-1β, the active transcription factor binds to hypoxia-responsive elements (HREs) in the promoter regions of target genes, among them CA IX [Figure 3.14] (Hockel & Vaupel, 2001). Renal cell carcinomas often carry mutations in the gene encoding pVHL leading to constitutive HIF-1α activation and as a consequence a strong upregulation of CA IX (Wykoff et al., 2000).

**Figure 3.14** Mechanism of hypoxia-induced gene expression mediated by the HIF-1 transcription factor. Under normoxia, prolyl-4-hydroxylase (PHD) hydroxylates P564 on hypoxia-inducible factor-1α (HIF-1α). The von Hippel-Lindau tumor suppressor protein (pVHL) binds hydroxylated HIF-1α and targets it for degradation by the ubiquitin-proteasome system. Under hypoxia, HIF-1α is not hydroxylated and hence not recognized by pVHL, which causes its stabilization and accumulation. HIF-1 transcription factor target genes include glucose transporters (GLUT1 and GLUT3), vascular endothelial growth factor (VEGF) that triggers neoangiogenesis, CA IX and CA XII involved in pH regulation and tumorigenesis, and additional genes with functions in cell survival, proliferation, metabolism and other processes. Adapted from (Supuran, 2008).
3.6.3. CA IX and CA XII in cancer

In 1993, Zavada et al. discovered the abundance of CA IX, first termed MN, in human carcinomas of ovary, endometrium and uterine cervix (Zavada et al, 1993). Later CA IX expression was found to be strongly increased in many types of tumors, such as gliomas/ependymomas (Pastorekova & Pastorek, 2004), mesotheliomas (Pastorekova & Pastorek, 2004), head and neck cancer (Koukourakis et al, 2001) and carcinomas of the bladder (Ord et al, 2007), uterine cervix (Swietach et al, 2007), nasopharynx (Sung et al, 2007), breast (Trastour et al, 2007), oesophagus (Pastorekova & Pastorek, 2004), lung (Swinson et al, 2003) and vulva (Pastorekova & Pastorek, 2004), among others.

CA XII and its role as a tumor-associated marker were discovered in 1998 by screening of an RCC cDNA expression library using autologous serum from an RCC patient (Tureci et al, 1998).

In VHL-defective renal cell carcinoma, both CA IX and CA XII are being constitutively expressed (Wykoff et al, 2000). In tumors other than renal cell carcinomas, CA IX and CA XII show the typical perinecrotic distribution pattern of HIF-1-regulated proteins (Chiche et al, 2009; Kivela et al, 2000; Leppilampi et al, 2003). Necrosis usually develops in regions most distant from blood vessels suffering from severe hypoxia or even anoxia. By contrast, cells that are localized in the perinecrotic area can adapt to these low oxygen conditions via the induction of HIF-1 targets including CA IX and CA XII. The strong overexpression of these two enzymes leads to an acidification of the hypoxic tumor by the production of carbonic acid (Chiche et al, 2009; Svastova et al, 2004). Tumor cells exposed to this hypoxic and acidic environment gain a survival advantage over cells that do not express CA IX and CA XII on their surface, because besides lowering pH_e, both contribute to maintain the intracellular pH (pH_i), an action that preserves ATP production, protein synthesis, cell proliferation and migration (Chiche et al, 2009).

In addition to the upregulation of CA IX and CA XII, HIF-1 activates membrane-located transporters, exchangers and pumps implicated in pH homeostasis. To survive in an acidic environment, the pH_i-regulating system of tumor cells actively extrudes acids via the Na\(^+/\)H\(^+\) exchanger 1 (NHE-1, (Sardet et al, 1989; Shimoda et al, 2006) and the
monocarboxylate transporters MCT1 and MCT4 (Ullah et al., 2006). In the opposite direction to H⁺ extrusion, HCO₃⁻ influx through Na⁺-HCO₃⁻ co-transporters and Cl⁻/HCO₃⁻ exchangers (AE) contributes to cytoplasmic alkalinization [Figure 3.15]. Recent in vivo experiments using tumor cells transfected with vectors for the Tet-inducible production of short hairpin RNAs to silence CA9, CA 12 or HIF-1α showed that CA9 silencing alone leads to a 40% reduction in xenograft tumor volume with up-regulation of CA12 mRNA levels, whereas invalidation of both CA IX and CA XII gave an impressive 85% reduction in tumor size (Chiche et al., 2009).

**Figure 3.15** Proteins and processes involved in pH regulation within a tumor cell. CA IX and CA XII, two transmembrane enzymes with an extracellular active site, catalyze CO₂ hydration to HCO₃⁻ and H⁺ in these cells. Other involved proteins include (a) monocarboxylate carrier; (b) Na⁺/H⁺ exchanger 1; (c) ATP-dependent Na⁺/K⁺ antiporter; (d) H⁺ channels; (e) plasma-membrane proton pump H⁺-ATPase; (f) aquaporins and (g) anion exchangers (AE). Adapted from (Supuran, 2008).
3.6.4. Ligands to carbonic anhydrases

3.6.4.1. Antibodies specific to carbonic anhydrase IX

Two monoclonal antibodies specific to CA IX have been studied extensively in the past for their \textit{in vivo} targeting performance of tumors: the M75 antibody, which binds to the extracellular proteoglycan-like domain (Chrastina \textit{et al}, 2003a; Chrastina \textit{et al}, 2003b) and the G250 antibody, which recognizes the extracellular CA domain (Brouwers \textit{et al}, 2004; van Dijk \textit{et al}, 1991; van Schaijk \textit{et al}, 2005). However, both antibodies were isolated by hybridoma technology and chimerized later on, in case of the G250 antibody, to perform radioimmuno therapy (RIT) studies in patients with RCC. However, in patients with progressive end-stage RCC, in the majority of patients no objective responses were observed and the MTD of the second $^{131}$I-cG250 treatment was 75\% of the MTD of the first RIT (Brouwers \textit{et al}, 2005). The chimeric antibody cG250 is currently being studied by Wilex in Phase III clinical trials for RCC therapy. Human or humanized monoclonal antibodies would thus be needed, as they are less immunogenic compared to rodent or chimeric antibodies upon sequential administrations (Hale \textit{et al}, 1988; Winter \textit{et al}, 1994).

3.6.4.2. Small organic molecules specific to carbonic anhydrases

At least 25 clinically used drugs have been reported to possess significant inhibitory properties to CAs [\textbf{Figure 3.16}]. These drugs are mainly derivatives belonging to the sulfonamide, sulphamate or sulphamide families. Carbonic anhydrase inhibitors (CAIs) include the classical inhibitors acetazolamide (compound 1), methazolamide (compound 2), ethoxozolamide (compound 3), sulthiame (compound 4) and dichlorophenamide (5). More recent drugs or investigational agents are dorzolamide (compound 6), brinzolamide (compound 7), indisulam (compound 8), topiramate (compound 9), zonisamide (compound 10), sulpiride (compound 11), COUMATE (compound 12), EMATE (compound 13), celecoxib (compound 14), valdecoxib (compound 15) and saccharin (compound 16). Compounds 17 and 18 are investigational agents for visualizing CA IX.
in *in vitro* cell cultures. Many of these compounds were initially developed years ago during the search for diuretics, among which the thiazides, compounds 19 a-e, as well as derivatives 20-25 are still widely clinically used (Supuran, 2008).

Few of the derivatives 1-16 show selectivity for a specific isoform: the classical inhibitors (compounds 1-5), the topically acting antiglaucoma sulphonamides (compounds 6 and 7) together with indisulam (compound 8), are promiscuous CAIs, with strong affinities for isoforms II, VA, VB, VI, VII, IX, XII, XIII and XIV (Supuran, 2008).

Specificity to the membrane-bound isoforms CA IX and CA XII has mainly been attempted to be achieved through the use of bioreductive pro-drugs (De Simone *et al.*, 2006) and membrane-impermeable glycoconjugates (Winum *et al.*, 2008). To our knowledge, none of these therapeutic approaches has been tested *in vivo* so far.

Acetazolamide (compound 1), sold as an anti-glaucoma agent under the tradename Diamox, displays $K_i$ values to all catalytically active CAs varying from 5.7 nM (CA XII), 25 nM (CA IX) to 200 μM (CA III) (Supuran, 2008).
Figure 3.16 Structures of carbonic anhydrase inhibitors 1-25. Adapted from (Supuran, 2008).
3.7. Aim of the thesis

The aim of this thesis was to study the molecular targeting of the tumor marker CA IX using both monoclonal antibodies and derivatives of acetazolamide.

Two high-affinity human monoclonal antibodies (A3 and CC7) specific to the extracellular CA domain of CA IX were isolated by phage technology. The performance of these antibodies to stain CA IX \textit{ex vivo} and to target the cognate antigen \textit{in vivo} in comparison to a chemical marker of hypoxia (pimonidazole) was assessed in two animal models of colorectal cancer (LS174T and SW1222). The new human anti-CAIX antibodies are expected to be non-immunogenic in patients with cancer and may serve as broadly applicable reagents for the non-invasive imaging of hypoxia and for pharmacodelivery applications.

Furthermore, the \textit{in vivo} targeting of the tumor-associated carbonic anhydrases IX and XII was studied using acetazolamide derivatives. \textit{In vivo} targeting was performed with a fluorescent acetazolamide derivative, while therapy studies in mice bearing subcutaneously-grafted tumors were carried out with a bispecific acetazolamide derivative capable of simultaneous binding to carbonic anhydrases and serum albumins. Acetazolamide was used as a building block, since this compound is able to inhibit a broad spectrum of carbonic anhydrases in the low nanomolar range, including CA IX and CA XII (Supuran, 2008).
4. RESULTS

4.1. Human monoclonal antibodies targeting carbonic anhydrase IX for the molecular imaging of hypoxic regions in solid tumors

4.1.1. Abstract

Hypoxia, which is commonly observed in areas of primary tumors and of metastases, influences response to treatment. However, its characterization has so far mainly been restricted to the ex vivo analysis of tumor sections using monoclonal antibodies specific to carbonic anhydrase IX (CA IX) or by pimonidazole staining, following the intravenous administration of this 2-nitroimidazole compound in experimental animal models. In this study, we describe the generation of high-affinity human monoclonal antibodies (A3 and CC7) specific to human CA IX. These antibodies were able to stain CA IX ex vivo and to target the cognate antigen in vivo. In one of the two animal models of colorectal cancer studied (LS174T), CA IX imaging closely matched pimonidazole staining, with a preferential staining of tumor areas characterized by little vascularity and low perfusion. By contrast, in a second animal model (SW1222), distinct staining patterns were observed for pimonidazole and CA IX targeting. We observed a complementary pattern of tumor regions targeted in vivo by the clinical-stage vascular targeting antibody L19 and the anti-CA IX antibody A3, indicating that a homogenous pattern of in vivo tumor targeting could be achieved by a combination of the two antibodies. The new human anti-CA IX antibodies are expected to be non-immunogenic in patients with cancer and may serve as broadly applicable reagents for the non-invasive imaging of hypoxia and for pharmacodelivery applications.
4.1.2. Introduction

Monoclonal antibodies and their derivatives are increasingly being used in anti-cancer therapeutic strategies for the selective delivery of bioactive agents (e.g., full immunoglobulins for Fc-mediated cell killing, drugs with cleavable linkers, radionuclides, photosensitizers, pro-coagulant factors, cytokines) to the tumor environment, thus sparing normal tissues (Adams & Weiner, 2005; Carter, 2006; Carter & Senter, 2008; Neri & Bicknell, 2005; Payne, 2003; Schliemann & Neri, 2007; Schrama et al., 2006). While originally monoclonal antibodies specific to membrane antigens on cancer cells have been used for tumor targeting applications, alternative targets such as markers of angiogenesis (Neri & Bicknell, 2005; Schnitzer, 1998; Thorpe, 2004), stromal antigens (Hofheinz et al., 2003; Rybak et al., 2007b; Schliemann & Neri, 2007) and intracellular proteins released at sites of necrosis (Miller et al., 1993; Street et al., 2006) are increasingly being considered. In all these cases, antibody-mediated pharmacodelivery options are particularly attractive in consideration of the fact that most conventional cytotoxic agents and many therapeutic proteins exhibit a reduced uptake at the tumor site, compared to normal organs (Bosslet et al., 1998; Tarli et al., 1999). Human or humanized monoclonal antibodies are preferred for targeting applications, as they are less immunogenic compared to rodent or chimeric antibodies (Hale et al., 1988; Winter et al., 1994).

Solid tumors are heterogenous masses and the characterization of the different microenvironments within a neoplastic lesion provides information about the tumor structures, which can be targeted in vivo with intravenously (i.v.) administered monoclonal antibodies to induce a therapeutic response. In this context, the characterization of hypoxic regions within solid tumor masses assumes a particular relevance, since hypoxic cancer cells are less sensitive to certain killing agents (e.g., radiation and cytotoxic compounds; (Bertout et al., 2008; Weinmann et al., 2004)). Hypoxic regions in tumors of experimental animal models can be assessed post-mortem by analysis of tissue sections, following i.v. administration of pimonidazole, a 2-nitroimidazole compound which is selectively reduced and binds to intracellular
macromolecules in hypoxic regions at pO$_2$ < 10 mm Hg (Dearling et al, 2004). However, it would be desirable to complement this invasive procedure with a molecular imaging approach based on selective ligands to accessible proteins over-expressed at sites of hypoxia. Based on transcriptomic profiling of cells exposed to different oxygen concentrations, our group and others had recognized that carbonic anhydrase IX (CA IX) is one of the most over-expressed genes in hypoxic conditions (Scheurer et al, 2004; Staller et al, 2003). In parallel, ex vivo staining of tumor sections with monoclonal antibodies specific to CA IX had revealed staining patterns overlapping (though somewhat broader) with the neoplastic regions stained with pimonidazole (Hoskin et al, 2003; Jankovic et al, 2006; Li et al, 2007; Olive et al, 2001; Sobhanifar et al, 2005).

The functional contribution of CA IX to tumor growth and progression has long been debated (Brahimi-Horn et al, 2007; Pouyssegur et al, 2006). Very recently, Pouyssegur and coworkers have reported that the simultaneous invalidation of CA IX and CA XII using short hairpin RNA technology led to a substantial growth retardation in transfected LS174T colorectal cancer xenograft models, while the individual knock-outs yielded a substantially lower tumor growth retardation (Chiche et al, 2009).

Monoclonal antibodies have also been used to achieve a selective in vivo localization on cells which display a high constitutive expression of CA IX (Brouwers et al, 2004; Chrastina et al, 2003a; Chrastina et al, 2003b; van Dijk et al, 1991; van Schaijk et al, 2005), especially kidney cancer cells, in which mutations in the gene encoding the von Hippel-Lindau tumor suppressor (pVHL) lead to a constitutive HIF-1$\alpha$ activation and, as a consequence, to a strong upregulation of CA IX on all tumor cells (Mazure et al, 2004; Wykoff et al, 2000). This target was also detected as one of the most prominent accessible markers of renal cell carcinoma (RCC) in a chemical proteomic study, based on the ex vivo perfusion of surgically resected human kidneys with cancer using an active ester derivative of biotin, followed by capture of biotinylated proteins and mass spectrometric analysis (Castronovo et al, 2006).
While hypoxic areas can be efficiently stained with CA IX antibodies \textit{ex vivo}, it was not known at the beginning of this study whether the same structures could be targeted \textit{in vivo}, considering that they are typically located at a 100-200 µm distance from the nearest tumor blood vessel (Kerbel & Folkman, 2002) and may be thus more difficult to reach. Indeed, while the antibody-based targeting of markers of tumor neo-vasculature is a rapid and efficient process [e.g., L19 antibody specific to the alternatively-spliced EDB domain of fibronectin; (Berndorff \textit{et al}, 2005; Borsi \textit{et al}, 2002; Borsi \textit{et al}, 2003; Tarli \textit{et al}, 1999; Tijink \textit{et al}, 2006; Viti \textit{et al}, 1999)], the tissue penetration of monoclonal antibodies to certain abluminal structures is often impaired by several factors, including molecular size (Adams \textit{et al}, 1998; Low \textit{et al}, 2008; Yokota \textit{et al}, 1992), antigen barrier (Dennis \textit{et al}, 2007; El-Emir \textit{et al}, 2007b) and tumor interstitial pressure (Jain, 1987). In this article, we describe the generation and characterization of two high-affinity human monoclonal antibodies (A3 and CC7) specific to the extracellular carbonic anhydrase (CA) domain of human CA IX. Both antibodies were shown to selectively recognize CA IX on the surface of tumor cells \textit{in vitro}, in tumor sections \textit{ex vivo} and to preferentially localize at sites of hypoxia \textit{in vivo} following i.v. administration.
4.1.3. Materials and Methods

4.1.3.1. Cell lines

Cell culture media and supplements were purchased from Invitrogen. The human colorectal adenocarcinoma cell lines LS174T (CL-188, ATCC) and HT-29 (HTB-38, ATCC) were maintained in DMEM and McCoy’s 5A medium, respectively, supplemented with 10% fetal bovine serum (FBS) and antibiotic-antimycotic at 37°C in an atmosphere of 5% CO₂. The human glioblastoma cell line U87 (HTB-14, ATCC) was cultured in MEM medium, supplemented as described above. The human RCC cell line SK-RC-52 (Ebert et al., 1990), a kind gift of Prof. E. Oosterwijk, was cultured in RPMI medium, supplemented as described above. The human colorectal adenocarcinoma cell line SW1222 was maintained in MEM medium supplemented with 10% FBS, 1% glutamine and 1% non-essential amino acids.

4.1.3.2. Cloning, expression and purification of recombinant human CA IX

The cDNA fragment encoding the carbonic anhydrase (CA) extracellular domain (aa 120-397) of CA IX was amplified from the full-length cDNA clone IRAUp969G1273D (imaGenes) used as template with primers BW_CA9 (5’-GGAGATCCTCAAGAACCA-3’) and FW_CA9_6xHis (5’-TTCCTCGAGTTAGTGATGGTGATGGTGATGACTGCTGTCCACTCCACG-3’) introducing a C-terminal 6xHis-tag. A secretion sequence, required for secretion into the extracellular medium, was amplified from the construct SIP(L19)-pcDNA3.1 (Borsi et al., 2002) using BW_SIP (5’-GATAAGCTTGTCGACCATGGGCTGGAG-3’) and FW_SIP (5’-TGGGGTTCTTGAGGATCTCCCGAGTGCACACCTGTG-3’) primers. The resulting PCR fragments were gel-purified, assembled by PCR and cloned into vector pCEP4 (Invitrogen) by means of HindIII and XhoI digestion. The sequence-verified plasmid was used to transfect HEK 293 EBNA cell line using FuGENE 6 transfection reagent (Roche). Transiently transfected cells were cultured in DMEM supplemented with 10% FBS and selected using 200 μg/ml of hygromycin (Invitrogen).
recombinant domain of CA IX was purified from supernatant by affinity chromatography on Ni-NTA agarose (Qiagen) by means of the C-terminal 6xHis-tag. The purified protein was analyzed by SDS-PAGE, size-exclusion chromatography using a Superdex 200 HR 10/30 column (GE Healthcare). The specific activity of 1 µg recombinant protein was measured at 400 nm by its esterase activity using 1 mM 4-Nitrophenyl acetate as substrate in 100 µl 50 mM Tris/SO₄⁺ pH 8.5. For biotinylation of recombinant CA IX EZ-Link Sulfo-NHS-LC-Biotin (Pierce) was used according to manufacturer’s instructions.

4.1.3.3. Selections of antibodies from the ETH-2-Gold library

Selections of antibodies were carried out on Immunotubes (Nunc) coated with recombinant CA IX at of 50 µg/ml in PBS (20 mM NaH₂PO₄, 30 mM Na₂HPO₄, 100 mM NaCl, pH 7.4), as described previously by our group (Silacci et al., 2005). Recombinant antibody fragments, which were positive in ELISA, in the scFv format were expressed in E.coli TG-1 and purified from culture supernatant by affinity chromatography using Protein A Sepharose Fast Flow resin (GE Healthcare), as described previously (Silacci et al., 2005).

Purified antibody fragments were analyzed by SDS-PAGE and by size exclusion chromatography on a Superdex 75 HR 10/30 column (GE Healthcare).

4.1.3.4. Construction of the affinity maturation libraries and selections of antibodies

The first affinity maturation library of scFv(A11) was cloned by introducing sequence variability in the CDR1 loops of both heavy and light chain. Antibody residues are numbered according to (Tomlinson et al., 1992) and (Williams et al., 1996). Mutations at positions 31, 32, 33 of VH and 31, 31a, 32 of VL were introduced by PCR using the same partially degenerate primers (Operon Biotechnologies) as described previously (Villa et al., 2008). A single round of selection on biotinylated antigen (final concentration 10⁻⁷ M) was carried out eluting bound phage with 100 mM triethylamine, as described previously
ELISA and Biacore screening of dissociation profiles of selected clones on a high-density coated chip yielded the antibody A3.

In the construction of the second affinity maturation library, scFv(A3) was used as template. Sequence variability was introduced in the CDR2 loops of both heavy and light chain. Mutations at positions 52, 52a, 53 and 56 of VH and at positions 50, 52 and 53 of VL were introduced using partially degenerate primers (Operon Biotechnologies), as described by (Silacci et al, 2006). A single round of selection on biotinylated antigen followed by a screening procedure, as described above, yielded the antibody CC7.

4.1.3.5. Biacore analysis

Affinity measurements were performed on a Biacore 3000 instrument (Biacore). 555 RU biotinylated recombinant CA IX were immobilized onto a streptavidin SA chip (Biacore). For the real-time interaction analysis peaks representing the monomeric fractions of scFv(A3) and scFv(CC7) were collected by size-exclusion on a Superdex 75 HR 10/30 column (GE Healthcare) and injected at a flow of 20 µl/min on the low-density coated antigen chip. All kinetic data were evaluated using the BIAevaluation 4.1 software (Biacore).

4.1.3.6. Cloning, expression and purification of antibodies in the SIP format

ScFvs were converted into the SIP format by cloning VH and VL into pcDNA3.1 (Invitrogen) using the same primers and strategy as described by (Silacci et al, 2006) (Figure 2). The plasmids were transfected into CHO-S cells (Invitrogen) using Cell Line Nucleofector Kit V (Amaxa), following the manufacturer’s protocol. Transfectomas were grown in RPMI supplemented with 10% FBS and selected by addition of 500 µg/ml Geneticin (G418) (Calbiochem). Monoclonal cultures were obtained by fluorescent-activated cell sorting after staining for secreted antibody, as described (Zuberbuhler et al, 2008). After 14 days of selection, cells were brought into suspension, and cultured in Power CHO-CD 2 (Lonza). SIP antibodies were purified from culture medium by affinity
chromatography using Protein A Sepharose Fast Flow resin (GE Healthcare), as described by (Zuberbuhler et al, 2008).

4.1.3.7. Fluorescence-activated cell sorting

Cells were harvested through incubation with 10 mM EDTA in PBS for 3 min at 37°C. After counting and a centrifugation step of 5 min at 1100 rpm, cells were re-suspended in PBS supplemented with 2% FBS to a final concentration of $5 \times 10^6$ cells/ml. $1 \times 10^6$ cells were then incubated for 30 min at room temperature with 1 µg/ml of SIP(A3) and SIP(CC7), respectively, in the presence of 2% FBS. For the detection, rabbit anti-human IgE antibody (Dako), followed by goat anti-rabbit IgG Alexa Fluor 488 antibody (Invitrogen) were used, diluted according to the manufacturer’s recommendation. Rinsing with PBS was performed in between all incubation steps. Fluorescence-activated cell sorting (FACS) was performed on a FACSCanto equipped with FACSDiva software (BD Biosciences). A typical cell area was gated and a total of 10,000 events per sample were acquired. Omission of the primary antibody, as well as an isotype matched control anti-lysozyme antibody, SIP(HyHEL-10) (Lavoie et al, 1992), at 9 µg/ml were used to define background staining. Results were expressed in percentage of the maximal FACS signal. Data were analyzed using FloJo software (Tree Star).

4.1.3.8. Immunofluorescence on frozen tissue sections

Healthy and tumor tissue was embedded in freezing medium (Microm), snap frozen in liquid nitrogen and stored at -80 °C until sectioned. Tissue sections (10 µm) were first fixed for 10 min in ice-cold acetone, re-hydrated with PBS, blocked with FBS and then double-stained for CA IX and CD31. SIP(A3) and SIP(CC7), used as primary binding reagents at 1 µg/ml, were detected with rabbit-anti-human IgE antibody (Dako), followed by goat anti-rabbit IgG Alexa Fluor 594 antibody (Invitrogen). Primary rabbit polyclonal anti-CA IX antiserum (sc-25599; Santa Cruz) was detected with goat anti-rabbit IgG Alexa Fluor 594 antibody (Invitrogen). Primary rat anti-mouse CD31 antibody (BD Pharmingen) was detected with donkey anti-rat IgG Alexa Fluor 488 antibody.
(Invitrogen). All commercial binding reagents were diluted according to the manufacturer’s recommendation. Rinsing with PBS was performed in between all incubation steps. Finally, slides were mounted with Glycergel mounting medium (Dako) and analyzed with a Zeiss Axioskop 2 mot fluorescence microscope (Zeiss). Images were captured with an AxioCam MRC using AxioVision 4.7 image analysis software (Zeiss).

4.1.3.9. Multi-fluorescence microscopy

5 $\times$ 10$^6$ LS174T or SW1222 human colorectal adenocarcinoma cells were injected subcutaneously into the left flank of 6-8-week-old female MF1 nu/nu mice. Tumors were allowed to grow for 14 days to a size of typically 1-1.5 cm$^3$.

All mice were injected i.v. into the tail vein with 100 µg of SIP(A3) (n=3) or SIP(CC7) (n=3) 6 h prior to sacrifice. To relate anti-CAIX SIP antibody distribution to tumor morphology/pathophysiology, the following parameters were studied by multi-fluorescence microscopy: (i) Perfusion: the in vivo DNA-binding dye Hoechst 33342 (10 mg/kg; Invitrogen) was injected i.v. 1 min prior to sacrifice. (ii) Blood vessels: an anti-CD31 antibody was used to stain for blood vessel distribution. (iii) Hypoxia: The hypoxic cell marker pimonidazole hydrochloride (1-[(2-hydroxy-3-piperidinyl) propyl]-2-nitroimidazole hydrochloride; 60mg/kg; Natural Pharmacia International Inc.,) was injected 30 min prior to sacrifice.

Sections (12 µm) were first fixed in acetone for 10 min at room temperature and blocked for 20 mins with 3% normal goat serum (Dako). Three serial sections from the same tumor were incubated with a 1:2 dilution of rat anti-mouse CD31 simultaneously with one of the following antibodies: (1) 1:200 dilution of rabbit anti-pimonidazole (Natural Pharmacia International Inc.), (2) 1:1000 dilution of rabbit anti-human IgE (Dako; to detect the injected SIP(A3) and SIP(CC7)) or (3) 1:10 dilution of rabbit polyclonal anti-CAIX antiserum (sc-25599; Santa Cruz). After rinsing with PBS, sections were incubated with a 1:200 dilution of goat anti-rat Alexa Fluor 594 antibody and goat anti-rabbit IgG Alexa Fluor 488 (Invitrogen). Sections were mounted in PBS and viewed using an AxioImager.Z1 microscope (Zeiss), fitted with a computer-controlled motorised stage. Images were captured by an AxioCam digital black and white camera using AxioVision.
4.6 Image analysis software (Zeiss). Image processing was performed as described previously (El-Emir et al., 2007a).

4.1.3.10. Biodistribution in LS174T human colorectal carcinoma model

1 x 10⁷ LS174T human colorectal adenocarcinoma cells were injected subcutaneously into the left flank of 6-8-week-old female Balb/c nu/nu mice (Charles River). Tumors were allowed to grow for 15 days to a size of typically 200 mg. SIP(A3), SIP(CC7), SIP(HyHEL-10) and SIP(L19) were conjugated to p-isothiocyanatobenzyl-DTPA (p-SCN-Bn-DTPA; Macrocyclics) in 0.1 M NaHCO₃ buffer at pH 8.2 by incubation with a 50-fold molar excess of p-SCN-Bn-DTPA for 1 h at room temperature. The p-SCN-Bn-DTPA-SIP conjugates were purified by size-exclusion on a PD-10 column (GE Healthcare). Mass spectrometric analysis revealed ~1.8 modifications/antibody molecule.

The p-SCN-Bn-DTPA-SIP conjugates were labeled with ¹⁷⁷Lu (Perkin Elmer) in a 125 mM ammonium acetate buffer pH 5.5 for 1 h at room temperature, as described by (Brouwers et al., 2004). Subsequently, free ¹⁷⁷Lu was complexed by addition of 2.5 mM EDTA. The radiolabeled antibody preparation was purified by size-exclusion on a PD-10 column (GE Healthcare) and eluted with PBS. For all preparations, the rate of radiolabel incorporation and the immunoreactivity were determined, as described (Tarli et al., 1999).

The in vivo targeting performance of the ¹⁷⁷Lu-labeled antibody preparations was evaluated by i.v. injection of 6-11 µg/mouse (7-11 µCi) in 200 µl into the tail vein of xenograft bearing nude mice. Each group contained four individuals. Mice were sacrificed 24 h after injection. Organs were excised, weighed and the radioactivity was counted. Targeting results are expressed as both tumor/organ ratios and as percentage of injected dose per gram of tissue (% ID/g).

All in vivo studies were carried out according to Swiss regulations under a project license granted by the Veterinäramt des Kantons Zürich (198/2005).
4.1.4. Results

4.1.4.1. Isolation of A3 and CC7, two human monoclonal antibodies specific to CA IX

The CA domain of CA IX (residues 120-397) was cloned and expressed as soluble protein in HEK EBNA 293 cells [Figure 4.1 A] and purified from the cell culture supernatant on NiNTA resin by means of a C-terminal 6xHis-tag. The native structure of CA IX on the cell membrane is reported to consist of cysteine-linked trimers (Pastorekova et al, 1992; Thiry et al, 2006). We observed the formation of a covalent homodimer (~75%) and of a monomeric protein (~25%) both by SDS-PAGE analysis [Figure 4.1 B] and by size-exclusion chromatography [Figure 4.1 C]. The specific esterase activity of the purified protein, as measured with 1 mM 4-nitrophenylacetate and 1.6 µg recombinant enzyme in 100 µl of 50 mM Tris-SO₄, pH = 8.5 containing 10 µM ZnSO₄ at 400 nm, was determined to be 1 nmole/min/µg [Figure 4.1 D].

The recombinant catalytic domain of CA IX was used for the isolation of human monoclonal antibodies from the ETH-2-Gold phage antibody library (Silacci et al, 2005). One of the clones isolated from the library (“A11”) [Table 4.1] was affinity-matured by combinatorial mutagenesis of residues in the CDR1 loops of VH and VL domains according to a procedure recently developed by our group (Villa et al, 2008), yielding clone A3. Additional mutagenesis of CDR2 loops led to the isolation of the daughter antibody clone CC7 [Table 4.1]. The A3 and CC7 antibodies were expressed as scFv fragment in E.coli and in small immunoprotein (SIP) format in CHO-S cells using published procedures (Borsi et al, 2002) and purified to homogeneity by Protein A affinity chromatography [Figure 4.2]. The scFv format is particularly suitable for affinity determination using Biacore technology, while the homobivalent SIP format has been shown to offer distinctive advantages for in vivo molecular imaging applications (Berndorff et al, 2005; Borsi et al, 2002; Olafsen et al, 2005; Sauer et al, 2009; Tijink et al, 2006; von Lukowicz et al, 2007).
Figure 4.1 Cloning, purification and quality control of recombinant CA IX. (A) Residues 120-397 corresponding to the extracellular CA domain of CA IX, flanked by a secretion sequence (SS) and a C-terminal 6xHis-tag, were cloned into pCEP4. (B) SDS-PAGE analysis of the purified recombinant CA IX. Under non-reducing (NR) conditions the protein mainly forms a covalent homodimer (d), whereas under reducing (R) conditions the monomeric protein (m) with a molecular weight of 31.2 kDa can be observed. (C) Size-exclusion chromatography of the purified recombinant CA IX. The retention volume (ml) of the major peak corresponds to a covalently formed homodimer (d). (D) The specific activity of the recombinant protein was measured at 400 nm by its esterase activity, using 1 mM 4-Nitrophenyl acetate as substrate (solid line). Spontaneous hydrolysis of the substrate is reported as dashed line.

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<th>scFv</th>
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<th>VL chain</th>
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<td></td>
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<td>95-100a</td>
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<tr>
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<td>SGSGGS</td>
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<tr>
<td>A3</td>
<td>WYA</td>
<td>SGSGGS</td>
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<tr>
<td>CC7</td>
<td>WYA</td>
<td>AGTGGH</td>
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Table 4.1: Relevant amino acid positions of antibody clones isolated from the designed synthetic libraries. Positions that are mutated in the primary antibody library (ETH-2-Gold) are underlined. Residues in A3 and CC7, mutated during the affinity maturation procedure, are in boldface. CC7 revealed an additional mutation (R instead of K) at position 39 in the VL chain sequence. Single amino acid codes are used according to standard IUPAC nomenclature.

a Numbering according to (Tomlinson et al., 1992) and (Williams et al., 1996).
4.1.4.2. *In vitro* characterization of A3 and CC7 antibodies

The monomeric fractions of the A3 and CC7 antibodies in recombinant scFv format were isolated by size exclusion chromatography and analyzed by real-time interaction analysis on a Biacore instrument, using a microsensor chip coated with the recombinant CA domain of CA IX. **Figure 4.3** illustrates sensograms for the two antibodies, revealing a $K_d$ dissociation constant of 2.4 nM for scFv(A3) [$k_{on} = 9.1 \times 10^5$ s$^{-1}$M$^{-1}$; $k_{off} = 2.2 \times 10^{-3}$ s$^{-1}$] and of 3.2 nM for scFv(CC7) [$k_{on} = 4.3 \times 10^5$ s$^{-1}$M$^{-1}$; $k_{off} = 1.4 \times 10^{-3}$ s$^{-1}$].
Figure 4.3 Biacore analysis of purified monomeric scFv preparations injected at different concentrations. (A) Binding of scFv(A3) and (B) scFv(CC7) to the extracellular CA domain of CA IX. Kinetic constants were calculated with the BIAevaluation 4.1 software.

The ability of the two antibodies in SIP format to recognize the native CA IX on the surface of tumor cells was investigated by fluorescence-activated cell sorting (FACS), in comparison to a recombinant SIP antibody of irrelevant specificity (HyHEL-10; Lavoie et al., 1992). Figure 4.4 shows that a partial shift in the FACS profile was observed for the colorectal cancer cell line LS174T, while a very marked increase of cell fluorescence was detected for the pVHL-defective human RCC cell line SK-RC-52, similar to what previously reported for the clinical-stage cG250 monoclonal antibody (Grabmaier et al., 2000).

A3 and CC7 were then tested by immunofluorescence for their ability to detect CA IX in a panel of xenografted human tumor tissue sections. Figure 4.5 shows the staining obtained with A3 only, since both antibodies performed equally well. As positive control, a commercial polyclonal anti-CA IX antiserum was used. A representative positive and negative control is shown for the LS174T tumor. A strong CA IX staining (green) was detectable in the colorectal cancer models LS174T and HT-29 at a distance of ~100 µm from tumor blood vessels (red). By contrast, virtually all tumor cells in SW 1222 colorectal carcinoma, U87 glioma and in SK-RC-52 renal cell carcinomas exhibited a strongly positive CA IX staining. However, while SW 1222 and U87 exhibited a defined vascular structure, CD31 staining in SK-RC-52 tumors yielded an irregular staining network. Other cancer types displayed a weaker level of CA IX staining (e.g., NCI-H460
human non small cell lung carcinoma), while MCF7 breast tumors and Ramos lymphomas were essentially negative (data not shown).

Figure 4.4 CA IX FACS histogram plots of two different human tumor cell lines. (A-C) Open curves indicate FACS histogram plots of LS174T human colorectal adenocarcinoma cells stained with SIP(A3), SIP(CC7) (both at 1 µg/ml) or the isotype-matched control SIP(HyHEL-10) (at 9 µg/ml), detected as described before. Solid curves represent FACS histogram plots of LS174T cells where the primary antibody was omitted for detection. (D-E) Open and solid curves indicate FACS histogram plots of SK-RC-52 human RCC cells stained as described for A-C.
Figure 4.5 Immunofluorescence analysis performed on (A-C, P-U) human colorectal adenocarcinoma LS174T, (D-F) human colorectal adenomacarcinoma SW1222, (G-I) human colorectal adenocarcinoma HT-29, (J-L) human glioblastoma U87 and (M-O) human RCC SK-RC-52 xenografted tumor tissue sections. Red staining in the left panels represents endothelial cells (anti-CD31 staining), whereas green staining in the middle panels represents expression of CA IX ((B, E, H, K, N) SIP(A3) or (T) polyclonal anti-CA IX antiserum staining). Overlay of red and green fluorescence is shown in the panels to the right. (Q) Primary antibody was omitted as a negative control. Scale bar = 100 µm.
4.1.4.3. A3 and CC7 selectively target hypoxic tumor regions in vivo

In order to investigate whether the new human anti-CA IX antibodies were able to selectively localize to the antigen in tumors, following i.v. administration in the tail vein, we used both fluorescence microscopy and radioactivity-based detection methods. As mouse models of human cancer, we chose LS174T and SW1222 tumors: two colorectal cancer models, which have previously been extensively studied using monoclonal antibodies specific to the carcinoembryonic antigen (El-Emir et al, 2007b; Fidarova et al, 2008) and with the vascular targeting anti-EDB antibody L19 (El-Emir et al, 2007a).

**Figure 4.6** shows representative results of a multi-colour fluorescence microscopy analysis of serial sections from LS174T tumors, following i.v. administration of SIP(A3) [6 hours prior to sacrifice of the mice], of pimonidazole [30 minutes prior to sacrifice] and Hoechst 33342 [1 minute prior to sacrifice]. The low magnification images (top panels) yield an informative impression of the heterogeneity of these tumors. One can recognize areas with dense vascular structures (red), some of which are well perfused (blue). The structures targeted in vivo by SIP(A3) closely match those stained ex vivo for pimonidazole modification, confirming that these hypoxic areas could be reached by the i.v. administered antibody. Importantly, these areas are superimposable to the structures stained ex vivo with a polyclonal anti-CA IX antiserum, thus indicating that CA IX-positive areas of the tumor could be reached by our reagent. A higher magnification view (bottom panels) shows details of well perfused areas with no detectable CA IX expression, as well as tumor regions efficiently targeted by the A3 antibody. Similar results were obtained with SIP(CC7) [**Figure 4.7**].

The selective tumor targeting of the human anti-CA IX antibodies was also evaluated by immunofluorescence, comparing sections of tumors and of normal organs, 6 hours after i.v. administration of SIP(A3). **Figure 4.8** shows that a much brighter fluorescence signal was observed in tumor lesions, compared to normal organs [heart, intestine, kidney, liver, lung and spleen].
Figure 4.6 Multi-fluorescence microscopy analysis in LS174T xenograft-bearing mice. (A, B) Representative overlays of multiple digital fluorescence images of a LS174T tumor injected with pimonidazole [30 min prior to sacrifice] and SIP(A3) [6 h prior to sacrifice] demonstrating perfusion with Hoechst 33342 [1 min prior to sacrifice] (blue), blood vessel staining (red), pimonidazole binding (green, left panel) and CA IX targeting by SIP(A3) (green, middle panel). (C) Ex vivo staining of an adjacent tumor section with a polyclonal anti-CA IX antiserum. (D-F) Higher magnification images of mainly perfused areas of the corresponding tumor sections. (G-I) Higher magnification images of mainly CA IX positive areas of the corresponding tumor sections. (A-C) Scale bar = 500 µm and (D-I) scale bar = 100 µm. N indicates necrosis.
**Figure 4.7** Multi-fluorescence microscopy analysis in LS174T xenograft-bearing mice. (A, B) Representative overlays of multiple digital fluorescence images of a LS174T tumor injected with pimonidazole [30 min prior to sacrifice] and SIP(CC7) [30 min prior to sacrifice] demonstrating perfusion with Hoechst 33342 [1 min prior to sacrifice] (blue), blood vessel staining (red), pimonidazole binding (green, left panel) and CA IX targeting by SIP(CC7) (green, middle panel). (C) *Ex vivo* staining of an adjacent tumor section with a polyclonal anti-CA IX antiserum. (D-F) Higher magnification images of mainly perfused areas of the corresponding tumor sections. (G-I) Higher magnification images of mainly CA IX positive areas of the corresponding tumor sections. (A-C) Scale bar = 500 µm and (D-I) scale bar = 100 µm.
Figure 4.8 *In vivo* immunofluorescence analysis in LS174T xenograft-bearing mice. (A) Endothelial cells of tumor blood vessels are shown in red (*ex vivo* anti-CD31 staining). (B) Green fluorescence staining represents CA IX targeting in the tumor 6 h after i.v. administration of SIP(A3) detected with immunofluorescence techniques. (C) Overlay of red (blood vessels) and green (targeted CA IX) staining in the tumor. (D) Heart, (E) intestine, (F) kidney, (G) liver, (H) lung and (I) spleen, when stained accordingly, showed a negligible uptake of SIP(A3). Scale bar = 100 µm.

Figure 4.9 presents a similar analysis for the *in vivo* targeting of SW1222 tumors. In this model, one can observe a striking difference between external, well-perfused areas (blue) and complementary, poorly perfused hypoxic regions in the tumor core. While an *ex vivo* analysis with the anti-CA IX antiserum yields a pattern with uniform staining intensity, the regions targeted *in vivo* by SIP(A3) mainly cluster around central vascular structures, with a gradient of staining intensity (green) which reflects a gradient in antibody diffusion. Interestingly, in this tumor model, pimonidazole staining and CA IX staining do not always overlap. Similar results were obtained with SIP(CC7) [Figure 4.10].
Figure 4.9 Multi-fluorescence microscopy analysis in SW1222 xenograft-bearing mice. (A, B) Representative overlays of multiple digital fluorescence images of a SW1222 tumor injected with pimonidazole [30 min prior to sacrifice] and SIP(A3) [6 h prior to sacrifice] demonstrating perfusion with Hoechst 33342 [1 min prior to sacrifice] (blue), blood vessel staining (red), pimonidazole binding (green, left panel) and CA IX targeting by SIP(A3) (green, middle panel). (C) Ex vivo staining of an adjacent tumor section with a polyclonal anti-CA IX antiserum. (D-F) Higher magnification images of mainly perfused areas of the corresponding tumor sections. (G-I) Higher magnification images of mainly CA IX positive areas of the corresponding tumor sections. (A-C) Scale bar = 500 µm and (D-I) scale bar = 100 µm.
Figure 4.10 Multi-fluorescence microscopy analysis in SW1222 xenograft-bearing mice. (A, B) Representative overlays of multiple digital fluorescence images of a SW1222 tumor injected with pimonidazole [30 min prior to sacrifice] and SIP(CC7) [6 h prior to sacrifice] demonstrating perfusion with Hoechst 33342 [1 min prior to sacrifice] (blue), blood vessel staining (red), pimonidazole binding (green, left panel) and CA IX targeting by SIP(CC7) (green, middle panel). (C) Ex vivo staining of an adjacent tumor section with a polyclonal anti-CA IX antiserum. (D-F) Higher magnification images of mainly perfused areas and CA IX positive areas of the corresponding tumor sections. (A-C) Scale bar = 500 µm and (D-F). Scale bar = 100 µm.

In order to assess the efficiency of in vivo targeting of CA IX, we performed a comparative biodistribution analysis with SIP(A3), SIP(HyHEL-10) used as negative control and SIP(L19), a clinical-stage antibody which recognizes tumor neo-vascular structures (Berndorff et al, 2005; Borsi et al, 2002; El-Emir et al, 2007a; Sauer et al, 2009; Tijink et al, 2006). We used antibody preparations labeled with lutetium-177 rather than radiiodine, as previous studies had suggested that anti-CA IX antibodies are deiodinated upon internalization (Brouwers et al, 2004). Table 4.2 presents the biodistribution results for the three antibodies in LS174T tumor-bearing mice, 24 hours after i.v. administration. Targeting data are expressed both as tumor:organ ratios and percent injected antibody dose per gram of tissue (% ID/g) ± standard error. At this time
point, SIP(L19) displayed a tumor accumulation of 9.3 %ID/g, with a tumor:blood ratio of 5.8. SIP(A3) exhibited a lower tumor uptake (2.4 %ID/g), but a better tumor:blood ratio (16.7). The negative control antibody displayed lower tumor uptake (1.1 %ID/g), in line with previous studies (Tarli et al, 1999).

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<tr>
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<th>SIP(L19)</th>
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<tr>
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<tr>
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<td>Kidney</td>
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<td>Intestine</td>
<td>2.7</td>
<td>2.3</td>
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Table 4.2 Biodistribution experiments of $^{177}$Lu-labeled anti-CA IX antibodies in nude mice bearing LS174T human colorectal adenocarcinoma xenografts.

Tumor/organ ratios, at 24 h after i.v. injection, are indicated in boldface. The numbers in brackets correspond to the percent injected antibody dose per gram of tissue (% ID/g) ± standard error.

Student’s $t$ test was applied to calculate significant differences. The tumor uptake of SIP(A3) is significantly higher than the one of the anti-lysozyme antibody SIP(HyHEL-10) ($P < 0.001$).
4.1.5. Discussion

In this study, we describe the isolation of the first fully human monoclonal antibodies (A3 and CC7) with high affinity to CA IX. Furthermore, the in vivo distribution and tumor-targeting properties of the two antibodies have been investigated using both fluorescence-based techniques and radiolabeled antibody preparations.

Monoclonal antibodies represent the most rapidly growing sector of Pharmaceutical Biotechnology (Walsh, 2006), particularly for cancer therapy applications. Since antibodies recognize their cognate antigens with exquisite selectivity, intense research efforts are devoted to the exploitation of these binding specificities for the development of superior therapeutic agents (Carter, 2006). As rodent and chimeric antibodies are immunogenic in patients, there is an increased need for good quality human monoclonal antibodies, which can be used for imaging and therapeutic purposes (Pendley et al, 2003; Presta, 2006; Winter & Harris, 1993). Both A3 and CC7 are fully human antibodies, which recognize human CAIX in its native conformation with affinities in the low nanomolar range. Interestingly, it was necessary to express the recombinant antigen in a mammalian system, as previous attempts to generate anti-CAIX antibodies from bacterially produced catalytic domain led to the isolation of antibody clones which did not recognize the antigen in its native conformation (data not shown). With the newly reported A3 and CC7 antibodies CA IX was shown to be expressed heterogeneously throughout the majority of the studied tumors, with exception of renal cell carcinomas in which all cancer cells express the antigen as a consequence of a mutated VHL gene (Rathmell & Chen, 2008) [Figure 4.5]. The hypoxia-related CA IX expression was usually detectable at a distance > 100 µm from the nearest oxygen-supplying blood vessel. This finding is in agreement with previous reports (Beasley et al, 2001; Hockel et al, 1996; Koukourakis et al, 2001; Loncaster et al, 2001; Wykoff et al, 2001; Wykoff et al, 2000) and in accordance with the diffusion limit of oxygen within tissues, which has been measured to be around 150 µm (Folkman et al, 2000; Vaupel, 2004).
By multi-fluorescence microscopy we subsequently demonstrated that the A3 and CC7 antibodies, in recombinant SIP format, preferentially localized to hypoxic areas of LS174T and SW1222 colorectal tumor xenografts 6 hours after i.v. administration. The tumor uptake was dramatically more efficient compared to normal organs, which displayed minimal staining at the same time point, using an immunofluorescence detection method [Figure 4.8].

In the LS174T model an excellent overlap was observed between pimonidazole staining and structures targeted in vivo by the anti-CA IX antibodies in all studied animals, although A3 and CC7 appeared to stain slightly closer to the vasculature than did the pimonidazole. The targeted structures were furthermore superimposable to the staining pattern of an ex vivo applied commercially available polyclonal anti-CAIX antiserum [Figures 4.6 and 4.7]. In the SW1222 model CA IX expression was consistently detected in a much broader area with the A3 and CC7 antibodies compared to the one covalently modified by pimonidazole [Figures 4.9 and 4.10]. In these tumors, both A3 and CC7 exhibited reduced accumulation compared to the ex vivo applied commercial anti-CA IX antiserum, and were in close proximity to both large and small vascular structures. This may indicate a barrier preventing a homogenous targeting of antigen within the tumor mass, although other tumor factors may also be involved. Interestingly, these large vascular structures were only poorly perfused with the Hoechst dye.

To our knowledge, this is the first study in which pimonidazole staining, CA IX targeting (both in vivo and ex vivo) and perfusion are simultaneously analyzed. As the time points for the injection of antibody, pimonidazole and Hoechst 33342 were different (6 hours, 30 min and 1 min prior to sacrifice, respectively), we cannot exclude that a transient occlusion of certain vascular structures took place (Chaplin et al, 2006).

Limitations in antibody diffusion within solid tumor masses have previously been reported for reagents used in the IgG format (Adams & Weiner, 2005; Dennis et al, 2007; El-Emir et al, 2007b), mainly in relation to the so-called “antigen barrier”. In this study, we have used antibodies in SIP format (Borsí et al, 2002; Villa et al, 2008), as this format and similar mini-antibody formats (Wu & Olafsen, 2008) have extensively been shown to offer distinctive advantages both for imaging applications (Leyton et al, 2008; von Lukowicz et al, 2007; Wei et al, 2008) and for radioimmunotherapy of cancer (Berndorff
et al, 2005; Kenanova et al, 2007; Sauer et al, 2009; Tijink et al, 2006). Recent publications suggest that smaller high-affinity ligands [MW < 2000 Daltons] may enjoy a much more rapid tissue distribution compared to antibodies and antibody fragments (Low et al, 2008). High-affinity low-molecular weight ligands to CA IX have been reported (Supuran, 2008) and it will be interesting to compare their in vivo tumor targeting properties with those of the human antibodies A3 and CC7 with a molecular weight of 76 kDa.

In our biodistribution studies we have used lutetium-177 as radionuclide, in light of previous reports indicating that dehalogenation takes place with internalizing anti-CA IX antibodies (Brouwers et al, 2004). With this radiometal, we observed high kidney and liver values [Table 4.2], similar to what we had previously reported for the 177Lu-labeled L19 antibody in SIP format (Tijink et al, 2006). In the pairwise comparison presented in this article, L19 appeared to yield higher tumor uptake values, yet at the expense of a slower blood clearance. Tumor pre-targeting strategies could also be considered, as they have yielded excellent tumor:organ ratios over a range of time points after injection (van Schaijk et al, 2005). By contrast, Fab and F(ab’)_2 preparations of the same antibody did not exhibit improved selectivity compared to the IgG format (van Dijk et al, 1991).

Our group has developed and brought to clinical trials human monoclonal antibody derivatives based on the L19 (Pini et al, 1998), F8 (Villa et al, 2008) and F16 (Brack et al, 2006) antibodies, specific to splice isoforms of fibronectin and of tenascin-C, respectively. These antibodies display comparable in vivo biodistribution results and can target a broad variety of tumors. However, their uptake within the solid tumor mass is confined to the subendothelial extracellular matrix (Borsi et al, 2002; Demartis et al, 2001; El-Emir et al, 2007a; Villa et al, 2008). The human anti-CA IX antibodies appear to target a similarly broad spectrum of cancers, yet with a four-times lower tumor uptake and a broader tissue distribution within the lesion. For this reason, one could envisage the simultaneous use of vascular-targeting and hypoxia-targeting antibodies for pharmacodelivery applications, in order to achieve a more homogenous distribution to
therapeutic agent (e.g., cytotoxic drug; (Carter & Senter, 2008; Wu & Senter, 2005)) within the tumor mass.
4.2. *In vivo* targeting of tumor-associated carbonic anhydrases using acetazolamide derivatives

4.2.1. Abstract

Membrane-associated carbonic anhydrases IX and XII are frequently over-expressed in cancer, either at sites of hypoxia or on pVHL-defective tumor cells. Various sulfonamides have previously been described which inhibit both intracellular and membrane-associated carbonic anhydrase activities. Here, we describe the synthesis and characterization of two acetazolamide derivatives, which contain either a charged fluorophore or an albumin-binding moiety, which restrict binding to carbonic anhydrase IX and XII present on tumor cells. The fluorescent acetazolamide derivative was shown to bind to cancer cells *in vitro* and to preferentially target tumors *in vivo*, using fluorescence-based detection methodologies. The albumin-binding acetazolamide (‘Albu-acetazolamide’) derivative was shown to simultaneously engage in binding interactions with both albumin and carbonic anhydrase IX, thus preventing internalization while inhibiting membrane-associated anhydrase activities. The ability of Albu-acetazolamide to cause tumor growth retardation was assessed in two xenograft models of cancer (LS174T and SK-RC-52) alone and in combination with chemotherapeutic drugs (5-fluorouracil and sunitinib).
4.2.2. Introduction

Virtually all tumors require new blood vessels, which provide nutrients and oxygen to the cancer cells, thus sustaining growth and offering an avenue for metastatic spread (Folkman, 1995). However, not all cells within the tumor mass receive a sufficient oxygen supply and cancer cells at a distance of 100-200 µm from the nearest blood vessel become hypoxic (Kerbel & Folkman, 2002). This pattern is particularly pronounced not only in experimental rodent models of cancer, but also in aggressive human solid tumors such as glioblastoma multiforme, where hypoxic cells form a “palisade”, delineating a border between cancer cells in proliferation around pseudoglomerular blood vessels and necrotic areas (Wippold et al, 2006).

Carbonic anhydrase IX (CA IX), a membrane-anchored enzyme, is one of the most prominent proteins, which become over-expressed in hypoxic conditions (Scheurer et al, 2004; Staller et al, 2003). CA IX catalyzes the reversible conversion of CO₂ to carbonic acid, thereby leading to an acidification of the extracellular tumor milieu (Brahimi-Horn et al, 2007). Additionally, it has been proposed that transport of bicarbonate into the cytoplasm of tumor cells through Cl⁻/HCO₃⁻ exchanger leads to the increase of the intracellular pH of cancer cells (Brahimi-Horn et al, 2007), resulting in the generation of alternatively spliced components of the extracellular matrix (Borsi et al, 1996; Borsi et al, 1995), which are now frequently used as targets for antibody-mediated pharmacodelivery strategies (Neri & Bicknell, 2005). Furthermore, by regulating the intracellular pH, CA IX and CA XII confer a growth and survival advantage to tumor cells exposed to the hypoxic and acidic microenvironment (Chiche et al, 2009).

The overexpression of CA IX in breast cancer has been found to be associated with reduced survival (Hussain et al, 2007). Furthermore, renal cell carcinoma (RCC) frequently carry a loss-of-function mutation in the VHL gene, the product of which is responsible for targeting the oxygen sensor HIF-1α for proteasomal degradation, thus leading to increased CA IX levels on all tumor cells, due to HIF-1α stabilization (Pugh & Ratcliffe, 2003). Indeed, CA IX has been used in the past as antigen for the generation of
tumor targeting monoclonal antibodies (Brouwers et al, 2004; Chrastina et al, 2003a; Chrastina et al, 2003b; van Dijk et al, 1991; van Schaijk et al, 2005) and the chimeric antibody cG250 is currently being studied by Wilex in Phase III clinical trials for the therapy of RCC.

Cells may express a number of different carbonic anhydrases. Carbonic anhydrase I, II, III, VA, VB, VII and XIII are expressed intracellularly, while carbonic anhydrase IV, IX, XII, XIV and XV are membrane-bound (Supuran, 2008). In tumors, not only CA IX but also CAXII is strongly over-expressed. Indeed, recent RNAi experiments indicate that only a simultaneous blockade of CA IX and CA XII leads to tumor growth retardation, as a result of impaired acidification of the tumor environment (Chiche et al, 2009).

Based on these considerations, it is not surprising that the development of carbonic anhydrase inhibitors has been a matter of intense medicinal chemical research, in an attempt to design selective inhibitors for the different CA isoforms (Supuran, 2008). As CA IX and CAXII are the most relevant carbonic anhydrases for the development of anti-cancer strategies, the use of bioreductive prodrugs (De Simone et al, 2006) or of membrane-impermeable glycoconjugates has been proposed (Winum et al, 2008). To our knowledge, none of these therapeutic approaches has been tested in vivo so far.

Dennis et al. have recently described a general strategy for the reduction of blood clearance of therapeutic proteins (such as tumor-specific antibody fragments) by means of a C-terminal albumin-binding peptide (Dennis et al, 2002). Interestingly, the longer circulatory half-life of anti-HER2-neu antibodies in stable non-covalent association with mouse serum albumin led to a homogenous decoration of tumor cells in vivo, which could not be obtained with the same antibody (Trastuzumab) in Fab or IgG format, that were confined to perivascular tumor cells (Dennis et al, 2007). In principle, the same strategy could be used for the modification of small organic enzyme inhibitors, with the additional advantage of preventing internalization, thus restricting their scope to membrane-bound and secreted enzyme. Recently, our lab has described derivatives of 2-amino-6-(4-(4-iodophenyl)butanamido)hexanoic acid as a general class of stable and
portable albumin-binding moieties, which can be conjugated to a variety of different
drugs and which prolong serum half-lives in vivo (Dumelin et al, 2008).

In this paper, we describe the synthesis and characterization (in vitro and in vivo) of a
novel bispecific acetazolamide derivative, capable of simulatenous binding to carbonic
anydrases and serum albumins (‘Albu-acetazolamide’). We used acetazolamide as a
building block, since this compound is able to inhibit a broad spectrum of carbonic
anhydrases in the low nanomolar range, including CA IX and CA XII (Supuran, 2008).
Furthermore, we investigated the conjugation of acetazolamide to a charged fluorophore
(‘FAM-acetazolamide’) as an alternative avenue to prevent internalization, thus
restricting binding to carbonic anhydrases overexpressed on the membrane of tumor cells.
FAM-acetazolamide binding to tumor cells was confirmed by fluorescence-activated cell
sorting, while preferential in vivo tumor targeting was assessed by fluorescence
microscopy analysis of tissue sections, 1h and 2h after intravenous (i.v.) administration.
The use of acetazolamide as targeting moiety for pharmacodelivery applications is
attractive since most tumors express CA IX and CA XII either at sites of hypoxia or on
pVHL-defective tumor cells. Small organic ligands may be advantageous for tumor
targeting applications, compared to monoclonal antibodies, in view of their rapid
extravasation and homogenous tissue distribution properties (Low et al, 2008).
4.2.3. Materials and Methods

All chemicals were purchased from Sigma-Aldrich unless stated otherwise.

4.2.3.1. Synthesis of FAM-acetazolamide

266 µmol of 5-carboxyfluorescein (Chemodex), 292 µmol of N-hydroxysuccinimide (NHS) and 292 µmol of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC-HCl) were dissolved in 3 ml of dry N,N-dimethylformamide (DMF) and stirred protected from light for 4 hours at 25°C. The reaction mixture was added to 319 µmol of 5-amino-1,3,4-thiadiazole-2-sulfonamide (Ramidus AB) and 3.2 mmol of triethylamine dissolved in 3 ml dry DMF. The reaction was stirred protected from light at 25°C for 16 hours followed by addition of 350 µl of 3 M Tris/Cl, pH 8 and stirring at 25°C for 30 minutes. HPLC purification was performed running a linear gradient from 40–60% acetonitrile. The eluting product [2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-5-(5-sulfamoyl-1,3,4-thiadiazol-2-ylcarbamoyl)benzoic acid, hereafter termed ‘FAM-acetazolamide’] was detected at λ=470 nm. The desired fractions were collected, dried under vacuum and the concentration was determined by UV / VIS spectrometry at 495 nm (ε = 72000 M⁻¹ cm⁻¹). ESI-MS m/z 538.71 ([M + H⁺], 100%), calcd: 538.03 Da.

4.2.3.2. Synthesis of Albu-acetazolamide

611 µmol of 5-amino-1,3,4-thiadiazole-2-sulfonamide (Ramidus AB) was stirred with 611 µmol of succinic anhydride in 1 ml dry N,N-dimethylformamide (DMF) for 24 hours at 50°C followed by HPLC purification running a linear gradient from 0-100% acetonitrile. After collection of the desired fractions, solvents and buffer were removed under vacuum. 366 µmol of the obtained intermediate 4-oxo-4-(5-sulfamoyl-1,3,4-thiadiazol-2-ylamino)butanoic acid, 366 µmol of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU, Novabiochem) and 366 µmol of N-ethyldiisopropylamine (DIPEA) were stirred for 10 minutes at 25°C in 3 ml dry DMF followed by addition of 366 µmol of 4-(p-iodophenyl)butyl lysine and 732 µmol of
triethylamine in dry DMF. The reaction was stirred for 24 hours at 30°C and purified by HPLC running a linear gradient from 40-60% acetonitrile. The eluting product [6-(4-(4-iodophenyl)butanamido)-2-(4-oxo-4-(5-sulfamoyl-1,3,4-thiadiazol-2-ylamino)butanamido)hexanoic acid, hereafter termed ‘Albu-acetazolamide’] was collected and solvents and buffer were removed under vacuum. ESI-MS m/z 680.86 ([M + H^+], 100%), calcd: 680.06 Da. ^1H NMR (DMSO-d_6): 7.61 (d, 8.3 Hz, 2H), 7.00 (d, 8.3 Hz, 2H), 4.14 (m, 1H (aH)), 3.01 (m, 2H) 2.74 (t, 6.6 Hz, 2H), 2.52 (m, 4H), 2.05 (m, 2H), 1.81-1.50 (m, 4H), 1.43-1.21 (m, 4H) (Bruker 400 avance instrument).

4.2.3.3. High Performance Liquid Chromatography Purification

All High Performance Liquid Chromatography (HPLC) purifications of small molecules were performed on a Waters 2795 Alliance HT with 2487 dual wavelength absorbance detector using a Phenomenex Synergi 4u POLAR-RP column (4 µM, 10 x 150 mm) with linear gradients over 15 minutes. The starting and end concentration of organic eluent is given for the individual purification steps. The solvents used were H_2O buffered by 0.1% trifluoroacetic acid and acetonitrile. Detection was generally performed at 210 nm and 260 nm or at 470 nm. The individual fractions were analyzed by mass spectrometry (Micromass Quattro micro API). After collection of the desired fractions, solvents and buffer were removed under vacuum.

4.2.3.4. Tumor mouse models

The human colorectal adenocarcinoma cell line LS174T (CL-188, ATCC) was maintained in DMEM, supplemented with 10% fetal bovine serum (FBS) and antibiotic-antimycotic at 37°C in an atmosphere of 5% CO_2. The human human renal cell carinoma (RCC) cell line SK-RC-52 (Ebert et al, 1990) was maintained in RPMI, supplemented as described above. 1 x 10^7 tumor cells were injected subcutaneously into the left flank of 6-8-week-old female Balb/c nu/nu mice (Charles River). Tumors were allowed to grow for 6 (LS174T)
to 10 days (SK-RC52) to a size of typically 80-100 mg for therapy applications. For biodistribution studies LS174T tumor of ~500 mg were used.

4.2.3.5. Cloning, expression and purification of recombinant CA IX

The cloning, expression and purification of the recombinant extracellular carbonic anhydrase (CA) domain of CA IX is described elsewhere (Ahlskog et al, 2009). Briefly, the cDNA fragment encoding aa 120-397 of CA IX was amplified from the full-length cDNA clone IRAUp969G1273D (imaGenes) used as template with primers introducing a C-terminal hexahistidine-tag. The resulting PCR fragment was assembled to an N-terminal secretion sequence. Subsequently, the amplicon was cloned into vector pCEP4 (Invitrogen) by means of HindIII and XhoI digestion. The sequence-verified plasmid was used to transfect HEK 293 EBNA cell line using FuGENE 6 transfection reagent (Roche). Transiently transfected cells were grown in DMEM supplemented with 10% FBS and selected using 200 µg/ml of hygromycin. The recombinant domain of CA IX was purified from supernatant by affinity chromatography on Ni-NTA agarose (Qiagen) by means of the C-terminal 6xHis-tag. The purified protein was analyzed by SDS-PAGE and size-exclusion chromatography using a Superdex 200 HR 10/30 column (GE Healthcare). The specific activity of the recombinant protein was measured by its esterase activity, as described below.

4.2.3.6. Isothermal Titration Calorimetry (ITC)

The $K_d$ values of Albu-Acetazolamide binding to CA IX and human serum albumin (HSA) were measured using a VP-ITC instrument (Microcal). A 4.7 µM solution of CA IX in PBS (20 mM NaH$_2$PO$_4$, 30 mM Na$_2$HPO$_4$, 100 mM NaCl, pH 7.4) was titrated with a 64 µM solution of Albu-Acetazolamide in PBS at 25°C (after an initial dummy injection of 1 µl, 29 injections of 10 µl each were performed). A 50 µM HSA (Sigma, fatty acid free) solution in PBS containing 2% (v/v) DMSO was titrated with a 500 µM solution of Albu-Acetazolamide in the same buffer under the conditions described above.
The resulting titration curves were then processed and fitted with the Origin 7 software (OriginLab) to obtain the corresponding $K_d$ values.

4.2.3.7. Enzymatic assay

The binding strength of CA IX ligands Albu-Acetazolamide and FAM-Acetazolamide was measured as $K_i$, detecting the esterase activity of the enzyme using 4-nitrophenylacetate as substrate, as described (Pocker & Stone, 1967). The assay was performed in 50 mM Tris-SO$_4$, pH 8.5 containing 1 % (v/v) DMSO and 10 µM ZnSO$_4$ using an enzyme concentration of 1 µM (pre-incubated for 30 min) with increasing concentrations of ligands) and a 4-nitrophenylacetate concentration of 2.5 mM. In case of the Albu-Acetazolamide, the assay was performed in the absence and presence of 10 µM mouse serum albumin (MSA). Autohydrolysis of the substrate was subtracted from the observed total initial reaction velocities. Absorption kinetics were measured with a VersaMax absorption plate reader (Molecular Devices) and data were fitted using the Sigma plot software (Systat Software).

4.2.3.8. Immunofluorescence on frozen tissue sections

Tumor tissue was embedded in freezing medium (Microm), snap frozen in liquid nitrogen and stored at -80 °C until sectioned. Tissue sections (10 µm) were first fixed for 10 min in ice-cold acetone, re-hydrated with PBS, blocked with FBS and then double stained for CA IX and CD31. Primary rabbit polyclonal anti-CA IX antibody (sc-25599, Santa Cruz) was detected with goat anti-rabbit IgG Alexa Fluor 594 antibody (Invitrogen). Primary rat anti-mouse CD31 antibody (BD Pharmingen) was detected with donkey anti-rat IgG Alexa Fluor 488 antibody (Invitrogen). All commercial binding reagents were diluted according to the manufacturer’s recommendation. Rinsing with PBS was performed in between all incubation steps. Finally, slides were mounted with Glycergel mounting medium (Dako) and analyzed with a Zeiss Axioskop 2 mot fluorescence microscope (Zeiss). Images were captured with an AxioCam MRC using AxioVision 4.7 image analysis software (Zeiss).
4.2.3.9. Transfection of HEK EBNA 293 with full-length CA IX

The cDNA fragment encoding full-length CA IX (aa 1-459) including the internal signal sequence was amplified from the full-length cDNA clone IRAUp969G1273D (imaGenes) used as template with primers BW_CAIX_fl (5’ GATAAGCTTGCTATGGCTCCCCTGTGCCCCAG 3’) and FW_CAIX_fl (5’ TTCCTCGAGTTAGGCTCCAGTCTCCTGGCTACCTCTGC 3’). The resulting PCR fragment was cloned into vector pCEP4 (Invitrogen) and subsequently used for transfection of HEK 293 EBNA cell line, as described above. Successful transfection was assessed by fluorescence-activated cell sorting (FACS) on non-transfected and transfected cells using primary rabbit polyclonal anti-CA IX antiserum (sc-25599; Santa Cruz) detected with goat anti-rabbit IgG Alexa Fluor 488 antibody (Invitrogen) (data not shown).

4.2.3.10. Cell internalization assay with tandem mass spectrometric quantification

5 x 10^6 LS 174T, HEK EBNA 293 and HEK EBNA 293 cells transfected with the full-length CA IX protein were incubated for 1 h at 37°C on an orbital shaker (55 rpm) with 500 μM Albu-acetazolamide in PBS, both in the absence and presence of 900 μM fatty acid free HSA. After 5 min centrifugation at 500×g, the cell pellets containing internalized ligand were washed with PBS, stripped with stripping buffer (50 mM glycine, 100 mM NaCl, pH = 2.5) to remove surface-bound ligand, and resuspended in 10 μl PBS. In order to protonate the carboxylate and thus to interrupt the compound-serum albumin interaction 240 μl of a 5:1 ethanol:0.1% trifluoroacetic acid mixture were added. After vortexing, samples were incubated on ice for one hour, centrifuged and the supernatant was transferred into a new vial. The supernatant was dried under vacuum and subsequently redissolved in 50 μl H_2O containing 50% (v/v) DMSO. Subsequently samples were analyzed by liquid chromatography tandem mass spectrometry. 50 μl of the sample were injected into an LC / MS / MS system (Micromass Quattro micro API) and run on a linear gradient in 0.1% formic acid from 5% - 95% acetonitrile in ten minutes on a Waters XTerra MS C_{18} column (3.5 μM, 1 x 50 mm) observing the daughter ions with
m/z of 84.15 and 356.27 of the parent ion 681.50 for Albu-acetazolamide. Comparing the area with a previously obtained calibration curve of Albu-acetazolamide with identical sample preparation allowed the quantification.

The establishment of an equivalent cell internalization assay followed by tandem mass spectrometric quantification failed in the case of acetazolamide, due to solubility problems during sample preparation and inferior ionization for acetazolamide compared to Albu-acetazolamide, resulting in substantially reduced sensitivity.

4.2.3.11. Fluorescence-activated cell sorting with FAM-acetazolamide

LS174T cells were harvested through incubation with 10 mM EDTA in PBS for 3 min at 37°C. After counting and centrifugation for 5 min at 1100 rpm, cells were re-suspended in PBS supplemented with 2% FBS to a final concentration of 5 x 10^6 cells/ml. 1 x 10^6 cells were then incubated for 90 min at room temperature with 0.26 µM or 3 µM of FAM-acetazolamide, in the presence of 1% FBS. As a positive control primary rabbit polyclonal anti-CA IX antibody (sc-25599, Santa Cruz) was used, followed by detection with goat anti-rabbit IgG Alexa Fluor 488 antibody (Invitrogen). After rinsing with PBS, FACS was performed on a FACSCanto equipped with FACSDiva software (BD Biosciences). A typical cell area was gated and a total of 10,000 events per sample were acquired. Results are expressed as percentage of the maximal FACS signal. Data were analyzed using FloJo software (Tree Star).

4.2.3.12. Biodistribution analysis with FAM-acetazolamide

LS174T tumor-bearing mice were injected i.v. into the tail vein with 680 µg of FAM-Acetazolamide 1h (n=2) and 2 h (n=2) prior to sacrifice. Tumor and normal organ tissue sections (10 µm) were processed as described above and analyzed for CA IX targeting with a Zeiss Axioskop 2 mot plus fluorescence microscope (Zeiss). Images of the various organs were captured at identical exposure times to guarantee comparability among organs.
4.2.3.13. Combination therapy studies in LS174T and SK-RC-52 tumor xenograft models

Tumor-bearing mice were grouped (n=5) and subsequently treated using a 5-weekly injection schedule for three, in case of the SK-RC-52 model, or two consecutive weeks, in case of the more rapidly growing LS174T model. Mice were injected i.v. in the lateral tail vein with saline containing 15 % (v/v) DMSO or 340 µg (17 mg/kg) of Albu-acetazolamide diluted in the same vehicle. 5-FU in saline (25 mg/kg) (Blumenthal et al, 1994; Ciccolini et al, 2001) and sunitinib (20 mg/kg) in DMSO (Osusky et al, 2004; Schueneman et al, 2003) were administered intraperitoneally (i.p.) either alone or in combination with Albu-acetazolamide. Injection volumes were 100 µl for i.v. administration and ~50 µl for i.p. administration. For combination studies, Albu-acetazolamide was injected first, immediately followed by the i.p. injection of the chemotherapeutic agent. Mice were monitored daily and tumors were measured three times per week. Tumor volume was estimated using the following formula: length×width²×π/6. Animals were sacrificed when tumors reached a volume >2000 mm³ or when tumors turned necrotic, according to Swiss regulations, under a project license granted by the Veterinäramt des Kantons Zürich (198/2005). Tumor volumes are expressed as mean ± SE.

4.2.3.13. Pharmacokinetics

Nude mice (n=2) were injected intravenously with 20 or 40 nmole of Albu-acetazolamide dissolved in 100 µl of 100 mM Tris/HCl, 105 mM NaCl, pH = 8.2. At the timepoints 1, 5, 15, 30, 60, 120, 240, 520 and 1500 minutes after injection 20-30 µl blood were taken from the vena saphena with EDTA coated capillaries (Sarstedt) and centrifuged for 10 minutes at 10000 × g. 2-10 µl of plasma were filled up with PBS to a final volume of 10 µl. The sample preparation was carried out as described in 4.2.3.10. The amount of Albu-acetazolamide in blood, expressed as % of the injected dose (% of I.D.), was determined by liquid chromatography tandem mass spectrometry, as described in 4.2.3.10.
4.2.4. Results

4.2.4.1. Design and synthesis of bifunctional acetazolamide derivatives

**Figure 4.11** (A) Schematic representation of the subcellular localization of the carbonic anhydrase isozymes. CA I, CA II, CA III, CA VII and CA XIII are cytosolic, CA VA and CA VB are mitochondrial, while CA VI is the only secreted isozyme. CA IV, CA IX, CA XII, CA IV and CA XV are membrane-bound isozymes (Supuran, 2008). CA XV is not expressed in humans or primates, but is abundant in rodents and other higher vertebrates (Hilvo et al., 2005). CA IX and CA XII are frequently found upregulated in tumors (Chiche et al., 2009). (B) Immunofluorescence analysis performed on human RCC SK-RC-52 (upper panel) and human colorectal adenocarcinoma LS174T (lower panel) xenografted tumor tissue sections. Overlay of red (endothelial cells, i.e. anti-CD31 staining) and green (anti-CA IX staining) fluorescence. In the pVHL-defective SK-RC-52 model CA IX is expressed constitutively, whereas in the LS174 T model the expression occurs in hypoxic regions in distance to blood vessels. Scale bar = 100 µm. (C) Structure of FAM-acetazolamide, used in this study for biodistribution analysis. (D) Structure of Albu-acetazolamide. This bispecific acetazolamide derivative is able to simultaneously bind to carbonic anhydrases and serum albumin, thereby preventing its internalization and restricting it to the extracellular space. In this study, Albu-acetazolamide was used for therapy experiments in tumor-bearing mice.
Figure 4.11A illustrates the rationale for the development of bispecific acetazolamide inhibitors. A charged fluorophore moiety or the stable non-covalent binding to serum albumin prevents the internalization of the derivatives, thus restricting binding of the acetazolamide moiety to the membrane-bound carbonic anhydrase isoforms. Figure 4.11B presents two-color fluorescence microscopy images of tumor sections, showing CA IX staining in green and blood vessels in red. Two extreme situations are presented. On one hand, virtually all cancer cells express CA IX in SK-RC-52 renal tumor cells due to a defective pVHL tumor suppressor (Wykoff et al, 2000). By contrast, CA IX is only detectable at sites of hypoxia (i.e., at > 100 µm from tumor blood vessels) in the LS174T colorectal adenocarcinoma xenograft model.

The structures of the two acetazolamide derivatives investigated in this study are depicted in Figure 4.11C, D. FAM-acetazolamide [2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-5-(5-sulfamoyl-1,3,4-thiadiazol-2-ylcarbamoyl)benzoic acid] is an amide derivative of the green fluorophore 5-carboxyfluorescein (FAM), and is negatively charged at neutral pH. Albu-acetazolamide [6-(4-(4-iodophenyl)butanamido)-2-(4-oxo-4-(5-sulfamoyl-1,3,4-thiadiazol-2-ylamino)butanamido)hexanoic acid] is a bispecific compound containing the acetazolamide moiety linked to the 2-amino-6-(4-(4-iodophenyl)butanamido)hexanoic acid moiety, which confers stable albumin binding and a slow blood clearance profile to the molecule (Dumelin et al, 2008).

4.2.4.2. In vitro and in vivo tumor cell targeting properties of FAM-acetazolamide

Figure 4.12 FACS histogram plots of the LS174T cell line. (A) FACS histogram plots of LS174T human colorectal adenocarcinoma cells stained with different concentrations of FAM-acetazolamide. (B) FACS histogram plot of LS174T cells stained with a polyclonal anti-CA IX (open curve). The solid curve represents the plot where the antiserum was omitted.
The ability of FAM-acetazolamide to bind to tumor cells was assessed by fluorescence-activated cell sorting, revealing a >10-fold average fluorescence increase upon incubation of LS174T cells with 3 µM FAM-acetazolamide solution [Figure 4.12]. In order to evaluate whether this compound was also able to reach tumor cells in vivo, 680 µg FAM-acetazolamide were injected in the tail vein of nude mice bearing LS174T tumors grafted subcutaneously. Figure 4.13 depicts fluorescence microscopy images of 10 µm-thick tissue sections, obtained from mice sacrificed 1h and 2h after i.v. injection. A strong and heterogenous tumor uptake was observed at both time points, in agreement with the immunohistochemical staining patterns presented in Figure 4.11B. At 1h negligible background fluorescence was detected for heart, lung, spleen and muscle, while organs involved in the clearance of the compound (kidney, liver and intestine) were homogenously bright. As expected, fluorescence signals in all organs but tumors substantially decreased at 2h.

Figure 4.13 Biodistribution analysis with FAM-acetazolamide. LS174T xenograft-bearing nude mice were injected i.v. with FAM-acetazolamide (A-H) 1h or (I-P) 2h prior to sacrifice. The accumulation of the fluorescent acetazolamide derivative in (A, I) tumor, (B, J) muscle, (C, K) heart, (D, L) lung, (E, M) intestine, (F, N) spleen, (G, O) kidney and (H, P) liver is shown in grayscale. Exposure times wer 900 ms for (A- H) and 1100 ms for (I-P). Scale bar = 100 µm.
4.2.4.3. *In vitro* characterization of Albu-acetazolamide binding and inhibition properties

The ability of Albu-acetazolamide to display high-affinity binding to both CA IX and human serum albumin (HSA) was first confirmed by isothermal titration calorimetry [Figure 4.14]. The compound exhibited dissociation constants $K_d = 3.2$ nM towards the recombinant catalytic domain of human CA IX and $K_d = 820$ nM towards HSA. These values are comparable to the $K_d$ constants of the individual binding moieties (Dumelin et al., 2008; Supuran, 2008), thus confirming that the construction of the bispecific molecule did not adversely affect binding.

![Figure 4.14 ITC characterization of (A) CA IX titrated with Albu-acetazolamide and (B) HSA titrated with Albu-acetazolamide.](image)

In order to show that Albu-acetazolamide was able to inhibit the enzymatic activity of CA IX alone and in the presence of serum albumin, we used a colorimetric assay based on the conversion of 4-nitrophenylacetate (Pocker & Stone, 1967). The albumin-binding moiety displays comparable affinities towards both human and murine serum albumin (Dumelin et al., 2008) and we used MSA in this assay, in view of the subsequent *in vivo* cancer therapy studies in tumor-bearing mice. As expected, Albu-acetazolamide displayed a comparable inhibitory activity when used alone or in the presence of 10 µM MSA [Figure 4.15].
**Figure 4.15** Esterase reaction velocity, catalyzed by CA IX, plotted as a function of the concentration of the inhibitory Albu-acetazolamide in the absence and presence of 10 µM MSA, using an enzyme concentration of 1 µM and a 4-nitrophenylacetate concentration of 2.5 mM. The simultaneous engagement of MSA did not influence the binding of acetazolamide moiety of Albu-acetazolamide to CA IX.

In order to demonstrate that the stable binding of Albu-acetazolamide to serum albumin prevents internalization of the drug into tumor cells, we used mass spectrometric methodologies to compare drug uptake in non-transfected HEK EBNA 293, full-length CA IX-transfected HEK EBNA 293 and in LS174T tumor cells, in the absence or presence of human serum albumin. A dramatic reduction of drug uptake in the presence of albumin was observed for all three cell lines **[Figure 4.16]**.

**Figure 4.16** Cell internalization assay with tandem mass spectrometric quantification of Albu-acetazolamide. Three different cell lines [full-length CA IX-transfected HEK EBNA 293 (tHEK EBNA 293), HEK EBNA 293 and LS174T] were incubated with 500 µM Albu-acetazolamide, both in the absence and presence of 900 µM HSA. The assay was performed in triplicate and the amount of internalized Albu-acetazolamide is expressed as % of the initial amount subjected to the cells. Significant differences are based on Student’s *t* test (two-tailed, two-typed), with *P < 0.005, **P < 0.0001 and ***P < 0.00005.
4.2.4.4. Characterization of the therapeutic activity of the bispecific acetazolamide derivative in two xenograft tumor models

We assessed the therapeutic activity of Albu-acetazolamide in mice bearing subcutaneously grafted SK-RC-52 or LS174T human tumors. While SK-RC-52 is a human RCC cell line, which displays a homogenous overexpression of CA IX due to the loss of pVHL, LS174T is a human colorectal cancer in which CA IX expression is confined to the membrane of hypoxic cells at a given distance to tumor blood vessels \[\text{Figure 4.11B}\]. Furthermore, the LS174T tumor model has recently been used to assess the anti-cancer activity of RNAi-based invalidation of CA IX and CA XII (Chiche \textit{et al}, 2009).

\textbf{Figure 4.17} shows the results of therapy experiments performed in the two tumor models, using Albu-acetazolamide alone or in combination with other drugs. We used 5-fluorouracil (5-FU) for the therapy of LS174T tumors, since this drug is the mainstay of colorectal cancer in most clinical protocols (Davies & Goldberg, 2008), while we used sunitinib for the therapy of SK-RC-52, since this compound is used as first-line therapy in kidney cancer (de Reijke \textit{et al}, 2009). For both therapy experiments, a dose of 340 µg (17 mg/kg) of Albu-acetazolamide was used for each injection, which was found to be well tolerated in preliminary mouse treatment studies (data not shown) and which corresponds to a 250 µM concentration in blood immediately at the end of the bolus i.v. injection \[\text{Figure 4.18}\].

In the SK-RC-52 therapy experiment, using a 5-weekly injection schedule for three consecutive weeks, we observed only a partial inhibition of tumor growth with Albu-acetazolamide \[\text{Figure 4.17A}\]. Sunitinib treatment showed a stronger inhibition of tumor growth. Tumor stabilizations for at least twenty days could be achieved with a combination of the two compounds. Both sunitinib and Albu-acetazolamide, alone and in combination, were well tolerated as reflected by the mouse weights, without evidence of cumulative toxicity. Only mice in the control treatment group lost weight, when the tumor burden became greater than 500 mg \[\text{Figure 4.17B}\].
Figure 4.17 Therapy experiments with Albu-acetazolamide alone or in combination with standard of care chemotherapeutics. (A) Tumor growth curve expressed as mean tumor volume ± SE of SK-RC-52 in Balb/c nude mice after treatment with saline (i.v.), 340 µg Albu-acetazolamide (i.v.), 20 mg/kg sunitinib (i.p.) or 340 µg Albu-acetazolamide (i.v.) + 20 mg/kg sunitinib (i.p.) (n=5). (B) Monitoring of the weight loss during therapy for the SK-RC-52 model expressed as % of body weight on day 1 of treatment. (C) Tumor growth curve expressed as mean tumor volume ± SE of LS174T in Balb/c nude mice after treatment with saline (i.v.), 340 µg Albu-acetazolamide (i.v.), 25 mg/kg 5-FU (i.p.) or 340 µg Albu-acetazolamide (i.v.) + 25 mg/kg 5-FU (i.p.) (n=5). The combination subgroup comprised only n=4 for the LS174T study, due to the unexpected death of one mouse (*). (D) Monitoring of the weight loss during therapy for the LS174T model expressed as % of body weight on day 1 of treatment. Arrows indicate days of treatment. Boxed arrows indicate 5-FU treatment, which was only administered in the first of the two consecutive weeks, due to its negative effect on the body weight.

The therapeutic results in the LS174T model were less favorable. This tumor model grows much more rapidly, thus forcing a treatment regimen of only two weeks [Figure 4.17C]. In this model, Albu-acetazolamide used as single agent led to a slight increase of
tumor growth, which however was not statistically significant, compared to the control group of mice treated with vehicle alone. 5-FU, which was administered at the 25 mg/kg/day dose commonly used in rodent therapy studies (Blumenthal et al., 1994; Ciccolini et al., 2001) yielded only a modest tumor growth retardation, yet at the expense of a substantial toxicity [Figure 4.17D]. The combination of Albu-acetazolamide with 5-FU showed no evidence of cumulative toxicity, but unfortunately did not offer a therapeutic synergy.

![Figure 4.18 Pharmacokinetic profile of 40 nmole (○) and 20 nmole (■) Albu-acetazolamide injected intravenously into nude mice (n=2). The amount of Albu-acetazolamide in blood is expressed as % of the injected dose (% of I.D.) The albumin-binding moiety 2-amino-6-(4-iodophenyl)butanamido)hexanoic acid, described originally by (Dumelin et al., 2008), confers a longer serum-half-life to the acetazolamide.](image-url)
4.2.5. Discussion

In this article, we have described the synthesis and properties of two novel acetazolamide derivatives, which preferentially target membrane-associated carbonic anhydrases on the surface of tumor cells.

For pharmacodelivery applications, small organic molecules are increasingly being considered as suitable delivery vehicles, in parallel to the more extensively characterized antibody-based approaches (Carter & Senter, 2008; Chari, 2008). Indeed, while the extravasation of both IgG’s and antibody fragments represents the rate limiting step for their efficient targeting of perivascular structures (Adams et al, 2004; Borsi et al, 2002; Leyton et al, 2008; Thurber & Wittrup, 2008), small organic molecules may extravasate in few seconds and homogenously distribute in tissues, as evidenced both by biodistribution studies and by recent intravital microscopic imaging of fluorescently-labeled folate derivatives (Low et al, 2008). However, while human monoclonal antibodies can be raised against virtually any antigen of choice (Winter et al, 1994), there are only few small organic molecules which can be used as portable tumor-targeting moieties for pharmacodelivery applications. In addition to certain naturally-occurring peptides and peptide derivatives (Reubi et al, 2005), folate derivatives may represent the only small targeting agents whose tumor targeting properties have extensively been characterized (Low et al, 2008). Alternatively, pre-targeting strategies may be considered, which rely on the injection of suitable antibody derivatives (e.g., antibody-streptavidin fusions (Paganelli et al, 1999; Su et al, 2005) or bispecific antibodies which recognize metal chelators (Goldenberg & Sharkey, 2007)), followed by the administration of a cognate small organic molecule. We expect that the promising tumor targeting behavior of the FAM-acetazolamide derivative presented in this study will stimulate pharmacodelivery investigations, both for imaging and for targeted delivery applications. Carbonic anhydrase targeting may facilitate not only the non-invasive imaging of hypoxia in vivo, but also the delivery of therapeutic moieties (e.g., cytotoxic drugs with cleavable linkers) to a variety of different tumors. It remains to be seen whether an improved tumor targeting performance can be obtained using sulfonamides with affinity higher than the one of acetazolamide, or using homobifunctional
acetazolamide derivatives. For recombinant antibodies, it is well established that multivalent binding may lead to improved tumor targeting (Adams et al, 2004; Berndorff et al, 2005; Borsi et al, 2002; Leyton et al, 2008; Viti et al, 1999).

In our study, the combined use of Albu-acetazolamide with standard chemotherapeutic agents led to substantial tumor growth retardation, yet without a substantial improvement compared to chemotherapy alone. It is not clear at present whether this situation may reflect a lack of functional activity of carbonic anhydrases for the tumors which we have investigated or rather an inability for the albumin-bound acetazolamide derivative to homogenously distribute within the tumor mass. RNAi-based therapy studies in stably-transfected LS174T tumors have shown that the simultaneous invalidation of both CA IX and XII is needed in order to achieve an 85% tumor growth retardation (Chiche et al, 2009). However, in that experimental setting, all tumor cells are genetically equivalent and pharmacokinetic considerations do not apply. Albumin-binding antibody fragments have been shown to homogenously reach tumor cells, while the parental IgG molecule was trapped on perivascular tumor cells (Dennis et al, 2007). Similar localization studies are more difficult to perform with Albu-acetazolamide and will require either microautoradiographic investigations with radiolabeled analogues or the use of trifunctional acetazolamide derivatives, capable of albumin binding and carrying, in addition, a suitable detection agent (e.g., a fluorophore, biotin). In spite of these considerations, it was reassuring to observe that Albu-acetazolamide treatment was well tolerated at doses up to 17 mg/kg/d and led to stabilizations of tumors, which are difficult to cure. This warrants future preclinical therapy studies, using different doses and schedules.
5. CONCLUSION

This thesis describes the generation of fully human monoclonal antibodies specific to CA IX. Following intravenous administration, the anti-CA IX antibodies were able to preferentially target hypoxic tumor regions \textit{in vivo}, despite the fact that these regions are usually located at distances >100 µm from the nearest blood vessel. Using multi-fluorescence analysis, the antibody distribution throughout the tumor tissue was compared to a common chemical marker of hypoxia, pimonidazole, in two human colorectal adenocarcinoma models (LS174T and SW1222). So far, a comparison between CA IX staining and chemical modification by pimonidazole had only been performed in an \textit{ex vivo} setting by others. Assessing the blood vessel distribution and perfusion parameters in parallel allowed us to conclude that tumors are highly heterogenic in terms of oxygen availability. Furthermore, it became clear, that a dynamic blood flow predominates the tumor and that tumor vessels are likely to transiently occlude. As a consequence, not all tumor regions may be targetable at a given time-point.

When performing biodistribution analysis using one of our $^{177}$Lu-labeled anti-CA IX antibodies, we observed a statistically significant preferential accumulation in the tumor in comparison to an antibody of irrelevant specificity (2.4 % ID/g versus 1.1 % ID/g and $P < 0.001$). The higher accumulation in the tumor of the anti-EDB antibody SIP(L19) (9.3% ID/g), which targets the subendothelial extracellular matrix of the tumor-neovascularure, suggests that the accessibility of the target from the blood stream plays a crucial role for targeting efficiency. Moreover, target abundance is a key factor in the determination of the targeting outcome.

It is well known that $^{177}$Lu leads to a higher uptake in organs of radioimmunoconjugate catabolism compared to $^{125}$I, namely kidney, liver and spleen (Tijink \textit{et al}, 2006) and radiation nephrotoxicity of antibody fragments and peptides is extensively described in the literature (Akizawa \textit{et al}, 2008; Behr \textit{et al}, 1998). Recent studies show that long residence times of radiolabeled end products from lysosomes are responsible for the renal radioactivity levels. Renal tubular reabsorption of radiolabeled antibody fragments and peptides has been shown to involve megalin-cubilin. Strategies to block tubular
reabsorption of radiolabeled antibody fragments and peptides include administration of competitive inhibitors, or charge modification and PEGylation of the radioimmunoconjugate (Akizawa et al, 2008). In pilot clinical trials the renal uptake in patients injected with Fab fragments and systemically given cationic amino acids could be decreased significantly, whereas the uptake by all other organs remained unaffected. In animal studies the systemic administration of lysine led to an almost 10-fold decreased uptake of antibody fragments in the kidney (Behr et al, 1998).

Tumor pre-targeting may be a further avenue to solve problems associated with the deficiencies in targeting directly radiolabeled antibodies. This strategy is based on the separation of the targeting moiety from the effector bearing the radionuclide, so as to permit administration of the radionuclide when optimal targeting has been accomplished. To pursue this strategy, there would be needs to construct bispecific antibody molecules that bind both the target antigen as well as the radiometal-chelate complex of the effector molecule (Goldenberg & Sharkey, 2007). Alternatively, an avidin-biotin based approach could be pursued (Goldenberg et al, 2006). To perform pretargeting, the optimal time-point of targeting would have to be further investigated.

Since the here described human anti-CA IX antibodies appear to target a similarly broad spectrum of cancers, one could envisage the simultaneous administration of vascular-targeting and hypoxia-targeting antibodies for pharmacodelivery applications, in order to achieve a homogenous distribution of therapeutic agent (e.g. a cytotoxic drug (Carter & Senter, 2008)) within the tumor mass.

In a second approach to target CA IX, we used small organic molecules with a ~70-fold lower molecular weight compared to the antibodies in the SIP format. This should in principle guarantee a better tissue penetration, yet at the cost of a faster blood clearance. This fact encouraged us to prolong the half-life of acetazolamide through the conjugation to the albumin-binding moiety 2-amino-6-(4-(4-iodophenyl)butanamido)hexanoic acid, that was isolated previously in our lab (Dumelin et al, 2008), yielding ‘Albu-acetazolamide’.
By appending an albumin-binding peptide to a tumor-specific antibody fragment, a group at Genentech not only observed a longer circulatory half-life, but also an enhanced tumor tissue penetration in comparison to the corresponding Fab fragment and IgG (Dennis et al., 2007). Albu-acetazolamide offers the additional benefit of preventing internalization of the drug, thus restricting its scope to membrane-bound isoforms like CA IX and CA XII, frequently found over-expressed in cancer. So far, isozyme-specific carbonic anhydrase inhibitors have been constructed through conjugation to sugar moieties (Winum et al., 2008) or by designing bioreducible prodrugs (De Simone et al., 2006). To our knowledge, none of these constructs has been tested in vivo so far.

We confirmed the targeting ability of acetazolamide in vivo by coupling it to 5-carboxyfluorescein (‘FAM-acetazolamide’). Encouraged by the preferential tumor accumulation of this targeting agent, we decided to test the effect of Albu-acetazolamide on tumor growth in two models of cancer (LS174T and SK-RC-52). In combination with 5-FU or sunitinib we were able to observe tumor growth retardation, but no substantial improvement could be observed compared to chemotherapy alone. It remains unclear, whether this finding reflects a lack of functional activity of carbonic anhydrases for the investigated tumors studied, or an impaired distribution of our bifunctional molecule. The construction of a trifunctional molecule, comprising an additional moiety for later detection (e.g. biotin or fluorophore) or the radiolabeling of the Albu-acetazolamide followed by micro-autoradiographic studies would allow to study the tumor penetration of this molecule. Different doses of both Albu-acetazolamide and chemotherapeutic drug, a different scheduling or the use of weakly basic anticancer drugs whose cellular uptake should be increased upon pH elevation (Raghunand et al., 1999; Vukovic & Tannock, 1997) may contribute to a better therapy outcome.

As shown, by Chiche et al. the combined invalidation of CA IX and CA XII using an RNAi-based approach in stably transfected LS174T tumors led to an 85% tumor growth retardation (Chiche et al., 2009). This approach may be useful for target validation, however, it does not take into account pharmacokinetics.

It will become clear in the future, whether the delivery of a toxic payload by a carbonic anhydrase specific antibody or the simultaneous inhibition of both CA IX and CA XII
enzyme activity through selective acetazolamide derivatives, or even a combined approach, represents the better therapeutic avenue for the treatment of hypoxic tumors that do not respond to classical chemo- and radiotherapy.
6. REFERENCES


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Huminiecki L, Gorn M, Suchting S, Poulsom R, Bicknell R (2002) Magic roundabout is a new member of the roundabout receptor family that is endothelial specific and expressed at sites of active angiogenesis. *Genomics* 79: 547-52


Kirkham PM, Mortari F, Newton JA, Schroeder HW, Jr. (1992) Immunoglobulin VH clan and family identity predicts variable domain structure and may influence antigen binding. EMBO J 11: 603-9


7. SUPPLEMENTARY MATERIAL

**HindIII**

SIP secretion sequence

A3 with VH, linker and VL

Positions mutated in ETH-2-Gold library (CDR3 of VH & VL)

Positions mutated during affinity maturation (CDR1 and CDR2 of VH & VL)

**Backbone mutation**

LINKER

eCH₄

Stop

**EcoRI**

7.1. Nucleotide sequence of SIP(A3)

```
CCC AAGCTT GTGCAACCAGCTGGAGGCTGGATCTCCTCCTGTGCCTCCTGGGCTGGAGCCTCCTCCCTGTTCCTCGTCGCTGTG
GCTACAGGTAAGGGCTCACAGTAGCAGGCTGTTGAGTGCTGACATATATATGGGT
GACAATGACATCCACCTTTTGCCTTTCTCTCCACAGGTGTGCACTCGGAGGTTGCAAGC
TGTTGAGTCTGGGAGGCTTGTGACAGCTTGGGGAGGTTGCCCCCTGAGACTCTCCT
CTGTGCAGCCTCTGGATTTACCTTTTAGCTGGATGCTATGAGCTGTGGGTCCGC
CAGGCTCCAGGAAAGGGCTGGGAGTGGGTCTGACGCTAAATAGTGGTAGTGGGTG
GTAGCACAATACTACGACAATCTGGTGAAGGGCCGTTCACCATCTCCAGAGA
CAATTCCAAGAACACGCTGTATCTGCAAAATGAACAGCTGAGAGGCGAGGAC
ACGGCGGTATATTACTGTCGAAAGGGAAGTGGAGGACTCTGAGTGTG
GGGGCCAGGGAACCCCTGCTCAACCTGTCGAGT

```

```
7.2. Amino acid sequence of SIP(A3)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSWYAMSWVRQAPGKLEWVSAISG
SGGSTYYADSVKGRFTISRDSKNTLYQLMNSLRAEDTAVVVCAYAKGKWRTDFD
YWGQGTLLVTSSGGSGGSSGGSSGGSSGSELTQDPAVSVALQTVRITCGDLSL
RRHLASWYQQKPGQAPVLIYIKNNRPGIPDRFSGGSSGNTASLTITGAQAEDE
ADYYCNSSPRGRDVVFQGGTTLTGLSGSGGPRPRAPEVAFYAFATPEWPGSRD
KRTLACLIQNFMPEDISVQLHNEVQLPDARHSTTQPRKTKGSFFVFSRLEV
RAEWEQKDEFICRAVHEAASPSQTVQRAVSVPNESRRGGC

7.3. Nucleotide sequence of SIP(CC7)

CCCAAGCTTGTGACCCATGGGCTGGAGGCCTTAGTCTCCTCTTGATCGTCGCTG
GCTACAGGTAAGGGGCTCACACTAGCAGGCTTGAGGTTGAGCGTACTCGAGGGT
GACAATGACAATCCACTTTGCTTCTCTCCACAGGTTGACTGACACTCGAAG
TGTTGAGTCTGGGGAGGCTTGGTACAGCTGGGAGGCTCCCTGGAGGCTCCCGC
CTGTCAGGCCTCGTACCTTCAAGCTGCTTAGCTGCTGAGGCTCCCGC

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CAGGCTCCAGGGAAGGGGCTTGAGTGGGCTCAGCTATTGCTGGTACGGGT
GGTCATACATACTACGAGACTCCGGTGAAGCCGGTTTACCACATCTCCAGAG
ACAATTCCAGAAACACGCTGTATCTGCAAATAGAACAGCTGAGGCGGAGGA
CACGGGCTGTATATATTCTGTGCAAAGGGAAGTGAGGACTGATTTTGACTAC
TGGGGCCAGGGAACCCTGGTCACCCGGTGCTCAGGGTACATTCCTCTCGAG
CAGACAGTGAGATCACATGACAGGACAGCCTCACAGGCAGCATCTGGGGA
AGCTGTTACCAGCAGAGGCCAGGAGACAGCCCCCTGATCTGACCAGCAGCAG
AAAACACCAGGGGCTTCAGGGATCCAGACAGCTGCTGCTCAGCTCCAGG
AAACACAGCTCCCTTTCAGGCACATACACTGGGGTCTCAGGCGGAAGTAGAGGTACGTAC
TATTACTGAACTCCTCTCCCGGTGGTGAGGAGCATGTGGTATTTCCGGGAGGG
GACCAAGCGTGAGGCTCTAGGTCTCCCCGGAAGGCTTGAGGGCCGGATCTGCTCC
CCGGAAGTGCTATGCCTGGTTGCGAAGCGACAGGGGAGTGCCGGGGAGGCGGGGACACG
GCACCCCTCGCTGGCTATGACAGATCCCCCTGAGGCTCGCCTAGCAGCAGCAG
TGGCCTGCAACAACGGAGGGTGCACTCCGGGACACGCGCAGCGACGCGCAGC
CCCAGAAGACAAGGGGCTCCGGGCTCTTCTCGTTCTCAGCAGGCCTGGAGGTGACC
AGGGCCGAATGGGAGCAGAAAGATGAGTTCATCGGTCGACGTCCAGTGGGGA
AGCGAGCCCCCTACAGACCGCCGGTGCAGCGGAGGCTGTCGTTACTATCCCGAGTCAT
CGAGGAGGGGTGGGTGCTGCTAGGTAAGCCGAGCTCGAGGCGTAGGGTTACCAGGCTCG

7.4. Amino acid sequence of SIP(CC7)

EVQLLESGGGLVQPGGSLRLSCAASGFTFYAMSWVRQAPGKGEW
VSAIAAGTGGHTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVY
YCAKGYCGLRTDFYWGQGTVSSGGGSGGGSGGSGGSGSSELTQDP
VSVALGQTVRITCGDSLRRHLASYYWPQERPQAPVLYIYGKNNRPG
PDRFSGSSSGNTASLTITGAQAEDEADYYCNSSPRGGRDVFGGNTKL
TVLGGSGGGPRAAPEYAFATPEWPGSRDKRTLACLIQNFMPEDISV
QWLHNEVQLPDARHSTTTQPRKTKGSGFFVFSRLEVTAREWEQKDEFI
CRAVHEAASPSQTYQARVSVPNPESSRRGGC
8. ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>AE</td>
<td>anion exchanger</td>
</tr>
<tr>
<td>CA</td>
<td>carbonic anhydrase</td>
</tr>
<tr>
<td>CDR</td>
<td>complementarity determining region</td>
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<tr>
<td>CHO</td>
<td>Chinese hamster ovary cell line</td>
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<tr>
<td>DIPEA</td>
<td>N-ethylidiisopropylamine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium (cell culture medium)</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EDC-HCl</td>
<td>N-(3-dimethylaminopropyl)-N' -ethylcarbodiimide hydrochloride</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FAM</td>
<td>carboxyfluorescein</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
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<td>HBTU</td>
<td>2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>HIF</td>
<td>hypoxia-inducible factor</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>HRE</td>
<td>hypoxia-responsive element</td>
</tr>
<tr>
<td>HSA</td>
<td>human serum albumin</td>
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<tr>
<td>HT-29</td>
<td>human colorectal adenocarcinoma cell line</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>ITC</td>
<td>isothermal titration calorimetry</td>
</tr>
<tr>
<td>$K_d$</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>$K_i$</td>
<td>inhibition constant</td>
</tr>
<tr>
<td>$k_{off}$</td>
<td>kinetic dissociation constant</td>
</tr>
</tbody>
</table>
\( k_{on} \) kinetic association constant

LC-MS liquid chromatography-mass spectrometry

LS174T human colorectal adenocarcinoma cell line

MCF-7 human breast adenocarcinoma cell line

MCT monocarboxylate transporter

MEM Minimum Essential Medium Eagle (cell culture medium)

\( \text{min} \) minute(s)

MS mass spectrometry

MSA mouse serum albumin

NCI-H460 human non small cell lung carcinoma cell line

NHE \( \text{Na}^+ / \text{H}^+ \) exchanger

NHS \( N \)-hydroxysuccinimide

\( p\text{-SCN-Bn-DTPA} \) \( p \)-isothiocyanatobenzyl-diethylene triamine pentaacetic acid

PBS phosphate buffered saline

PCR polymerase chain reaction

PHD prolyl-4-hydroxylase

\( \text{pH}_e \) extracellular pH

\( \text{pH}_i \) intracellular pH

pVHL von Hippel Lindau tumor suppressor protein

RAMOS human Burkitt’s lymphoma cell line

RCC renal cell carcinoma

RNAi inhibitory RNA

rpm rounds per minute

RPMI Roswell Park Memorial Institute (cell culture medium)

RT room temperature

SAGE serial analysis of gene expression

scFv single chain variable fragment (antibody format)

SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

SE standard error

SIP small immunoprotein (antibody format)

SK-RC-52 human renal cell carcinoma cell line
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>SW1222</td>
<td>human colorectal adenocarcinoma cell line</td>
</tr>
<tr>
<td>TEA</td>
<td>triethylamine</td>
</tr>
<tr>
<td>TEM</td>
<td>tumor endothelial marker</td>
</tr>
<tr>
<td>U87</td>
<td>human glioblastoma multiforme cell line</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VH</td>
<td>variable heavy chain</td>
</tr>
<tr>
<td>VL</td>
<td>variable light chain</td>
</tr>
</tbody>
</table>
9. ACKNOWLEDGEMENTS

Above all, I would like to express my sincere gratitude to Prof. Dr. Dario Neri for enabling me to perform my PhD in his laboratory and for supporting me throughout these four years. Despite his immense workload, Prof. Dr. Dario Neri always found the time for discussing my projects and providing valuable assistance.

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I would like to acknowledge Prof. E. Oosterwijk (Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands) for the provision of the human RCC cell line SK-RC-52.

The anti-CD31 antibody, used for the multi-fluorescence microscopy studies, was a kind gift from Prof. A. Mantovani (Instituto di Ricerche Farmacologiche, Milan, Italy).

I thank my dear friends for diverting my attention to the beautiful things in life.

Finally, I would like to thank my loved family, especially my parents, for their never-ending support and care.
10. CURRICULUM VITAE

Julia AHLSKOG

PERSONAL INFORMATION

Date of Birth  February 27, 1979, Villingen, Germany
Nationality  German
Marital status  Unmarried
Languages  German  native
Swedish  native
English  fluent
French  fluent
Catalan  intermediate
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EDUCATION

2004-present  PhD thesis in the group of Prof. Dr. Dario Neri on the topic
“Molecular Targeting of Carbonic Anhydrase IX using Human
Monoclonal Antibodies and Small Organic Ligands”
Institute of Pharmaceutical Sciences, ETH Zurich, Switzerland
2004  Diploma thesis in the group of Prof. Dr. Dario Neri on the topic “Exploring the magnitude of the chelate effect with the model system Streptavidin/Iminobiotin”
Institute of Pharmaceutical Sciences, ETH Zurich, Switzerland

2001-2004  Undergraduate studies in Biotechnology completed with Diploma Degree. Specialisation: Molecular Biology and Biotechnology
Ecole Supérieure de Biotechnologie, Strasbourg, France

1999-2001  Undergraduate studies in Biology completed with “Vordiplom”
Albert-Ludwigs-Universität Freiburg i. Br., Germany

1996-1999  “Abitur”
Wirtschaftsgymnasium Tuttlingen, Germany

1995-1996  Student exchange year with the American Field Service (AFS)
Manresa, Spain

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WORK EXPERIENCE
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2008  Organization of the 3rd Experimental Course on Antibody Phage Technology
Institute of Pharmaceutical Sciences, ETH Zurich

2006  Organization of the 2nd Experimental Course on Antibody Phage Technology
Institute of Pharmaceutical Sciences, ETH Zurich

2004-2008  Supervision of 4 diploma students during my PhD work

2004-2008  Teaching of the practical courses “Medicinal Chemistry” and “Anatomy of the rat” for pharmacy students
Institute of Pharmaceutical Sciences, ETH Zurich

2003  6 weeks of internship in the laboratory of Prof. Hellmut Augustin
Klinik für Tumoriologie, Freiburg. i. Br., Germany

2002  6 weeks of internship in the laboratory of Prof. Ola Myklebost,
The Norwegian Radium Hospital, Department of Tumor Biology, Oslo, Norway

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SCHOLARSHIPS

2004 DAAD (German Academic Exchange Service) scholarship for conducting the Diploma Thesis in the group of Prof. Dr. Dario Neri

ADDITIONAL TRAINING

“In Introductory Course in Laboratory Animal Science”
Institute of Laboratory Animal Science, University of Zurich, October 29 - November 08, 2007, Zurich, Switzerland

PUBLICATIONS


PRESENTATIONS AND POSTERS

Oral presentation at the “Doktorandentag” of the Institute of Pharmaceutical Sciences, ETH Zurich, September 19, 2007, Zurich, Switzerland

Poster at the Keystone Symposium on “Antibodies as Drugs: From Basic Biology to the Clinic”. February 1-6, 2006, Lake Louise, Canada

Poster at the EU Workshop “Molecular targets for cancer program”, September 28-29, 2007, Luxemburg, Luxemburg

Poster at the 2nd Monte Verità Conference on “Tumor Host Interaction and Angiogenesis: Basic Mechanisms and Therapeutic Perspectives”. October 1-5, 2005, Ascona, Switzerland
PERSONAL INTERESTS

Sports climbing, alpine skiing, ballet, playing the piano

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