A new anti-angiogenic cancer therapy approach: Tumour growth inhibition by depletion of tumour-associated macrophages (TAMs) in combination with anti-vascular endothelial growth factor (VEGF) antibody treatment

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A New Anti-Angiogenic Cancer Therapy Approach:

Tumour Growth Inhibition by
Depletion of Tumour-associated Macrophages (TAMs) in Combination
with Anti-Vascular Endothelial Growth Factor (VEGF) Antibody Treatment

A dissertation submitted to the
Swiss Federal Institute of Technology Zurich

for the degree of
Doctor of Natural Science

presented by
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Zurich, 2005
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SUMMARY

A tumour is a complex organ. Genetically heterogeneous cells define the tumour compartment and the epithelial parenchyma, and the interwoven stroma provides the connective framework of the tumour tissue. This framework includes a specific type of extracellular matrix (ECM), the tumour matrix, as well as a variety of cells such as fibroblasts, immune cells, and endothelial cells (ECs). The interactive signalling between tumour and stroma contributes to the formation of a complex multicellular organ, the tumour.

The formation of new blood vessels from pre-existing ones, termed angiogenesis, is fundamental for tumour growth and metastasis. Inhibition of tumour-induced angiogenesis seems to be a promising therapeutic option, due to targeting genetically stable cells and due to a high amount of fast growing tumour cells, which are depending on few ECs. Drugs and antibodies (Abs), which are blocking vascular endothelial growth factor (VEGF), one of the major inducers of angiogenesis, are already successfully used in clinical trials. Drawbacks of currently used cytotoxic drugs in cancer chemotherapy are non-specific side effects, short circulation times and development of resistance, requiring development of new therapies.

In this study, an anti-VEGF single chain fragment (scFv) Ab was engineered. This scFv was selected as a species cross-reactive Ab since syn- and xenogenic tumour mouse models are frequently used in preclinical anti-tumour trials. Using a human phage library, scFv-SZH9 Ab was selected recognising the mouse and human isoforms 164/5 of VEGF in the low nanomolar range. The scFv-SZH9 Ab is able to block VEGF induced angiogenesis in a model system, the chicken chorioallantoic membrane (CAM). Pharmacokinetic investigation in nude mice bearing xenografted tumours showed that approximately 3 % of the injected scFv Ab dose (% ID g⁻¹) localized in the tumour. In a therapeutic experiment tumour growth in a syngraft F9 teratocarcinoma mouse model was delayed by treatment with scFv-SZH9 Ab.

The first aim was to improve the obtained therapeutic effect by coupling the selected scFv Ab to stabilizing molecules. Consequently, the scFv-SZH9 Ab was linked to the surface of long-circulating vesicles. In this context poly-ethyleneglycol (PEG)-lipsomes show advantage in their long blood circulation time and they passively accumulate in the tumour, where VEGF needs to be neutralised. Although scFv-SZH9 Ab liposomes showed VEGF binding capacity in vitro, their therapeutic
application in vivo in a syngraft and a transgenic tumour mouse model failed to inhibit tumour growth. Unfortunately, due to low coupling efficiencies and limitations in protein production, the injected doses had to be reduced by a factor of 6 - 12 as compared to therapy with free scFv-SZH9 Ab.

Tumour-associated macrophages (TAMs) play a pivotal role in tumour growth and metastasis by e.g. promoting tumour angiogenesis. In immunology, liposome-encapsulated bisphosphonates, in particular clodronate, are used to deplete macrophages in many organs.

In a second approach, this technique was successfully translated to oncology, treating tumour-bearing mice with clodronate-containing liposomes (Clodrolip) which resulted in a significant reduction of tumour growth without causing toxic side effects in the animals. To be able to inhibit the angiogenic effect of VEGF, a combined anti-angiogenic therapy was applied, using anti-VEGF scFv Abs with Clodrolip. In a syngraft mouse model, the combination therapy led to a further significant tumour growth reduction compared to the single therapies. In a xenograft mouse model, only the combination therapy resulted in a sustained inhibition of tumour growth, whereas the tumours of the single treated mice started to grow immediately after end of therapy. Immunohistological investigation in the xenograft mouse model showed a significant depletion of TAMs, even 9 days after Clodrolip treatment. The evaluation of tumour vessel density by CD31 immunohistology in anti-VEGF scFv Ab or Clodrolip treated mice showed a significant reduction in vessel density. The application of anti-VEGF scFv-SZH9 Ab probably caused the blocking of macrophage recruitment by inhibition of tumour neo-angiogenesis, and a reduction of TAMs was also observed.

To summarise, in the presented work, a new highly effective anti-angiogenic cancer therapy approach was developed, using Clodrolip for the depletion of TAMs. The treatment with liposome-encapsulated bisphosphonate resulted in a significant reduction of angiogenesis (vessel density) and in a significant inhibition of tumour growth. Further experiments are needed to optimize dosing and treatment schemes to improve the therapeutic effect. In the future, the new therapy approach may be of value for the treatment of patients with solid tumours or inflammatory diseases.
ZUSAMMENFASSUNG


Die Blockierung der Tumorangiogenese, welche die Neubildung von neuen aus bestehenden Blutgefässen beschreibt, erscheint als eine aussichtsreiche Tumortherapie, da Blutgefäße eine genetisch homogene Zellpopulation bilden und viele Tumorzellen von wenigen ECs abhängig sind. Medikamente gegen den vaskulären endothelialen Wachstumsfaktor (VEGF), welcher einer der Hauptmediatoren in der Angiogenese darstellt, finden bereits erfolgreich klinische Anwendung.

Da die gegenwärtigen Chemotherapeutika zahlreiche Nachteile wie z.B. unspezifische Nebenwirkungen, kurze Zirkulationszeiten im Körper und die Entwicklung von resistenten Tumoren aufweisen, sind weitere neue Therapieansätze erforderlich. In der vorliegenden Arbeit wurde ein „Einfachketten Fragment Antikörper“ (scFv) gegen VEGF entwickelt. Da syn- und xenogene Tumor Mausmodelle in vorklinischen Studien zahlreiche Anwendung finden, wurde dieser als spezies-kreuzreaktiver Antikörper selektioniert. Zur Antikörperisolierung gegen die murinen und humanen VEGF Isoformen 164/5 wurde eine humane Phagen Bibliothek verwendet. Der generierte anti-VEGF (scFv')2-SZH9 Antikörper bindet lösliches VEGF im tiefen nanomolaren Bereich und ist in der Lage VEGF induzierte Angiogenese auf der Chorioallantois Membran (CAM) des Huhnes zu inhibieren. Pharmakokinetische Untersuchungen mit Tumor xenotransplantierten Nacktmäusen zeigten, dass sich etwa 3 % der injizierten (scFv')2-SZH9 Antikörpermengen (% ID g⁻¹) im Tumor anreicherten.

In einem syn-genen F9 Teratokarzinom Mausmodell führte die therapeutische Verabreichung des (scFv')2-SZH9 Antikörpers zu einer erfolgreichen Reduktion des Tumorwachstums.

Erstes Ziel war es, um den erzielten therapeutischen Effekt zu steigern, den selektionierten scFv-Antikörper an ein stabilisierendes Molekül zu koppeln. Der (scFv')2-SZH9 Antikörper wurde kovalent an Polyethylenglykol-Phospholipid (PEG)-Liposomen gekoppelt. Diese zeigen eine hohe Zirkulationsdauer im Blut und eine
bevorzugte passive Tumoranreicherung. Obwohl die scFv-SZH9-Liposomen in vitro VEGF-Bindung zeigten, führte die Applikation in vivo weder in einem syngenen noch in einem transgenen Tumormodell zu einer Unterdrückung des Tumorwachstums. Aufgrund niedriger Kopplungsausbeuten und limitierter Proteinproduktion, musste die applizierte Menge gegenüber freiem (scFv')₂-SZH9 Antikörper um den Faktor 6 - 12 reduziert werden.

Tumor assoziierte Makrophagen (TAMs) fördern die Entwicklung von Neoplasien. Um Makrophagen in unterschiedlichsten Organen zu depletieren werden in der Immunologie liposomal eingekapselte Bisphosphonate, speziell Clodronat, verwendet. In einer zweiten Zielsetzung wurde diese Technik erfolgreich in die Onkologie übertragen, indem tumortragende Mäuse mit clodronathaltigen Liposomen (Clodrolip) behandelt wurden. Dies führte zu einer signifikanten Hemmung des Tumorwachstums, ohne dass dabei toxische Nebenwirkungen beobachtet wurden.

Um den angiogenen VEGF-Effekt zu unterbinden wurde mittels anti-VEGF Antikörpern in Verbindung mit Clodrolip eine kombinierte anti-angiogene Therapie durchgeführt. In einem syngenen Mausmodell führte die Kombinationstherapie, verglichen zu den Einzeltherapien zu einer signifikant stärkeren Unterdrückung des Tumorwachstums. In einem xenogenen Mausmodell führte ausschließlich die Kombinationstherapie zu einer langanhaltenden Tumorwachstumsblockierung, wohingegen die Tumoren der Mäuse in Einzelbehandlung sofort nach Beendigung der Therapie anfingen zu wachsen.

Immunohistologische Untersuchungen in dem xenogenen Modell zeigten selbst 9 Tage nach Clodrolip-Therapieende eine signifikante TAM-Depletion. Die therapeutische Behandlung mit anti-VEGF Antikörpern oder Clodrolip resultierte in einer signifikanten Reduktion der Tumorgefässdichte. Die Verabreichung des anti-VEGF (scFv')₂-SZH9 Antikörpers führte ebenfalls zu einer Reduktion von TAMs, was möglicherweise durch Neutralisierung des VEGF-Aktivierungsignals oder durch die Blockierung der Neugefässbildung herrührte. Zusammengefasst, die vorliegende Arbeit beschreibt die Entwicklung einer neuen und hocheffizienten Methode zur Depletion von TAMs für die anti-angiogene Krebstherapie. Die Anwendung von Clodrolip führte zu einer signifikanten Reduktion der Tumorangiogenese und zu einer signifikanten Hemmung des Tumorwachstums. Für eine effiziente Tumorbehandlung sind weitere Experimente zur Optimierung der Dosierung und Therapieschemata nötig.
1. INTRODUCTION

1.1. CARCINOGENESIS AND CANCER TREATMENT

Based on observations in the early twentieth century that some cancers contain similarities, researchers began to look for genetic alterations that might underlie cancer pathogenesis. Over the following decades, enormous advances were made in identifying the molecular determinants of carcinogenesis and tumourigenesis became recognized as a multi-step process during which cancer cells accumulate multiple and consecutive genetic alterations [1]. These cancer-cell- and genome-focused models have led to the identification and characterization of many oncogenes and tumour-suppressor genes. However, these models have largely ignored the heterogeneous and structurally complex nature of the tissue, or “organ”, called the tumour. Even though Paget’s “seed and soil” hypothesis dates back to 1889 [2], the molecular determinants of the seed are still much better understood than those of the soil [3]. Only recently tumour progression has been recognized as the product of an evolving crosstalk between different cell types within the tumour and its surrounding supporting tissue, or tumour stroma [4]. Whereas genetically abnormal cells define the tumour compartment itself, the epithelial parenchyma of carcinomas, the surrounding and interwoven stroma, provides the connective-tissue framework of the tumour tissue. This framework includes a specific type of extracellular matrix (ECM), the tumour matrix, as well as cellular components such as fibroblasts, immune and inflammatory cells, such as macrophages, neutrophils, mast cells, and endothelial cells. As its constitution resembles that of the granulation tissue formed during wound healing, Hal Dvorak defined a tumour as “a wound that never heals” [5]. The relative mass of stroma and its composition vary considerably among tumours and does not correlate with the degree of tumour malignancy [6]. The interactive signalling between tumour and stroma contributes to the formation of a complex multicellular organ. In a manner similar to the development and function of normal organs, which occurs through reciprocal communication between different cell types, the interaction between cancer cells and their microenvironment can largely determine the phenotype of the tumour. For example, recent studies have shown that the establishment of human breast tumour xenografts in mice depends on the presence of human tumour derived stromal fibroblasts [7].
Cancer cells themselves can alter their adjacent stroma to form a permissive and supportive environment for tumour progression. Morphological evidence for this "reactive" tumour stroma has long been described in pathology textbooks [8] as "desmoplasia", which consists of fibroblast-like cells and specific ECM components, as well as of inflammatory and immune cells. Angiogenesis is another process involved in formation of the desmoplasia, as newly formed blood and lymph vessels support tumour growth and spread [9]. Taken together, these observations indicate that the tumour microenvironment is a potential therapeutic target (Tables 1.1 and 1.2). As summarized in Table 1.1, there have already been several exciting success stories in clinical targeting of the tumour stroma.

<table>
<thead>
<tr>
<th>Target</th>
<th>Approach</th>
<th>Clinical trial</th>
<th>Outcome / status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelial cells</td>
<td>Inhibition of VEGF signalling</td>
<td>Phase III for colon carcinoma, lung carcinoma and renal-cell carcinoma; Phase II for lung carcinoma and renal-cell carcinoma</td>
<td>Improved survival for colon carcinoma [11]; Phase II studies for lung and renal-cell carcinoma show slightly improved survival [12;13], and Phase III trials are ongoing‡</td>
</tr>
<tr>
<td></td>
<td>Inhibition of endothelial-cell proliferation by TNP-470, a fumagillin analogue</td>
<td>Phase I/II for lung carcinoma and advanced solid tumours</td>
<td>Drug well tolerated; some patients with partial response [13]</td>
</tr>
<tr>
<td></td>
<td>Induction of apoptosis in proliferating endothelial cells with tubulin-binding agents</td>
<td>Phase I for thyroid cancer</td>
<td>Ongoing‡</td>
</tr>
<tr>
<td>Inflammatory cells</td>
<td>NSAIDs</td>
<td>Phase II for colon carcinoma</td>
<td>Ongoing with first beneficial results [14]</td>
</tr>
<tr>
<td>ECM components</td>
<td>Local injection of radiolabelled Abs against tenascin</td>
<td>Phase I and II for glioma</td>
<td>Increased survival [15]</td>
</tr>
<tr>
<td>ECM/basement-membrane signalling</td>
<td>Abs against integrin</td>
<td>Phase I and II for lymphoma, melanoma and glioblastoma</td>
<td>Ongoing#</td>
</tr>
<tr>
<td>ECM integrity</td>
<td>MMP inhibitors</td>
<td>Phase I, II, III</td>
<td>Initial results were negative [16]; new components and combinations are in phase I ‡</td>
</tr>
<tr>
<td>ECM fragments</td>
<td>Injection of endostatin</td>
<td>Phase I</td>
<td>Ongoing [13]</td>
</tr>
</tbody>
</table>

‡ For further information, see the national Cancer Institute’s Angiogenesis Inhibitors in Clinical Trials web page in the online links.

¥ For further Information, see the Special Project Angiogenesis web page in the online links.
The inhibition of inflammatory cells and cytokines by treatment with non-steroidal anti-inflammatory drugs (NSAIDs) has been shown to lower the risk for colon and breast cancer and might help to prevent lung, oesophageal and stomach cancers [17]. Drugs designed to block VEGF signalling were successful in the treatment of colorectal cancer [18]. For example, Bevacizumab, a VEGF blocking monoclonal Ab, has been shown in Phase III trials in combination with first-line chemotherapy to significantly prolong the life of patients with colorectal and kidney cancer [19]. Blockade of signalling by the epidermal growth factor receptor ERB-B2 (also known as HER2) with a neutralizing Ab has been shown to down regulate tumour cell derived pro-angiogenic molecules [20]. In addition to its ability to inhibit proliferation of EGFR-expressing cancer cells, this effect on the tumour stroma could be a mechanism by which the ERB-B2-blocking Ab transtuzumab slows down tumour growth in patients with breast cancer [21]. However, there are also some disappointing results in targeting the stroma for cancer therapy. Clinical testing of MMP inhibitors has shown no efficacy in patients suffering from advanced stages of cancer, rather, severe intolerable side effects and even worsened prognosis were observed [22] (Tables 1.1 and 1.2).

Table 1.2: Potential therapeutic targets in the tumour stroma [10].

<table>
<thead>
<tr>
<th>Stromal element</th>
<th>Alteration</th>
<th>Effects and clinical and preclinical trial results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelial cells</td>
<td>Blocking endothelial-cell proliferation (such as through inhibition of VEGF)</td>
<td>Induces endothelial-cell apoptosis, angiogenesis (Table 1.1) and numerous experimental systems [23]</td>
</tr>
<tr>
<td>Tumour-associated fibroblasts</td>
<td>Inhibiting fibroblast proliferation and activation (such as through inhibiting of TGFβ signalling)</td>
<td>Promotes tumour progression; TGFβ overexpression, can promote malignancy in certain tumour types; tested in preclinical models of colon carcinoma, prostate and forestomach cancer [24;25]</td>
</tr>
<tr>
<td>Macrophages</td>
<td>Inhibiting macrophage recruitment by blocking recruitment factors</td>
<td>Reduces tumour malignancy in preclinical models of breast cancer [26;27]</td>
</tr>
<tr>
<td>Mast cells</td>
<td>Inhibiting mast-cell recruitment, such as by blocking recruitment factors</td>
<td>Reduces SCC skin tumour malignancy in mast-cell-deficient mice [16;28]</td>
</tr>
<tr>
<td>Stromal element</td>
<td>Alteration</td>
<td>Effects and clinical and preclinical trial results</td>
</tr>
<tr>
<td>----------------</td>
<td>------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td><strong>ECM molecules</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombospondin</td>
<td>Overexpression</td>
<td>Inhibits tumour invasion in experimental models for skin SCC and other tumours [29;30]</td>
</tr>
<tr>
<td>Tenascin</td>
<td>Inhibition with radio labelled Inhibitory Abs; inhibition of expression; blockade of tenasin binding inhibits tumour-cell migration</td>
<td>Prolonged patient survival in clinical trials with glioma patients (Table 1.2) [31]; inhibits angiogenesis and tumour-cell migration in preclinical models of melanoma and breast cancer [32]</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Inhibitory Abs that target extra domain B (ED-B)</td>
<td>Inhibits angiogenesis in various tumour models [33;34]</td>
</tr>
<tr>
<td>Decorin</td>
<td>Adenovirus-mediated expression</td>
<td>Suppresses tumourigenicity of colon and squamous carcinoma models [35]</td>
</tr>
<tr>
<td>Hyaluronate</td>
<td>Expression; degradation by hyaluronidase</td>
<td>Promotes motility of tumour cells [36]; reduces tumour growth in preclinical models of melanoma and breast cancer [37]</td>
</tr>
<tr>
<td>ECM cleavage products</td>
<td>Generation of endostatin, angiotatin, tumstatin and others</td>
<td>Inhibits angiogenesis in various solid tumour models [38;39] and in clinical trials (Table 1.2)</td>
</tr>
<tr>
<td><strong>Matrix-degrading proteases and inhibitors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMPs</td>
<td>Inhibition</td>
<td>Inhibit invasion and angiogenesis, also pro-angiogenic, by inhibiting the generation of reactive ECM fragments; negative results in clinical trials (Table 1.2), involving different treatment combinations [40]</td>
</tr>
<tr>
<td>ADAMs</td>
<td>Inhibition</td>
<td>Block release of growth factors from the ECM; increased expression observed in human tumour samples and preclinical tumours [41]</td>
</tr>
<tr>
<td>Serpin/PAI1</td>
<td>Altering expression levels</td>
<td>Enzymes are pro-angiogenic at high (therapeutic) concentrations and anti-angiogenic at low (physiological) concentrations in preclinical models of skin SCC and other tumour models [16;41]</td>
</tr>
<tr>
<td>TIMPs</td>
<td>Expression</td>
<td>Suppress tumour invasion and metastasis in tumour models, yet high expression levels correlate with poor prognosis in some human tumour types; expression studies performed in clinical samples and preclinical models [41;42]</td>
</tr>
<tr>
<td><strong>Regulatory molecules</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Integrins</td>
<td>Inhibition of signalling</td>
<td>Block malignant progression and angiogenesis in clinical trials (Table 1.2) and in preclinical models for breast and ovarian cancer [43-45]</td>
</tr>
<tr>
<td>Growth factors, cytokines produced by tumour cells (VEGF, PDGF, G-CSF, GM-CSF and others) or stromal cells (TGFβ, CSF1, HGF and IGF1 and others)</td>
<td>Inhibition of signalling</td>
<td>Inhibits tumour progression in preclinical models for colon carcinoma, breast, prostate and for stomach cancer [46-50] as well as in skin SCC [51;52]</td>
</tr>
<tr>
<td>Inflammation-associated growth factors and chemokines</td>
<td>Inhibition</td>
<td>Blocks inflammation, which is associated with poor patient prognosis in clinical and preclinical models [26;53]</td>
</tr>
</tbody>
</table>
This might be explained by the fact that trials designed for these broad spectrum inhibitors did not take into account the many and contradictory roles that MMPs are believed to have in the modulation of cell adhesive functions, integrin signalling and the activation of growth factors in the ECM. To overcome these problems, the development of more specific inhibitors is now underway. However, when developing microenvironment-based therapies, it needs to be kept in mind that targeting only one aspect of the tumour stroma, and doing this in patients with late-stage cancer, is not likely to be successful. Therefore, the aim has to be to combine drugs that target different compounds of the activated stroma with cytotoxic therapies that are directed against the tumour cells.

1.2. THE VASCULAR NETWORK FORMATION

The construction of a vascular network is a complex process that involves the extensive interplay between cells, soluble factors and ECM components (Figure 1.1). All these interactions occur in sequential steps mediated by a wide range of angiogenic inducers, including growth factors, chemokines, angiogenic enzymes, endothelial specific receptors and adhesion molecules or inhibitors that must be tightly regulated in a coordinated and temporal manner. In this chapter, the different steps leading to vascular development and their key regulators will be highlighted.

1.2.1. Vasculogenesis

Vessel formation can occur by a number of different processes [54-56]. Early during development, new vessels form by a process termed vasculogenesis, where endothelial precursors (angioblasts) differentiate and assemble in a primitive network. This primary network includes some of the major vessels in the embryo, such as the aorta and the major veins connected with each other by a "honeycomb-like" plexus of minor vessels (Figure 1.1 A). However, the resulting vasculature is at this stage immature and poorly functional. Fibroblast growth factor (FGF), which was shown in vitro to promote the differentiation of angioblasts is thought to play a crucial role in the initiation of vasculogenesis [57]. Likewise, gene inactivation studies indicate an essential function for VEGF [58-60] and the VEGF receptors 1 and 2 [61] in this process of vasculogenesis. Only a minority of vessels formed during embryonic development persist until adulthood [62]. Indeed, after the primitive vascular network
is set, it is remodelled to form the more mature appearing vascular patterns seen in the adult organism (Figure 1.1 B).

1.2.2. Angiogenesis

Angiogenesis, the formation of new blood vessels from pre-existing one occurs mainly during embryonic development. In adulthood it is restricted to the female reproduction cycle, hair growth and wound healing or is associated with pathological conditions [64].

In the embryo, from the pre-existing vascular plexus, new capillaries can form by sprouting or by splitting (intussusception) in a process termed angiogenesis. In angiogenesis by intussusception, the ECs in a vessel proliferate, producing a wide lumen that can be split by the formation of a trans-endothelial bridge composed of extracellular matrix. Sprouting angiogenesis (Figure 1.1 C) initiates with vasodilatation, a process involving release of nitric oxide (NO), followed by increased vessel permeability in response to VEGF [65]. Increased vascular leakiness involves
Ang-1 [66], Src family kinases [67] and the redistribution of the junctional molecules, vascular endothelial (VE)-cadherin [68] and platelet-EC-adhesion molecule (PECAM)-1 [56] that usually tighten vessel walls. Then, Ang-2 and proteases, such as u-PA (urokinase-type plasminogen activator) and MMPs (matrix metalloproteinases) [69] mediate dissolution of the existing vessel basement membrane and the interstitial matrix. Once the path has been cleared, proliferating ECs migrate to distant sites, and assemble in a tube-like shape to form a new capillary. Numerous molecules regulate these steps in a coordinated manner: VEGF [70], VEGFR-2, and VEGFR-3 [71], neuropilin-1 (NP), Ang-1 [72], Tie2, Ang-2 (in combination with VEGF), ephrin-B2 [63], αVβ3- and α5-integrins, PECAM-1 [73] and VE-cadherin [74] and other positive regulators [56]. Angiogenic sprouting is also balanced by a set of natural inhibitors that suppress proliferation or migration of the EC when necessary. Those include angiostatin (an internal fragment of plasminogen) [75], endostatin (a fragment of collagen XVIII) [76], PEDF [63], or interferon-β [77] and others. Circulating endothelial precursors, shed from the vessel wall or mobilised from the bone marrow, can also contribute to angiogenesis, as shown in some tumour angiogenesis models [78].

The vasculature is further remodelled into a more mature system containing larger and smaller vessels and excess branches are pruned. The process is termed "pruning" as the resulting vasculature pattern resembles a tree [54].

Vessels formed by sprouting are initially immature and with the exception of capillaries, most of them will further differentiate. Non-perfused blood vessels regress (Figure 1.1 D). Maturation of nascent vessels involves formation of a new basement membrane and stabilization of new vessels with pericytes and smooth muscle cells. The multi step process leading to vessel maturation is well documented. Ang-1 produced by mesenchymal cells activates the Tie2 receptor on ECs, which in turn leads to production and release of PDGF-BB (for platelet derived growth factor). PDGF-BB is a chemo-attractant for mesenchymal cells, that promotes their recruitment towards the forming vessel. Once mesenchymal cells arrive at and contact the endothelium, TGF-β is activated from its latent state, causing mesenchymal cells to differentiate into pericytes and smooth muscle cells (SMC) [56].

Following vessel maturation, Ang-1 continues to be important in maintaining the quiescence and stability of the mature vasculature. Disruption of this stabilization
signal coincides with re-initiation of vascular remodelling in the adult, seen in the adult's female reproductive system or in tumours. Such de-stabilization occurs under the influence of Ang-2 which acts as an antagonist of Ang-1 on the receptor Tie 2 [63].

The ephrin family members, especially Ephrin B2 and its receptor EphB4 seem also to be important for vessel maturation in distinguishing development of arterial versus venous vessels [79]. During vascular development Ephrin B2 and EphB4 present a remarkable reciprocal expression pattern, with Ephrin B2 present on future arterial vessels and EphB4 expressed on primordial venous vessels, where by both molecules are membrane bound. The molecular mechanism involved is unclear, albeit critical interactions and signalling at sites of cell-cell interactions are suggested between cells expressing the two molecules [80].

1.2.2.1. Angiogenesis during adult life

Most adult tissues have little ongoing angiogenesis. The endothelial cell proliferation is high during embryonic and postnatal development, but adult endothelial cell turnover is very low [54]. The endothelium is in a quiescent state during the entire life of a normal adult, yet it is able to proliferate in pathological conditions [62].

The ovary is one of the adult organs, in which angiogenesis normally occurs during the female reproductive cycle. The development and endocrine function of the ovarian corpus luteum (CL) is dependent on the growth of new capillary vessels. Progesterone release by CL is essential for implantation and maintenance of pregnancy [81]. During follicular growth, the theca interna becomes richly vascularized. Following ovulation, the thecal vessels invade the ruptured follicle and form a microvascular plexus that nourishes the developing CL [81]. Multiple mediators are involved in CL angiogenesis including VEGF, bFGF [82], Ang-1 and Ang-2 [83]. VEGF or a related factor is essential for CL angiogenesis since treatment with soluble VEGF receptor-1 extracellular domain results in complete suppression of CL angiogenesis [82]. At the same time endometrium fails to mature, probably reflecting suppression of ovarian steroid production and an inhibition of locally produced VEGF [82].

Angiogenesis during wound healing is a good example of pathophysiological neovascularization during adult life, where the capillaries regress after the termination of the process. Angiogenesis is part of the normal wound repair process, where it
provides a supply of nutrients, promotes granulation, tissue formation and clears debris. Neovascularization in wounds depends on cell-cell interactions, cell-ECM interactions and also on the balance between angiogenic agonists and antagonists. Tissue injury is followed by exudation of plasma constituents including fibrinogen. Fibrinogen provides the substrate for the generation of a fibrin-containing matrix, which is subsequently replaced by granulation tissue [84].

Many in vitro and in vivo studies suggest the involvement of bFGF in the angiogenic activity during wound healing. bFGF can be sequestered within cells and these stores can be released during cell injury, lysis or death [85]. Proteases found in early wounds are also able to release bFGF stored in the ECM [86]. Platelets at the wound site participate in coagulation and release several growth factors and enzymes. Platelet derived growth factor (PDGF), for example is a powerful chemoattractant for circulating neutrophils, monocytes and tissue macrophages [87]. By releasing bFGF and TNF-α, macrophages are able to exert angiogenic activity in the depths of a wound where oxygen tension is low and lactate is high due to anaerobic metabolism. For example, phagocytosis of fibrin increases macrophage angiogenic activity [5]. During the later stages of wound repair, macrophages stimulated by hypoxia, cytokines, or inflammatory mediators, synthesize new factors like TGF-β and VEGF [88]. During the final stages of healing the production of angiogenic factors is decreased when granulation tissue is formed and the area is less hypoxic [89].

Hypoxia is known to increase VEGF levels, but whether it stimulates wound angiogenesis is an open question. VEGF, produced by keratinocytes together with other mediators may play a role in wound angiogenesis [90]. VEGF is produced in many in vitro and in vivo systems under hypoxic conditions, supporting the view of VEGF production in hypoxic wounds [91].

1.2.2.2. Tumour angiogenesis and the angiogenic switch

The importance of angiogenesis for the growth of solid tumours is well recognized. A considerable body of research spanning almost three decades has documented that tumour growth and metastasis require persistent new blood vessel growth (Figure 1.2). The classical proof of this principle came from experiments where tumour fragments or cultured tumour cells were placed in an avascular site, the cornea of a rabbit eye [92]. The implants attracted new capillaries that grew in from the limbus to vascularize the expanding tumour mass. When the capillaries were
physically prevented from reaching the implant or were inhibited from undergoing angiogenesis, tumour growth was dramatically impaired. Subsequent experiments have confirmed these results and further revealed that in the absence of access to an adequate vasculature, tumour cells become necrotic [93] and/or apoptotic [94], restraining the increase in tumour volume that would result from continuous cell proliferation, the hallmark of cancer.

Figure 1.2: The angiogenic switch occurs prior to tumour formation as illustrated in three transgenic mouse models of tumourigenesis. (A) Expression of the Tag oncogene in the pancreatic islets elicits four sequential stages in tumour development: normal, oncogene-expressing islets; hyperplastic islets, populated by proliferating cells with the histological hallmarks of carcinoma in situ, CIS; angiogenic islets, in which new blood vessel growth has been activated; and solid tumours, which are islet cell carcinomas. (B) In transgenic mice carrying the BPV-1 oncogenes, the normal dermis is initially converted into a state of mild fibromatosis, revealed as focal accumulation of dermal fibroblasts. Angiogenesis is first evident in the next stage, aggressive fibromatosis, which is also marked by dense arrays of proliferating fibroblastic cells; both hyperproliferation and angiogenesis persist in the subsequent stage, protuberant fibrosarcoma. (C) Targeted expression of the HPV-16 oncogenes to basal cells of the epidermis induces multistage development of squamous cell carcinoma, beginning as hyperplasia of keratinocytes, with a mild increase in vessel density; which progresses to dysplasia, marked by morphologically aberrant keratinocytes with a high proliferation index and by abundant neovascularization; finally, two classes of squamous carcinoma arise, both evidencing extensive angiogenesis, (modified) [95].

Transgenic mice carrying dominant oncogenes and/or knockouts of tumour suppressor genes are now being widely utilized to study the process of tumourigenesis. In a number of these mouse models, cancers develop through temporally and histologically distinct stages, much as is apparent for many human cancers. The experimental manipulability of the mouse and the reproducibility afforded by genetic predisposition to specific cancers is allowing the individual stages
in tumourigenesis to be studied and perturbed, in efforts to establish functional significance of candidate genes and cellular processes. Specifically, Hanahan et al. [95] have investigated the pattern of angiogenesis in three diverse transgenic mouse models: islet cell carcinoma, dermal fibrosarcoma, and epidermal squamous cell carcinoma. In all three models, extensive vascularization and ongoing angiogenesis has been apparent in the end-stage tumours. Remarkably, in each case, an angiogenic switch could also be visualized during early stages preceding the appearance of the solid tumours, suggesting that activation of angiogenesis is a discrete event in tumour development.

The so-called "RIP-Tag" transgenic mice express the SV40 T antigen (or Tag) oncogene in the insulin-producing β cells [96] which are localized in approximately 400 islet nodules scattered throughout the pancreas. In several independent lines of mice, a few (2 – 10) of these 400 islets develop into solid tumours by 12 to 16 weeks of age. The distribution of this cell type into these natural focal nodules has allowed histological, temporal, and statistical analysis of tumourigenesis. A set of 4 discrete stages in the pathway to cancer is apparent by these criteria. Initially, every islet contains β cells, which express the oncogene but otherwise appear normal, with a low proliferation index. Then, focal activation of hyperproliferation occurs in individual islets [97]. This stage, while historically referred to as "hyperplastic," is composed of cells with the histological properties of a carcinoma-in-situ (CIS), wherein more than 20% of the cells are in S phase at any given time. Insulin-like growth factor (IGF)-II is activated at this stage and probably modulates apoptosis that accompanies the aberrant proliferation [98]. Yet, these hyperplastic islet nodules, while morphologically transformed in appearance, are not competent to proceed directly to a rapidly growing islet cell carcinoma, since 50% of the islets switch to the hyperproliferative stage, while only 1% – 2% progress to solid tumours. Hanahan et al. [95] have identified a discrete stage, referred to as "angiogenic islet," that appears to be an intermediate between these two stages, both statistically and temporally. They defined the angiogenic switch using an in vitro bioassay for angiogenic activity, in which capillary endothelial cells were co-cultured in a three-dimensional collagen gel with islets isolated at different ages from the RIP-Tag mice [99]. At early ages, none of the oncogene-expressing islets were angiogenic. Then, in older mice, individual islets scored as angiogenic, attracting a starburst of endothelial cells converging on the angiogenic islet. Over time, about 10% of the total islets scored as angiogenic.
The in vitro bioassay was substantiated by histological analysis, which revealed two hallmarks of angiogenesis, capillary sprouting and endothelial cell proliferation, in a subset of the hyperplastic islets and in all solid tumours in these transgenic mice [99].

The provocative result was that neither oncogene expression nor hyperproliferation appeared to be sufficient to activate the angiogenic switch, which rather appeared as a discrete, temporally separate step in this multistage pathway (Figure 1.2 A).

The induction of angiogenesis is mediated by many angiogenic molecules, as for example VEGF released by tumours (Figure 1.3) and host cells [100] (Chapter 1.3.1). Activation of endothelial cell proliferation and migration can be achieved by reduction of inhibitor levels or increase of activators [95].

![Diagram of angiogenesis](image)

**Figure 1.3:** The VEGF isoforms and their interaction with VEGF receptors. In response to a variety of stimuli, the diffusible VEGF isoforms, VEGF_{121} and VEGF_{165}, are released by a variety of normal and transformed cells (tumour cells shown) and may bind to VEGFR-1 (R1) and VEGFR-2 (R2). VEGF_{165}, but not VEGF_{121}, also interacts with the neuropilins NRP1 and NRP2. This binding results in enhancement of VEGFR-2-dependent signalling in endothelial cells (EC). Following plasmin generation and extracellular matrix (ECM) breakdown, VEGF_{165} is cleaved at the COOH-terminus and the resulting 110-amino acid NH2-terminal fragment is diffusible and bioactive [101].

The angiogenic antagonist Ang-2 and pro-angiogenic VEGF may be critical regulators of the balance between vascular regression and growth. This is supported by the increased expression of Ang-2 in the endothelium of destabilized tumour vessels and induced VEGF expression in the hypoxic tumour periphery [83]. Thus, the angiogenic properties of tumour-derived VEGF may be facilitated when vessels are destabilized by Ang-2 [102]. In established human tumours there is a significant fraction of vessels that have not yet recruited periendothelial cells because of ongoing angiogenesis and remodelling of tumour vessels. The dependence of these immature vessels on vascular survival factors leads to their regression following
VEGF withdrawal [103]. Yet, very recent findings describe a new mechanism by which some aggressive tumours may acquire a blood supply. According to these findings which still need to be verified, tumour cells metamorphose into vessels that either carry blood or connect to the host’s blood supply [104]. The generation of patterned vascular channels by deregulated aggressive tumour cells is observed in human melanomas in which the establishment of a microcirculation also facilitates metastasis [105].

Tumours have a complex morphology, differing in regional vasculature, host infiltrates, and connective tissue components [106]. Tumour vessels are anatomically heterogeneous structures, being relatively immature. They are lined by a simple endothelium, and have fewer pericytes and smooth muscle cells compared to normal tissue vessels. Tumour vessels are also more permeable to circulating plasma proteins and have differing capacities to leak macromolecules depending on their location [107].

Neovascularization takes place following different pathways, where tumour cells either synthesize their own angiogenic factors [108], or attract macrophages which release them [109]. Tumours secrete collagenases and heparanases [110] and degrade the ECM, which subsequently releases stored angiogenic factors [111]. Hypoxia-induced release of VEGF [91] contributes to the onset of tumour-associated angiogenesis. In tumours with a significant component of necrosis such as glioblastoma multiforme, VEGF mRNA expression is not uniform but occurs primarily in clusters of tumour cells at the border between viable tumour and necrotic areas [112]. This localization is consistent with local hypoxia being a major inducer of VEGF gene expression [113]. VEGF seems to play an essential role in tumour and wound angiogenesis, partly by inducing permeability with resulting plasma protein leakage from postcapillary venules. Subsequently, clotting of extravasated fibrinogen results in the formation of a fibrin gel substratum, an ideal environment for endothelial and tumour cell growth [114].

Antagonists of VEGF inhibit the growth of many tumours. For example, Abs against VEGF had a potent inhibitory effect on the growth of three human tumour cell lines injected subcutaneously in nude mice [115]. In a nude mouse model of liver metastasis VEGF Abs inhibited human colon carcinoma cell growth, thus decreasing the number of metastases [115;116]. Retrovirus-mediated expression of a dominant
negative VEGFR-2 mutant suppressed the growth of glioblastoma multiforme as well as other tumour cell lines in vivo [117].

1.2.2.3. Angiogenic stimulators

As described, angiogenesis is composed of a complex series of interdependent events, controlled by many different factors. The angiogenic process results from a shift in the balance of positive and negative mediators, cytokines and growth factors being the primary inducers (Tables 1.3 and 1.4) [95].

Table 1.3: Endogenous angiogenesis inducers [69].

<table>
<thead>
<tr>
<th>Inducers</th>
<th>Endothelial cells (ECs)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proliferation</td>
<td>Migration</td>
</tr>
<tr>
<td><strong>Heparin binding peptide growth factors</strong></td>
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</tr>
<tr>
<td>VEGF</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>PLGF</td>
<td>Weak</td>
<td>Yes</td>
</tr>
<tr>
<td>FGF-1, FGF-2</td>
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<td>Yes</td>
</tr>
<tr>
<td>Pleiotrophin</td>
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<td>?</td>
</tr>
<tr>
<td>HIV-tat</td>
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<td>Weak</td>
</tr>
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<td>PDGF</td>
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</tr>
<tr>
<td>HGF/SF</td>
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<td><strong>Non-heparin binding peptide growth factors</strong></td>
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</tr>
<tr>
<td>TGF-β</td>
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<td>Nitric oxide</td>
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<td>?</td>
</tr>
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<td>Ang-1</td>
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A variety of direct positive regulators of angiogenesis are identified and the most important are mentioned in the following text. There are factors that indirectly induce angiogenesis for example by the mobilization of macrophages, that release growth factors [145] or chemotactic factors for vascular endothelial cells [146], or both. Indirect angiogenic factors may also direct the release of immobilized angiogenic factors from the ECM or the basement membrane (BM) [147]. The ECM plays an important role in inducing an angiogenic response. BMs and ECMS are the substrates upon which cells migrate, proliferate and differentiate in vivo. Heparan sulfate proteoglycans (HSPGs) are very important for the binding of growth factors and enzymes that need to be immobilized and stored in the ECM for combined action with BM macromolecules. Heparan sulphate (HS) also contributes to the integrity and assembly of the ECM by binding to its fibrillar interstitial collagens, fibronectin, laminin, thrombospondin and tenascin as well as to different attachment sites on plasma membranes. HSPGs are also components of blood vessels [148], supporting proliferating and migrating endothelial cells as well as stabilizing the capillary wall.

1.2.2.3.1. Fibroblast growth factor

One class of angiogenic factors includes polypeptides like acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF, table 1.3). Both, aFGF and bFGF act as direct angiogenic factors, expressed by many tumours in vivo and tumour cell lines in vitro [149]. Low levels of bFGF are detected in the conditioned medium of endothelial cells [150] and of some tumour cell lines [151]. These two factors have served for a long time as the prototype of angiogenic factors, being in vitro mitogenic and chemotactic for endothelial cells and inducing capillary endothelial cells to migrate into three-dimensional collagen matrices to form capillary-like tubes [152]. Both factors also induce angiogenesis in vivo in the chick chorioallantoic membrane (CAM) and cornea bioassays [149]. FGFs stimulate endothelial cells to secrete collagenase and plasminogen activator proteases able to degrade the BM [149]. Binding of FGFs to the high affinity FGFRs is facilitated by their binding to heparin or heparin-like molecules which then present FGFs to their high affinity receptors [153]. FGF is sequestered in the ECM, BM, or on the cell surface upon binding to the heparan sulphate side chains of low-affinity, but high-capacity receptors [154]. Heparin enhances the mitogenic action of bFGF and protects aFGF and bFGF from degradation by heat, acid and proteases [155]. bFGF
can be mobilized in a biologically active form from the ECM and the BM by displacement with heparin or by degradation with heparanases and proteinases [111]. The role of bFGF in vivo was determined by generation of bFGF knockout mice. These mice are viable, fertile and phenotypically indistinguishable from the normal littermates [156].

1.2.2.3.2. Vascular endothelial growth factor

Another potent endothelial cell mitogen and angiogenesis inducer is VEGF (Figure 1.4 and table 1.3) which is devoid of consistent and appreciable mitogenic

![Figure 1.4: Schematic illustration of vascular endothelial growth-factor receptor 2 (VEGF-Receptor-2) intra-cellular signalling. Ligand binding to the extracellular domain induces dimerization and the autophosphorylation of specific intracellular tyrosin residues. Several intracellular proteins such as VEGFR-associated protein (VRAP), Sck and phospholipase C (PLC)-γ bind to specific tyrosine residues via their Src homology-2 (SH₂) domains, leading to the phosphorylation and activation of these proteins. PLC-γ activation results in hydrolysis of the membrane phospholipid phosphatidylinositol (4,5)-bisphosphate (PIP₂), which leads to the generation of the second messengers sn-1,2-diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (IP₃). DAG is a physiological activator of protein kinase C (PKC), whereas IP₃ binds to a specific receptor present on the endoplasmic reticulum (ER), resulting in the release of intracellular stored Ca²⁺ and an increase in the intracellular concentration of Ca²⁺ [Ca²⁺]. Many proteins are activated by the VEGFR-2 via an unknown mechanism, these include Src, phosphoinositide 3-kinase (PI3K), focal adhesion kinase (FAK) and p38 mitogen-activated protein kinase (p38 MAPK). Abbreviations: cPLA₂, cytosolic phospholipase A₂; eNOS, endothelial nitric oxide synthase; Erk, extracellular regulated kinase; HSP27, heatshock protein 27; MAPKAP 2/3, MAPK-activating protein kinase-2 and 3; NO, nitric oxide; PGI₂, prostacyclin; PIP₃, phosphatidylinositol (3,4,5)-trisphosphate; Sck, Sho-like protein; SPK, sphingosine kinase; VEGF, vascular endothelial growth factor [157].
activity for other cell types [158]. VEGF promotes angiogenesis in 3D in vitro models, inducing microvascular endothelial cells to invade collagen gels and to form capillary-like structures [159]. Studies provide evidence for a potent synergism between VEGF and bFGF in the induction of this effect [159]. VEGF induces sprouting from rat aortic rings embedded in collagen gel [160]. Furthermore, VEGF elicits a strong angiogenic response in a variety of in vivo models including the CAM [161], the rabbit cornea [162], the primate iris [163] and the rabbit bone [164]. Over the last few years, several members of the VEGF gene family were identified, including placenta growth factor (PIGF) [165], VEGF-B [166], VEGF-C [167], VEGF-D [168] and VEGF-E [169]. The human gene for VEGF resides on chromosome 6p21.3 [170]. The coding region spans ~14 kb and contains eight exons [158]. Alternative splicing of a single pre-mRNA generates several distinct VEGF species (Figure 1.5). To date, six isoforms have been identified with a length ranging from 121 to 206 amino-acid (aa) residues [171]. Moreover, a shorter form of 115 aa was described in murine immortal fibroblasts which differs from VEGF121 by 36 aas at the carboxyl terminus [172], but no human homologues have been identified. The most abundantly expressed isoforms are VEGF121, VEGF165 and VEGF189. Transcripts encoding VEGF183 are also largely distributed [173]. In contrast, VEGF145 and VEGF206 are comparatively rare, seemingly restricted to cells of placental origin. All these transcripts present a common basic structure, with domains encoded by exons 1 to 5 containing the information required for VEGF secretion (exon 1), and for recognition of VEGF-R1 and -R2 (exons 2 - 5). Woolard et al. showed that a new endogenous splice variant, VEGF165b is expressed as protein in normal cells and tissues and is circulating in human plasma where it binds VEGF receptor-2 with the same affinity as VEGF165 but does not activate or stimulate downstream signalling pathways. Moreover, VEGF165b prevents VEGF165-mediated VEGF receptor-2 phosphorylation and signalling in cultured cells [174]. The VEGF isoforms essentially differ by the presence or absence of sequences encoded by exons 6 and 7 that determine the basic character of the resulting protein, as well as their capacity to bind to heparin and HSPGs. VEGF121 that lacks these regions, is a weakly acidic protein that does not bind heparin [175]. In return, VEGF165, owing to the presence of 15 basic aas within the 44 residues encoded by exon 7, is secreted as ~46 kDa homodimer, which has basic character and moderate affinity for heparin [176]. The exon-6a encoded sequence of VEGF145 confers an affinity for heparin similar to that of the exon-7
encoded sequence of VEGF165 [177]. VEGF189, VEGF183 and VEGF206 contain additional sequences encoded by exon 6 and bind strongly to heparin.

Figure 1.4: Exon structure of the vascular endothelial growth factor (VEGF) mRNA splice variants. The gene encoding VEGF consists of eight exons that encode several different structural motifs. Alternative splicing of a single pre-mRNA species produces at least four different VEGF isoforms that vary in total amino-acid number. In humans these correspond to VEGF121, VEGF165, VEGF189 and VEGF206, of which VEGF165 is the predominant form. Sites of interaction with VEGFRs, neuropilin, heparin and plasmin cleavage site are indicated [178].

VEGF-C stimulates migration and mitogenesis of cultured endothelial cells [179]. In vivo, VEGF-C induces lymphangiogenesis in the differentiated CAM [179], whereas it stimulates angiogenesis in the early CAM [179]. In addition, VEGF-C promotes angiogenesis in the mouse cornea and in a setting of ischemic hindlimbs in rabbits [180]. VEGF-D is mitogenic for microvascular endothelial and bovine aortic endothelial cells (BAEs) [168]. In addition, treatment with VEGF-D induces the formation of an extensive network of capillary-like cords in a three-dimensional matrix [181]. VEGF-D is an angiogenic factor in rabbit cornea in vivo in a dose-dependent manner [181]. VEGF-E stimulates endothelial cell mitogenesis and migration in vitro [169] and it induces angiogenesis in vivo in the rabbit cornea [169] and when injected subcutaneously in a Matrigel plug [182]. VEGF-B stimulates endothelial cell proliferation of HUVE and bovine capillary endothelial (BCE) cells [166].

1.2.2.3.3. Placenta growth factor

Three alternative splice isoforms are identified for Placenta growth factor (PIGF), from which the large isoform PIGF-2 differs from the other two PIGF forms by
insertion of a highly basic 21-amino acid stretch at the carboxyterminal end of the protein (Table 1.3). PIGF-1 induces neovascularisation in the rabbit cornea and the CAM assay either as homodimers or as heterodimers with VEGF<sub>165</sub> [183]. PIGF-1 induces proliferation and chemotaxis of human umbilical vein endothelial cells (HUVEC) and capillary venule endothelial cells (CVEC) [183]. PIGF-2 stimulates mitogenesis of HUVEC [184].

1.2.2.3.4. Transforming growth factor

Transforming growth factor-alpha (TGF-α) is an angiogenic factor released by tumour-associated macrophages and many tumour cells (Table 1.3) [185]. TGF-α is structurally and functionally related to epidermal growth factor (EGF) released by macrophages and both induce endothelial cell DNA synthesis in vitro [186]. TGF-α also stimulates capillary-like tube formation in collagen gels [126]. The ability of TGF-α to promote tube formation is dependent on the release of substances like tissue-type plasminogen activator (tPA) and FGF by ECs [126]. In vivo, TGF-α is more potent than EGF in promoting angiogenesis in the hamster cheek pouch bioassay [186]. In addition, TGF-α and EGF are potent promoters of neoplastic cell growth in many carcinomas [187].

TGF-β which is not structurally related to TGF-α is synthesized by many normal and neoplastic cells [188;189]. TGF-β stimulates neovascularization in vivo when subcutaneously injected to mice [190]. However, several studies utilizing two-dimensional culture conditions found microvascular endothelial cell proliferation to be inhibited by TGF-β1, TGF-β2 and TGF-β3 raising doubt as to whether TGF-β functions as an angiogenic factor. It is possible that TGF-β promotes angiogenesis by differentiating ECs by indirect stimulation of angiogenesis [191]. Other studies also show that low concentrations of TGF-β1 enhance endothelial cell proliferation [192] and in vitro angiogenesis [193], whereas high concentrations inhibit proliferation and angiogenesis.

1.2.2.3.5. Tumour necrosis factor

An other important factor, TNF-α is a cytokine secreted by macrophages at sites of inflammation and it is toxic to tumour cells (Table 1.3) [194]. TNF-α acts on endothelial cells to exert a procoagulant effect [195]. Also, it inhibits proliferation and antagonizes the mitogenic effect of bFGF in HUVE cells and also inhibits the
proliferation of cultured capillary endothelial cells [196]. However, TNF-α does not inhibit mitogenesis of dermal and lung microvascular endothelial cells [197;198]. These differences may be due to the different concentrations of cytokines used in the \textit{in vitro} assays, or to the difference in the sensitivity of endothelial cells originating from distinct locations. Other \textit{in vitro} and \textit{in vivo} studies revealed that TNF-α stimulates angiogenesis at low concentrations, whereas it inhibits angiogenesis at high concentrations [199]. The concentration of TNF-α is low in tissues \textit{in vivo} so that the overall effect of TNF-α is stimulatory to angiogenesis [200]. Other reports show that TNF-α stimulates endothelial cell migration and tube formation, but that it inhibits proliferation of these cells. TNF-α stimulates angiogenesis in the rabbit cornea and in the CAM [201]. Hypoxia induces \textit{in vitro} the release of TNF-α by macrophages \textit{in vitro} [202].

\subsection*{1.2.2.3.6. Granulocyte / macrophage colony stimulating factor}

Granulocyte-colony stimulating factor (G-CSF) and granulocyte/macrophage-colony stimulating factor (GM-CSF) are myeloid growth factors required for the survival, growth, and differentiation of hemopoietic precursor cells (Table 1.3). Both are produced in many different tumour types [203] and are chemotactic for macrophages \textit{in vitro} [204;205]. Recombinant G-CSF and GM-CSF stimulate the migration and proliferation of human endothelial cells [206].

\subsection*{1.2.2.3.7. Insulin-like growth factor I and interleukin 8}

Insulin-like growth factor I (IGF-I) is secreted by activated macrophages and it is suggested to have a role in inflammatory angiogenesis (Table 1.3) [207;208]. IGF-I induces endothelial cell mitogenesis, migration and tube formation [209]. Interleukin-8 (IL-8) is produced by activated macrophages, [210] is mitogenic \textit{in vitro} and stimulates angiogenesis in the rat corneal assay [211].

\subsection*{1.2.2.3.8. Hepatocyte growth factor / scatter factor}

Hepatocyte growth factor/scatter factor (HGF/SF) is a mesenchyme-derived cytokine that stimulates motility and invasiveness of epithelial and cancer cells (Table 1.3). HGF/SF is a potent angiogenic molecule \textit{in vivo} and its angiogenic activity is mediated primarily through direct actions on vascular endothelial cells. These include
stimulation of cell migration, proliferation, protease production, invasion, and organization into capillary-like tubes [123].

1.2.2.3.9. Angiopoietins

The angiopoietins have recently joined the members of the VEGF family as the only known growth factors largely specific for the vascular endothelium (Table 1.3). Ang-1 is an angiogenic factor that signals through the endothelial cell-specific Tie-2 receptor receptor tyrosine kinase [212]. Ang-1 is essential for normal vascular development in the mouse. An Ang-1 relative, termed angiopoietin-2 (Ang-2) acts as a naturally occurring antagonist for Ang-1 and Tie-2 [83]. Ang-1 has a role during later stages of vascular development. Coming into play after VEGF in angiogenesis, it enhances vessel branching and remodeling and promotes maturation and stabilization of vessels [79]. Ang-1 is potent in inducing sprouting angiogenesis of adrenal-cortex derived microvascular endothelial cells in vitro in 3D fibrin gels and it is only a weak mitogen for HUVEC [144]. Instead, Ang-2 must act as a destabilizing protein being expressed at sites of vascular remodelling, leading to vessel regression in the absence of VEGF or causing vessel outgrowth in the presence of VEGF [83].

1.2.2.3.10. Heparin

Heparin was first found to induce endothelial cell migration and proliferation in vitro, but it was later shown that it is not angiogenic in vivo by itself but potentiates the angiogenic activity of tumour cell extracts [213]. Species of heparin and HS promote dimerization and receptor binding of bFGF [214] as well as of bFGF-mediated mitogenesis [214], suggesting that bFGF-HSPG complexes serve as the biologically active form of this growth factor. Cell surface heparin-like molecules are also involved in binding of VEGF to its high affinity receptor sites [215]. Released FGF can be sequestered from its site of action by binding to HS in the ECM [216] to be kept for emergencies, such as wound repair and neovascularization [217].

1.2.2.4. Angiogenic inhibitors

There are a number of peptidic growth regulators, that inhibit vascular endothelial cell proliferation (Table 1.4). Many of these peptide growth factors have both stimulatory and inhibitory activity on a single cell, depending on the context of other signalling molecules present [218]. Thrombospondin, an extracellular matrix protein,
suppresses neovascularization *in vivo* in the rat cornea [219] and it also inhibits endothelial cell proliferation, migration and angiogenesis *in vitro* [220]. Another factor able to down-regulate or inhibit angiogenesis is INF-α, being potent in regressing.

Table 1.4: Endogenous inhibitors of angiogenesis [69].

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Mechanism of action</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein fragments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angiostatin (fragment of plasminogen)</td>
<td>↓ EC proliferation, ↑ EC apoptosis</td>
<td>[221;222]</td>
</tr>
<tr>
<td>Endostatin (fragment of collagen XVIII)</td>
<td>↓ EC proliferation, ↑ EC apoptosis</td>
<td>[76]</td>
</tr>
<tr>
<td>aaAT (fragment of antithrombin 3)</td>
<td>↓ EC proliferation, ↑ EC apoptosis</td>
<td>[223]</td>
</tr>
<tr>
<td>Prolactin (16 kDa fragment)</td>
<td>↓ EC proliferation</td>
<td>[224]</td>
</tr>
<tr>
<td></td>
<td>↓ FGF-2-induced angiogenesis</td>
<td></td>
</tr>
<tr>
<td>Soluble mediators</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSP-1</td>
<td>↓ EC proliferation, ↑ EC apoptosis</td>
<td>[225]</td>
</tr>
<tr>
<td>Troponin I</td>
<td>↓ EC proliferation</td>
<td>[226]</td>
</tr>
<tr>
<td>IFN-α</td>
<td>↓ EC proliferation, ↑ EC apoptosis</td>
<td>[227]</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>↓ EC proliferation, ↑ IP-10</td>
<td>[228]</td>
</tr>
<tr>
<td>PEDF</td>
<td>↓ EC proliferation</td>
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<tr>
<td>IP-10</td>
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<td>[128]</td>
</tr>
<tr>
<td>PF-4</td>
<td>↓ EC proliferation</td>
<td>[128]</td>
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<tr>
<td>IL-12</td>
<td>↓ IFN-γ, ↑ IP-10</td>
<td>[230]</td>
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<tr>
<td>IL-4</td>
<td>↓ EC migration</td>
<td>[231]</td>
</tr>
<tr>
<td>VEGI</td>
<td>↓ EC proliferation</td>
<td>[232]</td>
</tr>
<tr>
<td>TIMP-1, -2</td>
<td>↓ MMP activity</td>
<td>[233]</td>
</tr>
<tr>
<td>PAI-1</td>
<td>↓ uPA activity</td>
<td>[234]</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>↓ EC migration, transcription factor</td>
<td>[235,236]</td>
</tr>
<tr>
<td>ANG-2</td>
<td>↓ Blood vessel maturation, antagonist of Ang-1</td>
<td>[83]</td>
</tr>
<tr>
<td>2-Methoxyoestradiol</td>
<td>↓ EC proliferation and migration, ↑ EC apoptosis</td>
<td>[237]</td>
</tr>
<tr>
<td>Tumour suppressor genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>↑ TSP-1 synthesis, ↓ VEGF synthesis</td>
<td>[238]</td>
</tr>
<tr>
<td>VHL</td>
<td>↓ VEGF synthesis</td>
<td>[239]</td>
</tr>
</tbody>
</table>

hemangiomas [240] and the highly vascularized Kaposi sarcomas [241]. INF-γ inhibits aFGF-induced endothelial cell proliferation [242], capillary formation [243] and endothelial cell growth *in vitro* [244]. INF-γ is implicated as an anti-angiogenic agent *in vivo* [245]. Platelet factor 4 is a strong anti-angiogenic factor *in vitro* [246] and inhibits growth of solid tumours [247]. TGF-β1 induces cells to secrete thrombospondin, in this way enhancing its own effects [248]. Protamine is an inhibitor of angiogenesis in the embryo, in tumourigenesis, in inflammation and immune responses [249].

Angiostatin and endostatin inhibit specifically endothelial cell proliferation and both factors are derived from proteolytic cleavage of native proteins. Angiostatin, an
internal fragment of plasminogen produced by primary tumours, was identified as an endogenous inhibitor of endothelial cell proliferation that inhibits angiogenesis in vivo. When the primary tumour is present, it suppresses in certain cases metastatic growth by secreting this angiogenesis inhibitor into the circulation. After tumour removal, metastases are able to neovascularize and grow [94]. Endostatin, a C-terminal fragment of collagen XVII is another endogenous inhibitor of angiogenesis. It suppresses endothelial cell proliferation in vitro and angiogenesis in vivo in the CAM [76]. Several other endogenous inhibitors of angiogenesis have been identified, such as an interferon-inducible protein. It is able to inhibit angiogenesis in vivo, but has no effect on endothelial cell growth, attachment and migration in vitro [250]. Two chemokines, gro-α and gro-β specifically inhibit growth factor stimulated proliferation of capillary endothelial cells and gro-β is potent in inhibiting in vivo blood vessel formation in the CAM [251]. The 16-kDa N-terminal fragment of prolactin is a potent inhibitor of angiogenesis [252]. Steroids administered in combination with heparin inhibit angiogenesis in the CAM and in rabbit corneas [253]. The non-anticoagulating derivative of heparin, heparin adipic hydrazide (HAH) covalently linked to the anti-angiogenic steroid cortisol has an antiproliferative effect on murine pulmonary capillary endothelial cells [254].

Collagenase inhibitors inhibit angiogenesis, which may depend on the importance of BM synthesis and degradation in the formation of new capillaries [255]. Other protease inhibitors like plasminogen activator inhibitor 1 (PAI-1) or 2 (PAI-2) and tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2) inhibit the degradation of the BM and endothelial cell invasion of the ECM [256]. Simultaneous expression of enzyme and inhibitor stimulates neovascularization, while protecting newly vascularized tissues. Endothelial cells secrete proteolytic enzymes such as plasminogen activator (PA) enabling them to penetrate the BM and migrate into surrounding tissues. Macrophages secrete enzyme inhibitors and produce cytokines that alter enzyme secretion by endothelial cells [257]. Recently, a fragment of MMP-2 which comprises the C-terminal hemopexin-like domain, termed PEX was shown to prevent the binding of this enzyme to αvβ3 integrin and block cell surface collagenolytic activity [258]. PEX blocks MMP-2 activity on the CAM where it disrupts angiogenesis and tumour growth [258].
1.3. MACROPHAGES

Macrophages belong to the mononuclear phagocyte system. They form a heterogeneous cell population because of different developmental and functional stages [259]. Macrophages share a common progenitor cell with granulocytes in the bone marrow. Commitment to the macrophage lineage gives rise to the macrophage colony-forming cells, which are succeeded by monoblasts, the first characteristic phagocytic cells. They further differentiate into pro-monocytes and bone marrow monocytes [259]. Monocytes enter the blood stream and migrate into tissues, where they undergo final differentiation to tissue macrophages, often assuming tissue-specific properties as histiocytes, alveolar macrophages, Kupffer cells, osteoclasts, peritoneal macrophages, synovia type A cells, or microglia. Developmental stages and differentiation steps of macrophages have been defined according to the intracellular localisation pattern of peroxidase activity or the expression of distinct antigens. Subsequently, it was shown, that inflammatory infiltrates contain several differentiation stages of macrophages [260]. Macrophages are thus present ubiquitously in all tissues and in case of inflammation certain subtypes are recruited from blood-borne monocytes.

A multitude of functions are performed by the cells of the mononuclear phagocyte system (MPS), such as endocytosis, cytotoxicity, and secretion of more than 100 cell products [257;260]. However, macrophages do not carry out all these functions at the same time. Their activities are rather dependent on the (patho)physiological situation. In order to become angiogenic, the versatile secretory potential of macrophages needs to be activated. What is less appreciated is their important role in tissue morphogenesis during development. Nevertheless, analysis of mice that are deficient for macrophages and other mononuclear phagocytic cells (such as osteoclasts) because of a null mutation in the gene that encodes colony-stimulating factor 1 (Csf-1) showed that these cells have a significant role in the morphogenesis of many tissues [261]. Taken together, these experiments demonstrated that cells of the mononuclear phagocytic lineage have important developmental roles through their remodelling and trophic functions. It seems likely that tumours co-opt the normal developmental roles of macrophages to promote their own development and invasion through the surrounding stroma [262]. In contrast to normal epithelia, however, tumour cells owing to intrinsic
transforming mutations, have lost positional identity, and so they continue to send out "help me" signals that result in invasion into the vasculature [26].

1.3.1. Tumour-associated macrophages (TAMs) in angiogenesis

It is becoming clear that tumours are largely recognized as self and lack strong foreign antigens. Instead, they appear to have been selected to manipulate the host immune system to prevent rejection [263] and to use it to facilitate their own growth and spread [26;264]. In addition, there is a growing body of clinical data on a wide range of solid tumour types that has correlated a high density of leucocytic infiltration with poor outcome [28]. It was also understood that cells containing DNA alterations caused by viral or chemical carcinogens do not progress to become cancerous until they are exposed to a stimulus by chronic irritants or inflammatory agents or by infectious or other agents that induce chronic inflammation [28].

In contrast to leucocyte recruitment in response to wounding, inflammatory or pathogenic stimuli, tumour cells shed continuous signals for the attraction of leucocytes to continue to support the tumour's development. This concept has led to the term that tumours are "wounds that never heal" [5].

It is widely recognized that tumours require angiogenesis to grow beyond a certain size. As pioneered by Folkman [9] angiogenesis has been found to be the crucial process for tumour progression by providing oxygen and nutrients and removal of waste products (Chapter 1.2.2.2 and figure 1.7). Moreover, angiogenesis provides an expanding endothelial surface for the tumour cells to enter the circulation and it also involves degradation of basement membranes followed by migration of endothelial cells into the tumour stroma.

Recent studies have shown that the tumour-associated macrophages (TAMs) produce factors that promote all these steps associated with malignancy within tumours. These factors are both stimulatory and inhibitory, including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), the angiopoietins (ANG-1 and ANG-2), IL-1, IL-8, tumour necrosis factor-α (TNF-α), thymidine phosphorylase (TP), the matrix metalloproteinases MMP-9 and MMP-2, nitric oxide (NO) and others. The coordinated spatial and temporal expression of these molecules result in the proliferation and migration of endothelial cells, the remodelling of the extracellular matrix and the eventual formation of stabilized vessels. Macrophages are perfectly designed to promote these processes, as their
monocytic precursors can migrate into sites where they differentiate into macrophages, and these wandering cells can synthesize the required angiogenic molecules on demand in specific locations (Figure 1.5) [10].

There is strong evidence in support of a positive impact of macrophages on tumour progression. For example, in more than 80% of clinical studies, an increase in TAMs density was correlated with poor prognosis, with less than 10% of studies showing the contrary [265]. Similarly, over-expression of macrophage chemoattractants within tumours has also been shown to correlate with poor prognosis. Other chemokines, have been identified for macrophage recruitment in several human tumours, including the bladder, cervix, ovary, lung and breast [266]. The strong correlation of over-expression of these macrophage chemoattractants with macrophage recruitment and poor prognosis suggests that TAMs can play a major role in the progression of tumours to metastasis.

Macrophages also indirectly enhance blood vessel formation by possessing a pro-coagulant activity through fibrin deposition [267]. In addition, many macrophages produce factors, proteases and protease activators such as transforming growth factor-β (TGF-β), platelet-derived growth factor, interleukin-6 (IL-6), urokinase plasminogen activator (uPa) and tissue-type plasminogen activator (t-Pa) that may cause degradation of extracellular matrix to facilitate tumour cell invasion and migration and induce angiogenesis [268].

Thus, for example, the ablation of macrophage recruitment in transplantable breast cancers by a chemokine receptor antagonist significantly inhibited tumour development [269;270]. These data further underscore the importance of the involvement of macrophages in tumour development in mouse models in general.

Conversely, TAMs contribute greatly to the growth of tumours by producing pro-angiogenic and tumour-stimulating chemokines such as CCR2 ligands [271]. Macrophages can display tumour cytotoxicity and can potentially present tumour antigens to induce specific immune reactions against tumours. However, these cells are believed to have primarily a pro-tumour function since both tumours and TAMs produce potent immunomodulating agents that suppress macrophage tumoricidal activity. Such tumour-produced molecules, including IL-4, IL-6, IL-10, CSF-1, TGF-β and prostaglandin E2 (PGE2) and TAM-produced factors such as IL-10 and PGE2, contribute to the general immunosuppression of the host as well as the antitumour activity of macrophages [272]. This has led to the idea of tumour-educated
macrophages whose functions are modified by the local cytokine/chemokine environment [26]. In most cases, these effects enhance tumour development and directs the local immune system away from an anti-tumour response [273].

The evidence described above suggests that cells of the myeloid lineages, particularly macrophages, play an active role in enhancing tumour progression and metastatic capacity. This is through their ability to promote angiogenesis and tissue remodeling as well as to exert direct effects on epithelial cell viability, growth and migration.

**Figure 1.5: Pro-tumourigenic functions of TAMs.** Macrophages are recruited to tumours by chemotactic factors and provide many trophic functions that promote tumour progression and metastasis. These TAMs migrate to hypoxic areas within the tumour, where they stimulate angiogenesis by expressing factors such as VEGF, ANG-1 and ANG-2, and recruit other haematopoietic cells, mast cells and neutrophils, that can perform similar tasks. TAMs also promote tumour invasion by producing proteases, such as uPA, MMP-9 and cathepsins, that break down the basement membrane and remodel the stromal matrix. MMP-9 also contributes to angiogenesis. Various growth factors and chemokines, e.g. EGF, TGF-β, IL-8 and TNF-α, contribute to the migration of tumour cells towards vessels and provide proliferative and anti-apoptotic signals to these cells. Macrophages that are attracted to sites of inflammation or tissue breakdown can also initiate or promote tumourigenesis through synthesis of oestrogens and the generation of mutagens as a by-product of the production of reactive oxygen and nitrogen-oxide radicals [26].
1.4. ANTIBODIES FOR TARGETED CANCER THERAPY

The clinical use of chemotherapeutic agents against malignant tumours is successful in many cases but still suffers from major drawbacks. One drawback is the lack of selectivity, which leads to severe side effects and limited efficacy, another is the emergence/selection of drug-resistance. To limit non-specific toxicity and to improve the efficiency of cancer therapy, “tumour markers”, which are proteins generally overexpressed on the surface of tumour cells, can be selectively targeted. Growth factor receptors are of the most extensively studied tumour markers. The implication of growth factor receptors in the pathogenesis and evolution of cancer has clearly been established and provides therefore, a rationale for therapeutic intervention. The targeting of cytotoxic substances to tumour markers with “magic bullets” is an old idea that raised high expectations but also disappointment. Over the past decade, newly gained understanding of mechanisms for targeted therapy has brought new hopes. Pharmacological agents that selectively target and block the action of growth factors and their receptors have been prepared, such as monoclonal Abs or Ab fragments, bispecific Abs, Abs conjugated to drugs, toxins or radioisotopes, small peptidic and peptidomimetic molecules in free form or conjugated to drugs, antisense oligonucleotides, immunoliposome-encapsulated drugs, and small molecule inhibitors. As summarized in Table 1.5 clinical trials are underway to test the safety and efficacy of many of these strategies.

Several monoclonal Abs directed against the extracellular domain of receptors have been approved. Mechanistically, there are four ways in which monoclonal Abs can be active:

I) Antagonistic monoclonal Abs may have a pharmacological effect by themselves because they competitively block ligand-receptor interaction, inhibit signalling and accelerate receptor desensitization or downregulation [274];

II) Agonistic monoclonal Abs may have a pharmacological effect by themselves by inducing terminal differentiation or apoptosis, or by increasing desensitization or internalization of the receptor [275];

III) Monoclonal Ab binding to certain epitopes on cancer cells may induce immune-mediated responses and trigger Ab-dependent cellular cytotoxicity (ADCC) by macrophages or natural killer cells [276], or engineered bispecific Abs can bridge a
selected tumour target cell with an immune effector cell (e.g. an activated T cell) such that the tumour cell is killed [277]; and

IV) Complement pathway can be activated by certain monoclonal Abs [278].

Table 1.5: Selected targeted therapies in clinical trials [279].

<table>
<thead>
<tr>
<th>Agent</th>
<th>Target</th>
<th>Phase</th>
<th>Indication</th>
<th>Company</th>
<th>Ref.</th>
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<td>Sandostatin (LAR)</td>
<td>IGF-IR</td>
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<td>Pituitary neoplasm</td>
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<td>Herceptin (Trastuzumab)</td>
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<td>Approved</td>
<td>Metastatic breast cancer</td>
<td>Genentech</td>
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<td>MDX-H210</td>
<td>HER2</td>
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<td>Advanced cancers</td>
<td>Medarex</td>
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<tr>
<td>Cetuximab (C225)</td>
<td>EGFR</td>
<td>III</td>
<td>Otopharyngeal, hypopharyngeal, laryngeal cancers</td>
<td>ImClone Systems</td>
<td>[283]</td>
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<td>Abx-EGF</td>
<td>EGFR</td>
<td>I/II</td>
<td>Solid tumours</td>
<td>Immunex</td>
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<td>EGFR</td>
<td>I/II/III</td>
<td>Variety of cancers</td>
<td>AstraZeneca</td>
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<td>Erlotinib (OSI-774)</td>
<td>EGFR</td>
<td>I/III</td>
<td>Breast, NSCL, ovarian, head and neck, pancreatic cancers and metastatic carcinoma of the endometrium</td>
<td>OSI/Genentech/Roche</td>
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<td>TarcevaTM</td>
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<td>Semaxanib (SU5416)</td>
<td>VEGFR</td>
<td>II/III</td>
<td>Kaposi sarcoma, NSCL, breast, and metastatic colorectal cancers</td>
<td>Sugen</td>
<td>[287]</td>
</tr>
<tr>
<td>HuMV833</td>
<td>VEGF</td>
<td>I</td>
<td>Variety of cancers</td>
<td></td>
<td>[288]</td>
</tr>
<tr>
<td>ZD 4674</td>
<td>VEGF</td>
<td>I</td>
<td>Advanced cancers</td>
<td>Astra Zeneca</td>
<td>[289]</td>
</tr>
<tr>
<td>PTK 787/ZK 22584</td>
<td>VEGF</td>
<td>I/II</td>
<td>Solid tumours</td>
<td>Novartis/Schering AG</td>
<td>[290]</td>
</tr>
<tr>
<td>CPG 4125</td>
<td>VEGF</td>
<td>I/II</td>
<td>Solid tumours</td>
<td>Novartis</td>
<td>[291]</td>
</tr>
<tr>
<td>SU 6668</td>
<td>VEGFR</td>
<td>I/II/III</td>
<td>Variety of cancers</td>
<td>Sugen</td>
<td>[292]</td>
</tr>
<tr>
<td>Interferon alpha</td>
<td>bFGFR, PDGFR</td>
<td>II</td>
<td>Renal cell, pancreatic, colorectal cancers and neuroblastoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTR 571 (Gleevec)</td>
<td>VEGF, PDGFR, ckit, Bcr-Abl kinase</td>
<td>I/II/Approved</td>
<td>Recurrent malignant gliomas, childhood soft tissue sarcoma, bone cancer, Unresectable and/or metastatic malignant gastrointestinal stromal tumours (GISTO and chronic myelogenous leukemia)</td>
<td>Novartis [294;295]</td>
<td></td>
</tr>
</tbody>
</table>

Monoclonal Abs directed at two RTKs, EGFR and HER2 have shown to be effective with satisfactory tolerance [296]. These findings represent the proof-of-concept that the use of monoclonal Abs against growth factor receptors is a valid and promising approach in therapy. Indeed, many monoclonal Abs are currently in use in the clinic or being evaluated in pre-clinical trials (Table 1.5).
Certain monoclonal Abs have low therapeutic efficacy. An attractive alternative is to use monoclonal Abs as carriers for drugs, toxins or radio-isotopes to deliver the effector drugs selectively. The first approaches utilized recombinant immunotoxins, consisting of a toxin (from bacteria, plants or fungi) fused or chemically conjugated to Fv regions of tumour-targeting Abs [297]. Also, conventional chemotherapeutics have been coupled to monoclonal Abs as carriers [298]. Monoclonal Abs or small peptides can also be used as targeting agents in combination with liposome encapsulation technology to generate ligand-targeted liposomes. Encapsulation of drugs with liposomes and derivatisation of liposomes with targeting agents such as monoclonal Abs or Ab fragments can increase the efficacy against receptor-bearing tumour cells [299].

Numerous strategies for improving the efficacy of anti-tumour Abs are now being tested, including the representative examples shown in figure 1.6. Enhancing effector functions involve improving Ab-dependent cellular cytotoxicity and/or complement-dependent cytotoxicity by means of site-directed mutations or manipulation of Ab glycosylation (Figure 1.6 A).

**Figure 1.6: Strategies for enhancing the potency of anti-tumour Abs [300].**

Direct arming of Abs entails their covalent linkage to a killing machinery, such as radionuclides or toxins (e.g., small molecules or proteins). Alternatively, arming Abs with cytokines is intended to create high intratumour concentrations of cytokines to stimulate the antitumour immune response (T cells, B cells or natural killer cells),
while avoiding the toxicities associated with systemic cytokine delivery (Figure 1.6 B). Indirect arming of Abs can be achieved by attaching engineered Ab fragments to the surface of liposomes loaded with drugs or toxins for tumour-specific delivery (Chapter 1.5.1). Bispecific Abs that bind to two different antigens can be preloaded with the cytotoxic machinery before administration (indirect arming) or alternatively, pre-targeted to the tumour before delivery of the cytotoxic payload (Figure 1.6 C). Pre-targeting strategies aim for the selective delivery of radionuclides to tumours or selective intra-tumour activation of pro-drugs, thereby diminishing the systemic toxicities of these agents. For pro-drug pre-targeting, an Ab-fragment-enzyme fusion protein is typically allowed to localize to a tumour and to be cleared from the system. A pro-drug is then administered and ideally converted to an active drug solely within the tumour. For radionuclide pre-targeting, e.g. an Ab-streptavidin conjugate is allowed to accrue within a tumour and is then used to capture a biotin-chelator-radionuclide complex (Figure 1.6 D) [300].

1.5. LIPOSOMES

Liposomes are spherical biomembranes that are formed spontaneously, when phospholipids, e.g., phosphatidylcholine molecules, are dispersed in water. The phospholipid molecules attain a conformation in which their hydrophobic fatty acid chains are protected from the surrounding water. Thus, phospholipid bilayers are formed in which the hydrophilic head groups form both outer parts of each bilayer, whereas the hydrophobic fatty acid groups are located directly opposed to each other within the membrane.

Depending on the preparation method and the lipid composition liposomes have a diameter between 25 nm (SUV, small unilamellar vesicles) to 1 μm (MLV, multilamellar vesicles). The advantages of using liposomes as drug delivery systems have been well documented and include: (a) Liposomes are biodegradable and nontoxic; (b) Molecules can be entrapped into the liposomes without modification; (c) Liposomes can carry a higher amounts of drugs compared to the direct conjugation of the drug to Abs; (d) Compounds are protected from enzymatic degradation or immunological reaction in the blood stream; (e) The host cells are protected from the drug; and (f) Liposomes can interact with the target cells and are therefore able to promote the intracellular delivery of different molecules [301].
Apart from liposomes that have been developed to avoid their uptake by macrophages (so called “stealth” liposomes), their usual fate is ingestion and digestion by macrophages. For this reason, liposomes form a suitable tool to manipulate macrophage function [302].

**Figure 1.7:** Liposomes – state of the art. A) Simple phospholipids "plain" liposomes with water soluble drug (a) entrapped into the aqueous liposome interior, and water-insoluble drug (b) incorporated into the liposomal membrane (these designations are not repeated on other figures). B) Ab-targeted immunoliposome with Ab covalently coupled (c) to the reactive phospholipids in the membrane, or hydrophobically anchored (d) into the liposomal membrane after preliminary modification with a hydrophobic moiety. C) Long-circulating liposome grafted with a protective polymer (e) such as PEG, which shields the liposome surface from the interaction with opsonizing proteins (f). D) Long-circulating immunoliposome simultaneously bearing both protective polymer and Ab, which can be attached to the liposome surface (g) or, preferably, to the distal end of the grafted polymeric chain (h). E) New-generation liposome, the surface of which can be modified (separately or simultaneously) by different ways. Among these modifications are: the attachment of protective polymer (i) or protective polymer and targeting ligand, such as Ab (j); the attachment/incorporation of a diagnostic label (k); the incorporation of positively charged lipids (l) allowing for the complexation with DNA (m); the incorporation of stimuli-sensitive lipids (n); the attachment of stimuli-sensitive polymer (o); the attachment of cell-penetrating peptide (p); the incorporation of viral components (q). In addition to a drug, liposomes can be loaded with magnetic particles (r) for magnetic targeting and/or with colloidal gold or silver particles (s) for electron microscopy [303].

Since the pioneering observation of Alec Bangham roughly 40 years ago that phospholipids in aqueous systems can form closed bilayered structures, liposomes have moved a long way from being just another exotic object of biophysical research to become a pharmaceutical carrier of choice for numerous practical applications. The real breakthrough developments in the area during the past 15 years have resulted in the approval of several liposomal drugs (see table 1.6) and the appearance of many unique biomedical products and technologies involving liposomes. The interest in the field remains high - almost 2 000 papers and more than 150 reviews on various aspects of liposome research were published in 2003 alone [303].
Table 1.6: Liposomal drugs approved for clinical application or undergoing clinical evaluation [303].

<table>
<thead>
<tr>
<th>Active drug</th>
<th>Product name</th>
<th>Indications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daunorubicin</td>
<td>DaunoXome</td>
<td>Kaposi's sarcoma</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Mycet</td>
<td>Combination therapy of recurrent breast cancer</td>
</tr>
<tr>
<td>Doxorubicin in PEG-liposomes</td>
<td>Doxil/Caelyx</td>
<td>Refractory Kaposi's sarcoma; ovarian cancer; recurrent breast cancer</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>Ambisome</td>
<td>Fungal infections</td>
</tr>
<tr>
<td>Cytarabine</td>
<td>DepoCyt</td>
<td>Lymphomatous meningitis</td>
</tr>
<tr>
<td>Vincristine</td>
<td>DepoCyt</td>
<td>Non-Hodkin's lymphoma</td>
</tr>
<tr>
<td>Lurtotecan</td>
<td>Onco TCS</td>
<td>Ovarian cancer</td>
</tr>
<tr>
<td>Nystatin</td>
<td>NX211</td>
<td>Topical antifungal agent</td>
</tr>
<tr>
<td>All-trans retinoic acid</td>
<td>Nyotran</td>
<td>Acute promyelocytic leukaemia; non-Hodkin's lymphoma; renal-cell carcinoma; Kaposi's sarcoma</td>
</tr>
<tr>
<td>Platinum compounds</td>
<td>Altron</td>
<td>Solid tumours</td>
</tr>
<tr>
<td>Annamycin</td>
<td>Platar</td>
<td>Doxorubicin-resistant tumours</td>
</tr>
<tr>
<td>E1A gene</td>
<td>Allovecin-7</td>
<td>Various tumours</td>
</tr>
<tr>
<td>DNA plasmid encoding HLA-B7 and α2 microglobulin</td>
<td></td>
<td>Metastatic melanoma</td>
</tr>
</tbody>
</table>

The clinical applications of liposomes are well known (Table 1.6). The initial success achieved with many liposome-based drugs has fuelled further clinical investigations. Recently, the selective delivery of the anticancer agent doxorubicin in polyethylene glycol (PEG) liposomes for the treatment of solid tumours in patients with breast-carcinoma metastases was shown, which resulted in a subsequent improvement in survival [304;305]. The same set of indications was targeted by a combination therapy comprising liposomal doxorubicin and paclitaxel [306] or Caelyx (Schering-Plough, doxorubicin in PEG liposomes) and carboplatin [307]. Caelyx is also in Phase II clinical trials for patients with squamous cell cancer of the head and neck [308] and ovarian cancer [309]. Clinical research showed the impressive effect of doxorubicin in PEG liposomes against unresectable hepatocellular carcinoma [310], cutaneous T-cell lymphoma [311] and sarcoma [312]. Liposomal lurtotecan was found to be effective in patients with topotecan-resistant ovarian cancer [313]. Other indications targeted by liposomal formulations include amphotericin B for the treatment of visceral leishmaniasis [314] and long-acting analgesia with liposomal bupivacaine in healthy volunteers [315].

1.5.1. Liposomes in targeted drug delivery

To increase liposomal drug accumulation in the desired tissues and organs, the use of targeted liposomes with surface-attached ligands capable of recognizing and
binding to cells of interest has been suggested (Figure 1.7 B). Immunoglobulins (Ig) of the IgG class and their fragments are the most widely used targeting moieties for liposomes, which can be attached to liposomes without affecting liposomal integrity or the Ab properties by covalent binding to the liposome surface or by hydrophobic insertion into the liposomal membrane after modification with hydrophobic residues [316]. Still, despite improvements in targeting efficacy, the majority of immunoliposomes accumulate in the liver as a consequence of insufficient time for the interaction between the target and targeted liposome. Better target accumulation can be expected if liposomes can be made to remain in the circulation long enough.

Different methods have been suggested to achieve long circulation of liposomes in vivo, including coating the liposome surface with inert biocompatible polymers, such as PEG, which form a protective layer over the liposome surface and slow down liposome recognition by opsonins and therefore subsequent clearance of the liposomes [317;318]. The circulation time is proportional to the PEG chain length (PEG5000-PE > PEG2000-PE > PEG750-PE). The half-life for liposome blood clearance increased from 0.7 h, 1.7 h to 6.2 h by inclusion of PEG750-PE, PEG2000-PE and PEG5000-PE in the lipid composition, respectively. The highest possible amount of PEG to include into liposomes is 7 mol% [319] (Figure 1.7 C). Long-circulating liposomes are now being investigated in detail and are widely used in biomedical in vitro and in vivo studies and they have also found their way into clinical practice [320]. An important feature of the protective polymers is their flexibility, which allows a relatively small number of surface-grafted polymer molecules to create an impermeable layer over the liposome surface [321]. Long-circulating liposomes demonstrate dose-independent, non-saturable kinetics and increased bioavailability [322]. Current research on PEG liposomes focuses on attaching PEG in a removable fashion to facilitate liposome capture by cells. After PEG-liposomes accumulate at the target site, through the enhanced permeability and retention (EPR) effect [323], the PEG coating is detached under the action of local pathological conditions (e.g., decreased pH in tumours). New, detachable PEG conjugates have been described [324] in which the detachment process is based on the mild thiolysis of the dithiobenzylurethane linkage between PEG and an amino-containing substrate (such as PE). Although, PEG remains the gold standard for the steric protection of liposomes, attempts continue to identify other polymers that could be used to prepare long-circulating liposomes. Earlier studies with various water-soluble flexible
polymers have been summarized elsewhere [325]. More recent papers describe long-circulating liposomes that were prepared using poly-N-vinylpyrrolidones [326], L-amino-acid-based biodegradable polymer-lipid conjugates [327] and polyvinyl alcohol [328]. Investigation of the relative roles of the liposome charge and protective polymer molecular mass revealed that opsonins with different molecular masses might be involved in the clearance of liposomes containing differently charged lipids [329].

Continuing interest in using long-circulating liposomes in cancer chemotherapy [330;331] is supplemented by their potential use for other purposes, such as carrying imaging agents and the treatment of infection [332]. Interestingly, recent evidence showed that PEG liposomes, previously considered to be biologically inert, could still induce certain side reactions through activation of the complement system [333].

1.5.2. Liposome surface-modification chemistry

The further development of liposomal carriers involved the attempt to combine the properties of long-circulating liposomes and immunoliposomes in one preparation [334]. Early experiments have been performed by simple co-immobilization of an Ab and PEG on the surface of the same liposome, although the protective polymer can create steric hindrances for target recognition with the targeting moiety [335]. To achieve better selectivity of PEG-coated liposomes, it is advantageous to attach the targeting ligand via a PEG spacer arm, so that the ligand is extended outside of the dense PEG brush, thereby reducing steric hindrance of binding to the target. Currently, various advanced technologies are used, and the targeting moiety is usually attached above the protecting polymer layer by coupling it with the distal water-exposed terminus of the activated liposome-grafted polymer molecule [334] (Figure 1.7 D).

The preparation of modified liposomes with controlled properties requires the chemical conjugation of proteins, peptides, polymers and other molecules to the liposome surface. One of the most efficient coupling methods consists of the conjugation of thiolated proteins to liposomes containing either thiol or maleimide groups [336;337].

Although various monoclonal Abs have been shown to deliver liposomes to many targets, the optimization of properties of immunoliposomes is an ongoing concern. The mainstream of research in this area relates to cancer targeting, which takes
advantage of a variety of Abs. Internalizing Abs are required to achieve an improved therapeutic efficacy with Ab-targeted liposomal drugs, as shown for example with B-lymphoma cells and internalizable epitopes (CD19) [338]. An interesting concept was developed to target HER2-overexpressing tumours using anti-HER2 liposomes [339]. The Ab CC52, which is directed against rat colon adenocarcinoma CC531 lines was attached to PEGylated liposomes resulting in the specific accumulation of liposomes in a rat model of metastatic CC531 tumours [340]. Nucleosome-specific Abs, capable of recognizing various tumour cells through tumour-cell-surface-bound nucleosomes improved Doxil (Alza) targeting to tumour cells and increased its cytotoxicity [341]. Immunoliposomes containing the novel antitumour drug fenretinide, and targeting the ganglioside GD2 induced apoptosis in neuroblastoma and melanoma cell lines and demonstrated strong antineuroblastoma activity both in vitro and in vivo in mice [342]. A combination of immunoliposome and endosome-disruptive peptide improves the cytosolic delivery of the liposomal drug, increases cytotoxicity and opens up new avenues for constructing targeted liposomal systems. This was shown with the diphtheria toxin A chain, which was combined with pH-dependent fusogenic peptide diNF-7 into integrated liposomes specifically targeted to ovarian carcinoma [343]. Recently, Marty et al. [344] prepared small unilamellar liposomes derivatised with single chain Ab fragments specific for the ED-B domain of B-fibronectin [345;346]. This extracellular matrix associated protein is expressed around newly forming blood vessels in the vicinity of many types of tumours. The single chain Ab fragments (scFv) were modified by introduction of C-terminal cysteines and linked to liposomes via maleimide groups located at the terminal ends of poly(ethylene glycol) modified phospholipids. The properties of these anti-ED-B scFv Ab-liposomes were analysed in vitro on ED-B fibronectin expressing Caco-2 cells and in vivo by studying their biodistribution and their therapeutic potential in mice bearing subcutaneous F9 teratocarcinoma tumours. Radioactively labelled (114Indium) scFv Ab-liposomes accumulated in the tumours at 2 to 3-fold higher concentrations during the first 2 h after i.v. injection compared to unmodified liposomes. After 6–24 h both liposome types, scFv Ab-PEG- and PEG liposomes were found in similar amounts (8 to 10%ID g−1) in the tumours. Animals treated i.v. with scFv Ab-liposomes containing the cytotoxic agent 2'-deoxy-5-fluorouridyl-N4-octadecyl-1-ß-D-arabinofuranoslycytosine showed a reduction of tumour growth by 62–90%, determined on days 5 and 8, respectively and compared to animals receiving control liposomes. Histological
analysis revealed a marked reduction of F9 tumour cells and excessive deposition of fibronectin in the extracellular matrix after treatment with the cytotoxic immunoliposomes. ScFv Ab-liposomes targeted to ED-B fibronectin positive tumours therefore represent a promising and versatile novel drug delivery system for cancer therapy [344].

The search for new ligands for liposome targeting is focused on specific receptors that are overexpressed on target cells (particularly cancer cells) and certain specific components of pathological cells. For example, liposome targeting to tumours has been achieved by using vitamin and growth factor receptors [347]. Vasoactive intestinal peptide (VIP) has been used to target PEG liposomes with radionuclides to VIP receptors on the surface of tumour cells and resulted in enhanced breast cancer inhibition in rats [348]. In addition, PEG liposomes loaded with doxorubicin have been targeted by RGD peptides to the integrins of tumour vasculature and demonstrated increased efficiency against C26 colon carcinoma in murine model [349]. RGD peptides were also used for targeting liposomes to integrins on activated platelets and could therefore be used to specifically target the cardiovascular system [350]. Similarly, an angiogenic homing peptide was used for targeted delivery of drug-loaded liposomes to vascular endothelium in the experimental treatment of tumours in mice [351]. Epidermal growth factor receptor (EGFR)-targeted immunoliposomes have been specifically delivered to variety of tumour cells that overexpress EGFR [352]. The incorporation of mitomycin C into long-circulating hyaluronan-targeted liposomes increases its activity against tumours that overexpress hyaluronan receptors [353]. Research also continues on the use of galactosylated liposomes to target drugs to the liver for the treatment of liver tumours or metastases [354]. The ability of galactosylated liposomes to accumulate specifically in parenchymal cells led to their application in gene delivery to these cells [355]. Finally, cisplatin-loaded liposomes that specifically bind to chondroitin sulphate, which is overexpressed in many tumour cells, have been used for the successful suppression of tumour growth and metastases in vivo [356].
1.6. BISPHOSPHONATES

Bisphosphonates are state of the art compounds used to prevent or inhibit development of bone metastases or excessive bone resorption, and for the therapy of inflammatory diseases such as rheumatoid arthritis and osteoarthritis [357]. However, the cellular and molecular mechanisms by which bisphosphonates inhibit bone resorption are only just becoming clear [358].

The bisphosphonates are compounds with a chemical structure similar to that of inorganic pyrophosphate (PPI), an endogenous regulator of bone mineralization (Figure 1.8).

While PPI is composed of two phosphate groups linked by phosphoanhydride bonds (a P-O-P structure), bisphosphonates are composed of two phosphonate groups linked by phosphoether bonds to a central carbon atom (a P-C-P structure). Unlike the unstable nature of the P-O-P bonds, the P-C-P structure is highly resistant to hydrolysis under acidic conditions or by pyrophosphatases. Two additional covalent
bonds to the central carbon atom of bisphosphonates can be formed with carbon, oxygen, halogen, sulfur, or nitrogen atoms, giving rise to an enormous range of possible structures. Like PPi, bisphosphonates form a three-dimensional structure capable of binding divalent metal ions such as Ca, Mg, and Fe in a bidentate manner, by coordination of one oxygen atom from each phosphonate group with the divalent cation. The affinity for calcium can be increased further if one of the side chains is a hydroxyl (-OH) or primary amino (-NH2) group, because this allows the formation of a tridentate conformation that is able to bind Ca more effectively. The high avidity of bisphosphonates for Calcium ions is the basis of the bone-targeting property of these compounds. Rogers et al. [358] have found that clodronate can be metabolised by amoebae of the eukaryotic microorganism Dictyostelium dicoideum into non-hydrolysable, adenine-containing analogues of adenosine triphosphate (ATP). This was followed by the recent discovery that also mammalian cells, such as J774 macrophages, can also metabolise clodronate [360]. The metabolite of clodronate, adenosine 5'-β,γ-dichloromethylene)triphosphate (AppCp), can be detected using mass spectrometry as described by Auriola et al. [360] (Figure 1.9).

Figure 1.9: Structures of bisphosphonate metabolites. The structure of adenosine triphosphate (ATP) and the AppCp-metabolite of clodronate first identified in Dictyostelium amoebae. The formation of AppCp metabolites of clodronate is catalyzed by aminoacyl-tRNA synthetases. An amino acid (Aa) condenses with ATP (Appp) to form an aminoacyl-adenylate (Aa-AMP), releasing pyrophosphate (pOp) in a reversible reaction (I). The aminoacyl-adenylate then condenses with a molecule of tRNA to form aminoacyl-tRNA (reaction II). Since clodronate (pCp) resemble pyrophosphate in structure, the reverse reaction of (I) can occur with pCp in place of pOp, to form an analog of ATP (AppCp) containing clodronate (reaction III) [359].
Two classes of bisphosphonates, the simple bisphosphonates (clodronate, etidronate) and the amino-bisphosphonates (tiludronate, alendronate, pamidronate, ibandronate, neridronate, risedronate, zoledronic acid and derivatives thereof) are defined according to their mechanisms of action. The simple bisphosphonates are metabolized to non-hydrolizable analogs of adenosine triphosphate and diadenosine tetraphosphates [361;362] whereas, the amino-bisphosphonates are potent inhibitors of the farnesyl diphosphate synthase, the major enzyme of the mevalonate pathway [359]. Newer bisphosphonates, such as ibandronate and zoledronate, show 10 000-100 000-fold greater potency than do the older agents such as etidronate.

Bisphosphonates are well-known and are reviewed or described, for example, in: Fleisch [363], Vasikaran [364] or Ross et al. [365], respectively. Use of bisphosphonates as anti-angiogenic agents to embolize newly formed blood vessels has been found to lead to suppression of solid tumours and metastases, e.g. bone metastases and even reduction in size of tumours, after appropriate periods of treatment. It has been observed that newly formed blood vessels disappear after bisphosphonate treatment, but that normal blood vessels remain intact [366]. Further it has been observed that the embolized blood vessels are not restored following cessation of the bisphosphonate treatment. Also it has been observed that bone metastasis, rheumatoid arthritis and osteoarthritis patients experience decreased pain following bisphosphonate treatment.

Liposome-encapsulated clodronate is toxic to macrophages in vitro and in vivo following phagocytic uptake of the liposomes and intracellular release of clodronate [367;368]. Internalised clodronate can inhibit the release of nitric oxide and proinflammatory cytokines from activated macrophages in vitro [367;369;370]. Liposome-encapsulated clodronate is effectively used in immunologic studies and for the treatment of arthritis and rheumatoid arthritis in inflammatory animal models [371-373].

1.7. THE MACROPHAGES AS TARGET IN LIPOSOME-BASED THERAPY STRATEGIES

Macrophages can be found in all organs of the body. For that reason, the majority of liposomes intended for drug- or antigen-targeting in vivo, will encounter macrophages on their way. Phagocytosis of the liposomes followed by disruption of their
phospholipid bilayers and release of the entrapped molecules will be the consequence. This capability of macrophages may be an advantage when these cells themselves are the targets of the liposomes (Chapter 1.5).

In several organs, liposomes cannot be targeted to macrophages, because they are not able to cross the vascular barriers formed by capillary walls. In other organs, liposomes can be targeted to defined macrophage (sub)populations provided that they are applied along appropriate administration routes (Table 1.7).

Table 1.7: Influence of administration routes on liposome-mediated depletion of macrophages [374].

<table>
<thead>
<tr>
<th>Organ/macrophages</th>
<th>iv</th>
<th>sc</th>
<th>lt/in</th>
<th>loc</th>
<th>ia</th>
<th>ip</th>
<th>Model</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow МΦ</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Rat</td>
<td>[375]</td>
</tr>
<tr>
<td>Knee joint/phagocytic synovial lining cells</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Mouse</td>
<td>[376;377]</td>
</tr>
<tr>
<td>Liver/Kupffer cells</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Mouse</td>
<td>[378]</td>
</tr>
<tr>
<td>Lung/alveolar МΦ</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Mouse</td>
<td>[375]</td>
</tr>
<tr>
<td>Lymph nodes/subcapsular sinus МΦ; medullary МΦ</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Mouse</td>
<td>[379]</td>
</tr>
<tr>
<td>Peritoneal МΦ</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Rat</td>
<td>[380]</td>
</tr>
<tr>
<td>Spleen/red pulp МΦ; marginal zone МΦ; marginal metallophilic МΦ</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Mouse</td>
<td>[381]</td>
</tr>
<tr>
<td>Testis macrophages</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Rat</td>
<td>[382]</td>
</tr>
<tr>
<td>Thymus macrophages</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Rat</td>
<td>[383]</td>
</tr>
</tbody>
</table>

Abbreviations: ia, intra-articular injection in synovial cavity; in, intranasal; ip, intraperitoneal; lt, intratracheal instillation; iv, intravenous; loc, local administration in thymus or in testis; sc, subcutaneous injection (in draining area of lymph node); + macrophage depletion induced by liposome-encapsulated clodronate.
1.8. AIM OF THE PROJECT

Hypoxic tumours secrete many mediators of inflammation e.g. CCLs, bFGF, members of the VEGF family, PDGF, CSF-1 (see abbreviations, chapter 8) and many more, which lead to infiltration and stimulation of different leucocytes, in particular macrophages, mast cells and neutrophils. It is known, that tumour-associated macrophages (TAMs) play a key role in tumour progression and metastasis as they produce cytokines and chemokines which promote many effects associated with tumour malignancy. These factors include bFGF, VEGFs, angiopoietins, interleukins, TNF-α, thymidine phosphorylase (TP), matrix metalloproteinases, nitric oxide (NO) and many others. These molecules, expressed by TAMs in a coordinated spatial and temporal fashion, result in the proliferation and migration of ECs, matrix remodelling and the eventual formation of stabilized vessels. It is well known that the liposome-encapsulated bisphosphonate clodronate (Clodrolip) is highly cytotoxic to macrophages, a property that is extensively exploited in immunology research. Here it was hypothesized that the elimination of TAMs and thus the abolishment of cytokine production by Clodrolip could inhibit tumour growth.

The major current problems in the treatment of cancer are: (a) the non-specificity of cytotoxic agents leading to unwanted toxic side effects; (b) the high diversity in development of cancer; and (c) the rise of resistance against one single regime of therapy.

To attempt to circumvent these disadvantages, therapies are needed that could eliminate as many pro-angiogenic factors from the tumour environment as possible. The goal of this work was to neutralize two major sources which are responsible for tumour angiogenesis: VEGF secreted by hypoxic tumour cells or by the stroma, and tumour-associated macrophages, which are also known to express high amounts of pro-angiogenic mediators.

To reach this goal, a new therapeutic approach with an anti-VEGF single chain Ab fragment (scFv) in combination with liposome-encapsulated clodronate for depletion of tumour-infiltrated macrophages was developed.
2. SELECTION, PRODUCTION, PURIFICATION AND IN VITRO CHARACTERISATION OF SPECIES CROSS-REACTIVE ANTI-VEGF (SCFV')₂-SZH9 ANTIBODY FRAGMENT

2.1. ABSTRACT

Human vascular endothelial growth factor isoform 165 (hVEGF₁₆₅) and murine VEGF isoform 164 (mVEGF₁₆₄) share 88.5% amino acid sequence identity. A human derived synthetic Ab phage-display library was used for the in vitro selection of species cross-reactive single chain Ab fragments (scFvs). The C-terminus of a bacterial scFv Ab clone, which showed the best cross-reactivity in ELISA was genetically modified for the expression as a (scFv')₂ Ab homodimer and for the attachment to liposomes. The modification contains a FLAG-tag sequence, a hydrophilic spacer and 3 C-terminal cysteines. For high yield expression, the plasmid was transformed into the yeast P. pastoris. Expression and culture conditions were analysed and optimised in shaker-flasks and for large-scale production in a bioreactor. The soluble anti-VEGF (scFv')₂-Ab was purified by protein-A affinity chromatography. Anti-VEGF (scFv')₂-Ab bound specifically to immobilized human, mouse and dog VEGF and neutralized soluble human and dog VEGF in a competitive ELISA, resulting in Kₐ values in the low nanomolar range. These results indicate that phage display technology is an efficient tool for the in vitro selection of species cross-reactive scFv Abs provided that purified antigen is available.

2.2. INTRODUCTION

Angiogenesis is the process by which new blood vessels arise from the pre-existing vasculature. VEGF is a multifunctional cytokine that plays a major role in angiogenesis [385]. Neoangiogenesis is essential for tumour growth and VEGF has been implicated with a major role in angiogenesis as inhibition of VEGF with neutralizing Abs can result in inhibition of tumour growth and even tumour regression [386]. Increased VEGF expression has been detected in many human tumours, in tumour-associated stroma, and in some cancer cell lines [387]. Recent studies have linked VEGF-rich tumours to poorer prognosis and early relapse [388].
Humanized mAbs have proven to be effective therapeutic agents [389]. However, their large size prevents optimal tumour uptake and penetration [390]. The development of single chain Ab fragments (scFvs) in combination with phage display technology offers new avenues for anti-tumour therapy [391]. This technology allows in vitro selection of human derived scFv Abs. Large collections of scFv Abs can be displayed on the surface of filamentous phage particles as fusion proteins with the phage minor coat protein (gIII). Each phage having a scFv Ab protein on its surface also contains the gene encoding for the Ab fragment. ScFv Abs with desired specificities can be selected from these phage-display libraries by repeated rounds of panning on antigen [392]. Selections are typically performed using purified antigen, although direct panning on cells has also been described [393]. In addition to the possibility to isolate Abs by immunization, the scFv Abs selected from phage libraries offer several advantages over mAb derived from hybridomas. The genes of selected scFv Abs are already cloned and encoded by a single DNA fragment simplifying their production and further modifications. Moreover, there is no need to humanize the Ab, obtained e.g. from rabbits or sheep. ScFv Ab phage-display libraries can be assembled from the variable regions expressed by the B-cells of an individual known to have mounted a particular immune response as a result of immunization or exposure to an infectious agent. The exposure of the donor to the antigen results in a repertoire of B cells expressing Abs specific for the antigen and allows the isolation of high affinity Abs [394]. However, libraries derived from immunized individuals allow the isolation of scFvs against a limited number of antigens. Alternatively, highly diverse Ab repertoires can be derived from semisynthetic V-genes, created by PCR extension of V-germline genes using primers containing degenerated sequences corresponding to the complementarity determining region 3 (CDR3) of the variable region of immunoglobulins (synthetic repertoires) [391]. This strategy does not require immunization and the diversity of these semi-synthetic libraries is not constrained by forces of selection acting in the natural immune system. The number and affinity of the Abs generated to a particular antigen is a function of library size and diversity, larger libraries yielding a greater number of high-affinity Abs. The typical affinity of Abs isolated form libraries of a size of $5 \times 10^8$ different clones is in the range of $10^{-6} - 10^{-8}$ M. About 1/6 the size of a native Ab, the 25 kDa scFv Ab retains the specificity of an Ab but has a decreased avidity due to its monovalent nature. Pharmacokinetic studies with scFv Abs have shown that their small size leads
to rapid blood clearance by renal glomerular filtration [395]. Covalent dimeric (scFv')₂ Ab molecules have been engineered by C-terminal addition of a peptide [396] or a cysteine [395] or by cross-linking with chemical linkers [397].

The methylotrophic yeast *P. pastoris* has been described to be appropriate for high yield expression of various proteins by secretion into the culture supernatant or by intracellular localisation. *P. pastoris* is capable of metabolizing methanol as its sole carbon source and as a eukaryote, *P. pastoris* has many of the advantages of higher eukaryotic expression systems such as protein processing, protein folding and posttranslational modification, while being as easy to manipulate as *E. coli* or *S. cerevisiae* [398].

The primary objective of this work was to couple species-cross reactive VEGF-specific scFv Abs as neutralizing molecules attached to poly(ethylene) glycol (PEG)-liposomes. The first aim was to stabilize the scFv Ab-molecules *in vivo* and second to combine anti-angiogenic- with chemotherapy. The covalent attachment of proteins to PEG-liposomes has been used in many therapeutic applications to extend plasma half-life, reduce immunogenicity, and decrease proteolysis [344]. Long-circulating PEG-derivatized liposomes are widely used as stabilizing and delivery systems with a broad spectrum of agents including chemotherapeutics, imaging agents, antigens, lipids and DNA [399] (See also chapter 1.5).

The *in vitro* selection of mouse/human cross-reactive anti-VEGF scFv Abs, the derivatisation of one scFv Ab clone with cysteines, the high yield expression in *P. pastoris* and the binding to purified recombinant VEGFs *in vitro* is described in this chapter.

2.3. MATERIAL AND METHODS

2.3.1. Reagents

Recombinant cfVEGF₁₆₄ and VEGF-E (originating from the orf-family of pox viruses) were produced in *P. pastoris* and purified by ion exchange chromatography as described previously [400]. Recombinant hVEGF₁₆₅ was kindly provided by Dr. A. Zisch (Institute of Obstetrics, University Hospital Zurich, Switzerland). It was produced in 293T cells transiently transfected with the expression plasmid VEGF₁₆₅SR as described previously [401]. Recombinant mVEGF₁₆₄ (purity > 95%) was purchased from Reliatech (Braunschweig, Germany), expressed in insect cells.
The synthetic ETH-2 human Ab phage library was a kind gift of Prof. D. Neri (Institute of Pharmaceutical Sciences, ETH Zurich, Switzerland). The construction of the library was previously described [392]. *E. coli* suppressor strain TG1 for propagation of phage particles and HB2151 non-suppressor strain for expression of scFv Abs were obtained from Invitrogen (Leek, the Netherlands). Helper phage VCS M13 for the rescue of phagemid libraries was purchased from Stratagene (Cedar Creek, TX). Antibiotics and isopropyl-ß-D-thiogalactopyranoside (IPTG) were obtained from Invitrogen and Fermentas (Ontario, Canada), respectively.

For detection of scFv Abs the mouse M2 anti-FLAG was obtained from Sigma and an alkaline phosphatase (AP)-conjugated goat anti-mouse mAb was obtained from Serotec (Oxford, UK).

The plasmid sequence of pCM3 for introduction of C-terminal cysteines was obtained from Dr. C. Marty [344;402]. For sub-cloning XL10-Gold ultra competent *E. coli* were purchased from Stratagene. The yeast expression vector pPICZαA, the X-33 yeast strain, the 5'AOX1 and 3'AOX1 primers and the antibiotic zeocin were obtained from Invitrogen. All yeast culture media were from Difco (Detroit, USA). The oligonucleotides were synthesised by Microsynth (Balgach, Switzerland). Restriction endonucleases were from Roche Diagnostics Mannheim (Mannheim, Germany) and Life Technologies, Inc. (Gaithersburg, MD). All buffer salts and other chemicals were from Fluka or Sigma (Buchs, Switzerland).

2.3.2. Phage display: *In vitro* selection of species cross-reactive scFv Ab Abs

For the selection of species cross-reactive scFv Abs from the synthetic ETH-2 scFv Ab-phage library 3 rounds of panning with recombinant mouse (isoform 164) and human (isoform 165) VEGF were performed. Nunc-ImmunoTubes (Maxisorp, Nunc GmbH, Wiesbaden, Germany) were coated with 25 µg ml⁻¹ mVEGF₁₆₄ (in PBS, 0.137 M NaCl, 2.68 mM KCl, 8.09 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4) (panning round 1 and 2) and hVEGF₁₆₅ (round 3) under identical conditions for 12 h at RT. The scFv Ab-selection steps were performed as described [392]. At the end of the third round of panning the phages eluted from the immunotube were used to infect HB2151 (non-suppressor) *E. coli* bacteria and plated on 2xTYE (yeast extract tryptone medium, 1.5% (w/v) agar) plates containing 100 µg ml⁻¹ ampicillin and 1% glucose. Single clones were screened by ELISA for binding to recombinant
hVEGF$_{165}$ and mVEGF$_{164}$ using the M2 anti FLAG-Ab for signal detection (Chapter 2.3.3 and 2.3.4).

2.3.3. Expression of soluble scFv Ab Ab fragments in 96 well plates

Single bacterial colonies were grown in a shaker for 2 h at 37°C and 180 rpm in 2xTY medium containing 100 µl ml$^{-1}$ ampicillin and 0.1% glucose. The production of soluble scFv Ab was induced by the addition of IPTG to a final concentration of 1 mM and the bacteria were further incubated for 16 - 24 h at 30°C. After centrifugation at 1800 g for 10 min the supernatant was collected and used for ELISA.

2.3.4. Detection of VEGF in ELISA

Immuno-plates (Maxisorb, Nunc, Live Technology) were coated for 12 - 16 h at 4°C with the following antigens: 5 µg ml$^{-1}$ mVEGF$_{164}$, 5 µg ml$^{-1}$ hVEGF$_{165}$ and 5 µg ml$^{-1}$ BSA in PBS. After all incubation steps 3 PBS wash cycles using a Tecan multi-well plate-washer (Salzburg, Austria) were performed. Unspecific binding was blocked with PBS containing 2% milk powder (w/v) for 2 h. Bacterial supernatants containing soluble scFv Ab were diluted 1 : 1 with PBS containing 4% milk. After 2 h at RT the wells were first incubated with mouse anti-FLAG tag mAb (M2, 1 : 3 000, v/v in PBS containing 2% milk powder) for 1 h and with alkaline phosphatase (AP)-conjugated goat anti-mouse mAb (diluted 1 : 30 000, v/v in PBS containing 2% milk powder) for 1 h. The ELISA was developed using 4-nitrophenyl phosphate (4-NPP; 3 mM 4-nitrophenyl phosphate x H$_2$O, 50 mM NaHCO$_3$, 50 mM MgCl$_2$) as the substrate and the OD$_{410nm}$ was measured.

2.3.5. Subcloning into the yeast expression vector pPICZaA

The DNA sequence of the anti-VEGF scFv Ab (E. coli clone: H9) was modified by PCR using isolated plasmid pDN332 containing the sequence of H9 as a template. The forward primer (CCG GAA TTC CAG GTG CAG CTG GTG CAG TCT GGG) included an EcoRI site for cloning into the P. pastoris expression vector pPICZaA. The reverse primer (pCM3: GCTTGTTCTAGATCAACATGAACACCCACATGA CCCTGATGA CCC CCC TGATGA CCC CCC CTT GTCATC GTC GTC CTT GTA GTC ACC TAG GAC GGT CAG) contained the sequence for a FLAG-tag, for hydrophilic spacer amino acids and for 3 cysteines, a stop codon and an Xbal site for cloning into the P. pastoris expression vector. The PCR reactions were performed in
100 μl sterilized water containing 1× reaction buffer (20 mM Tris-HCl (pH 8.8), 2 mM MgSO₄, 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton®X-100, 0.1 mg ml⁻¹ nuclease-free BSA, Stratagene, Cedar Creek, TX), 50 ng of DNA template, 500 nM of each primer, 100 μM dNTP (Boehringer) and 2.5 U PFU DNA Polymerase (Stratagene). Thirty cycles were carried out in a DNA thermocycler (TECHNE Progene, Cambridge, UK) each consisting of 1 min at 95°C, 1 min at 48°C, and 2 min at 72°C. The PCR products were digested with EcoRI and XbaI and purified by PCR-DNA purification kit (Qiagen). The modified DNA constructs were ligated into the pPICZαA vector which had been digested with EcoRI and XbaI, treated with alkaline phosphatase, purified by electrophoresis and isolated with DEAE nitrocellulose paper. The ligations were performed in 10 μl containing 1 μl ligase buffer (Boehringer), 100 ng vector, 300 to 500 ng insert and 0.5 U of T4 DNA ligase (Boehringer) for 12 h at 16°C. The DNA samples were stored at -20°C. Amplified PCR products were verified on 0.8% agarose gels (0.5 x TBE).

2.3.6. Transformation of pPICZαA into E. coli

DNA (50 ng) was added to 100 μl of XL10-Gold ultracompetent cells (Stratagene) and 25 mM mercaptoethanol, incubated 30 min on ice, 30 sec. at 42°C in a water bath, 2 min on ice and incubated 1 h (37°C, 240 rpm) after 900 μl of SOC (0.5% (w/v) yeast extract, 2% (w/v) tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 20 mM MgSO₄, 20 mM glucose) had been added. Transformed colonies were selected on low salt LB plates (1% (w/v) tryptone, 50 mM MgCl₂, 0.5% (w/v) yeast extract, 1.5% (w/v) agar, pH 7.5) containing 25 μg ml⁻¹ zeocin. Zeocin resistant colonies were inoculated in 5 ml low salt LB containing 25 μg ml⁻¹ zeocin and grown for 12 h at 37°C and 250 rpm.

2.3.7. Isolation of plasmid-DNA

Over night E. coli cultures containing pPICZαA were centrifuged for 5 min at 5000 rpm (Eppendorf, Centrifuge 5417C) and resuspended in 300 μl buffer PI (25 mM Tris Cl (pH 8.0), 10 mM EDTA, 4% (w/v) RNAseA, Sigma). After addition of 300 μl buffer PII (0.2 N NaOH, 1% (w/v) sodium dodecyl sulphate (SDS)), inverting and incubation (3 - 5 min), 300 μl of chilled buffer PIII (5 M potassium acetate solution, pH 4.8) was added and the lysed bacteria centrifuged 10 min at 14 000 rpm at 4°C (Eppendorf, Centrifuge 5417C). To the supernatant 700 μl isopropanol was
added and centrifuged 15 min The precipitated DNA was washed with 70% ethanol and centrifuged (2 min), the supernatant removed and after drying on air (10 min) the pellet was resuspended in 30 μl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

The plasmids containing an insert were identified by restriction digest analysis using BamHI. Clones containing the required insert were identified by DNA sequencing using the 5’AOX1 (GAC TGG TTC CAA TTG ACA AGC) and 3’AOX (GCA AAT GGC ATT CTG ACA TCC) primers.

The plasmids (pPICZαA anti-VEGF scFv-H9 Ab and pPICZαA unspecific scFv-A1 Ab) containing the correct insert were amplified in 500 ml low salt LB containing 25 μg ml⁻¹ zeocin, grown for 12 h at 37°C at 180 rpm and plasmid DNA isolated using a plasmid maxiprep kit (Qiagen).

2.3.8. Transformation of pPICZαA into P. pastoris

The plasmids were linearised with SsaI and transformed into the P. pastoris strain X-33 by electroporation. The X-33 yeast cells were grown for 12 h at 30°C in 5 ml yeast extract peptone dextrose medium (YPD; 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose). Fresh YPD medium (500 ml) was inoculated with the cultures and grown for 12 - 16 h to reach an OD₆₀₀ₙₐₙ of 1.3. The cells were centrifuged (5 min, 1 500 g) and the pellets resuspended in 500 ml sterile ice cold water, followed by sequential centrifugation steps with resuspension of the pellet in 250 ml sterile ice cold water, 20 ml sterile ice cold 1 M sorbitol and 1.5 ml sterile ice cold 1 M sorbitol, respectively. The competent cells were electroporated by pulse discharge (1500 V, 25 μF, 200 Ω; Genepulser, Bio Rad Laboratories, Hercules, CA) in the presence of 10 μg of each linearised plasmid. After adding 1 ml 1 M sorbitol the cultures were incubated for 1 h at 30°C without shaking. The electroporation mixtures were plated on YPDS (YPD with 1 M sorbitol) plates containing 100 μg ml⁻¹ zeocin. After 72 h incubation at 30°C 30 clones of each construct were transferred on minimal methanol medium (MM) agar plates (1.34% (w/v) yeast nitrogen base, 4 x 10⁻⁵% biotin, 0.5% (v/v) methanol), on minimal dextrose medium (MD) agar plates (1.34% (w/v) yeast nitrogen base, 4 x 10⁻⁵% biotin, 2% (w/v) dextrose) and on YPDS plates containing different amounts of zeocin (100, 500, 1000 and 2000 μg ml⁻¹). After growing for 3 days at 30°C transformants that were growing on MD medium and on medium containing 2000 μg zeocin but only very slowly on MM medium were retained for further screening for high expression clones.
2.3.9. Selection for high expression yeast clones

Ten positive clones were used to inoculate 5 ml BMGY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 1% (v/v) glycerol, 100 mM potassium phosphate (pH 6), 1.34% (w/v) yeast nitrogen base with ammonium sulphate and without amino acids) in 15 ml tubes for 12 h at 30°C. The cells were centrifuged (5 min, 2 000 g) and the pellets resuspended in 5 ml BMMY medium (BMGY containing 1% (v/v) methanol instead of glycerol) and incubated for 3 to 5 h. Culture supernatants were sampled at various time points to monitor the scFv Ab expression by Western blot analysis. The best clones of each construct were stored at -80°C in BMGY medium containing 25% (v/v) glycerol.

2.3.10. Scaling-up of (scFv')2 antibody expression in shaker flasks

Clones were transferred from the -80°C glycerol stocks and grown in 5 ml BMGY at 250 rpm for 12 h. These cultures were used to inoculate 500 ml BMGY in 1 litre shaker flasks for 24 h at 30°C and 180 rpm. To induce the scFv Ab production the cells were centrifuged (5 min, 2000 g) and the pellets resuspended in 500 ml BMMY. The cells were shaken in a 1 litre baffled flask for 96 h at 28°C and 180 rpm. Cultures were supplemented every 24 h with 1% (v/v) methanol.

2.3.11. Expression of (scFv')2 antibodies in a bio-reactor

(ScFv')2 antibodies were produced at large quantities in a 3.7 litre bio-reactor (Bioengineering, Wald, Switzerland) as described [337]. Briefly, a single cryo-stock clone was transferred in 5 ml BMGY and grown at 250 rpm, 30°C for 12 h. This culture was used to inoculate 200 ml BMGY in a 1 litre shaker flask for 24 h at 30°C and 180 rpm. This inoculum was transferred into the bio-fermenter, containing 1.5 litres BMGY medium (pH 6.0). During 48 h of glycerol (50%) feed, the temperature was kept at 30°C and dissolved oxygen maintained between 20 - 40% air saturation. The pH was regulated with 25% NaOH and 3 M H₃PO₄. Protein expression was induced by addition of methanol (100% supplemented with PTM1 trace salts) and carried out under reduced temperature conditions (28°C, to reduce proteolytic activity) for 48 h.
2.3.12. Purification of soluble (scFv')2 antibodies produced in 
*P. pastoris*

To inhibit proteolytic digestion, all steps were performed at 4°C. Culture supernatants (3000 g, 20 min) containing the soluble (scFv')2 antibodies were further clarified by 0.8 µm (Millipore, Bedford, MA) filtration, concentrated (Schleicher & Schuell, Dassel, Germany) and dialysed (20 mM Tris, pH 8.0). For elimination of remaining yeast contaminants, the protein solution was ultracentrifuged (50 000 g, 30 min; Beckman Coulter Optima XL Ultracentrifuge) and filtrated (0.2 µm, Millipore) before performing affinity chromatography using a 5 ml protein-A column (Amersham Pharmacia Biotech, Uppsala, Sweden). After washing the column with 20 mM Tris-buffer, pH 8.0 the protein was eluted with 100 mM glycine, pH 2.5 and directly collected in 1 M Tris-buffer, pH 8.0. Fractions of 2 ml were collected and analysed on 13% SDS-PAGE gels. The fractions containing the protein were pooled, dialysed (PBS, pH 7.4), quantified with a ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and stored at concentrations of 1 - 2.5 mg ml⁻¹ at -80°C.

2.3.13. Detection of VEGF by Western immunoblotting

For protein analysis 13% SDS-PAGE was carried out according to Laemmli [403] and gels were stained by the Fairbanks Coomassie method [404].

For scFv Ab recognition of VEGF-variants, proteins were resolved with 13% SDS-PAGE and transferred to PVDF membranes (Millipore). Blots were incubated with scFv Ab in various concentrations in 2% (w/v) skimmed milk in PBS at RT for 2 h. Reactivity of (scFv')2 antibodies was detected by anti-FLAG M2 Ab (Sigma; diluted 1 : 4 000, v/v) and alkaline-phosphatase (AP) conjugated goat anti-mouse IgG (Sigma; diluted 1 : 30 000, v/v). After extensive washings with PBS, secondary AP-Ab was detected using 4-nitrophenyl phosphate (4-NPP) as the substrate (3 mM 4-nitro-phenyl phosphate x 6 H₂O, 50 mM NaHCO₃, 50 mM MgCl₂) and the OD₄₁₀nm was measured.

2.3.14. Affinity determination

The antigen binding constants against mVEGF₁₆₄ and cfVEGF₁₆₄ of the (scFv')₂-SZH9 Ab were determined based on a competitive ELISA. The affinity constants of antigen-Ab equilibria in solution were estimated according to Friguet *et al.* [405]. Varying concentrations of mVEGF₁₆₄ or cfVEGF₁₆₄ in PBS supplemented with
0.02% BSA were incubated with the \((\text{scFv'})_2\) Ab at \(10^{-9}\) M overnight at RT until equilibrium was reached. The equilibrated solution was transferred into a cfVEGF\(_{164}\)-coated microtiter plate (5 \(\mu\)g ml\(^{-1}\) in PBS). Free \((\text{scFv'})_2\) Ab at equilibrium was determined by ELISA and the affinity constants were calculated using the Scatchard equation as described in detail by Friguet et al. [405].

2.4. RESULTS

2.4.1. Selection and bioengineering of species cross-reactive anti-VEGF (scFv')\(_2\)-SZH9 Ab homodimer

Here, the selection and engineering of anti-VEGF human/mouse cross-reactive scFv Ab-dimers from a human synthetic phage-display library is described. Based on the 88.5% amino acid (aa) sequence identity between human VEGF\(_{165}\) and murine VEGF\(_{164}\) (Figure 2.1), the likelihood of obtaining cross-reacting scFv Abs was high and resulted after three rounds of selection in almost exclusively species cross-reactive scFv Abs (Figure 2.2).

<table>
<thead>
<tr>
<th>Human VEGF(_{165})</th>
<th>APMAEGGGQNHHEVVKFMDEVYQRSYCHPIETLVDIFQEQYDIEIEY</th>
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<tbody>
<tr>
<td>Murine VEGF(_{164})</td>
<td>APTTEGE-QKSEVHIKFMDEVYQRSYCRPIETLVDIFQEQYDIEIEY</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Human VEGF(_{165})</th>
<th>IFKPSCVPLRMCGCCNDEGECVPTEESNITMQIMRIKPHQGQH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine VEGF(_{164})</td>
<td>IFKPSCVPLRAGCCNDEALECVPTSESNITMQIMRIKPHQGQH</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Human VEGF(_{165})</th>
<th>IGEMSLQHNSCECRKKDRQENPCGCSERRKLHDFVQDPQTC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine VEGF(_{164})</td>
<td>IGEMSLQHNSCECRKKDRTPKPHENCEPCERRKLHDFVQDPQTC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Human VEGF(_{165})</th>
<th>KCSCKTDSRCARQLEBRETURNCRDCRRK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine VEGF(_{164})</td>
<td>KCSCKTDSRCARQLEBRETURNCRDCRRK</td>
</tr>
</tbody>
</table>

**Figure 2.1**: Aa-alignment of human VEGF\(_{165}\) and murine VEGF\(_{164}\), indicating 88.5% sequence identity. Differences in aa-sequences of human VEGF\(_{165}\) and murine VEGF\(_{164}\) are indicated in bold letter type.

Bacterial scFv Ab-expression levels were revealed by Western blot analysis and showed comparable scFv Ab-levels (data not shown). Sequence analysis of the plasmid DNAs revealed that each of the 5 chosen clones (B10, E4, E9, G8 and H9) encoded a different scFv Ab (data not shown). In order to increase the *in vivo* half-life of the selected scFv Ab and to allow to couple scFv Abs to the surface of PEG-
Figure 2.2: Species cross-reactivity analysis of selected clones by ELISA. Binding properties of several bacterial clones after phage display selection using unpurified bacterial supernatants containing soluble scFv Ab to human VEGF\textsubscript{165}, BSA and murine VEGF\textsubscript{164}. Data are expressed as ± SD of the mean (n = 3).

liposomes, the bacterial scFv Ab clone H9 (Figure 2.2) and an unspecific control, scFv-A1 Ab were functionalized with 3 cysteines using PCR. The DNA-sequences of the (scFv')\textsubscript{2} Abs were subcloned into the \textit{P. pastoris} expression vector pPICZ\textalpha A containing a sequence for extracellular protein secretion and zeocin selection. Sequencing was performed on plasmid DNA. Alignment analysis of the anti-VEGF (scFv')\textsubscript{2}-SZH9 Ab and the unspecific control (scFv')\textsubscript{2}-A1 Ab, shown in figure 2.3, demonstrates differences in the variable CDR3 region of the heavy and light chain and also in other locations.

Figure 2.3: Aa-alignment of anti-VEGF (scFv')\textsubscript{2}-SZH9 Ab and the unspecific control (scFv')\textsubscript{2}-A1 Ab in the monomeric form. The variable complementarity determining region 3 (CDR3) of the heavy chain is the largest and most diverse loop of the antigen recognition site of the Ab. Random loops of 4, 5 or 6 amino acids: C98, A, K, (X)4-6, F, D, Y are introduced for diversity. The diversity in 6 amino acid positions in the CDR3 of light chains is assembled with the DPL-16 or DPK-22 germline genes. The heavy and light variable CDR3 regions are indicated in bold letter type, connected by a linker sequence, indicated in italic letters. The introduced C-terminal FLAG-tag, the hydrophilic spacer sequence and three cysteines are underlined.
At the C-terminal end they show the modifications introduced by PCR. The scheme of the engineered construct with the corresponding protein product is shown below (Figure 2.4).

![Diagram of engineered construct](image)

**Figure 2.4:** Antiparallel representation of bioengineered anti-VEGF (scFv')2-SZH9 Ab. VL: variable light chain; VH: Variable heavy chain; FLAG-tag is indicated in italic letters, hydrophilic spacer sequence is underlined and 3 terminal cysteines are shown in bold letters (Parallel alignment is also likely to occur).

### 2.4.2. Expression and purification of (scFv')2-SZH9 antibody

Plasmid-PICZαA constructs of anti-VEGF (scFv')2-SZH9 Ab and control (scFv')2-A1 Ab were subcloned into the eucariotic high expression system _P. pastoris_ and (scFv')2 Abs were produced in a 3.7 litre bio-reactor. Expression levels of 40 to 50 mg l⁻¹ culture medium were obtained and the proteins, containing a DP47-sequence were purified by protein-A affinity chromatography (Figure 2.5 A and B).

![Graph of purification](image)

**Figure 2.5:** Purification of cystein-derivatized scFv Abs. (A) Purification of cys-derivatized anti-VEGF (scFv')2-SZH9 Ab. Elution profile on a protein-A sepharose column. After column equilibration with 20 mM Tris, pH 8.0 binding buffer (1, red line), the protein was loaded (2, blue line). The column was washed with 20 mM Tris, pH 8.0 (3, red line) and the (scFv')2 Ab was eluted with 20 mM Tris, pH 2.5 (4, blue line). The column was washed with 0.1 M Na-acetate, 0.5 M NaCl (5, red line). Eluted protein was immediately dialysed in PBS and stored at -80°C. (B) Fairbanks-Coomassie stained SDS-PAGE of cystein-derivatized and purified (scFv')2-SZH9 Ab under reducing (lanes 1 and 2) and non-reducing (lanes 4 and 5) conditions. Amounts loaded: 0.5 µg (lanes 1 and 4), 1 µg (lanes 2 and 5) of (scFv')2-SZH9 Ab and 1 µg BSA (lane 3). ScFv Ab monomers (approx. 29 kDa) and dimers (approx. 58 kDa) are indicated by m and d, respectively.
Table 2.1 summarizes important biochemical characteristics of the monomeric forms of the anti-VEGF (scFv')$_2$-SZH9 and the unspecific control (scFv')$_2$-A1 Abs.

Table 2.1: Properties of the anti-VEGF (scFv')$_2$-SZH9 monomer and the control (scFv')$_2$-A1 monomer Abs.

<table>
<thead>
<tr>
<th></th>
<th>Anti-VEGF scFv-SZH9 Ab (monomer)</th>
<th>Control scFv-A1 Ab (monomer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length</td>
<td>277 aa</td>
<td>280 aa</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>28667 Da</td>
<td>28708 Da</td>
</tr>
<tr>
<td>1 µg of protein corresponds to</td>
<td>34.883 pMoles</td>
<td>34.833 pMoles</td>
</tr>
<tr>
<td>Molar extinction coefficient</td>
<td>49770</td>
<td>41520</td>
</tr>
<tr>
<td>One absorption unit (AU, 280 nm) corresponds to</td>
<td>0.58 mg ml$^{-1}$</td>
<td>0.69 mg ml$^{-1}$</td>
</tr>
<tr>
<td>One mg ml$^{-1}$ of protein corresponds to</td>
<td>1.74 AU (at 280 nm)</td>
<td>1.45 AU (at 280 nm)</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>4.42</td>
<td>4.52</td>
</tr>
<tr>
<td>Charge at pH 7</td>
<td>-11.29</td>
<td>-10.28</td>
</tr>
</tbody>
</table>

2.4.3. In vitro binding properties of (scFv')$_2$-SZH9 antibody

In Western-blot analysis (scFv')$_2$-SZH9 Ab recognized the dimeric form of murine, human and dog VEGF and did not show any cross-reactivity to VEGF-E that also binds to VEGF-R2 or unspecific binding to BSA (Figure 2.6 A). Similar results were obtained in ELISA with human, murine and dog VEGF immobilized on plastic (Figure 2.6 B).

In competitive ELISA experiments, (scFv')$_2$-SZH9 Ab showed almost equal binding affinity for cfVEGF (Figure 2.6 C) and hVEGF (Figure 2.6 E). Addition of up to 10 µM VEGF-E or BSA did not reduce the ELISA signal in the competitive assay, indicating that (scFv')$_2$-SZH9 Ab was not cross-reactive with VEGF-E that has binding and signalling properties to VEGF-R2 like VEGF-A, despite their shared sequence homology [169]. The apparent calculated dissociation constant ($K_d = A^{-1}$) of (scFv')$_2$-SZH9 Ab was for dogVEGF$_{164} = 16$ nM and for humanVEGF$_{165} = 20$ nM, respectively (Figure 2.6 D and F, respectively), reflecting binding affinities in the low nM range, similar to most scFv Abs selected from this phage-display library [392].
Figure 2.6: In vitro (scFv)2 Ab-binding assays. (A) Western blot analysis of (scFv)2-SZH9 Ab binding to VEGFs. BSA (line 1), purified recombinant VEGF-E (lane 2), dog VEGF164 (lane 3), human VEGF165 (lane 4), and murine VEGF164 (lane 5) were used (0.25 μg lane\(^{-1}\)) for SDS-PAGE on a 13% gel in non-reducing conditions. Anti-VEGF (scFv)2-SZH9 Ab was used as primary Ab. VEGF dimers (d) are indicated (approx. 43 kDa). (B) Binding curves of anti-VEGF (scFv)2-SZH9 Ab to immobilized mouse, dog, and human VEGF in ELISA. As negative controls BSA and VEGF-E were used. (C and E) Competitive binding inhibition of (scFv)2-SZH9 Ab to ELISA plates pre-coated with VEGF in the presence of soluble VEGF. Competitive ELISAs were performed according to Friguet et al. [405] using BSA, orfVEGF-E, hVEGF165, and cfVEGF164. Data are expressed as the mean ± SD of the mean (n = 3). The total concentration of (scFv)2-SZH9 Ab was 10\(^{-9}\) M. Equilibrium was achieved at 4°C after 12 h of incubation (D and F). Scatchard binding plots of cfVEGF164 and hVEGF165 to (scFv)2-SZH9 Ab were calculated from the binding data according to Friguet et al.[405], where \(\nu\) is the fraction of bound (scFv)2 Ab and \(a\) the concentration of free antigen at equilibrium. The total concentration of (scFv)2-SZH9 Ab was 10\(^{-9}\) M. All samples were analysed at least in duplicate. Equilibrium was achieved at 4°C after 12 h of incubation.
2.5. DISCUSSION

The key motivation and advantages for the generation of therapeutic species cross-reactive scFv Abs is that preclinical evaluation is facilitated [406]. To inhibit tumour xenograft growth and neovascularisation, blocking of both tumour cell and host expressed VEGF is required [407]. Notably, Bevacizumab (rhMAb VEGF; Avastin™; Genentech, South San Francisco, CA), a humanized monoclonal Ab directed at hVEGF [19], the most advanced anti-VEGF Ab in clinical development, does not display species cross-reactivity [407]. For this purpose species cross-reactive anti-VEGF scFv Abs able to recognize mouse and human VEGF were selected from a human derived phage-display library (Chapter 2.4.1, figure 2.2). These small scFv Ab molecules have several advantages. The genes of selected scFv Abs are already cloned simplifying their production and further modifications. Moreover, there is no need to humanize the Ab, obtained e.g. from rabbits or sheep [391;394]. One drawback is their very short half-life \textit{in vivo} [395]. In order to increase the \textit{in vivo} circulation time of the produced Ab 3 C-terminal cysteines were introduced to express the Ab in a (scFv')2-homodimeric (Chapter 2.4.1, figure 2.4) form. The cysteines modification would also allow to couple the scFv Ab to the surface of PEG-liposomes [408]. Because for therapeutic applications high amounts of protein are needed, the scFv Ab was subcloned in the methyloxytrophic expression system \textit{P. pastoris} and the Ab produced in a 3.7 liter bio-reactor. After selection of high expression clones and optimizing production conditions in the bio-reactor, yields of 40 – 50 mg litre\(^{-1}\) culture medium of soluble (scFv')2 Ab were obtained. The purified (scFv')2-SZH9 Ab has \(K_d\) values to VEGF in the low nanomolar range. Phage Ab display is an efficient method for generating Abs \textit{in vitro}, particularly if purified protein as antigen is available. (ScFv')2-SZH9 Ab will be a useful tool for specific targeting and neutralization of human and mouse VEGF in tumour mouse models to investigate the role of this multi-functional factor in tumour angiogenesis.
3. *IN VIVO* CHARACTERISATION OF THE SPECIES CROSS-
REACTIVE ANTI-VEGF (SCFV')2-SZH9 ANTIBODY

3.1. ABSTRACT

The selection, bioengineering and expression of a species cross-reactive anti-VEGF (scFv')2 Ab homodimer recognizing VEGF *in vitro* in the low nanomolar range has been described in chapter 2.4. The question arises whether this anti-VEGF Ab could show an inhibitory effect on angiogenesis *in vivo*, an important issue for the development of therapeutic applications.

The (scFv')2-Ab showed a strong and reproducible inhibition effect of VEGF-induced blood vessel formation in the chicken chorioallantoic membrane (CAM) assay. Pharmacokinetic and biodistribution experiments in tumour-bearing mice showed that the (scFv')2-Ab is rapidly removed from the blood with a typical biphasic profile. Almost 3% of the injected (scFv')2 Ab dose per gram of tissue (%IDg⁻¹) localized in xenografted human A673 rhabdomyosarcoma tumours after injection of the ¹²⁵I-labelled Ab. Therapeutic injection of the (scFv')2-Ab in a F9 teratocarcinoma syngraft mouse model inhibited tumour growth. *In vitro* cultivation of F9 cells in presence of (scFv')2-Ab resulted in no significant reduction of growth indicating that the inhibition of tumour progression was not caused by cytotoxic effects but by the inhibition of tumour-induced angiogenesis.

Here, the results of angiogenesis inhibition, pharmacokinetics and biodistribution of ¹²⁵I-labelled anti-VEGF (scFv')2-Ab in the xenograft human A673 rhabdomyosarcoma CD1 nude mouse model and the therapeutic application of (scFv')2-Ab in the syngraft F9 teratocarcinoma SV129 mouse model are presented.

3.2. INTRODUCTION

Most aggressive solid tumours cannot grow beyond a certain mass without the formation of new blood vessels (angiogenesis), and a correlation between microvessel density and tumour invasiveness has been reported [64].

A direct role of VEGF in tumourigenesis has been clearly demonstrated *in vivo* by various means. Treatment with either VEGF-neutralizing monoclonal Abs [115;409;410], or antisense VEGF oligonucleotides [411] strongly inhibited the growth of tumours transplanted in mice. Inhibition of endogenous expression of VEGF leads
to reduced tumourigenicity [115;412] or even to a complete inhibition of xenograft tumour progression, when both tumour and host VEGF can be neutralized [407].

The primary objective was to study the properties of (scFv')$_2$-SZH9 Ab in vivo, in particular whether it would be possible to block VEGF-induced angiogenesis in the CAM assay and whether tumour progression could be inhibited by performing anti-angiogenic therapy in tumour-bearing mice. The CAM assay is widely used because pro-and anti-angiogenic effects can easily be investigated in vivo. The murine F9 teratocarcinoma SV129 tumour mouse model was used as a first test system. This highly vascularized syngraft model was chosen to compare the selected (scFv')$_2$-SZH9 Ab with the anti-VEGF scFv-V65 Ab that was selected against murine VEGF$_{164}$ and known to inhibit tumour progression in syngraft mouse models [413;414]. The standard models for testing cancer strategies in preclinical studies normally include cultured human tumour cell lines and rodent xenografts that comprise many of the same human lines grown subcutaneously in immuno-compromised animals. It must be taken into account that these models contain tumour cell lines that are passaged for numerous generations in culture and which might not be representative of the tumour in its native state. Cells in culture lack the architecture and cellular complexity of real tumours, which include inflammatory cells, vasculature and other stromal components. Whereas normal tumours in humans develop over a number of years, mouse grafts are chosen in a way that they can be assayed in the timeframe of days or weeks, as e.g. F9 teratocarcinoma cells form a primary tumour of 1 cm$^3$ within 8 – 10 days [344]. Perhaps most importantly, even if a syngraft represents significant aspects of the tumour from which it was derived, it probably captures only a fraction of the total genetic heterogeneity of a given tumour subtype.

In this chapter the in vivo properties of the anti-VEGF (scFv')$_2$-SZH9 Ab are described performing inhibition studies of VEGF-induced angiogenesis on the CAM assay and investigating tumour growth inhibition in a syngraft tumour mouse model. Pharmacokinetic properties were studied by injection of radiolabelled (scFv')$_2$-SZH9 Ab in a xenograft human A673 tumour mouse model.
3.3. MATERIAL AND METHODS

3.3.1. Reagents, cells and animals

Rat 208F fibroblast, murine F9 teratocarcinoma (CRL-1720) and human A673 rhabdomyosarcoma (CRL-1598) cells were obtained from American Type Culture Collection (ATCC; Rockville, MD) and human umbilical vein endothelial (HUVEC) cells were obtained from PromoCell (Heidelberg, Germany). Female SV 129 mice and CD1 nude mice were purchased from Charles River Wiga (Sulzfeld, Germany) and kept in standard housing and normal diet at the animal facility. Animal studies were performed under the licenses (No. 75533 and 75534) issued to R. A. Schwendener by the Veterinary Department of the Canton Aargau, Switzerland. The ethical guidelines that were followed meet the standards required by the UKCCCR guidelines [415]. White fertilized chicken eggs Lohman LSL strain were purchased from Animalco (Staufen, Switzerland) and kept in a Elvatotto 120 humidified incubator at 37.9°C. Cell viability was tested with WST-1 solution from Roche Diagnostics (Mannheim, Germany). Matrigel and 20% Lipovenös were obtained by Pharmacia and Fresenius, respectively. For radioactive scFv Ab-labelling, \(^{125}\)Iodine (2 mCi, 20 μl\(^{-1}\)) was obtained from Amersham Pharmacia. For preparation of cDNA the RNeasy Kit was obtained from Qiagen, and the cDNA Synthesis Kit was from Invitrogen/Life Technologies (San Diego, CA), dNTPs were from Roche Diagnostics, PFU DNA polymerase from Stratagene and primers from Microsynth (Balgach, Switzerland). All buffer salts and other chemicals were from Fluka or Sigma (Buchs, Switzerland).

3.3.2. Ex ovo chick chorioallantoic membrane (CAM) assay

Experiments were performed on chicken embryos grown by the ex ovo culture method [416]. Methylcellulose discs (0.5%, w/v; 5 mm in diameter) supplied with 3 μg of dog VEGF\(_{164}\) in presence of 50 μg (scFv')\(_2\) Ab were grafted on top of the growing CAM at day 10 of embryonic development. Parallel grafting experiments were performed with plain discs, discs supplied with PBS or 3 μg of cfVEGF\(_{164}\). After 48 h, a lipid emulsion (20% Lipovenös) was injected under the allantoic membrane to obtain a better contrast. The angiogenic response was evaluated by 3 independent observers under a stereomicroscope and recorded with a digital camera.
3.3.3. *In vitro* cytotoxicity of anti-VEGF (scFv')<sub>2</sub>-SZH9 antibody

For determining cytotoxic effects, murine F9 teratocarcinoma, human A673 rhabdomyosarcoma and human umbilical vein endothelial (HUVEC) cells were seeded at a density of 10<sup>4</sup> well<sup>-1</sup> in sterile 96 well plates in the presence of DMEM and HUVEC growth-medium, supplemented with 10% FBS and antibiotics. Cells were cultivated in presence of anti-VEGF (scFv')<sub>2</sub>-SZH9 Ab at concentrations from 0.5 – 250 µg ml<sup>-1</sup> at 37°C, 5% CO<sub>2</sub>. After 48 h the supernatants were removed and 100 µl well<sup>-1</sup> freshly diluted WST-1 solution in medium (1 : 10 v/v) were added and incubated for 60 min at 37°C and 5% CO<sub>2</sub>. As a typical tetrazolium salt, WST-1 is degraded to formazan by the mitochondrial succinoxidase system. Formazan can be detected photometrically with a maximum of extinction at 450 nm wavelength. The color change corresponds directly to the number of living cells. Cell viability was determined by measuring the absorption at 410 nm using a Dynatech MR5000 plate reader (Microtec Produkte, Embrach, Switzerland).

3.3.4. RNA isolation and cDNA synthesis

Murine F9 teratocarcinoma and rat 208F fibroblast cells (4 x 10<sup>6</sup>) were maintained in culture as described above for 24 h and total RNA was isolated with RNeasy kit and cDNA synthesized by RT-PCR using cDNA synthesis kit. Samples were stored at -20°C or immediately used for PCR. The oligonucleotide primers used for VEGF cDNA amplification were 5' ATG AAC TTT CTG CTC TCTTG 3' as sense primer and 5' TCA CCG CCT CGG CTT GTC 3' as anti-sense primer. Oligonucleotide primers used for the GAPDH gene amplification were 5' ATG GTGAAG GTC GGTGTCAACG 3' as sense primer and 5' CGC CAG TAG ACT CCA CGA CAT ACT CAG 3' as anti-sense primer. GAPDH mRNA was chosen as internal standard RNA for PCR. Three µl of cDNA obtained from 208F and F9 samples were brought to a final volume of 50 µl containing the following compounds: sterilized water, 1 x reaction buffer (20 mM Tris-HCl (pH 8.8), 2 mM MgSO<sub>4</sub>, 10 mM KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton®X-100, 0.1 mg ml<sup>-1</sup> nuclease-free BSA, Stratagene), dNTPs 0.5 mM each, 2.5 U PFU DNA polymerase, sense and antisense primers (0.2 pmol µl<sup>-1</sup> each). Amplification was carried out in a thermal DNA cycler (TECHNE Progene, Cambridge, UK) with an initial denaturation step of 5 min at 95°C, followed by 30 cycles. Each cycle consisted of 1 min at 95°C
(denaturation step), 1 min at 60°C (hybridisation step), 90 s at 72°C (elongation step). A final elongation step was carried out for 10 min at 72°C. The PCR samples were stored at -20°C. The amplified PCR products were verified on 0.8% agarose gels (0.5 x TBE) and compared with PCR products derived using cDNA templates for VEGF 121, 164, and 188 respectively.

3.3.5. Determination of soluble VEGF in cell-culture supernatants
Murine F9 teratocarcinoma and rat 208F fibroblast cells (4 x 10^6) were maintained in DMEM plus 10% heat-inactivated fetal bovine serum, 1% L-glutamine, penicillin 100 U ml^-1 and streptomycin 100 μg ml^-1 in gelatine coated wells. Supernatant was prepared by centrifugation (2 000 g, 10 min) of freshly collected cells and stored at -70°C until the VEGF assay was performed. Soluble VEGF was measured by ELISA (Quantikine, R & D Systems, Minneapolis, MN), according to the manufacturers instructions.

3.3.6. Therapeutic application of (scFv')_2-SZH9 antibody in the syngraft
F9 teratocarcinoma SV129 mouse model
Exponentially growing F9 cells (7 x 10^6/50 μl) were injected s.c. on the abdominal side of SV 129 mice. The mice (6 - 8 per group) received 25 mg kg^-1 of (scFv')_2 Abs in 100 μl by i.v. injection into the tail. Tumour growth was measured in a blinded manner with a calliper every day and volumes calculated using the following equation: \( V = \pi a b^2 / 6 \) (a = largest tumour diameter, b = perpendicular diameter). Mice were sacrificed 8 days after onset of treatment.

3.3.7. Biodistribution of ^{125}\text{I}-(scFv')_2-SZH9 antibody in the xenograft A673 rhabdomyosarcoma mouse model
For determination of pharmacokinetic parameters (scFv')_2-SZH9 Ab was iodinated (^{125}\text{I}) using the iodogen method [417] (Pierce, Rockford, IL). Briefly, 200 μCi ^{125}\text{I} was added to 200 μg (scFv')_2-SZH9 Ab (in 100 μl PBS, pH 7.4) and incubated for 1 h at RT. Unbound ^{125}\text{I} was removed on a 8 ml PD-10 column (Amersham Pharmacia) equilibrated in PBS. Fractions of 500 μl were collected and the radioactivity determined by liquid scintillation countering (Packard Instruments, Groningen, Netherlands).
The radiolabelled Ab fragment was analysed by non-reducing 13% SDS-PAGE and by radioactive counting of gel-fractions. The labelling efficiency of (scFv')2-SZH9 Ab was 115 μCi, corresponding to 58% of the initial radioactivity. Female CD1 nude mice with subcutaneously implanted A673 cells at each flank, were injected i.v. with 3 μg (5 μCi) of (scFv')2 Ab in 100 μl PBS. Tumour size ranged from 0.5 - 1.8 g. Three animals were used for each time point. Mice were killed at 0.17, 0.5, 1, 2, 4, 6, and 24 h after injection, the organs were weighed and the radioactivity counted. Results of representative organs are expressed as percent injected dose of Ab per gram of tissue (%ID g⁻¹) or per organ (%ID organ⁻¹), respectively. Pharmacokinetic parameters (t½ and AUC) were calculated using the GraphPad Prism (Version 4.0) software.

3.3.8. Statistical analysis
All values were presented as means ± SE. Unpaired t-test was performed for comparison between PBS controls and treated groups: P < 0.05 was considered significant. Calculations were made using the GraphPad Prism (Version 4.0) software.

3.4. RESULTS

3.4.1. Inhibition of VEGF-induced angiogenesis on the chicken chorioallantoic membrane (CAM) using (scFv')2-SZH9 antibody
To evaluate whether the selected (scFv')2-SZH9 Ab exhibits neutralizing activity against VEGF and as consequence can block angiogenesis in vivo, it was applied in presence of VEGF to methylcellulose discs on the CAM ex ovo. Neovascularisation of the CAM is maximal at day 10 of the developmental stage. The effect of the Ab on blood vessel formation was monitored at different time points by microscopic inspection. (ScFv')2-SZH9 Ab showed a strong and reproducible anti-angiogenic effect in the CAM assay (Figure 3.1). Compared to the unspecific control Ab (scFv')2-A1 Ab (Figure 3.1 C), (scFv')2-SZH9 Ab inhibited the sprouting of vessels (Figure 3.1 D), initialised by 7 μM dog VEGF164 which was pre-incubated for 24 h at 4°C with the corresponding Abs before applying onto the CAM. The maximal inhibitory effect was observed with a 10 x molar increased concentration of anti-VEGF (scFv')2-SZH9 Ab at day 3. The established vessels, which had formed before

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application of the (scFv')$_2$ Abs, were also affected as could be seen at higher magnifications (data not shown). The vessel structure had a diffuse and unsharp appearance. This destabilisation of existing blood vessels indicates that (scFv')$_2$-SZH9 Ab was also able to recognize and neutralize chicken VEGF, which is known to be required for stabilizing the pre-existing blood vessels.

Taken together, these results indicate that (scFv')$_2$-SZH9 Ab is able to recognize dog and probably chicken VEGF$_{164}$ and neutralize its angiogenic activity.

**Figure 3.1**: In vivo chicken CAM assay of angiogenesis. Representative CAMs from 10 days old chick embryos were incubated for 72 h with PBS (A), VEGF (B) and cfVEGF in presence of a control (scFv')$_2$-A1 Ab (C) and the anti-VEGF (scFv')$_2$-SZH9 Ab (D). Up-regulation of angiogenesis was seen by sprouting of new blood vessels from larger pre-existing vessels (indicated by arrows) in (B) and (C). In contrast, no up-regulation of angiogenesis was visible in presence of the anti-VEGF (scFv')$_2$-SZH9 Ab. The grafts remained essentially vessel-free (D). Dashed circles delineate the methylcellulose disc, the bar represents 2.5 mm.

### 3.4.2. Biodistribution of $^{125}$I-(scFv')$_2$-SZH9 antibody in the xenograft human A673 rhabdomyosarcoma mouse model

To investigate the circulation time of the (scFv')$_2$-SZH9 Ab in vivo after intravenous injection, a biodistribution experiment in the A673 rhabdomyosarcoma mouse model was performed (Table 3.1).

**Table 3.1**: Biodistribution of anti-VEGF (scFv')$_2$-SZH9 Ab (%ID per organ ± SE of the mean).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Blood</th>
<th>Tumour</th>
<th>Liver</th>
<th>Heart</th>
<th>Kidney</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>39.5 ± 1.4</td>
<td>0.6 ± 0.2</td>
<td>8.4 ± 0.3</td>
<td>0.3 ± 0.0</td>
<td>11.5 ± 0.4</td>
<td>0.8 ± 0.0</td>
</tr>
<tr>
<td>30</td>
<td>27.4 ± 1.6</td>
<td>1.0 ± 0.2</td>
<td>3.9 ± 0.2</td>
<td>0.3 ± 0.0</td>
<td>6.4 ± 0.7</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>60</td>
<td>23.4 ± 3.5</td>
<td>1.3 ± 0.2</td>
<td>2.7 ± 0.2</td>
<td>0.2 ± 0.0</td>
<td>3.7 ± 0.4</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td>120</td>
<td>15.7 ± 2.9</td>
<td>1.4 ± 0.4</td>
<td>2.0 ± 0.3</td>
<td>0.2 ± 0.0</td>
<td>2.0 ± 0.3</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>240</td>
<td>11.8 ± 0.6</td>
<td>1.0 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>0.2 ± 0.0</td>
<td>1.6 ± 0.1</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>480</td>
<td>7.9 ± 1.2</td>
<td>0.9 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>0.1 ± 0.0</td>
<td>1.0 ± 0.2</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>1440</td>
<td>0.3 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

Human A673 rhabdomyosarcoma xenografted female CD1 nude mice (n = 3) were injected i.v. with $^{125}$I-labelled anti-VEGF (scFv')$_2$-SZH9 Ab and killed after the indicated time points. Results are expressed as % of injected radioactivity per total organ (± SE of the mean).
Radioiodinated (scFv')2-SZH9 Ab was injected in the tail vein of mice with subcutaneously implanted A673 tumours, which are known to express high levels of VEGF [115]. Ab distribution in various organs was obtained at different time points (Figure 3.2, table 3.1).

(ScFv')2-SZH9 Ab is rapidly eliminated from the blood through the kidneys, with a typical biphasic profile resulting in a blood distribution half-life of $t_{1/2a} = 0.2$ h and an elimination half-life of $t_{1/2ß} = 3.3$ h. Almost 3% percent of the injected (scFv')2 Ab dose per gram of tissue (%IDg$^{-1}$) localized in the tumour 4 h after injection (Figure 3.2).

![Figure 3.2: Pharmacokinetic profile of 125I-labelled (scFv')2-SZH9 Ab in A673 tumour-bearing mice. Tumour, liver, kidney and blood levels are expressed as percent injected dose per gram (%IDg$^{-1}$).](image)

Tumour to blood ratios at 1, 2, 6 and 24 h after injection were 0.2, 0.3, 0.4 and 0.9, respectively, the tumour to blood ratios remained for SZH9 below 1 at any time. (ScFv')2-SZH9 Ab did not accumulate in the liver or other organs, with %ID g$^{-1}$ values at 24 h for the main organs ranging between 0.2 and 0.7.

Based on the AUC$_{0-24h}$ values the total Ab recovery was 9.8%, in particular 6.9% for blood, 0.6% for tumour, 1.0% for liver, 0.1% for heart, 0.1% for spleen and 1.1% kidney, respectively (Table 3.2).

<table>
<thead>
<tr>
<th>Distribution compartments of intravenously injected (scFv')2-SZH9 Ab</th>
<th>Normalized AUC$_{0-24h}$ values (%)</th>
<th>Estimated Ab (µg) ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>100</td>
<td>500</td>
</tr>
<tr>
<td>Blood</td>
<td>6.9</td>
<td>34.5</td>
</tr>
<tr>
<td>Tumour</td>
<td>0.6</td>
<td>3</td>
</tr>
<tr>
<td>Liver</td>
<td>1.0</td>
<td>5</td>
</tr>
<tr>
<td>Heart</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.1</td>
<td>5.5</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

‡ Ab distribution and circulation after intravenous injection of 500µg of (scFv')2-SZH9 Ab from estimated AUC$_{0-24h}$ values. Ab distribution was calculated using normalized AUC$_{0-24h}$ values and amount of total injected Ab.
3.4.3. Therapeutic application of (scFv')$_2$-SZH9 antibody in the syngraft F9 teratocarcinoma SV129 mouse model

Next, it was tested whether the (scFv')$_2$-SZH9 Ab could inhibit murine F9 teratocarcinoma growth in mice. First, it was verified *in vitro* that F9 cells do indeed transcribe different VEGF mRNA isoforms, mainly isoform 121 and 164 (Figure 3.3 A) and express and secrete VEGF (Figure 3.3 B). The demonstrated expression of VEGF by these tumour cells makes them a good model system to test the anti-angiogenic effect of anti-VEGF scFv Abs. Anti-VEGF (scFv')$_2$-SZH9 Ab was injected into tumour-bearing SV 129 mice (Figure 3.3 C and D) and compared with the homodimeric (scFv')$_2$-V65 Ab, an anti-VEGF scFv Ab selected against mouse VEGF$_{164}$ that was shown to block tumour-induced angiogenesis in a xenograft rat FE-8 CD1 nude mouse model [413;414]. The s.c. injection of F9-cells results in the formation of well-vascularized tumours of reproducible size within 5 to 10 days. As shown in figure 3.3 C, daily i.v. injection of (scFv')$_2$-V65 Ab and (scFv')$_2$-SZH9 Ab, beginning 24 h after tumour cell implantation, decreased tumour volumes by 56% and 46%, respectively (Figure 3.3 D and table 3.3).
Figure 3.3: Properties and therapeutic application of the anti-VEGF (scFv')2-V65 and SZH9 Abs in the syngraft F9 teratocarcinoma SV 129 mouse model. (A) VEGF isoform transcription in 208F fibroblast and F9-teratocarcinoma cells after 24 h cell culture. Using cDNA templates for VEGF isoforms 121 (lane 1), 164 (lane 2), 188 (lane 3), and 121/164/188 (lane 4), or total cDNA isolated from 208F (lane 5) and F9 (lane 6) cell cultures. (B) VEGF levels in the supernatant of 4 x 10⁶ 208F and F9 cells after 8 h and 24 h, using the Quantikine mouse VEGF immunoassay. (C) F9 cells (7x10⁶) were injected s.c. in SV 129 mice (n = 6 - 8 mice per group) and treated i.v. at days 1 - 5 (S) with 25 mg kg⁻¹ unspecific control (scFv')2-A1 Ab, anti-VEGF (scFv')2-SZH9 Ab, anti-VEGF (scFv')2-V65 Ab or the same volume PBS. Relative tumour growth was normalized to day 1. (D) Bar graph of tumour sizes at day 8 show the calculated averages of tumour volume reduction.

Table 3.3: Tumour volume reduction at day 8, 3 days after the last therapy.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumour volume ± SEM (cm³)</th>
<th>P-value</th>
<th>Normalized tumour volume ± SEM (%)</th>
<th>Change of tumour volume compared to PBS control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0.46 ± 0.17</td>
<td>-</td>
<td>100 ± 37</td>
<td>-</td>
</tr>
<tr>
<td>(ScFv')2-A1</td>
<td>0.53 ± 0.14</td>
<td>0.5394</td>
<td>114 ± 30</td>
<td>+ 14</td>
</tr>
<tr>
<td>(ScFv')2-V65</td>
<td>0.20 ± 0.04</td>
<td>0.1795</td>
<td>44 ± 8</td>
<td>- 56</td>
</tr>
<tr>
<td>(ScFv')2-SZH9</td>
<td>0.25 ± 0.03</td>
<td>0.2969</td>
<td>54 ± 7</td>
<td>- 46</td>
</tr>
</tbody>
</table>
Figure 3.4: Absence of in vitro cytotoxicity of anti-VEGF (scFv')₂-SZH9 Ab on murine F9 teratocarcinoma, human A673 rhabdomyosarcoma and human umbilical vein endothelial (HUVEC) cells. For the proliferation assays, cells were seeded at a density of $10^4$ per well in 96 multiwell plates and cultured as described (Chapter 3.3.3) in the presence of (scFv')₂-SZH9 Ab at the indicated concentrations. After 48 h, cell viability was determined by adding WST-1 solution, $n = 3$ ± SE of the mean.

3.5. DISCUSSION

As described in the literature for different recombinant Abs, dimerisation resulted in prolonged Ab retention in the blood and in higher %ID g⁻¹ concentrations retained in tumour and in normal tissues, compared with the corresponding monomeric Abs [395]. In this study comparisons between scFv Ab monomers and engineered dimers were not performed. Unlike scFv Abs which show vascular or stromal targeting properties [345;418], the tumour to blood ratios remained for SZH9 below 1 at any time. Dissimilar to IgG Abs, (scFv')₂-SZH9 Ab did not accumulate in the liver or other organs, with %ID g⁻¹ values at 24 h for the main organs ranging between 0.2 and 0.7. Based on the AUC₀-2₄h values the total Ab recovery was 9.8%, in particular 6.9% for blood, 0.6% for tumour, 1.0% for liver, 0.1% for heart, 0.1% for spleen and 1.1% kidney, respectively (Table 3.2).

It was shown by Muller et al. that the neutralization mechanism of Bevacizumab involves steric blocking of the receptor site, with an induction of conformational changes in VEGF [419]. The neutralization mechanisms on the molecular level of (scFv')₂-SZH9 Ab and (scFv')₂-V65 Ab are not known yet. However, to verify that the obtained tumour growth inhibition is an anti-angiogenic effect and not caused by direct cytotoxicity of the (scFv')₂-V65 Ab and (scFv')₂-SZH9 Ab is shown here and also by Vitaliti et al. [414]. The investigated scFv SZH9 Ab can inhibit the formation of new blood vessels from pre-existing ones (Figure 3.1 D). F9-teratocarcinoma cells in culture do not show any growth inhibition in presence of (scFv')₂-SZH9 Ab.
(Figure 3.4). If the tumours are allowed to reach a certain size prior to the onset of the treatment, the effect of inhibition is less complete and tumours may escape from the anti-angiogenic treatment. It is noteworthy, that these results argue against the possibility that a partial blockade of VEGF might be sufficient for optimal inhibition of VEGF-dependent tumour angiogenesis. This is in apparent contrast to the findings in gene knockout experiments in mice where partial inactivation of the VEGF gene resulted in embryonic lethality [59]. This phenomenon might reflect molecular and cellular alterations resulting from genomic instability and/or increased mutation rates in the tumour cells. This might provide the molecular framework for a compensatory up-regulation of angiogenic molecules other than VEGF, or alternatively, for the downregulation of anti-angiogenic genes under growth selective conditions. However, rapid blood clearance, which is common to small molecules, with a half-life shorter than 30 min for the α-phase results in low quantitative tumour retention [346]. Thus, it is reasonable to assume that the therapeutic effect of anti-VEGF (scFv')$_2$ Abs is mainly dictated by the relation between serum concentration and dissociation constant $K_d$. Further it was attempted to increase the avidity of (scFv')$_2$-SZH9 and the circulation half-life by coupling of the (scFv')$_2$-scFv Abs to the surface of PEG-liposomes for target specific drug delivery and to combine an anti-angiogenic action with a cytotoxic action using immunoliposomes loaded with cytotoxic drugs reviewed in chapter 1.5.
4. IMMUNOLIPOSOME THERAPY APPROACH IN THE SYNGRAFT F9 TERATOCARCINOMA AND IN THE TRANSGENIC RIP1TAG2 MOUSE MODEL

4.1. ABSTRACT

A new type of immunoliposomes for the neutralization of VEGF in solution was developed. The anti-VEGF (scFv')2-Ab was coupled by the C-terminal thiol groups via the bifunctional linker molecule sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) to the distal end of 1,2-dipalmitoyl-snglycero-3-phosphoethanolamine-N-amino(polyethylene glycol)2000 (DPPE-PEG-NH2) on small unilamellar phospholipid vesicles (liposomes). Up to 100 scFv-SZH9 molecules could be attached to the surface of one liposome with a mean diameter of 100 nm. The coupling efficiency of scFv-Ab-immunoliposomes varied in the range of 10 to 30%. The binding properties of these immunoliposomes were tested in a competitive ELISA showing comparable binding properties as uncoupled anti-VEGF (scFv')2-Ab. Because of the passive tumour targeting properties of liposomes in vivo, they appeared attractive as carriers of anti-angiogenic molecules. The effects of anti-VEGF scFv Ab-immunoliposomes were investigated in two therapeutic tumour mouse models. Unfortunately, neither in the syngraft F9 teratocarcinoma SV129 mouse model nor in a transgenic Rip1Tag2 model did the therapeutic application of scFv-SZH9-immunoliposomes result in a reduction of tumour growth. The therapy was not successful, probably because of the low dosage. In the Rip1Tag2 model, the most likely reason was that the duration of the therapy was too short.

4.2. INTRODUCTION

Bevacizumab (Avastin®), a recombinant humanized monoclonal Ab to VEGF, is a new anti-cancer drug that was approved in 2004 by the American Food and Drug Administration (FDA) for clinical use. Preclinical research revealed, that Bevacizumab specifically inhibits VEGF, has activity in multiple cancer cell lines and is synergistic with several cancer chemotherapeutic agents. In humans, Bevacizumab has a long circulation half-life, allowing intravenous administration once every 2 to 3 weeks [19]. Recent progress in Ab phage-display technology has improved the possibility to
select for human-derived scFv Abs that have desirable properties for immunotherapy, for example the selection of species-cross reactive Abs as demonstrated in chapter 2.4.1. The use of scFv Abs rather than mAbs offers several advantages. Modification of the Ab, such as fusion with molecules or toxic protein sequences, can be performed easily by gene technology methods. However, due to their small size and their monovalence the major drawback of scFv Abs is their low affinity and their fast clearance from the blood as shown in chapter 3.4.2 [420].

The in vivo circulation time of Abs and Ab fragments can be altered by modification with PEG [421] or by coupling to the surface of PEG-liposomes [422]. Such modifications lead to extended plasma half-lifes, reduced immunogenicity and increased resistance to proteolysis [423]. One method of attaching proteins to the distal end of maleimide modified PEG chains on liposomes (Figure 4.3) is by coupling of C-terminal thiol groups on the protein (Figure 4.4) [408].

It has been shown in tumour models that i.v. administrated liposomes of sizes between 100 to 200 nm are able to passively extravasate from the bloodstream via the fenestrated leaky tumour vasculature and accumulate in the tumour stroma [424]. This behaviour makes liposomes a promising tool for stabilizing small molecules, which should preferentially neutralize soluble antigens, e.g. VEGF expressed in growing tumours.

Anti-VEGF scFv-SZH9 immunoliposomes were used for a first therapeutic approach in the syngraft F9 teratocarcinoma SV129 mouse model. This tumour mouse model is well suited as a test system for tumour angiogenesis due to the high vascularisation and its fast growth (Chapter 3.4.3). In the second in vivo trial immunoliposomes were applied in transgenic Rip1Tag2 mice that develop between 10-14 weeks β-cell tumours in the pancreatic islets of Langerhans [96] (Figure 4.1).

![Figure 4.1](image_url)

**Figure 4.1:** Illustration of the transgenic islet cell carcinoma model in which expression of the simian virus 40 large T oncprotein (Tag) under control of the rat insulin promoter elicits genesis of insulinomas. Expression of Tag begins in the β cells of the islets of Langerhans during embryogenesis. Four distinct stages of tumourigenesis are discernible: (1-3 weeks) normality, (3-5 weeks) hyperproliferation (hyperplasia), (5-7 weeks) onset of angiogenesis, and (10-14 weeks) formation of solid, encapsulated tumours [425].
Although islet cell insulinomas are rare in humans, this transgenic tumour model represents an important tool for studying development and progression of common human epithelial cancers, such as breast, prostate and bladder carcinoma and tumour angiogenesis.

In this chapter the construction and in vitro binding capacity of anti-VEGF scFv-SZH9 immunoliposomes are described. Further, in two tumour mouse models, i.e. the syngraft F9 teratocarcinoma model and the transgenic Rip1Tag2 model, the immunoliposomes were applied to inhibit tumour-induced angiogenesis.

4.3. MATERIAL AND METHODS

4.3.1. Reagents, cells and animals

Hydrogenated soy phosphatidylcholine (HSPC) was obtained from L. Meyer (Hamburg, Germany) and cholesterol recrystallised from methanol was purchased from Fluka (Buchs, Switzerland). 1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)2000] (DPPE-PEG-NH₂) was synthesized by Shearwater Polymers, Inc. (Enschede, The Netherlands). Crystalline Sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) was purchased from Pierce (Lausanne, Switzerland). The 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester (BODIPY) was used for fluorescence labelling of the scFv Abs and was obtained from Molecular Probes, (Leiden, The Netherlands). FICOLL P70 was obtained from Amersham Pharmacia Biotech.

Murine F9 teratocarcinoma was from the American Type Culture Collection (ATCC; Rockville, MD). Female SV 129 mice were purchased from Charles River Wiga (Sulzfeld, Germany) and Rip1Tag2 mice were a gift from G. Christofori (Institute of Biochemistry and Genetics, Department of Clinical-Biological Sciences, University of Basel) and kept in standard housing and normal diet at the animal facility. Animal studies were performed under the licenses (No. 75533 and 75534) issued to R. A. Schwendener by the Veterinary Department of the Canton Aargau, Switzerland and under the license (No. 1878) issued to G. Christofori by the Veterinary Department of the Canton Basel, Switzerland. The ethical guidelines that were followed meet the standards required by the UKCCCR guidelines [415].
4.3.2. PEG-liposome preparation by extrusion

Multilamellar vesicles composed of HSPC (80 mg ml\(^{-1}\)), cholesterol (8 mg ml\(^{-1}\)) and DPPE-PEG-NH\(_2\) (19.6 mg ml\(^{-1}\)) corresponding to 1 : 0.2 : 0.07 mol parts were prepared with the extrusion method described by Marty et al. [408] (Figure 4.2 and table 4.1). Briefly, the lipid mixture was dissolved in methanol / methylenechloride (1 : 1, v/v) and dried on a rotation evaporator (Büchi Labortechnik, Flawil, Switzerland). The dry lipid film was dispersed in PB (13 mM KH\(_2\)PO\(_4\), 54 mM NaHPO\(_4\), pH 7.4) and small unilamellar vesicles (SUV) were prepared by sequential (5 - 10 x) filter extrusion through Nuclepore membranes (Sterico, Dietikon, Switzerland) of 0.4 \(\mu\)m and 0.1 \(\mu\)m pore diameter with a Lipex extruder (Lipex Biomembranes Inc., Vancouver, Canada). Size and stability of the liposomes were analysed at a dilution of 1 : 100 (v/v) in PB with a submicron particle sizer (Nicomp Model 370, Santa Barbara, USA).

![Figure 4.2: Structures of lipids used for liposome preparation: A) HSPC; B) cholesterol and C) DPPE-PEG-NH\(_2\).](image)

![Table 4.1: Lipid composition used for the preparation of liposomes](table)

<table>
<thead>
<tr>
<th>Lipid</th>
<th>HSPC (mg ml(^{-1}))</th>
<th>Cholesterol (mg ml(^{-1}))</th>
<th>DPPE-PEG-NH(_2) (mg ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 mg ml(^{-1})</td>
<td>80 mg ml(^{-1})</td>
<td>8 mg ml(^{-1})</td>
<td>19.6 mg ml(^{-1})</td>
</tr>
<tr>
<td>100 mol%</td>
<td>20 mol%</td>
<td>7 mol%</td>
<td></td>
</tr>
<tr>
<td>104 (\mu)mol</td>
<td>20 (\mu)mol</td>
<td>7 (\mu)mol</td>
<td></td>
</tr>
</tbody>
</table>

4.3.3. Derivatisation of PEG-liposomes with sulfo-SMCC

The PEG-liposomes were modified with sulfo-SMCC to introduce maleimide groups according Marty et al. [408] (Figure 4.3). Briefly, liposomes containing 80 mg ml\(^{-1}\) HSPC and 7 mol% DPPE-PEG-NH\(_2\) corresponding to 2 \(\times\) 10\(^{18}\) amino groups on the surface of the liposomes in 1 ml PB were incubated with 7.2 mg crystalline sulfo-SMCC (9.4 \(\times\) 10\(^{18}\) molecules) for 30 min at 30°C. The molar ratio of amino groups to
sulfo-SMCC was 1:5. Unreacted sulfo-SMCC was removed by dialysis 12 h in HBSE (10 mM Hepes, 150 mM NaCl, 9.1 mM EDTA, pH 7.5).

Figure 4.3: Scheme of the modification of PEG-liposomes containing amino groups with sulfo-SMCC for the introduction of maleimide groups.

4.3.4. Coupling of scFv antibodies to sulfo-SMCC-PEG-liposomes for *in vivo* studies

Anti-VEGF (scFv')<sub>2</sub>-SZH9 Ab and control (scFv')<sub>2</sub>-A1 Ab (4 mg ml<sup>-1</sup>) in HBSE were reduced with a 2 mM final concentration of tributylphosphine (TBP) for 4 h at 4°C under a nitrogen atmosphere. The scFv Abs were coupled to the surface of sulfo-SMCC-PEG-liposomes according Marty et al. [344] (Figure 4.4). Briefly, after removing of excess sulfo-SMCC by dialysis, sulfo-SMCC modified PEG-liposomes corresponding to 7.2 x 10<sup>19</sup> maleimide groups in 8 ml HBSE were incubated with 30 mg scFv Abs (corresponding to 18 x 10<sup>17</sup> thiol groups) at a molar ratio of 40:1 in the presence of 2 mM TBP for 72 h at 4°C under a nitrogen atmosphere. The aminoreactive dye BODIPY FL®, SE was used for the determination of binding efficiency of the scFv Abs on liposomes. 100 µg scFv Ab in 100 µl HBSE were coupled with 5 µl BODIPY FL®, SE (10 mg ml<sup>-1</sup> in dimethylformamide (DMF)) for 3 h at RT. Unreacted BODIPY was removed by dialysis for 12 h at 4°C. BODIPY-labelled scFv Abs were added to the liposome scFv Ab-coupling reaction as trace labels and fluorescence was analysed in a TECAN fluorescence micro plate reader. ScFv Ab-derivatized PEG-liposomes and unreacted scFv Ab were separated on a discontinuous FICOLL P70 gradient. The immunoliposome coupling solution (200 µl) was mixed with 200 µl FICOLL (60% in HBSE (w/v)) in ultracentrifuge tubes (Beckman, Fullerton, CA) and overlaid with 2 ml FICOLL (10% in HBSE (w/v)) followed by HBSE as a top layer. The density gradient was centrifuged (16 h, 50 000 g, 4°C) using a Ti60 rotor in a Beckman ultracentrifuge. Fractions of 1 ml were carefully removed.
from the bottom to the top. For analysis of the fractions aliquots were taken for SDS-PAGE and Western blot analysis as described (Chapter 2.3.13). For quantitative analysis of the scFv Ab-coupling efficacy, fluorescent-labelled (BODIPY) scFv Abs were used for trace labelling. Control liposomes were obtained by coupling cysteines to maleimide modified liposomes (cys-PEG-liposomes).

4.3.5. Determination of lipid concentration in liposome solutions

For determination of liposomal concentrations a method based on the phosphorous assay for the determination of organic phosphate according Ames et al. and Chan et al. was used [426;427]. Briefly, samples were incubated in borosilicate tubes (Kimble, Vineland, NJ) with 0.9 M H₂SO₄, 0.16 M HClO₄ at 250°C for 30 min. After cooling to RT, samples were supplemented with 500 µl ddH₂O, 80 µl 3.15 M H₂SO₄, 160 µl 2.5% (w/v) ammonium heptamolybdate tetrahydrate and 80 µl 10% (w/v) ascorbic acid. The stable blue complex was measured in a spectrophotometer (Kontron, Milan, Italy) at 820 nm and lipid concentrations were calculated using a calibration curve.

![Scheme of the coupling reaction of scFv Abs to maleimide modified amino-PEG-liposomes.](image)

4.3.6. Competitive ELISA with scFv-antibody-PEG immunoliposomes

Varying concentrations of cfVEGF₁₆₄ in PBS supplemented with 0.02% BSA were incubated with anti-VEGF scFv-SZH9 Ab and control scFv-A1 Ab PEG-immunoliposomes, respectively at 10⁻⁹ M for 12 h at 4°C until equilibrium was reached. The equilibrated solution was transferred to a cfVEGF₁₆₄-coated microtiter plate (5 µg ml⁻¹ in PBS) and unbound scFv Abs at equilibrium were determined by ELISA.
4.3.7. ScFv antibody PEG-immunoliposomes in the syngraft F9-teratocarcinoma SV129 mouse therapy model

Exponentially growing F9 cells (1.4 x 10^7 50 µl^-1) were injected s.c. on the abdominal side of SV 129 mice (6 - 8 per group). Starting 24 h after tumour cell injection, the mice received 8 x every 24 h free anti-VEGF (scFv')2-SZH9 Ab or scFv-SZH9 Ab coupled to PEG-liposomes (4 mg scFv Ab kg^-1, in 100 µl of PBS) i.v. into the tail. Mice injected with free anti-VEGF (scFv')2-SZH9 Ab in presence of cys-liposomes and unspecific scFv-A1 Ab PEG-immunoliposomes were used as controls. Tumour growth was measured in a blinded manner with a calliper every day and volumes calculated using the following equation: V = π a b^2 / 6 (a = largest tumour diameter, b = perpendicular diameter).

4.3.8. ScFv antibody immunoliposomes in the transgenic Rip1Tag2 tumour model

Starting at week 10 the mice (3 per group) received 5 x every 48 h free anti-VEGF (scFv')2-SZH9 Ab or scFv Ab coupled to PEG-liposomes each (2 mg scFv Ab kg^-1, in 100 µl of PBS) i.v. into the tail. Mice injected with free anti-VEGF (scFv')2-SZH9 Ab in presence of cys-PEG-liposomes, unspecific scFv-A1 Ab PEG-immunoliposomes and PBS were used as controls.

4.3.9. Histology and immunohistochemistry

Tumours and pancreata of Rip1Tag2 mice were fixed overnight in 4% paraformaldehyde in PBS (1.37 M NaCl, 27 mM KCl, 100 mM Na2HPO4, 20 mM KH2PO4, pH 7.4) and immersed in 30% sucrose in PBS for 12 h. Finally, they were embedded in optimal cutting temperature, OCT (Tissue Tek, Torrance, CA) and snap frozen in liquid nitrogen. Sections (10 µm thick) were cut, mounted on silane-coated slides and immunostained. Vessel density was determined by CD31 (rat anti-mouse CD31 Ab, 1 : 50, v/v, PharMingen, San Diego, CA) immunostaining. Specific Ab staining was visualized using the ABC-Vector horseradish peroxidase kit according to the manufacturer’s recommendations (Vector Laboratories, Burlington, CA). For quantification blood vessels were counted in at least 10 comparable fields per section (magnification, x 100).
4.3.10. Statistical analysis

All measurements are shown as average ± SE of the mean. Statistical analysis of data from inhibition trials was performed using a two-tailed, unpaired t-test. A \( P < 0.05 \) was considered statistically significant. Calculations were made using the GraphPad Prism (Version 4.0) software.

4.4. RESULTS

4.4.1. Coupling of anti-VEGF scFv-SZH9 antibodies to the surface of PEG-liposomes

Anti-VEGF (scFv')_2-SZH9 Abs form dimers in the absence of reducing agents due to the introduced cysteines (Figure 2.2 C). Therefore, the disulfide bridges had to be cleaved for the coupling reaction of the scFv Ab to maleimide modified PEG-liposomes. The reduction of the anti-VEGF (scFv')_2-SZH9 Ab and unspecific (scFv')_2-A1 Ab with a 2 mM final concentration of TBF for 4 h at 4°C produced active and stable monomers. For the introduction of reactive amino-groups on the liposome surface 7 mol% DPPE-PEG-NH\(_2\) was used (Table 4.1). The incubation of H\(_2\)N-PEG-liposomes with a 5 x molar excess of bifunctional sulfo-SMCC resulted in about 1670 maleimide groups per liposome of 100 nm mean diameter [408].

For the preparation of the PEG-immunoliposomes the molar ratio of maleimide groups on the liposomes to the thiol groups on the proteins was 40 : 1, resulting according Marty et al. in an average of 100 scFv Abs per one liposome of 100 nm mean diameter [408].

Unreacted scFv Ab was separated from the immunoliposomes on a FICOLL gradient. One ml fractions from the gradient were collected and analysed by 13% reducing SDS-PAGE (Figure 4.5 A) and by Western blot analysis (Figure 4.5 B). In figure 4.5 A gradient fractions separated under denaturing conditions (in presence of \( \beta \)-mercaptoethanol) from the top to the bottom (1 to 9) are shown. Sample 2 corresponds to the liposome fraction and sample 9 to the fraction containing the uncoupled scFv Ab. Fractions containing sulfo-SMCC liposomes (Figure 4.5 B, lanes 1 and 2) gave under reducing conditions a ~33 kDa band on Western blots corresponding to the molecular weight of the scFv Ab monomer plus the attached PEG-lipid (lane 1). Separating immunoliposomal fractions without \( \beta \)-mercaptoethanol
(lane 2) resulted in 3 bands at 70, 100 and 130 kDa for dimer, trimer and tetramer forms of coupled scFv Ab, respectively.

As determined by BODIPY labelling the yield of coupled scFv Abs varied between 10 and 30%. The recovery of liposomes after scFv Ab coupling determined using the phosphorous assay ranged from 39 to 82%, corresponding to 31 to 66 mg ml\(^{-1}\) HSPC. For functional tests and therapeutic experiments, immunoliposomes were diluted according to the scFv Ab-amount coupled to the liposome surface.

**4.4.2. ScFv antibody-PEG-immunoliposomes in competitive ELISA**

PEG-immunoliposomes were tested *in vitro* in competitive ELISA (Figure 4.6). Varying concentrations of cfVEGF\(_{164}\) in PBS supplemented with 0.02% BSA were incubated with the anti-VEGF scFv-SZH9 Ab PEG-immunoliposomes and free (scFv')\(_2\)-SZH9 Ab at 10\(^{-9}\) M overnight at RT until equilibrium was reached. As negative control, anti-VEGF scFv\(_2\)-SZH9 Ab PEG-immunoliposomes were incubated under same conditions with BSA and as a further control unspecific scFv-A1 Ab PEG-immunoliposomes were incubated with increasing concentrations of cfVEGF\(_{164}\) as described above. The equilibrated solutions were transferred into cfVEGF\(_{164}\)-
coated microtiter plates and free scFv Ab PEG-immunoliposomes and uncoupled scFv Abs at equilibrium were determined by ELISA.

ScFv-SZH9 Ab PEG-immunoliposomes showed binding in presence of a 10 x molar excess of VEGF and an inhibition of binding when incubated with a > 10 x molar excess of competing VEGF. In contrast, uncoupled (scFv')2-SZH9 Ab showed more than a 2-fold inhibition of binding in presence of a 10 x molar excess of VEGF and in presence of a 100 x molar excess of VEGF compared to the scFv-SZH9 Ab PEG-immunoliposomes. Incubating scFv-SZH9 Ab PEG-immunoliposomes in presence of increasing amounts of BSA did not reduce the binding signal. Incubating unspecific scFv-A1 Ab immunoliposomes with increasing amounts of VEGF did not result in any specific ELISA signal.

![Graph](image.png)

**Figure 4.6**: Competitive binding inhibition of scFv SZH9Ab-immunoliposomes to ELISA plates pre-coated with VEGF in the presence of soluble VEGF. Competitive ELISA was performed according to Friguet et al. [405] and ctVEGF164 and BSA were added in concentrations from 10^{-11} to 10^{-5} M. The total concentrations of anti-VEGF scFv-SZH9 Ab liposomes and free scFv-SZH9 Ab and control scFv-A1 Ab liposomes were 10^{-9} M. Equilibrium was reached at 4°C after 12 h.

### 4.4.3. Therapeutic application of scFv-SZH9 antibody PEG-immunoliposomes in the syngraft F9 teratocarcinoma model

Stimulated by the described high in vivo circulation time and the accumulation of PEG-liposomes in tumours [428], the anti-angiogenic activity of scFv-SZH9 Ab PEG-immunoliposomes was tested *in vivo*. Mice, bearing subcutaneously syngrafted F9 teratocarcinomas were treated for eight days every 24 h with scFv-SZH9 Ab PEG-immunoliposomes, scFv-A1 Ab PEG-immunoliposomes, (scFv')2-SZH9 Ab alone and (scFv')2-SZH9 Ab in presence of Cys-PEG-liposomes. The administration of immunoliposomes was not toxic for the animals, since the body weight remained constant during the experiment. However, as shown in figure 4.7 and table 4.2 the
therapy with SZH9 PEG-immunoliposomes resulted in no reduction of tumour growth. Neither directly after treatment nor six days after the end of therapy tumour growth was inhibited compared to the control groups.

![Graph](image)

**Figure 4.7:** *In vivo* effects of scFv-SZH9 Ab PEG-immunoliposome treatment on F9 tumour growth. (A) Tumour-bearing mice were treated 8 times as indicated with (scFv')2-SZH9 Ab, control scFv-A1 Ab PEG-immunoliposomes (IL), (scFv')2-SZH9 Ab plus Cys PEG-liposomes (L), or scFv-SZH9 Ab PEG-immunoliposomes every 24 h, i.v.. Each treatment consisted of 4 mg kg⁻¹ free anti-VEGF (scFv')2 Ab or equal amounts of scFv Ab PEG-immunoliposomes. Values represent the mean of 5 to 7 treated mice ± SE of the mean. Relative tumour growth was normalized to day 2 (B) Bar graph of tumour sizes at day 14 indicating the calculated averages. Error bars represent ± SE of the mean.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumour volume ± SEM (cm³)</th>
<th>Normalized tumour volume ± SEM (%)</th>
<th>Change of tumour volume compared to scFv-A1 Ab PEG-IL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ScFv-A1 PEG-IL</td>
<td>1.52 ± 0.34</td>
<td>100 ± 23</td>
<td>-</td>
</tr>
<tr>
<td>(scFv')2-SZH9</td>
<td>2.08 ± 0.47</td>
<td>137 ± 31</td>
<td>+37</td>
</tr>
<tr>
<td>(scFv')2-SZH9 + Cys PEG-L</td>
<td>2.56 ± 0.64</td>
<td>169 ± 42</td>
<td>+69</td>
</tr>
<tr>
<td>ScFv-SZH9 PEG-IL</td>
<td>2.03 ± 0.17</td>
<td>134 ± 11</td>
<td>+34</td>
</tr>
</tbody>
</table>

**4.4.4. ScFv-SZH9 antibody PEG-immunoliposomes in the transgenic Rip1Tag2 model**

To address the question of being able to affect tumour-induced angiogenesis inhibition in a mouse model with a less aggressive tumour-progression, the transgenic Rip1Tag2 model was chosen. Rip1Tag2 mice were injected 5 x i.v. every 48 h with 2 mg scFv Ab kg⁻¹ during a period when animals were between 10 and
12 weeks of age, a time period during which angiogenesis is known to be highly active in β-cell tumourigenesis. No apparent effects on the general condition of the animals were observed during the course of the experiments. At 12 weeks of age, the mice were sacrificed and the pancreata prepared for IHC. Analysis of tumour vessel density revealed no decrease in vessel number of scFv-SZH9 Ab PEG-immunoliposome treated mice as compared with control mice treated with PBS, unspecific control (scFv')2-A1 Ab, free (scFv')2-SZH9 Ab or (scFv')2-SZH9 Ab plus cys-PEG-immunoliposomes (Figure 4.8 and table 4.3).

**Figure 4.8**: Application of anti-VEGF scFv-SZH9 Ab PEG-immunoliposomes (IL) to Rip1Tag2 mice. (A) Treatment scheme of mice between age 10 and 12 weeks using 2 mg kg\(^{-1}\) every 48 h. (B) Two days after end of treatment mice were sacrificed, tumours and pancreata isolated and vessel density evaluated using CD31-Ab staining. Bar graphs represent averages of vessel density of 3 mice per group. Error bars represent ± SE of the mean.

**Table 4.3**: Angiogenesis inhibition in Rip1Tag2 mice, 2 days after end of therapy.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CD31(^+) cells per field ± SEM</th>
<th>CD31(^+) cells ± SEM (%)</th>
<th>Normalized ± SEM (%)</th>
<th>Change of angiogenesis vs PBS controls (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>27.7 ± 5.9</td>
<td>2.2 ± 0.6</td>
<td>100 ± 27</td>
<td>-</td>
</tr>
<tr>
<td>(ScFv')2-A1</td>
<td>25.8 ± 4.5</td>
<td>1.9 ± 0.5</td>
<td>86 ± 23</td>
<td>- 14</td>
</tr>
<tr>
<td>ScFv-A1 PEG-IL</td>
<td>24.0 ± 3.9</td>
<td>1.8 ± 0.5</td>
<td>82 ± 23</td>
<td>- 18</td>
</tr>
<tr>
<td>ScFv-SZH9 PEG-IL</td>
<td>29.8 ± 2.5</td>
<td>2.4 ± 0.4</td>
<td>109 ± 18</td>
<td>+ 9</td>
</tr>
<tr>
<td>(ScFv')2-SZH9</td>
<td>26.9 ± 1.6</td>
<td>2.1 ± 0.6</td>
<td>95 ± 27</td>
<td>- 5</td>
</tr>
<tr>
<td>(ScFv')2-SZH9 + Cys PEG-L</td>
<td>24.4 ± 3.0</td>
<td>1.9 ± 0.6</td>
<td>86 ± 27</td>
<td>- 14</td>
</tr>
</tbody>
</table>

92
4.5. DISCUSSION

In chapter 2 the selection of anti-VEGF \((\text{scFv'})_2\)-SZH9 Ab was described (Chapter 2.4.1) and in chapter 3 in vivo characterisation experiments were carried out (Chapter 3.4.1-3). The aim of the experiments described in this chapter was to improve blood circulation time and tumour uptake of the anti-VEGF \((\text{scFv'})_2\)-SZH9 Ab in order to increase the anti-angiogenic effects in tumour progression. To achieve this objective, the anti-VEGF \((\text{scFv'})_2\)-SZH9 Ab was coupled to the surface of PEG-liposomes, since they show longer blood circulation compared to small molecules and they also passively accumulate in tumours. This concept is based on results of Bendas et al. and Marty et al. [402;429]. Bendas et al. carried out pharmacokinetic and biodistribution studies comparing PEG-immunoliposomes with unmodified liposomes. Although plain liposomes displayed a longer blood circulation time with a 50% value after 6 h compared to the elimination of Ab-modified PEG-immunoliposomes with a 50% value after 2.5 h [429], the superior blood circulation time compared to free \((\text{scFv'})_2\)-SZH9 Ab as reported in chapter 3.4.2 with a 50% value of < 10 min is obvious. Coupling the anti-E-DB scFv CGS-1 Ab to the surface of PEG-liposomes by Marty et al., resulted in 8.5 ± 4.7 ID% g⁻¹ in F9 tumours after 24 h compared to 4.7 ± 0.6% of scFv-L19 Ab which is an affinity matured version of CGS-1 that has a 1000 fold lower dissociation constant [344;345]. It is remarkable that non-targeted PEG-liposomes showed about the same tumour accumulation values compared to immunoliposomes as described before.

Because the liposome size is a critical parameter for tumour uptake, scFv-SZH9 Abs were coupled to the surface of PEG-liposomes ranging in size from 82 to 113 nm. Charrois and Allen have examined this parameter by performing biodistribution studies of liposomes in tumour-bearing mice [430]. Liposomes with the 3 smallest out of 4 tested diameters of 80, 100, 150 or 240 nm showed similar tumour accumulation profiles that were significantly higher than the largest liposomes. Liposome levels peaked in tumours within 24 h, whereby the 100 nm liposomes were superior to all other liposomes differing in size. A therapeutic application of liposomal doxorubicin against the 4T1 mouse mammary carcinoma resulted in optimal tumour growth inhibition when using 100 nm liposomes [430]. The capillary permeability of the endothelial barrier in newly vascularized tumours is significantly greater than that of normal organs. Thus, liposomes can extravasate through the leaky endothelium by
passive transport. Liposomes larger than 300 nm in diameter accumulate in the spleen and those smaller than 80 nm primarily in the liver due to the capture by the cells of the mononuclear phagocytose system (MPS) [303;431]. The MPS is the physiological system responsible for the elimination of foreign macromolecules and particles from the body and mainly macrophages of the liver (Kupffer cells), spleen and the lymphatic system play a key role in this elimination process [303]. Because the effective numbers of coupled molecules and their location on the surface of the liposomes is crucial for the elimination from the blood circulation by the MPS, not more than 100 scFv Ab molecules were coupled to the distal ends of PEG liposomes [424]. For Ab coupling a C-terminal cysteine protein modification via a bifunctional coupling molecule to PEG-liposomes was chosen as described before [337;432]. After the successful C-terminal modification with 3 cysteins, a FLAG-tag and a hydrophilic spacer-sequence, the scFv-SZH9 Ab was expressed in a soluble homodimeric form (Figure 2.2 C). In the absence of reducing agents the modified scFv Abs formed dimers due to the introduced C-terminal cysteines. Therefore, the disulfide bridges had to be cleaved for the coupling reaction of the scFv Abs to maleimide modified liposomes. The reduction can be achieved by using reducing agents containing thiol groups themselves (e.g. β-mercaptoethanol or dithiothreitol) or with agents without thiol groups (TBF or dithionite). The advantage of working with an agent without thiol groups is that the reducing agent has not to be removed before the coupling reaction takes place. Agents with thiol groups could react with the maleimide groups on the liposomes. Marty et al. showed that dithionite is a very aggressive reducing agent which reduced the binding of a scFv Ab to ED-B fibronectin [402]. In contrast to dithionite, the reduction of the scFv Ab dimers with a 2 mM final concentration of TBF for 4 h at 4°C produced active and stable monomers [433]. In a chemical reaction the molar ratios of active groups are important. Labelling of proteins with small molecules or modification of liposomes with proteins should be carried out with a molar excess of the smaller molecules. The major problem of the liposome modification with (scFv')2-SZH9 Ab was the limited amount of available protein and due to the low coupling efficiency of 10 to 30%, the yield of obtained immunoliposomes was low, respectively the loss of scFv Abs very high.

The Western blot analysis showed, that during coupling under reducing conditions not only monomers, but also stable dimers, trimers and possibly tetramers were formed on the surface of PEG-liposomes (Figure 4.5 B). This can be explained by the
three cysteines, that have been introduced at the C-terminal end of the Ab. Depending on how many cysteines are reduced by tributylphosphine, scFv Abs can either react with the sulfo-SMCC spacer on the liposome or interact with additional scFv Ab monomers.

The binding properties of the scFv-SZH9 Ab PEG-immunoliposomes were tested by competitive ELISA using the M2 anti-FLAG Ab, to detect the scFv Abs on the liposomes. When comparing the capacity to neutralize soluble VEGF with immunoliposomes and free (scFv')₂ Abs no differences resulted (Figure 4.6). Immobilized VEGF was recognized by free (scFv')₂-SZH9 Ab and by scFv-SZH9 Ab PEG-immunoliposomes, but 10 x more soluble VEGF competitor was required to neutralize immunoliposome binding to immobilized VEGF as compared to free (scFv')₂-SZH9 Ab. This result indicates, that all scFv-SZH9 Abs coupled to the surface of the liposomes needed to be saturated by VEGF to inhibit the binding of the immunoliposomes to immobilized VEGF.

Based on the encouraging results obtained in vitro, the VEGF neutralizing effect of scFv-SZH9 Ab PEG-immunoliposomes was tested in vivo using the syngraft F9 teratocarcinoma and the transgenic Rip1Tag2 tumour mouse models. Although SZH9 is a human-mouse anti-VEGF cross-reactive Ab, the use of a syngeneic model was preferred for better evaluation of the anti-tumour effect as already evaluated in previous experiments (Chapter 3.4.3).

Because of limitations in the protein production and the poor scFv Ab coupling efficiency to the surface of PEG-liposomes (Figure 3.3 C, compared to figure 4.7 A) for the therapeutic treatment of F9 tumour-bearing mice, the amount of daily or total injected liposomal scFv Abs was 6.25 times (25 versus 4 mg scFv Ab kg⁻¹) or 8 times (total dose: 2.5 versus 0.6 mg scFv Ab mouse⁻¹) less than in the initial therapeutic experiment (Chapter 3.4.3). Due to the increased blood circulation time of PEG-immunoliposomes compared to small molecules it was intended to compensate this disadvantage. However, although bioactive anti-VEGF scFv-SZH9 Ab immunoliposomes were injected into mice bearing F9 teratocarcinoma, no tumour growth inhibition could be recorded neither directly nor 6 days after end of treatment (Figure 4.7). It is assumed, that due to the rapid growth of the F9 tumours, it is not possible to block tumour-induced angiogenesis with such small amounts of VEGF-neutralizing scFv Abs neither with free (scFv')₂ Abs nor coupled to the surface of PEG-liposomes.
Several anti-angiogenic agents have been reported to block tumour-induced angiogenesis in Rip1Tag2 mice [425;434]. Since the syngeneic Rip1Tag2 model is less aggressive in tumour progression compared to the F9 tumour model anti-tumour effects of anti-VEGF treatment with immunoliposomes should be investigated. Parangi et al. demonstrated in this model that tumour progression can be impaired by treatment with a combination of angiogenesis inhibitors in a regimen that begins prior to the emergence of solid tumours. Mice received a combination of three anti-angiogenic drugs, consisting of TNP470 (30 mg kg$^{-1}$, s.c.), minocycline (10 mg kg$^{-1}$, i.p.) and murine IFN (1x 10$^6$ units, i.p.) every 24 h, respectively. The animals were treated starting at 6 weeks of age and euthanized at 13.5 weeks. After 7.5 weeks of daily anti-angiogenic treatment, the capillary density was more than halved, and cumulative tumour volume was about 1/10 of untreated controls [425].

Due to the described limitations in scFv Ab production and due to the low scFv Ab coupling efficiency to liposomes, only 2 mg kg$^{-1}$ of liposomal scFv Ab could be applied every second day during a period of 10 days (total dose: 0.2 mg scFv Ab mouse$^{-1}$). Because of this limitation, we expected an anti-angiogenic response by a reduction of vessel density within the tumour but no change in tumour progression. But no difference in tumour vessel density compared to the control groups was found (Figure 4.8). In conclusion, anti-VEGF scFv-SZH9 Ab could not be delivered at sufficient amounts, neither free nor coupled to the surface of PEG-liposomes that would have been required to neutralize the angiogenic activity of VEGF.

The repetitive therapy schedule (every 24 and 48 h) of immunoliposome application is probably disadvantageous with regard to the influence of repeated liposome application on their in vivo circulation time. Although the scFv Ab fragments do not contain the main immunogenic constant part of IgG or Fab' Abs it was shown in the literature that repetitive injections of immunoliposomes cause immunogenic reactions. Harding et al. have shown, that PEG-grafted IgG-immunoliposomes are more immunogenic than the free IgG component and more importantly, that repeated injections of immunoliposomes (~ 18 IgG per 100 nm liposome) even at an interval of 2 weeks resulted in rapid clearance of the liposomes, which was accompanied by a significant increase in anti-IgG specific Ab titers [435]. Upon repeated injection or coinjection with the parent liposomes free IgG consistently exhibited prolonged circulation without any increase in IgG-specific antisera. But they raised significantly when administered into animals that had been pretreated with the immunoliposomes.
Screening of the immunoliposome induced antisera against human polyclonal IgG and IgG-derived Fab' fragments revealed that the immune response was specifically triggered by the constant region of IgG [435]. A method to improve the circulation time of immunoliposomes could consist in blocking the MPS by pre-treatment with conventional liposomes. The immunoliposomes would then remain in the circulation for longer time periods and thus have a higher probability of neutralizing soluble molecules such as VEGF in the bloodstream. Increased diffusing rates through the fenestrations of newly formed vessels would result in a higher probability of neutralizing soluble molecules in the abluminal compartment.

Although PEG-immunoliposomes have been successfully used in several in vivo applications, in this experimental setup we could not benefit from this properties. In this chapter it was shown, that the binding capacity of scFv-SZH9 Ab PEG-immunoliposomes to soluble VEGF was comparable to free (scFv')<sub>2</sub> Abs in vitro (Figure 4.6). But due to limitations in protein production and in low scFv Ab coupling efficiencies to PEG-liposomes, injected scFv Ab PEG-immunoliposomes could not be delivered in sufficient amounts to block VEGF-dependend tumour angiogenesis resulting in no inhibition of tumour growth in vivo in the both tumour modells used (Figure 4.7 and 4.8).
5. DEPLETION OF TUMOUR-ASSOCIATED MACROPHAGES (TAMs) USING CLODRONATE-CONTAINING LIPOSOMES (CLODROLIP): A NEW ANTI-ANGIOGENIC CANCER THERAPY APPROACH

5.1. ABSTRACT
The selective cytotoxicity of macrophages after application of clodronate-containing liposomes (Clodrolip) was evaluated in vitro and in vivo. Incubation of isolated peritoneal macrophages in presence of Clodrolip resulted in a cytotoxic reaction in a concentration-dependent manner. Endothelial and tumour cells were not affected. Treating mice with Clodrolip resulted in a selective depletion of splenic macrophages whereas splenic dendritic cells (DC), B and T lymphocytes were not affected. Therapeutic Clodrolip treatment in the syngraft F9 teratocarcinoma mouse model resulted in significant tumour growth inhibition without developing side effects. Although free clodronate did not result in depletion of splenic macrophages, it had an intermediate effect on tumour growth inhibition. Qualitative immunohistological evaluation of isolated tumours revealed an almost complete elimination of MOMA1+ and F4/80+ macrophages after clodronate therapy.

5.2. INTRODUCTION
Solid tumours are not only composed of malignant cells, but are complex structures comprising many cell types including a wide range of haematopoietic cells. The role of infiltration of white blood cells (leucocytes) into solid tumours was already noticed more than 100 years ago. Then, it was suggested that these cells had a causal role in carcinogenesis. These cell infiltrates are composed of myeloid cells (neutrophils, dendritic cells, macrophages, eosinophils and mast cells) as well as of lymphocytes (See general introduction chapter 1.1).
Recent studies have shown that tumour-associated macrophages (TAMs) produce factors that promote many steps associated with malignancy of tumours. These factors are either stimulatory and inhibitory, including bFGF, VEGF, ANG-1 and ANG-2, IL-1, IL-8, TNF-α, TP, MMP-9 and MMP-2, and NO as described in more detail in
chapter 1.3.1. The coordinated spatial and temporal expression of these molecules results in the proliferation and migration of ECs, the remodelling of the ECM and the formation of stabilized blood vessels. Macrophages are perfectly designed to support these processes, as their monocytic precursors can migrate into sites where they differentiate into macrophages, and these migrating cells can synthesize the required angiogenic molecules on demand once they reached their tumour target locations [10;26].

In order to block macrophage dependent tumour angiogenesis new techniques are needed for clinical applications.

It has been found in several immunological studies that certain bisphosphonates (in particular clodronate) when encapsulated in liposomes have the ability to deplete macrophages allowing to investigate their role [436]. In this chapter it was analysed whether TAMs which are recruited by growing solid tumours can be depleted by application of liposome-encapsulated clodronate (Clodrolip) and whether this macrophage depletion would lead to inhibition of tumour growth.

Here, the selective cytotoxicity of Clodrolip to macrophages is shown in vitro and in vivo. By therapeutic application of Clodrolip in the syngraft F9 teratocarcinoma mouse model, tumour growth was significantly reduced.

5.3. MATERIAL AND METHODS

5.3.1. Reagents, cells and animals

Murine F9 teratocarcinoma and human A673 rhabdomyosarcoma cells were obtained from American Type Culture Collection (ATCC; Rockville, MD) and human umbilical vein endothelial cells (HUVECs) were obtained from PromoCell (Heidelberg, Germany). Female SV 129 mice were obtained from Charles River Wiga (Sulzfeld, Germany) and kept in standard housing and normal diet at the animal facility. Animal studies were performed under the licenses (No. 75533 and 75534) issued to R. A. Schwendener by the Veterinary Department of the Canton Aargau, Switzerland. The ethical guidelines that were followed meet the standards required by the UKCCCR guidelines [415].

SPC was obtained from L. Meyer (Hamburg, Germany), Cholesterol from Fluka, D,L-α-tocopherol from Merk (Darmstadt, Germany), mannitol from Sigma, Clodronate from Bioindustria L.I.M. (Novi Ligure, Italy) and 45CaCl2 from Amersham Pharmacia
Biotech UK Ltd. (Little Chalfont, UK). Media and reagents for tissue culture as Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), trypsin, antibiotics or Hank’s balanced salt solution (HBSS) were obtained from Sigma or Invitrogen. HUVEC growth medium was obtained from PromoCell. Primary rat or rabbit anti-mouse Abs specific for macrophage and lymphocyte were obtained as indicated in table 5.1. Staining of apoptotic cells was performed with Apop Tag Peroxidase-detection Kit from (Norcross, GA). Alkaline phosphatase-labelled species-specific secondary Abs were obtained from Jackson ImmunoResearch Labs (West Grove, PA.). Alkaline phosphatase substrate AS-BI (6-bromo-2-hydroxy-3-naphtholic acid-2-methoxy anilide) phosphate and fuchsin were from Sigma (Buchs, Switzerland). Buffer salts and other chemicals were from Fluka or Sigma.

Table 5.1: Monoclonal Abs used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Distributor</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>American Type Culture Collection (Manassas, Va.)</td>
</tr>
<tr>
<td>F4/80</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>MOMA-1</td>
<td>BMA Biomedicals (Augst, Switzerland)</td>
</tr>
<tr>
<td>ER-TR 9</td>
<td>BMA Biomedicals</td>
</tr>
<tr>
<td>CD68</td>
<td>Rat-anti-mouse CD68, Serotec (Dusseldorf, Germany)</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>CD31, BD PharMingen (San Diego, CA)</td>
</tr>
<tr>
<td>CD45R/B220</td>
<td>RA3-6B2, BD PharMingen</td>
</tr>
<tr>
<td>FDC</td>
<td>Rat-anti-mouse FDC, BD PharMingen</td>
</tr>
<tr>
<td>CD11b</td>
<td>M1/70, BD PharMingen</td>
</tr>
<tr>
<td>CD11c</td>
<td>HL3, BD PharMingen</td>
</tr>
</tbody>
</table>

5.3.2. Preparation of clodronate-liposomes (Clodrolip)

Liposomes composed of soy phosphatidylcholine (SPC, 40 mg ml⁻¹), cholesterol (6 mg ml⁻¹) and D,L-α-tocopherol (0.2 mg ml⁻¹) corresponding to 1 : 0.3 : 0.01 mol parts (Figure 5.1 and table 5.2) were prepared by freeze-thawing and filter extrusion. The dry lipid mixture was solubilized in a physiologic phosphate buffer (20 mM, pH 7.4) supplemented with mannitol (230 mM) as cryoprotectant, allowing freezing of the liposomes and 66 mg ml⁻¹ clodronate (clodronic acid disodium salt tetrahydrate, CH₂Cl₂Na₂O₆P₂ x 4 H₂O, Mol. Wt. 361). The resulting multilamellar vesicles were freeze-thawed in 3 cycles of liquid nitrogen and water at 40°C, followed by repetitive (5–10 x) filter extrusion through 400 nm membranes (Nuclepore, Sterico, Dietikon, Switzerland) using a LipexTM extruder (Lipex Biomembranes Inc., Vancouver, Canada).
For the determination of clodronate encapsulation efficiency the preparations were trace labelled with $^{45}\text{CaCl}_2$ and 1 mM CaCl$_2$ as carrier. Non-encapsulated clodronate was removed by dialysis (Spectrapore tube, 12 – 14 000 mol. wt. cut-off) with mannitol-phosphate (67 mM, pH 7.4) as dialysis buffer (1 : 100 v/v). All preparations were sterile filtrated through a 0.45 μm filter and aliquots of 1 ml were stored at -80°C. Liposome size and homogeneity were routinely measured with a Nicomp laser light scattering particle sizer (Nicomp 370, Sta. Barbara, CA). Routinely prepared liposomes contain approximately 20 mg ml$^{-1}$ clodronate and have a mean diameter of 135 ± 70 nm.

Table 5.2: Lipid composition of the liposomes.

<table>
<thead>
<tr>
<th>SPC (40 mg ml$^{-1}$)</th>
<th>Cholesterol (6 mg ml$^{-1}$)</th>
<th>D,L-α-tocopherol (0.2 mg ml$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mol%</td>
<td>30 mol%</td>
<td>1 mol%</td>
</tr>
<tr>
<td>52 μmol</td>
<td>15.6 μmol</td>
<td>0.52 μmol</td>
</tr>
</tbody>
</table>

5.3.3. Selective cytotoxic effects of Clodrolip on macrophages, endothelial cells and tumour cells

For a selective cytotoxic in vitro assay, freshly isolated and cultured intraperitoneal macrophages, HUVECs, human A673 rhabdomyosarcoma and murine F9 teratocarcinoma cells were seeded at a density of 10$^4$ cells per well in sterile 96 well plates in the presence of DMEM or HUVEC growth medium supplemented with 10% FBS and antibiotics and cultivated for 48 h at 37°C, 5% CO$_2$. The cells were washed with PBS and incubated for 4, 6, and 12 h in the corresponding medium containing the clodronate dissolved in PBS and Clodrolip with final concentrations of 0.00277 – 13.85 mM (0.001 – 5 mg ml$^{-1}$). The supernatants were removed at the corresponding
times and 100 μl well⁻¹ freshly diluted WST-1 solution in medium (1 : 10 v/v) (La Roche Diagnostics, Mannheim, Germany) added and incubated for 5 h at 37°C and 5% CO₂. Cell viability was determined by measuring the absorption at 410 nm using a Dynatech MR5000 plate reader (Microtec Produkte, Embrach, Switzerland).

5.3.4. In vivo depletion of splenic macrophages using Clodrolip in SV 129 mice

Depletion of splenic macrophages in 5 - to 7 -week-old female SV 129 mice (4 - 5 per group) was achieved by 3 x i.p. injections of Clodrolip initially 100 mg kg⁻¹, followed by 50 mg kg⁻¹ every 96 h. Control groups received PBS and free clodronate. Three days after the last treatment, macrophage depletion efficiency was monitored by leucocyte specific IHC analysis of collected spleens.

5.3.5. Anti-tumour therapy using Clodrolip in the syngraft F9 teratocarcinoma SV 129 mouse model

Exponentially growing F9 cells (5 x 10⁶ in 50 μl) were injected s.c. on the abdominal side of SV 129 mice. Starting at different time points (day 0, 4, and 8) after tumour cell injection the mice (6 - 8 per group) received free clodronate or Clodrolip at an initial dose of 100 mg kg⁻¹, followed by 50 mg kg⁻¹ by i.p. application. Mice injected with the same volume of empty liposomes or PBS were used as controls. Tumour growth was measured in a blinded manner with a calliper every day and volumes calculated using the following equation: \( V = \pi ab^2 / 6 \) (\( a \) = largest tumour diameter, \( b \) = perpendicular diameter).

5.3.6. Immunohistochemistry

Histological and immunohistochemistry (IHC) assays were done as described previously [437]. Briefly, mice were euthanized with CO₂, and the appropriate organs (spleen and tumour) were removed. Tissue specimens for IHC analysis were collected at the times indicated, immersed in Hanks balanced salt solution, and snap frozen in liquid nitrogen. Two- to three-micrometer-thick tissue sections were cut in a cryostat, fixed with acetone, and stored at -70°C. For the staining of cell differentiation markers, the following primary rat or rabbit anti-mouse monoclonal Abs were used: Abs against CD3 (KT3), CD11b (M1/70), CD11c (HL3), CD31, FDC,
CD68, F4/80 macrophages (A3-1), B220 (RA3-6B2), marginal metallophilic or marginal zone macrophages (MOMA1 or ERTR9).

Primary Abs were revealed by sequential incubation with alkaline phosphatase-labelled species-specific secondary Abs. Alkaline phosphatase was visualized using naphthol AS-BI (6-bromo-2-hydroxy-3-naphtholic acid-2-methoxy anilide) phosphate and new fuchsin as a substrate, yielding a red reaction product. Cell nuclei were counterstained with hemalum.

5.3.7. Statistical analysis

All measurements are shown as average ± SE of the mean. Statistical analysis of data from inhibition trials was performed using a two-tailed, unpaired t-test. A value of \( P < 0.05 \) was considered statistically significant. Calculations were made using the GraphPad Prism (Version 4.0) software.

5.4. RESULTS

5.4.1. Selective cytotoxicity of Clodrolip on macrophages in vitro

Clodrolip has been reported in immunology research to eliminate macrophages in the MPS, mainly in the liver, spleen and other organs.

To investigate whether tumour infiltrating macrophages could also be depleted in order to inhibit tumour growth, Clodrolip was used as a therapeutic agent in a tumour mouse model. First, the cytotoxic effect of Clodrolip was tested on freshly isolated murine peritoneal macrophages in vitro. Due to the phagocytotic uptake, treatment with Clodrolip was found to kill peritoneal macrophages in a concentration dependent manner resulting in an IC\(_{50}\) of 2.8 mM or 1 mg ml\(^{-1}\) (Figure 5.2).
Figure 5.2: In vitro cytotoxicity of Clodrolip on murine peritoneal macrophages. For proliferation assays, freshly isolated macrophages were seeded at a density of $10^6$ cells per well in sterile 96 multi well plates and cultured as described (Chapter 5.3.3) for 48 h. After 6 h incubation in presence of different concentrations of Clodrolip, cell viability was determined by light microscopic analysis (A a-e) and by adding WST-1 solution ($n = 3$), ± SE of the mean (B). As controls PBS (A a and B: ◊) or empty liposome solutions (A b and c; F: ○) at lipid concentrations as were used for Clodrolip at 5 mg ml$^{-1}$ or 10 mg ml$^{-1}$ (A d and e; B: ●) were analysed.

Endothelial cells, murine F9 teratocarcinoma or human A673 rhabdomyosarcoma cells were not affected upon incubation with Clodrolip in the concentration range used above (Figure 5.3).

Figure 5.3: In vitro cytotoxicity of Clodrolip on freshly isolated peritoneal macrophages, HUVE cells, murine F9 teratocarcinoma and human A673 rhabdomyosarcoma cells. For the proliferation assays, cells were seeded at a density of $10^4$ cells per well in 96 multi-well plates and cultured as described (Chapter 5.3.3) in presence of 1 mg ml$^{-1}$ free clodronate, Clodrolip or corresponding amounts of empty liposomes. After 6 h, cell viability was determined by adding WST-1 solution; ($n = 3$) ± SE of the mean. $P$ (t test): *, $P < 0.05$.

It is notable, that free clodronate and Clodrolip showed the same significant cytotoxic effect on macrophages. But only the co-incubation with free clodronate resulted in
proliferation of tumour cells, whereas this effect was not found with Clodrolip as shown in figure 5.3.

5.4.2.Selective cytotoxicity of Clodrolip towards splenic macrophages

In a next step the cytotoxic effect of Clodrolip on leukocytes in fully immunocompetent SV129 mice was evaluated (Figure 5.4). Clodrolip treatment showed a selective cytotoxic effect on splenic marginal zone metallophilic MOMA1+ macrophages (Figure 5.4 S), marginal zone ER-TR9+ macrophages (Figure 5.4 T) and activated F4/80+ macrophages in the red pulp (Figure 5.4 U) while CD68+ macrophages in the red and the white pulp could not be depleted (Figure 5.4 V). Whereas Clodrolip depleted MOMA1+, ERTR9+ and F4/80+ macrophages, application of free clodronate showed no cytotoxic effect in vivo (Figure 5.4 J, K and L).

Splenic white pulp FDC+ dendritic cells, red pulp CD11b+ and CD11c+ dendritic cells (Figure 5.4 N, O, P and W, X, Y), white pulp B220+ B-cells (Figure 5.4 Q and Z) and white pulp CD3+ T-cells (Figures 5.4 R and A1) were not affected neither after treatment with free clodronate nor by Clodrolip.

Figure 5.4: Depletion of spleen macrophage subpopulations after i.p. treatment with Clodrolip. Splenic tissues obtained from mice injected with PBS (A to I), with free clodronate (J to R), or with Clodrolip (S to A1) are shown. Three days after 3 treatments (initially 100 mg kg\(^{-1}\), followed by 50 mg kg\(^{-1}\), every 96 h), spleens were removed and sections IHC stained for marginal zone metallophilic MOMA1+ macrophages (A, J, and S), marginal zone ER-TR 9+ macrophages (B, K, and T), red pulp F4/80+ macrophages (C, L, and U), red and white pulp CD 68+ macrophages (D, M and V), white pulp FDC+ dendritic cells (E, N, and W), red pulp CD11b+ dendritic cells (F, O and X), red pulp CD11c+ dendritic cells (G, P and Y), white pulp B220+ B-cells (H, Q, and Z), and white pulp CD3+ T-cells (I, R and A1). Bar indicates 100 \(\mu m\).
5.4.3. Anti-tumour therapy using Clodrolip in the syngraft F9 teratocarcinoma mouse model

Tumour-associated angiogenesis in part resulting from macrophage infiltration plays an important role in tumour growth and expansion. Therefore, the effects of Clodrolip on tumour progression and tumour growth in a syngraft mouse model was studied. In SV129 mice, $8 \times 10^6$ tumour cells were injected s.c. abdominally (5-7 per group). Mice were treated i.v. with PBS, free clodronate, empty liposomes or Clodrolip. For clodronate treatment we used initially 100 mg kg$^{-1}$, followed by 50 mg kg$^{-1}$. Therapy was started for all four groups at the same day after tumour cell inoculation, followed by further injections every four days. For two additional groups the Clodrolip therapy was started at day four and eight, respectively. Tumour volumes were monitored every day (Figure 5.5 A).

![Graph A](image)

Figure 5.5: In vivo effects of Clodrolip treatment on F9 tumour growth in SV 129 mice. (A) Tumour-bearing mice (6 - 8 per group) were treated by i.p. injection of PBS, empty-liposomes, free clodronate, and Clodrolip, starting at day 0 every 96 h. In two Clodrolip groups therapy was started at a delayed manner at days four and eight after tumour cell inoculation. Each treatment contained initially 100 mg kg$^{-1}$, followed by 50 mg kg$^{-1}$ Clodrolip. Values represent the tumour growth ± SE of the mean. Relative tumour growth was normalized to day 1 (B) Bar graph of tumour sizes at day 14 ± SE of the mean. * Treated versus PBS < 0.05.

Clodrolip treatment was found to significantly decrease tumour volume, estimated two days after end of experiment at day 14 (Figure 5.5 B and table 5.3). These observations suggest that Clodrolip inhibits carcinoma growth in SV129 mice by depleting the TAMs which are secreting high levels of pro-angiogenic factors.
Table 5.3: Tumour volume at day 14 i.e., 2 days after end of therapy.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumour volume ± SEM (cm³)</th>
<th>P-value</th>
<th>Normalized tumour volume ± SEM (%)</th>
<th>Change of tumour volume vs PBS control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0.99 ± 0.23</td>
<td>-</td>
<td>100 ± 23</td>
<td>-</td>
</tr>
<tr>
<td>Empty liposomes (day 0, 4, 8, 12)</td>
<td>0.92 ± 0.35</td>
<td>0.8694</td>
<td>93 ± 35</td>
<td>- 7</td>
</tr>
<tr>
<td>Free clodronate (day 0, 4, 8, 12)</td>
<td>0.55 ± 0.24</td>
<td>0.2090</td>
<td>55 ± 24</td>
<td>- 45</td>
</tr>
<tr>
<td>Clodrolip (0, 4, 8, 12)</td>
<td>0.26 ± 0.10</td>
<td>0.0185</td>
<td>26 ± 10</td>
<td>- 74</td>
</tr>
<tr>
<td>Clodrolip (4, 8, 12)</td>
<td>0.50 ± 0.23</td>
<td>0.1670</td>
<td>51 ± 23</td>
<td>- 49</td>
</tr>
<tr>
<td>Clodrolip (8, 12)</td>
<td>0.79 ± 0.26</td>
<td>0.2354</td>
<td>80 ± 26</td>
<td>- 20</td>
</tr>
</tbody>
</table>

Immunohistological examinations revealed almost complete depletion of tumour infiltrated MOMA1⁺ and F4/80⁺ macrophages after Clodrolip treatment (Figure 5.7 D-F and J-L). Although free clodronate does not show any cytotoxic effect on MOMA1⁺ and F4/80⁺ macrophages in the spleen (Figure 5.6 C and I), MOMA1⁺ and F4/80⁺ macrophages in the tumour could be fully depleted (Figure 5.7 C and I).

Figure 5.6: Depletion of spleen macrophage after i.p. treatment with Clodrolip. Spleen tissues obtained from mice injected with PBS (A, G, and M), empty liposomes (B, H, and N), free clodronate (C, I, and O), Clodrolip started therapy at day 0 (D, J, and P), or day 4 (E, K, and Q) or day 8 (F, L, and R) are shown. Two days after the last treatment, spleens were removed and sections IHC stained for marginal zone metallophilic MOMA1⁺ macrophages (A to F), red pulp F4/80⁺ macrophages (G to L) and apoptotic cells (TUNEL-assay; M to R). Bar indicates 100 μm.
However, when free clodronate was used for murine F9 tumour growth inhibition in SV 129 mice tumour growth was not significantly inhibited (Figure 5.5 B). When the therapy with Clodrolip was started at a timepoint the tumours were already established (day 4 or 8), the inhibitory effect was less pronounced as compared when the onset of therapy was at the day of tumour cell inoculation (Figure 5.5 A and B). Although macrophages are known to be essential for several immunological functions, the depletion therapy using Clodrolip did not cause cytotoxic side effects. Treatment with total of 250 mg kg$^{-1}$ Clodrolip per mouse did not significantly change body weight during the monitored time period of 14 days compared to untreated control mice (Figure 5.8).
5.5. DISCUSSION

In the presented work, liposome encapsulated clodronate (Clodrolip) was applied to deplete tumour associated macrophages (TAMs). The depletion of TAMs led to a significant inhibition of tumour growth.

First, the cytotoxic effect of Clodrolip on freshly isolated murine peritoneal macrophages has been studied in vitro. Due to the phagocytotic uptake, treatment with Clodrolip was found to selectively inhibit the survival of peritoneal macrophages in a concentration-dependent manner with an IC_{50} of 2.8 mM or 1 mg ml^{-1} Clodrolip, whereas endothelial cells, F9 and A673 tumour cells, remained unaffected (Figures 5.2 and 5.3).

In this in vivo study, treatment with Clodrolip showed a selective cytotoxic effect on splenic marginal zone metallophilic MOMA1^{+} macrophages (Figure 5.4 S), marginal zone ERTR9^{+} macrophages (Figure 5.4 T) and activated F4/80^{+} macrophages in the red pulp (Figure 5.4 U), whereas CD68^{+} macrophages in the red and the white pulp were not depleted (Figure 5.4 U). CD68^{+} macrophages are known to eliminate apoptotic cells and cell debris. This could explain why only few apoptotic macrophages could be detected by the TUNEL-assay (Figure 5.6, M to R). Splenic white pulp FDC^{+} (Figure 5.4 W), red pulp CD11b^{+} (Figure 5.4 X) and CD11c^{+} (Figure 5.4 Y) dendritic cells, white pulp B220^{+} B-cells (Figure 5.4 Z) and white pulp CD3^{+} T-cells (Figure 5.4 A1) were not affected by Clodrolip treatment. Treating mice with free clodronate did not result in any depletion of macrophages in the spleen.

Figure 5.8: Absence of systemic toxicity of Clodrolip (CL) and free clodronate (FC) treatment on SV 129 mice as shown in figure 5.5. Bar graph of relative body weight change during 14 days. Values represent the mean of 6 - 8 treated mice, ± SE of the mean. Calculated P values (t test) resulted in insignificant values of body weight change.
(Figure 5.4 J-R) and was comparable to the PBS-control group (Figure 5.4 H-I). This result indicates, that for depletion of macrophages in the spleen the concentration of injected free clodronate was either too low and/or the elimination from the blood too fast.

The effects of Clodrolip on tumour progression of F9 teratocarcinoma syngrafts in SV129 mice was evaluated. Preventive treatment of clodronate was found to significantly decrease tumour size (Figure 5.5 and table 5.3). The anti-tumour effect was less effective, when the Coldrolip treatment was started after the tumour was already established, indicating that this approach is less a curative but rather a preventive therapy. Histological examination revealed an almost complete depletion of tumour infiltrated MOMA1+ and F4/80+ macrophages after Clodrolip treatment (Figure 5.7 D-F and J-L). There was no qualitative difference in macrophage depletion detectable, when the mice were treated 2, 3 or 4 times, corresponding to 3, 4 or 5 mg of Clodrolip injected in total. Remarkably, although 5 mg of free clodronate in total did not show any cytotoxic effects on MOMA1+ and F4/80+ macrophages in the spleen (Figure 5.6 C and I), MOMA1+ and F4/80+ macrophages in the tumour could be effectively depleted (Figure 5.7 C and I). It seems that macrophage populations in the spleen differ in their cytotoxic response to the application of free clodronate compared with TAMs. When free clodronate was used for F9 teratocarcinoma growth inhibition in SV 129 mice the tumour volumes were only insignificantly reduced, two days after the end of treatment (Figure 5.5 B). Although treatment with free clodronate did not reduce macrophages in the spleen, TAMs were efficiently depleted. This was accompanied by a reduction in tumour growth by about 45% (Figure 5.5 B). The anti-tumour effect obtained with a total dose of 5 mg free clodronate given on days 0, 4, 8 and 12 was comparable to Clodrolip therapy applied on days 8 and 12 with 3 mg in total dose. (Figure 5.5 A). Until now, the mechanisms through which macrophages evade the cytotoxic effect of bisphosphonates is not understood. But it is evident that different bisphosphonates have different biological properties, since e.g. the potent amino-bisphosphonates such as alendronate, pamidronate and zoledronate can stimulate cytokine production in mononuclear cells in vitro and cause a transient, acute-phase response in vivo, whereas clodronate does not show these properties [438-440]. Free bisphosphonates are known to inhibit the growth of human myeloma, melanoma, breast and prostate carcinoma cell lines in vitro [441]. Concentrations of $10^{-5}$ to $10^{-4}$ M or higher of free clodronate,
Pamidronate, ibandronate or zoledronate are required to inhibit tumour cell proliferation. The treatment of ECs with free bisphosphonates e.g. clodronate, risedronate, ibandronate and zoledronate reduces proliferation, induces apoptosis, and decreases capillary-like tube formation \textit{in vitro} [441]. Based on these anti-tumour properties of free bisphosphonates \textit{in vitro}, their effect on growth of tumour xenografts has been investigated obtaining contradictory results. Free amino-bisphosphonates, e.g. risedronate, alendronate, ibandronate and zoledronate did not inhibit tumour growth in mice when human tumour cells (MDA-MB-231, PC-3, PC3-ML) were injected subcutaneously or orthotopically [442-444]. However, treatment of ovarian cancer, melanoma and carcinoma in mouse models with free zoledronate, alendronate, and minodronate, respectively, led to significant reduction in tumour progression [440;445;446]. Giraudo \textit{et al.} treated mice by s.c. injection with 100 \( \mu \text{g} \) zoledronate kg\(^{-1} \) every 24 h for four and six weeks resulting in significant suppression of MMP-9 expression by TAMs, resulting in significant inhibition of tumour-associated angiogenesis and a reduction of 55-61\% of squamous cell carcinoma (SCC) volume [440]. Yamagishi \textit{et al.} recently investigated how minodronate, a newly developed nitrogen-containing bisphosphonate, inhibited melanoma growth and improved survival in nude mice by suppressing angiogenesis. Mice received i.p. 5 \( \mu \text{g} \) of minodronate every 24 h for 40 days. The application of minodronate significantly inhibited TAM infiltration and tumour growth and repressed the VEGF-induced increase in DNA synthesis and tube formation of ECs. Furthermore, minodronate inhibited VEGF-induced expression of intercellular adhesion molecule-1 and monocyte chemoattractant protein-1 in ECs [446]. Although the functional tumour-inhibition mechanisms of free bisphosphonates are not completely understood so far, it seems that extensive and repetitive treatment schemes are needed to obtain anti-cancer responses.

Colony-stimulating factor (CSF)-1 and VEGF are among the major chemotactic and differentiation regulators of tissue macrophages. CSF-1 expression is correlated with poor prognosis in breast cancer and is believed to enhance mammary tumour progression and metastasis through recruitment and regulation of TAMs [27;447]. Blocking CSF-1 expression by intratumourally injected anti-sense oligonucleotides resulted in suppression of mammary carcinoma growth by 50\% as shown by Aharinejad \textit{et al.} [448]. The present study shows that TAMs can be depleted by Clodrolip treatment, resulting in decreased tumour growth. This approach is
technically less demanding than the method based on anti-sense technology described above.

Several lines of evidence implicate VEGF as the key factor involved in tumour growth and metastasis. VEGF expression is associated with macrophage infiltration and angiogenesis, the extent of which being correlated with tumour prognosis. The anti-tumour therapy performed in this study is highly effective although not all stromal sources of pro-angiogenic mediators, for example neutrophils and mast cells, can be eliminated. One of the main inducers of VEGF and other pro-angiogenic factors, the hypoxic tumour cell itself, can not be inhibited to express and secrete high levels of angiogenic stimulators.

This raised the question, whether suppression of growing tumours would be even more effective by combination of the Clodrolip therapy with an anti-VEGF Ab therapy approach. This combination of two anti-cancer therapies would take into account that a tumour as a complex organ needs to be treated with a multi-drug approach.

Thus, it may be reasonable to consider incorporating Clodrolip therapy into conventional therapeutic regimens for additional tumour types and in other diseases where inflammatory and angiogenic processes play a role.
6. COMBINATION THERAPY WITH ANTI-VEGF (SCFV')\textsubscript{2} ANTIBODIES AND CLODROLIP FOR IMPROVED ANTI-ANGIOGENIC ACTIVITY

6.1. ABSTRACT

It was shown that the preventive application of anti-VEGF Abs and the treatment of liposome-encapsulated clodronate (Clodrolip) inhibited tumour growth as single modality therapies (Chapter 3.4.3 and 5.4.3). In this chapter these two anti-angiogenic regimes were combined in order to further improve inhibition of tumour progression. In a first experiment, an anti-VEGF (scFv')\textsubscript{2}-Ab was injected in combination with Clodrolip into F9 tumour-bearing mice significantly decreasing tumour volumes. This tumour inhibition was significantly superior compared to the single treatments. The second approach for a combination therapy using an anti-VEGF (scFv')\textsubscript{2}-Ab and Clodrolip in the xenograft A673 tumour mouse model led to a similar tumour volume reduction. In this model, the combination treatment was not significantly different compared to the single therapies. Immunohistochemical investigation of isolated A673 tumours showed significant depletion of MOMA1\textsuperscript{+} and F4/80\textsuperscript{+} TAMs after Clodrolip treatment and intermediary reduction after anti-VEGF (scFv')\textsubscript{2}-Ab treatment. Blood vessel staining of tumour specimens showed an almost total inhibition of CD31\textsuperscript{+} blood vessel growth after Clodrolip therapy and an intermediary effect after anti-VEGF (scFv')\textsubscript{2}-Ab treatment. Due to the almost complete inhibition of angiogenesis no further reduction after combination therapy was detectable. Strong positive correlation was found for F4/80\textsuperscript{+} TAM and MOMA1\textsuperscript{+} TAM densities versus vessel density. H&E and TUNEL-staining showed increased levels of necrotic and apoptotic cell populations in all therapy groups.

6.2. INTRODUCTION

Angiogenesis is essential for tumour progression and metastasis [449]. The newly formed blood vessels deliver the nutritional and respiratory requirements of an expanding tumour, which otherwise cannot grow larger than several millimetres (For more details see general introduction chapter 1.2.2.2).
Here, blocking of VEGF in combination with depletion of TAMs in the syngraft F9 teratocarcinoma SV129 mouse model (see also chapter 3.2) and in the human xenograft A673 rhabdomyosarcoma mouse model was examined. The A673 cell line was established from a patient with a primary rhabdomyosarcoma (RMS) [450]. This cell line has been widely used as an important tool in tumour biology. A673 cells are known to produce several growth factors with oncogenic potential, as well as cell growth-inhibitory factors [451]. The A673 cell line is referred to in the literature both as Ewing tumour (ET) or sarcoma (ES) and as RMS [452]. Tumour morphology and TAM depletion efficiency as well as tumour vessel density were analysed by immunohistochemistry.

6.3. MATERIAL AND METHODS

6.3.1. Reagents, cells and animals

Murine F9 teratocarcinoma and human A673 rhabdomyosarcoma tumour cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Female SV 129 and CD1nude mice were obtained from Charles River Wiga (Sulzfeld, Germany) and kept in standard housing and normal diet at the animal facility. Animal studies were performed under the licenses (No. 75533 and 75534) issued to R. A. Schwendener by the Veterinary Department of the Canton Aargau, Switzerland. The ethical guidelines that were followed meet the standards required by the UKCCCR guidelines [415]. DNA-Plasmid of anti-VEGF scFv V65 was obtained from R. Klemenz (Division of Cancer Research, Department of Pathology, University Hospital, Zurich, Switzerland) and modified at the C-terminal end as described previously (Chapter 2.4.1). Clodrolip was prepared as described in chapter 5.3.2.

6.3.2. Anti-tumour therapy using Clodrolip in combination with anti-VEGF (scFv')2 antibodies in the syngraft F9 and the xenograft A673 mouse models

Exponentially growing A673 cells where mixed with Matrigel (1 : 1; v/v) or with F9 cells (7 x 10^8 50 μL⁻¹) were injected s.c. on the abdominal side of CD1nude or SV 129 mice. Starting at different time-points after tumour cell injection the mice (6 - 8 per group) received 25 mg kg⁻¹ of anti-VEGF (scFv')2 Abs in 100 μl PBS by tail-vein injection and/or Clodrolip in 50 or 100 μl PBS (100 or 50 mg kg⁻¹) by i.p. application
as indicated. Tumour growth was measured in a blinded manner with a calliper every
day and volumes calculated using the following equation: \( V = \pi ab^2 / 6 \) (\( a \) = largest
tumour diameter, \( b \) = perpendicular diameter).

6.3.3. Immunohistochemistry

Spleens and tumours were removed and prepared for immunohistological
examination as described in chapter 5 (Chapter 5.3.6, table 5.1). For general
histological analysis, sections were stained with hematoxylin-eosin (H&E) staining
solution. Further primary macrophage specific mouse Abs used in this chapter were
obtained as indicated in table 6.1.

Table 6.1: Monoclonal Ab used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Marker</th>
<th>mAb</th>
<th>Distributor</th>
</tr>
</thead>
<tbody>
<tr>
<td>LYVE-1</td>
<td>Anti-mouse LYVE-1</td>
<td>ReliaTech (Braunschweig, Germany)</td>
</tr>
<tr>
<td>F4/80</td>
<td>A3-1</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>MOMA-1</td>
<td>MOMA-1</td>
<td>BMA Biomedicals (Augst, Switzerland)</td>
</tr>
<tr>
<td>ER-TR 9</td>
<td>ER-TR 9</td>
<td>BMA Biomedicals</td>
</tr>
<tr>
<td>CD68</td>
<td>Rat-anti-mouse CD68</td>
<td>Serotec (Dusseldorf, Germany)</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>CD31</td>
<td>BD PharMingen (San Diego, CA)</td>
</tr>
<tr>
<td>CD45R/B220</td>
<td>RA3-6B2</td>
<td>BD PharMingen</td>
</tr>
<tr>
<td>FDC</td>
<td>Rat-anti-mouse FDC</td>
<td>BD PharMingen</td>
</tr>
<tr>
<td>CD11b</td>
<td>M1/70</td>
<td>BD PharMingen</td>
</tr>
<tr>
<td>CD11c</td>
<td>HL3</td>
<td>BD PharMingen</td>
</tr>
</tbody>
</table>

Quantification of macrophage depletion and vessel density was done with the
analySIS software.

6.3.4. Statistical analysis

All data are shown as average ± SE of the mean. Statistical analysis of data from
inhibition trials and quantifications of immunostainings was performed using a two-
tailed, unpaired t-test. A \( P < 0.05 \) was considered statistically significant.

6.4. RESULTS

6.4.1. Therapeutic application of Clodrolip in combination with anti-VEGF
(scFv’)2-Abs in a syngraft and a xenograft tumour mouse model

In the syngraft F9 teratocarcinoma model, tumour cells were injected s.c. and mice
were treated as indicated in figure 6.1 while in the xenograft A673 model, tumour
cells were injected s.c. in a 1 : 1 mixture with Matrigel [115;407]. Mice were treated
as described before with species cross-reactive (scFv')$_2$-SZH9 Abs, unspecific control Abs and Clodrolip as shown in figure 6.2.

In the xenograft model, one mouse was removed from each group at day 16, 3 days after the end of therapy, the tumours and spleens were excised and snap frozen for immunohistochemistry. Finally, at day 22 all mice were sacrificed and the tumours removed and prepared for immunohistochemistry (Figure 6.3). As shown in figure 6.1 and 6.2, Clodrolip treatment was found to significantly decrease tumour size by 82% ($P < 0.005$, figure 6.1 B and table 6.2) as assessed by calculating the absolute tumour volume in the F9 model, and by 63% ($P < 0.05$, figure 6.2 B and table 6.3) in the A673 model, respectively.

Treatment with anti-VEGF (scFv')$_2$ Abs significantly reduced tumour size by 65% ($P < 0.05$, figure 6.1 B and table 6.2) in the F9 model, and by 59% ($P < 0.05$, figure 6.2 B and table 6.3) in the A673 model, respectively.

![Figure 6.1: Tumour growth inhibition by Clodrolip treatment in combination with anti-VEGF (scFv')$_2$-V65 Ab in the syngraft F9 teratocarcinoma model. (A) Normalized F9 tumour growth. Tumour-bearing mice were treated with PBS, unspecific control (scFv')$_2$-A1 Ab, anti-VEGF (scFv')$_2$-V65 Ab, Clodrolip plus unspecific control (scFv')$_2$-A1 Ab, and Clodrolip plus anti-VEGF (scFv')$_2$-V65 Ab as indicated. Clodrolip was injected i.p. and the (scFv')$_2$ Abs i.v. in the tail. Each Clodrolip treatment contained initially 100 mg kg$^{-1}$, followed by 50 mg kg$^{-1}$ and the (scFv')$_2$ Abs were (S in the graph) injected at 25 mg kg$^{-1}$. Values represent the mean of 5 treated mice ± SE of the mean. Relative tumour growth was normalized to day 2 (B) Bar graph of absolute tumour volumes measured at day 14, indicating the calculated averages of 5 treated mice ± SE of the mean. $P$ (unpaired, two-tailed t test), combination treatment versus single treatment (bar): *, $P < 0.05.$]
Table 6.2: Tumour volume reduction at day 14 i.e., 4 days after end of therapy.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumour volume ± SEM (cm³)</th>
<th>P-value</th>
<th>Normalized tumour volume ± SEM (%)</th>
<th>Change of tumour volume vs PBS controls (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>1.59 ± 0.30</td>
<td></td>
<td>100 ± 19</td>
<td>-</td>
</tr>
<tr>
<td>(scFv')₂-A1</td>
<td>1.76 ± 0.27</td>
<td>0.7143</td>
<td>111 ± 17</td>
<td>+ 11</td>
</tr>
<tr>
<td>Clodrolip + (scFv')₂-A1</td>
<td>0.29 ± 0.03</td>
<td>0.0027</td>
<td>18 ± 2</td>
<td>- 82</td>
</tr>
<tr>
<td>(scFv')₂-V65</td>
<td>0.55 ± 0.15</td>
<td>0.0466</td>
<td>35 ± 9</td>
<td>- 65</td>
</tr>
<tr>
<td>Clodrolip + (scFv')₂-V65</td>
<td>0.12 ± 0.04</td>
<td>0.0013</td>
<td>8 ± 3</td>
<td>- 92</td>
</tr>
</tbody>
</table>

Combination therapy was superior over the single treatments in both tumour models. Clodrolip treatment, combined with anti VEGF (scFv')₂ Ab therapy significantly inhibited tumour growth by 92% ($P < 0.005$, figure 6.1 B and table 6.2) in the F9 model, and by 74% ($P < 0.01$, figure 6.2 B and table 6.3) in the A673 model, respectively. The combined therapy approach in the syngraft tumour mouse model revealed a significantly decreased tumour size compared to the two single treatments with $P$ values of $< 0.05$ for (scFv')₂-V65 Ab and Clodrolip (Figure 6.1).

Figure 6.2: Tumour growth inhibition of Clodrolip treatment in combination with anti-VEGF (scFv')₂-SZH9 Ab in A673 rhabdomyosarcomas. (A) Normalized graph of tumour growth. Tumour-bearing mice were treated with PBS, unspecific control (scFv')₂-A1 Ab, anti-VEGF (scFv')₂-SZH9 Ab, Clodrolip plus unspecific control (scFv')₂-A1 Ab and Clodrolip plus anti-VEGF (scFv')₂-SZH9 Ab as indicated. Clodrolip was injected i.p. and the (scFv')₂ Abs i.v. in the tail. Each Clodrolip treatment contained initially 100 mg kg⁻¹, followed by 50 mg kg⁻¹ and the (scFv')₂ Abs were injected at 25 mg kg⁻¹. Values represent the mean of 5 treated mice ± SE of the mean. Relative tumour growth was normalized to day 1. (B) Bar graph of absolute tumour volumes measured at day 16 shows the calculated averages of 5 treated mice ± SE of the mean. $P$ (unpaired, two-tailed $t$ test), combination treatment versus single treatment (bar): not significant, NS, $P \geq 0.05$. 

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Table 6.3: Tumour volume reduction at day 16 i.e., 3 days after end of therapy.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumour volume ± SEM (cm³)</th>
<th>P-value</th>
<th>Normalized tumour volume ± SEM (%)</th>
<th>Change of tumour volume vs PBS controls (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0.50 ± 0.09</td>
<td>-</td>
<td>100 ± 18</td>
<td>-</td>
</tr>
<tr>
<td>(scFv')₂-A1</td>
<td>0.63 ± 0.10</td>
<td>0.8644</td>
<td>127 ± 19</td>
<td>+ 27</td>
</tr>
<tr>
<td>Clodrolip + (scFv')₂-A1</td>
<td>0.19 ± 0.04</td>
<td>0.0148</td>
<td>37 ± 9</td>
<td>- 63</td>
</tr>
<tr>
<td>(scFv')₂-SZH9</td>
<td>0.20 ± 0.06</td>
<td>0.0390</td>
<td>41 ± 12</td>
<td>- 59</td>
</tr>
<tr>
<td>Clodrolip + (scFv')₂-SZH9</td>
<td>0.13 ± 0.02</td>
<td>0.0093</td>
<td>26 ± 3</td>
<td>- 74</td>
</tr>
</tbody>
</table>

Figure 6.3: Photographs from CD1 nude mice bearing abdominal A673 rhabdomyosarcomas at day 16 (arrows), 3 days after end of treatment (A to E) and isolated tumours at the end of experiment at day 22 (F to J). Mice were treated as indicated in figure 6.2 A with PBS (A and F), unspecific control (scFv')₂-A1 Ab (B and G), anti-VEGF (scFv')₂-SZH9 Ab (C and H), Clodrolip plus unspecific control (scFv')₂-A1 Ab (D and I) and Clodrolip plus anti-VEGF (scFv')₂-SZH9 Ab (E and J).

In contrast, the combination therapy in the xenograft tumour mouse model did not result in a significant decrease of tumour volume when compared with the individual treatments with (scFv')₂-SZH9 Ab and Clodrolip (Figure 6.2). This suggests that Clodrolip efficiently inhibits tumour growth in both tumour models by depleting TAMs. Combination therapy with anti-VEGF (scFv')₂ Abs and Clodrolip resulted in the syngraft F9 model in significantly ($P < 0.05$) better tumour growth reduction. In the xenograft A673 model the combination treatment led to almost complete inhibition of tumour growth, even 9 days after end of treatment.

6.4.2 Immunohistochemical investigation of TAM depletion

Immunohistochemical analysis of A673 tumour sections from mice sacrificed 3 and 9 days after the end of the Clodrolip treatment revealed significant inhibition of macrophage infiltration and tumour angiogenesis (Figures 6.5 to 6.9 and tables 6.4-6.6). Depletion of splenic MOMA1⁺ and F4/80⁺ macrophages is shown in figure 6.4. Strong depletion effects of tumour-associated MOMA1⁺ and F4/80⁺ macrophages, were observed 3 days after Clodrolip treatment (Figures 6.5 and 6.6, D and E). Only
a modest repopulation of MOMA1⁺ and F4/80⁺ macrophages was observed at day 22, 9 days after the end of the therapy (Figure 6.5 and 6.6, I and J). Repopulation of macrophages in the spleen requires more time, since MOMA1⁺ and F4/80⁺ macrophages in the Clodrolip-treated mice at day 22 were only scarcely detected (Figure 6.4, I and J). For quantification, 4 to 5 tumour sections per treatment group were analysed immunohistologically and the positively stained areas in 3 microscopic pictures using image analysSIS software were determined. Clodrolip treatment significantly depleted tumour-associated MOMA1⁺ macrophages by 93% (P < 0.005, figure 6.5 and 6.7, table 6.4) and F4/80⁺ macrophages by 90% (P < 0.005, figure 6.6 and 6.7, table 6.4), respectively. Anti-VEGF (scFv')₂ Ab treatment was found to have an intermediate effect on tumour-associated MOMA1⁺ macrophages with a reduction of 52% and F4/80⁺ macrophages by 30%, respectively. This is either due to inhibition of VEGF as a macrophage-stimulating factor and/or by blocking the formation of new blood vessels via macrophage infiltrate into the tumour (Figure 6.5, 6.6 and 6.7, tables 6.4 and 6.5).

Figure 6.4: Depletion of MOMA1 and F4/80 positive spleen macrophage populations in CD1nude mice after i.p. treatment with Clodrolip as shown in figure 6.2 A. Spleen sections obtained from mice treated with PBS (A and F), control (scFv')₂-A1 Ab (B and G), anti-VEGF (scFv')₂-SZH9 Ab (C and H), Clodrolip (D and I), or Clodrolip in combination with anti-VEGF (scFv')₂-SZH9 Ab (E and J) are shown. At day 22, 9 days after the last treatment, sections were IHC stained for marginal zone metallophilic MOMA1⁺ macrophages (A to F) and red pulp F4/80⁺ macrophages (G to L). Arrows show populations of macrophages that were not depleted or that were repopulated after end of depletion therapy (day 22). Bar indicates 200 µm.
Figure 6.5: Depletion of MOMA1 positive tumour-associated macrophages with Clodrolip in xenografted CD1nude mice. A 673 rhabdomyosarcoma tissue sections obtained from mice injected with PBS (A and F), control (scFv')2-A1 Ab (B and G), anti-VEGF (scFv')2-SZH9 Ab (C and H), Clodrolip (D and I) or Clodrolip in combination with anti-VEGF (scFv')2-SZH9 Ab (E and J) as indicated (Figure 6.2 A) are shown. At day 16 (A to E), 3 days after end of therapy, and at day 22 (F to J), 9 days after end of treatment, sections were IHC stained for marginal zone metallophilic MOMA1+ macrophages. Arrows (in D, E, I and J) show populations of metallophilic MOMA1+ macrophages that were not be depleted or that were repopulated after end of depletion therapy (day 16). Bar indicates 200 μm.

Figure 6.6: Depletion of F4/80 positive activated tumour-associated macrophages with Clodrolip in xenografted CD1nude mice. A 673 rhabdomyosarcoma tissue sections obtained from mice injected with PBS (A and F), unspecific control (scFv')2-A1 Ab (B and G), anti-VEGF (scFv')2-SZH9 Ab (C and H), Clodrolip (D and I) or Clodrolip in combination with anti-VEGF (scFv')2-SZH9 Ab (E and J) as indicated (Figure 6.2 A) are shown. At day 16 (A to E), 3 days after end of treatment, and at day 22 (F to J), 9 days after end of treatment, sections were IHC stained for activated F4/80+ macrophages. Arrows (in D, E, I and J) show populations of F4/80+ macrophages that were not be depleted or that were repopulated after end of depletion therapy. Bar indicates 200 μm.
Due to the almost quantitative depletion of MOMA1+ and F4/80+ macrophages by Clodrolip, the combination therapy was not superior then the single treatments. The most striking therapy was Clodrolip treatment combined with anti VEGF (scFv')2 Ab. That significantly depleted MOMA1+ TAMs by 94% (P < 0.001) and F4/80+ TAMs by 90% (P < 0.0001), respectively (Figures 6.5, 6.6 and 6.7; tables 6.4 and 6.5).

![Depletion Graph](image)

**Figure 6.7**: Quantification of depletion of MOMA1+ and F4/80+ macrophages in A673 tumours of CD1 nude mice at day 22. Three to five cryostat sections of tumour tissues were stained. MOMA1+ and F4/80+ areas in three different fields of each section were measured and quantified using the analySIS software. The bar graph indicates the calculated averages ± SE of the mean. P (unpaired, two-tailed t test). Treated versus PBS: *, P < 0.05.

**Table 6.4**: Quantification of depletion effect, MOMA1+ macrophages at day 22.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Positively stained area ± SEM (%)</th>
<th>P-value</th>
<th>Normalized ± SEM (%)</th>
<th>Depletion effect vs PBS controls (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0.19 ± 0.05</td>
<td>-</td>
<td>100 ± 24</td>
<td>-</td>
</tr>
<tr>
<td>(scFv')2-A1</td>
<td>0.16 ± 0.05</td>
<td>0.7115</td>
<td>86 ± 27</td>
<td>-14</td>
</tr>
<tr>
<td>(scFv')2-SZH9</td>
<td>0.09 ± 0.05</td>
<td>0.1828</td>
<td>48 ± 27</td>
<td>-52</td>
</tr>
<tr>
<td>Clodrolip</td>
<td>0.01 ± 0.00</td>
<td>0.0034</td>
<td>7 ± 3</td>
<td>-93</td>
</tr>
<tr>
<td>Clodrolip + (scFv')2-SZH9</td>
<td>0.01 ± 0.00</td>
<td>0.0006</td>
<td>6 ± 2</td>
<td>-94</td>
</tr>
</tbody>
</table>

**Table 6.5**: Quantification of depletion effect, F4/80+ macrophages at day 22.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Positively stained area ± SEM (%)</th>
<th>P-value</th>
<th>Normalized ± SEM (%)</th>
<th>Depletion effect vs PBS controls (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>1.29 ± 0.26</td>
<td>-</td>
<td>100 ± 20</td>
<td>-</td>
</tr>
<tr>
<td>(scFv')2-A1</td>
<td>1.29 ± 0.26</td>
<td>0.9805</td>
<td>100 ± 19</td>
<td>-</td>
</tr>
<tr>
<td>(scFv')2-SZH9</td>
<td>0.90 ± 0.16</td>
<td>0.2850</td>
<td>70 ± 10</td>
<td>-30</td>
</tr>
<tr>
<td>Clodrolip</td>
<td>0.13 ± 0.07</td>
<td>0.0011</td>
<td>10 ± 5</td>
<td>-90</td>
</tr>
<tr>
<td>Clodrolip + (scFv')2-SZH9</td>
<td>0.12 ± 0.03</td>
<td>0.0001</td>
<td>10 ± 2</td>
<td>-90</td>
</tr>
</tbody>
</table>
6.4.3. CD31 and LYVE-1 staining of A673 tumour specimens

Histological examination using the endothelial cell specific (PECAM-1) Ab CD31 revealed a reduction of vessels three days after the end of therapy. A significant reduction of vessel density was still observed nine days after the last applications. After (scFv')\_2-SZH9 Ab treatment vessel density was reduced by 48\% (P < 0.05) and treatment with Clodrolip alone even resulted in a 89\% (P < 0.0001) reduction of CD31 positive endothelial cells at day 22. A combination of (scFv')\_2-SZH9 Ab and Clodrolip application resulted in a reduction of 85\% (P < 0.0001) in CD31\(^+\) cells which was statistically not different from Clodrolip treatment alone (Figure 6.8, table 6.6 and figure 6.9).

**Figure 6.8**: Vessel density in histopathologic analysis. Human A 673 rhabdomyosarcoma tissues obtained from mice injected with PBS (A and F), control (scFv')\_2-A1 Ab (B and G), anti-VEGF (scFv')\_2-SZH9 Ab (C and H), Clodrolip (D and I), or Clodrolip in combination with anti-VEGF (scFv')\_2-SZH9 Ab (E and J) as indicated (Figure 6.2 A) are shown. At day 16 (A to E), 3 days after end of treatment and at day 22 (F to J), 9 days after end of treatment, sections were IHC stained for CD31\(^+\) endothelial cells (vessel density). Arrows (in C and H-J) show very small vessels. Bar indicates 200 \(\mu\)m.

**Table 6.6**: Quantification of vessel density by CD31 staining.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Positively stained area ± SEM (%)</th>
<th>P-value</th>
<th>Normalized ± SEM (%)</th>
<th>Inhibition of angiogenesis vs PBS controls (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0.76 ± 0.10</td>
<td>-</td>
<td>100 ± 13</td>
<td>-</td>
</tr>
<tr>
<td>(scFv')_2-A1</td>
<td>0.74 ± 0.07</td>
<td>0.6997</td>
<td>98 ± 9</td>
<td>-2</td>
</tr>
<tr>
<td>(scFv')_2-SZH9</td>
<td>0.39 ± 0.08</td>
<td>0.0174</td>
<td>52 ± 10</td>
<td>-48</td>
</tr>
<tr>
<td>Clodrolip</td>
<td>0.08 ± 0.03</td>
<td>0.0034</td>
<td>11 ± 3</td>
<td>-89</td>
</tr>
<tr>
<td>Clodrolip + (scFv')_2-SZH9</td>
<td>0.11 ± 0.04</td>
<td>0.0001</td>
<td>15 ± 6</td>
<td>-85</td>
</tr>
</tbody>
</table>
Figure 6.9: Quantification of vessel density in A673 tumours at day 22. Three to five cryostat sections were stained. CD31-positive areas in 3 different fields of each section were quantified using the analySIS software. Bar graph indicates the calculated averages ± SE of the mean. Ps (unpaired, two-tailed t test): Treated versus PBS: *, P < 0.05.

By plotting F4/80+ and MOMA1+ TAM density versus microvessel counts a significant (P < 0.001) positive correlation with r = 0.755 of tumour-associated F4/80+ macrophages and with r = 0.735 of tumour-associated MOMA1+ macrophages was found (Figure 6.10). Compared with the strong invasive vascularization by blood vessel of the tumour, there were almost no lymphatic vessels found by LYVE-1 staining (Table 6.7 and in appendix figure 10.3).

Figure 6.10: Correlation of tumour-associated macrophages versus microvessel counts in human A673 rhabdomyosarcoma. Tumour-bearing mice were treated as indicated in figure 6.2 A and at day 22, 9 days after end of treatment, tumours were prepared for IHC. Correlation of F4/80+ macrophages (—, r = 0.755, P < 0.0001) and MOMA1+ macrophages (—, r = 0.735, P < 0.0001) density versus microvessel counts were plotted.
6.4.4. H&E and TUNEL staining of A673 tumour specimens

Staining (H&E and TUNEL) of apoptotic and necrotic A673 cells showed larger affected areas in the treated compared to the control groups. The tumours of mice treated with Clodrolip showed only a narrow peripheral ring of living tumour cells surrounding a large necrotic and apoptotic centre (Figures 6.11 D and E and 6.12 C, D and E). In the (scFv')2-SZH9 Ab treated group an increase in apoptotic cells was observed (Figure 6.12 C and H).

Figure 6.11: Histopathologic analysis of H&E-positive A673 tumour cells. A 673 RMS tissue sections obtained from mice injected with PBS (A and F), control (scFv')2-A1 Ab (B and G), anti-VEGF (scFv')2-SZH9 Ab (C and H), Clodrolip (D and I) or Clodrolip in combination with anti-VEGF (scFv')2-SZH9 Ab (E and J) as indicated (Figure 6.2 A) are shown. At day 16 (A to E), 3 days after end of treatment, and at day 22 (F to J), 9 days after end of treatment sections were IHC stained. Arrows in A to C and F to H show areas of necrotic and apoptotic cells and large regions of apoptotic cell clusters in C, H, I and J. Bar indicates 500 μm.

Figure 6.12: Histopathologic analysis of TUNEL-positive, apoptotic A673 tumour cells. A 673 RMS tissue sections obtained from mice injected with PBS (A and F), control (scFv')2-A1 Ab (B and G), anti-VEGF (scFv')2-SZH9 Ab (C and H), Clodrolip (D and I) or Clodrolip in combination with anti-VEGF (scFv')2-SZH9 Ab (E and J) as indicated (Figure 6.2 A) are shown. At day 16 (A to E), 3 days after end of treatment, and at day 22 (F to J), 9 days after end of treatment sections were IHC stained for apoptotic cells using the TUNEL assay (Apoptosis). Arrows in A and G show some areas of apoptotic cells and large regions of apoptotic cell clusters in C, H, I and J. Bar indicates 500 μm.
Macrophages are essential for several immunological functions and phagocytosis of dead cells. Macrophage depletion did not cause systemic toxic side effects in the mice such as weight loss, weakness or loss of activity. As shown in figure 6.13, the treatment with a total of 5 mg clodronate-liposomes per mouse resulted in no significant change of body weight during the monitored time period of 22 days.

![Figure 6.13](image)

**Figure 6.13**: Body weight changes during treatment of a 673 tumour-bearing CD1 nude mice. The Bar graphs show relative change of body weight during the 21 day observation period. Values represent the mean of 5 treated mice, ± SE of the mean. Calculated P (Student’s t test) resulted in no significant values of change in body weight (not shown).

Staining of tumour sections with additional markers (ER-TR 9, CD68, Lyve-1, CD11b and CD11c) are summarized in table 6.7. In the tumour center only few ER-TR 9⁺ TAMs (appendix, figure 10.1) could be detected, whereas in the outer tumour layers CD68⁺ macrophages (appendix, figure 11.2) were found in a widely spread area and were not affected after Ab or Clodrolip treatment.

![Table 6.7](image)

**Table 6.7**: Effect of Clodrolip on leucocytes, blood and lymphatic vessel density. Scoring of positively stained areas after immunohistologic investigation of A673 tumour specimens taken at day 22, 9 days after end of therapy with different Abs.

<table>
<thead>
<tr>
<th>Marker</th>
<th>PBS</th>
<th>(ScFv)²-A1</th>
<th>(ScFv)²-SZH9</th>
<th>Clodrolip</th>
<th>Clodrolip + (scFv)²-SZH9</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOMA1</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ER-TR 9⁺</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F4/80</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD68⁺</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>CD11b⁺</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>CD11c⁺</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD31</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LYVE-1⁺</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Symbol code used in table: +++ very strong staining; ++ moderate staining; weak or inconsistent staining, - no staining. †, Pictures in appendix of this work.
6.4. DISCUSSION

Clodronate-liposomes (a bisphosphonate encapsulated in small phospholipid vesicles) have previously been shown to deplete macrophages in different organs in a variety of immunological studies [375;378;453]. In the work presented here this technique was used to deplete macrophages in tumours. The application of clodronate-liposomes (Clodrolip), suppressed tumour growth by significantly depleting tumour infiltrated macrophages. Depletion of TAMs by Clodrolip is very efficient, since the inhibition of tumour growth in the syngraft F9 mouse model was about 75% (Chapter 5.4.3, figure 5.5 B) and 82% (Figure 6.1 B), and in the xenograft A673 mouse model 63%, respectively (Figure 6.2 B). Efficient elimination obtained by Clodrolip-application could be shown by IHC studies which revealed a nearly complete depletion of MOMA1+ and F4/80+ TAMs (Figure 6.7, tables 6.4 and 6.5). The depletion of tumour-associated macrophages was accompanied by inhibition of neo-vascularisation (Figure 6.9 and table 6.6), resulting in large necrotic and apoptotic areas mainly in the centers of the tumours (Figure 6.11 and 6.12).

Several lines of evidence implicate VEGF as one of the key factors involved in tumour growth and metastasis. VEGF expression levels are associated with macrophage infiltration and angiogenesis, the extent of which being correlated with tumour prognosis. The application of anti-VEGF (scFv)2-SZH9 Ab in the xenograft A673 tumour mouse model resulted in an intermediate reduction of MOMA+ and F4/80+ TAMs, respectively (Figure 6.7, tables 6.4 and 6.5). This implies either, that due to the decreased number of blood vessels TAMs can not populate the tumour mass or that VEGF was efficiently blocked, preventing macrophage attraction. In this study, two anti-angiogenic therapies were combined to eliminate VEGF and TAMs, one of the major sources of angiogenic factors. It is conceivable, that the best therapeutic outcome could be achieved by combination of an anti-angiogenic with a macrophage depletion therapy. Although the anti-angiogenic combination therapy was successful to inhibit tumour growth, it was not able to eliminate the tumours completely, resulting in re-growth after the end of therapy (Figures 6.1 A and 6.2 A).

It must be pointed out, that depletion of TAMs does not remove all the stromal sources of angiogenic mediators, e.g. those produced by mast cells and neutrophils. However, in combination with neutralising anti-VEGF single-chain Abs, the tumours...
were starved and shrank to nearly undetectable size. Combination therapy was superior in both tumour models compared to the single modality therapies.

As discussed in the previous chapter CSF-1 is, together with VEGF, one of the major chemotactic factors of tissue macrophages. Aharinejad et al. showed that blocking CSF-1 or the CSF-1 receptor, suppressed macrophage infiltration and mammary tumour growth by 40 to 50%. Most importantly they demonstrated, that host macrophage infiltration, host MMPs, VEGF expression and EC proliferation within tumours of treated mice were decreased as compared to tumours in control groups. In addition, mouse survival significantly increased after blocking CSF-1 indicating that macrophages contribute to tumour progression and are important targets for the treatment of solid tumours [448].

Although a comparison of the two tumour models used here is complex, it seems that the fast growing F9 cells are more susceptible to anti-angiogenic drugs than the slowly growing A673 tumours. TAM depletion is therefore particularly well suited to block fast growing tumours (Figure 6.1 B and table 6.2). Furthermore, by removing an additional angiogenic factor, the VEGF secreted by the tumour cell, with an anti-VEGF Ab, a further significant decrease in tumour size resulted in the F9 tumour model (Figure 6.1 B). In the A673 tumours only a modest additional decrease in tumour size was observed (Figure 6.2 B). The two tumour models have in common that once the anti-angiogenic therapy was discontinued, the tumours started to regrow. This disadvantage could only be circumvented by combining anti-angiogenic strategies with cytotoxic anti-cancer approaches. Bevacizumab (Genentec: Avastin®), a recombinant humanized monoclonal Ab to VEGF, is a very successful method for anti-cancer therapy that was approved last year by the American Food and Drug Administration (FDA) for clinical application [19]. In clinical studies Bevacizumab was successfully applied as an anti-angiogenic drug complementing classical chemotherapy. Preclinical research showed, that Bevacizumab inhibits human VEGF with a $K_d$ value in the low nanomolar range, has activity in multiple cancer cell lines, and is synergistic with several cancer chemotherapeutic agents. In humans, Bevacizumab has a long blood circulation half-life, allowing intravenous administration once every 2 to 3 weeks [19]. Dose-limiting toxicities, the formation of Abs to Bevacizumab, and problems with wound healing after surgery have not been observed in clinical trials. Bevacizumab is a good example for the successful combination of anti-angiogenic with cytotoxic therapy. A
phase II study of Bevacizumab in combination with 5-fluorouracil and leucovorin in patients with metastatic colorectal cancer showed promising results, i.e., a good therapeutic response and prolonged disease-free patient survival. Taken together with further phase III studies, the anti-VEGF Ab Bevacizumab has shown promise in promoting a synergism in combination with other chemotherapeutic agents for the treatment of various cancers [19].
7. FINAL DISCUSSION

Tumour neo-vascularisation can be repressed with anti-angiogenic compounds. One of the critical mediators of angiogenesis is vascular endothelial growth factor (VEGF). It plays a pivotal role in the regulation of vasculogenesis and angiogenesis by inducing endothelial cell proliferation and migration, increases microvascular permeability and acts as a survival factor for newly formed blood vessels (see general introduction). This multifunctional role of VEGF renders it an attractive target for anti-angiogenic therapeutic intervention.

In a carcinoma mouse model treatment with newly anti-VEGF (scFv')_2-Ab homodimers showed inhibition of tumour volume. Although the (scFv')_2-Ab was rapidly removed from the blood, almost 3% of the injected (scFv')_2-Ab dose per gram of tissue (%IDg⁻¹) localized in A673 rhabdomyosarcoma tumours. Because of the passive tumour targeting properties of liposomes in vivo [428] and their long in vivo circulation times they appeared attractive as carriers of anti-angiogenic molecules such as (scFv')_2-SZH9 Abs. Several experimental approaches have demonstrated that liposomes with a diameter between 100 – 200 nm accumulate passively in solid tumours upon extravasation from the blood stream through the leaky tumour vasculature [424]. In addition, immunoliposomes could be loaded with cytotoxic and/or anti-angiogenic drugs [344].

The coupling process of molecules to the outer surface of liposomes is well established. The use of PEG as stabiliser against rapid elimination of immunoliposomes results in steric hindrance of the Ab-antigen interaction, especially when the Abs are coupled directly to the surface of the liposomes. To solve this problem, scFv Abs were attached to the distal end of the PEG chain on the surface of PEG-liposomes according to Marty et al. [344;402]. This method leads to the covalent binding of an average of 100 scFv Ab molecules to the surface of one liposome of 100 nm diameter.

The binding properties of scFv-SZH9 Ab immunoliposomes and free (scFv')_2-SZH9 Ab to VEGF were similar in vitro, but a therapeutic effect was not observed in the animal models (see chapter 4.5). Application of SZH9-immunoliposomes in the syngraft F9 teratocarcinoma and the transgenic Rip1Tag2 mouse model revealed no inhibition in tumour growth and no reduction of tumour vessel density, respectively (Chapter 4.4.3 and 4.4.4). These results can be attributed to a number of factors, e.g.
to limited dosage of the scFv Ab due to the rather low coupling efficiency to liposomes of 10 to 30%. Only 4 - 2 mg kg\(^{-1}\) of liposomal anti-VEGF scFv Ab could be applied in the syngraft or the transgenic tumour mouse model, respectively. Because of this limitation, a change in tumour progression in the Rip1Tag2 model was not expected. A possible anti-angiogenic response would have been manifested by reduction of vessel density. However, no difference in capillary density was found compared to the control groups. In contrast to these results, a decrease in vessel density has been reported in Rip1Tag2 mice treated repetitively with anti-angiogenic agents such as TNP470, minocycline and murine IFN in a comparable schedule [425;434]. Although in this approach scFv antibody liposomes were used the highly repetitive application mode probably resulted in immunogenic effects. Harding et al. showed, that systemic and repetitive application of immunoliposomes resulted in a immune response, mainly caused by the constant IgG part of the attached Abs [435]. In conclusion, anti-VEGF scFv-SZH9 Ab liposomes could not be delivered in amounts that would have been necessary to neutralize the angiogenic activity of VEGF in the two models investigated. However, the long blood circulation and the tumour accumulation properties still make them a suitable tool for the stabilization of therapeutic molecules.

The infiltration of leucocytes into solid tumours was noticed many years ago and it was suggested that they had a causal role in carcinogenesis. (See general introduction) These infiltrates are now known to contain myeloid cells (neutrophils, dendritic cells, macrophages, eosinophils and mast cells) as well as lymphocytes [26].

To increase the uptake by phagocytic macrophages unpegylated liposomes containing clodronate (Clodrolip) were prepared. \textit{In vitro} studies showed only a selective cytotoxic effect of Clodrolip to peritoneal macrophages, but not to HUVECs, F9 or A673 tumour cells. This selective cytotoxicity was also obtained \textit{in vivo} after Clodrolip treatment, where splenic macrophages were depleted and lymphocytes, e.g. B- and T- cells were not affected.

Preventive treatment with Clodrolip in a syngraft tumour mouse model was found to significantly decrease tumour size (Chapter 5.4.3) whereas the anti-tumour effect was less effective, when the treatment was started after the tumour was established. This indicates, that the approach is less a curative but rather a preventive therapy.
Histological examination revealed an almost complete depletion of tumour infiltrated MOMA1⁺ and F4/80⁺ macrophages after Clodrolip treatment (Figure 5.7). Remarkably, although free clodronate did not show any cytotoxic effects on macrophages in the spleen (Figure 5.6), TAMs were effectively depleted, indicating that macrophage populations in the spleen differ in their cytotoxic response. Although free clodronate did not reduce the tumour volume it showed a modest inhibition effect. So far, the exact cytotoxic mechanisms of bisphosphonates have not been identified. It was shown by Frith et al. that clodronate encapsulated in liposomes was phagocytosed by macrophages and metabolized to a toxic metabolite, AppCp, providing a mechanism by which macrophages, but not non-phagocytic cells, can be selectively removed following ingestion of such liposomes [454].

It is obvious that different bisphosphonates have different biological properties, since the amino-containing bisphosphonates such as alendronate, pamidronate and zoledronate can stimulate cytokine production by mononuclear cells in vitro and cause a transient, acute-phase response in vivo, whereas clodronate does not show these properties [438-440]. Because of the described anti-tumour effects of free bisphosphonates in vitro, their effect on growth of tumour xenografts in animals has been investigated. Whereas amino-bisphosphonates, e.g. risedronate, alendronate, ibandronate and zoledronate did not inhibit tumour growth in mice when human tumour cells (MDA-MB-231, PC-3, PC3-ML) were injected subcutaneously or orthotopically [442-444], treatment of ovarian cancer, melanoma and carcinoma in mouse models with free amino-bisphosphonates, led to significant reduction of tumour growth [440;445;446]. Although the mechanism of action responsible for tumour inhibition of free bisphosphonates is not completely understood, it seems that repetitive application schemes are needed to achieve anti-cancer responses.

Clauss et al. showed, that VEGF is not only an endothelial mitogen but that also stimulates macrophage migration [455]. Leek et al. suggested that, in addition to stimulating angiogenesis directly, VEGF may have an indirect effect on tumour angiogenesis by attracting macrophages into tumours [456]. The anti-tumour approach developed in this work is highly effective although stromal cells, contributing angiogenic mediators were not fully depleted and the hypoxic tumour cells, one of the major producers of VEGF and other angiogenic factors, were not inhibited.
This raised the issue, whether it would be even more successful to combine the anti-VEGF Ab therapy with Clodrolip therapy. The combination of two anti-cancer therapies would take into account that a tumour, as a complex organ, needs to be treated in a multimodal manner (Figure 7.1).

Elimination of TAMs by Clodrolip treatment is very efficient, since it causes significant decrease of tumour size in the syngraft F9 (Chapter 5.4.3 and 6.4) and in the xenograft A673 mouse model. Efficient depletion by Clodrolip-application could be shown by IHC studies which revealed a nearly complete depletion of MOMA1+ and F4/80+ TAMs. Depletion of TAMs blocked neo-angiogenesis almost entirely, resulting in large necrotic and apoptotic areas mainly in the centres of the tumours.

Inhibition of tumour angiogenesis by therapeutic application of anti-VEGF scFv Abs led to a significant decrease of tumour size in the syngraft F9 (Chapter 3.4.3 and 6.4) and in the xenograft A673 model (Chapter 6.4). In the xenograft A673 model the application of anti-VEGF Abs resulted in a modest reduction of TAMs. This implies either that, because of the decreased number of blood vessels, TAMs cannot infiltrate into the tumour, or that VEGF can efficiently be neutralized and thereby inhibit macrophage attraction.

**Figure 7.1:** Pathways of tumour-induced angiogenesis and possibilities of inhibition. Macrophages are recruited to hypoxic tumours by chemotactic factors, e.g. soluble colony-stimulating factor-1 (CSF-1) or VEGF and provide many trophic functions that promote tumour progression and metastasis. These TAMs migrate to hypoxic areas within the tumour, where they stimulate angiogenesis by expressing factors such as VEGF, bFGF, Ang-1 and Ang-2, IL-1 and IL-8, TNF-α, tyrosine phosphorylase (TP), MMP-2 and MMP-9, nitric oxide (NO) and others. In this study, 2 anti-angiogenic therapies were combined to eliminate VEGF, by anti-VEGF Abs (1), and TAMs, using liposome-encapsulated clodronate (2) in order to block tumour progression.
In two tumour mouse models Clodrolip application in combination with anti-VEGF single-chain Abs led to significantly decreased tumour growth (Figures 6.1 and 6.2). The presented anti-angiogenic therapy seems to be more effective in fast growing, highly angiogenesis dependend tumours since the combination therapy in the fast growing F9 model led to significantly decreased tumour volume in comparison to the single therapies. In contrast, in the slowly growing A673 model only an insignificant increased tumour size inhibition was observed. In both tumour models, once the anti-angiogenic therapy is stopped, the tumours regrow, indicating that this therapy is not curative. Depletion of tumour-associated macrophages does not remove all stromal sources of pro-angiogenic mediators, e.g. mast cells and neutrophils and tumour cells are inhibited in growth but are not killed. However, this disadvantage could only be circumvented by combining anti-angiogenic strategies with cytotoxic anti-cancer approaches.

In conclusion, a highly effective anti-angiogenic anti-cancer therapy using Clodrolip to inhibit tumour growth was established. In the future, this method needs to be further investigated in order to evaluate the maximal tolerated and the minimal effective dose. It will be important to study the synergistic effects of free clodronate in combination with liposome-encapsulated clodronate, and whether other bisphosphonates encapsulated in liposomes show similar or even improved tumour growth inhibition. It has been shown here, that combination of anti-angiogenic drugs significantly improves therapeutic results compared to single drug treatments. Additionally, this information can lead to the design of multi-drug therapies, e.g. combination of anti-angiogenic with cytotoxic drugs, which are needed for a successful anti-tumour therapy in the future.
# 8. ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAMA</td>
<td>disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>AOX1</td>
<td>Alcohol oxidase gene</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BMGY</td>
<td>Buffered glycerol-complex medium</td>
</tr>
<tr>
<td>BMMY</td>
<td>Buffered methanol-complex medium</td>
</tr>
<tr>
<td>BODIPY FL®, SE</td>
<td>4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-daza-S-indacene-3-propionic acid, succinimidyl ester</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCLs</td>
<td>Chemokine ligands</td>
</tr>
<tr>
<td>CSF1</td>
<td>Colony stimulating factor 1</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetra acetate</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbant assay</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>IGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>IGF1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropylthiogalactoside</td>
</tr>
<tr>
<td>I.v.</td>
<td>Intravenously</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani medium</td>
</tr>
<tr>
<td>MD</td>
<td>Minimal dextrose</td>
</tr>
<tr>
<td>MM</td>
<td>Minimal methanol</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>PAI1</td>
<td>Plasminogen activator inhibitor 1</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>P. pastoris</td>
<td><em>Pichia pastoris</em></td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBSM</td>
<td>PBS containing 2% milk</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphadidylethanolamine</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>DPPE-PEG-NH₂</td>
<td>1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)2000]</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>S.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous-cell carcinoma</td>
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<tr>
<td>ScFv</td>
<td>single chain Fv Ab fragment</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SPC</td>
<td>soy phosphatidylcholine</td>
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<tr>
<td>sulfo-SMCC</td>
<td>Sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate</td>
</tr>
<tr>
<td>SUV</td>
<td>Small unilamellar vesicles</td>
</tr>
<tr>
<td>TBP</td>
<td>Tributylphosphine</td>
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<tr>
<td>TGFβ</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TUNEL</td>
<td>TdT-mediated dUTP Nicked End Labeling</td>
</tr>
<tr>
<td>TY</td>
<td>Trypton yeast extract</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>V₇</td>
<td>Variable heavy chain</td>
</tr>
<tr>
<td>V₈</td>
<td>Variable light chain</td>
</tr>
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<td>WST-1</td>
<td>(4-[3-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast extract peptone dextrose</td>
</tr>
<tr>
<td>YPDS</td>
<td>Yeast extract peptone dextrose sorbitol</td>
</tr>
</tbody>
</table>
9. ONLINE LINKS

Angiogenesis Inhibitors in Clinical Trials web site:
http://cancer.gov/clinicaltrials/developments/anti-angio-table

Special Project Angiogenesis:
http://www.med.unibs.it/~airc/therapy.html
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11. APPENDIX

Figure 11.1: Detection of ER-TR 9 positive tumour-associated macrophages in xenografted CD1 nude mice. A 673 rhabdomyosarcoma tissue sections obtained from mice injected with PBS (A and F), control (scFv')-A1 Ab (B and G), anti-VEGF (scFv')-SZH9 Ab (C and H), Clodrolip (D and I) or Clodrolip in combination with anti-VEGF (scFv')-SZH9 Ab (E and J) as indicated (Figure 6.2 A) are shown. At day 16 (A to E), 3 days after end of therapy, and at day 22 (F to J), 9 days after end of treatment, sections were IHC stained for ERTR9 macrophages. Arrows (in A, B, C and F) show only some tiny populations of ERTR9 macrophages at the outer surface but not any in the tumour center. Bar indicates 200 μm.

Figure 11.2: No depletion of CD68 positive tumour-associated macrophages with Clodrolip in xenografted CD1 nude mice. A 673 rhabdomyosarcoma tissue sections obtained from mice injected with PBS (A and F), control (scFv')-A1 Ab (B and G), anti-VEGF (scFv')-SZH9 Ab (C and H), Clodrolip (D and I) or Clodrolip in combination with anti-VEGF (scFv')-SZH9 Ab (E and J) as indicated (Figure 6.2 A) are shown. At day 16 (A to E), 3 days after end of therapy, and at day 22 (F to J), 9 days after end of treatment, sections were IHC stained for CD68 macrophages. Bar indicates 200 μm.
Figure 11.3: Lymph vessel density in histopathologic analysis. Human A 673 rhabdomyosarcoma tissues obtained from mice injected with PBS (A and F), control (scFv')2-A1 Ab (B and G), anti-VEGF (scFv')2-SZH9 Ab (C and H), Clodrolip (D and I), or Clodrolip in combination with anti-VEGF (scFv')2-SZH9 Ab (E and J) as indicated (Figure 6.2 A) are shown. At day 16 (A to E), 3 days after end of treatment and at day 22 (F to J), 9 days after end of treatment, sections were IHC stained for LYVE1+ lymphatic vessels. Arrows (in B) show the only lymphatic vessels that could be detected in all investigated sections. Bar indicates 200 μm.

Figure 11.4: Depletion of CD11b positive tumour-associated dendritic cells (DCs) with Clodrolip in xenografted CD1nu nude mice. A 673 rhabdomyosarcoma tissue sections obtained from mice injected with PBS (A and F), control (scFv')2-A1 Ab (B and G), anti-VEGF (scFv')2-SZH9 Ab (C and H), Clodrolip (D and I) or Clodrolip in combination with anti-VEGF (scFv')2-SZH9 Ab (E and J) as indicated (Figure 6.2 A) are shown. At day 16 (A to E), 3 days after end of therapy, and at day 22 (F to J), 9 days after end of treatment, sections were IHC stained for CD11b+ DCs. Bar indicates 200 μm.
Figure 11.5: Depletion of CD11c-positive tumour-associated dendritic cells (DCs) with Clodrolip in xenografted CD1 nude mice. A 673 rhabdomyosarcoma tissue sections obtained from mice injected with PBS (A and F), control (scFv')2-A1 Ab (B and G), anti-VEGF (scFv')2-SZH9 Ab (C and H), Clodrolip (D and I) or Clodrolip in combination with anti-VEGF (scFv')2-SZH9 Ab (E and J) as indicated (Figure 6.2 A) are shown. At day 16 (A to E), 3 days after end of therapy, and at day 22 (F to J), 9 days after end of treatment, sections were IHC stained for CD11c⁺ DCs. Arrows (in A and J) show very tiny populations of CD11c⁺ DCs. Bar indicates 200 μm.

Figure 11.6: Quantification of CD11c⁺ stained cells in A673 tumours at day 22. Three to five cryostat sections were stained. CD11c-positive areas in 3 different fields of each section were quantified using the analySIS software. Bar graph indicates the calculated averages ± SE of the mean. Ps (unpaired, two-tailed t test): Treated versus PBS: *, P < 0.05.
Table 11.1: Quantification of depletion effect, CD11c+ macrophages at day 22.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Positively stained area ± SEM (%)</th>
<th>P-value</th>
<th>Normalized ± SEM (%)</th>
<th>Depletion effect compared to PBS controls (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0.12 ± 0.19</td>
<td>-</td>
<td>100 ± 2.3</td>
<td>-</td>
</tr>
<tr>
<td>(scFv')2-A1</td>
<td>0.22 ± 0.26</td>
<td>0.8702</td>
<td>102 ± 4.4</td>
<td>+2</td>
</tr>
<tr>
<td>(scFv')2-SZH9</td>
<td>0.20 ± 0.06</td>
<td>0.0189</td>
<td>3.7 ± 1.1</td>
<td>-96</td>
</tr>
<tr>
<td>Clodrolip</td>
<td>0.24 ± 0.02</td>
<td>0.0055</td>
<td>0.7 ± 0.3</td>
<td>-99</td>
</tr>
<tr>
<td>Clodrolip + (scFv')2-SZH9</td>
<td>0.63 ± 0.51</td>
<td>0.0019</td>
<td>0.5 ± 0.2</td>
<td>-99</td>
</tr>
</tbody>
</table>

Figure 11.7: Correlation of tumour-associated CD11c+ DCs versus microvessel counts in human A673 rhabdomyosarcoma. Tumour-bearing mice were treated as indicated in figure 6.2 A and at day 22, 9 days after end of treatment, tumours were prepared for IHC. Correlation of CD11c+ DC density versus microvessel counts were plotted (·, r = 0.636, P < 0.0019).
11.1. LIST OF PUBLICATIONS

Zeisberger S. M.; Odermatt B.; Marty C.; Zehnder-Fjällman A.; Ballmer-Hofer K. and Schwendener R. A.: Clodronate-liposome mediated depletion of tumor associated macrophages (TAMs) is a new and highly effective anti-angiogenic therapy approach (submitted, 2005)


11.1.1. Poster Presentations


Steffen M. Zeisberger and Reto A. Schwendener: Immunoliposomal inhibition of tumour-induced angiogenesis; ETH Pharma-day 2002, July 18, 2002, Zurich, Switzerland.

Steffen M. Zeisberger and Reto A. Schwendener: Immunoliposomal inhibition of tumour-induced angiogenesis; Cancer Research Retreat, November 9, 2002, Zurich, Switzerland.

11.1.2. Oral Presentations
Targeting and neutralization of VEGF using scFvAb immunoliposomes for the inhibition of tumour-induced angiogenesis; Doktorandentag des Departementes Pharmazie; ETH Zurich, Switzerland.
11.2. CURRICULUM VITAE

2001 – 2005 Ph. D. student at the Institute of Molecular Cell Biology, Department of Live Science, Paul Scherrer Institut (PSI) and Swiss Federal Institute of Technology (ETH) Zürich, under the guidance of Prof. Dr. H. Wunderli-Allenspach and Prof. Dr. R.A. Schwendener

2000 Diploma in Biology

1999 – 2000 Diploma student at the University Children’s Hospital of Zurich, Switzerland, Division of Metabolism and Molecular Pediatrics under the guidance of Prof. Dr. B. Steinmann

1994 – 1999 Studies in Biology at the University of Mainz, Germany

1993 Graduation diploma at the Max-Planck-Gymnasium of Russelsheim, Germany