Doctoral Thesis in Biotechnology

Targeting Human Epidermal Growth Factor Receptors with Drug Conjugates Based on Affibody Molecules

JIE ZHANG

Stockholm, Sweden 2023
Targeting Human Epidermal Growth Factor Receptors with Drug Conjugates Based on Affibody Molecules

JIE ZHANG

Academic Dissertation which, with due permission of the KTH Royal Institute of Technology, is submitted for public defence for the Degree of Doctor of Philosophy on Friday 22nd September 2023 at 10:15 in Oskar Kleins Auditorium, Roslagstullsbacken 21, AlbaNova University Center, Stockholm.

Doctoral Thesis in Biotechnology
KTH Royal Institute of Technology
Stockholm, Sweden 2023
Abstract

Cancer is a major public health challenge and the second leading cause of death in the world, with millions of new cases being diagnosed each year. Traditional cancer treatments such as surgery, radiation therapy, and chemotherapy are many times effective, but may also cause damage to healthy cells, leading to side effects. Targeted therapy is a more precise and focused approach to cancer treatment, where the aim is to target the cancer cells while leaving the normal cells unaffected. It is particularly effective in cancers where specific molecular targets are known, such as the subset of breast cancer patients with HER2 over-expression or in the subset of patients with pancreatic cancer with HER3 over-expression.

Antibody-drug conjugates (ADCs) are an important addition to tumor-targeted therapy, with twelve drugs approved for clinical use by the FDA. They utilize the high specificity of monoclonal antibodies conjugated with highly cytotoxic small molecules to enhance the accumulation of the drugs in the tumor, for highly specific and efficient killing. However, traditional ADCs may not be the optimal delivery format for the directed delivery of cytotoxic drugs. They are limited by their relatively large molecular weights, resulting in relatively low penetration of solid tumors.

Recently, a novel type of drug conjugates, affibody-drug conjugates, has been described. These combined an engineered scaffold affinity protein, an affibody molecule, with an albumin binding domain (ABD) for half-life extension, to which a cytotoxic payload has been conjugated. Previous studies show that these novel drug conjugates have a potent and tumor-cell-specific cytotoxic effect. In the future, they may therefore become complementary or alternatives to current targeted cancer therapies.

This thesis focuses on the optimization of affibody-drug conjugates targeting HER2 and HER3, members of the human epidermal growth factor receptor family. The thesis presents in vitro and in vivo preclinical data, showing the potential for further clinical development. In paper I, we investigated the influence of the drug-to-affibody ratio (DAR) on the pharmacokinetic profile of affibody-drug conjugates targeting HER2. Increasing the drug load resulted in an elevated delivery of the DM1 drug to the tumors; however, it also led to increased uptake by the liver. Further optimization of the molecular design is necessary to enable highly efficient delivery to tumors while minimizing the uptake in normal organs and tissues. In paper II, the effect of the length and composition of the linker between the HER2 targeting affibody molecule and the ABD was investigated. The use of a 12 amino acids linker reduced hepatic uptake compared with the use of a 5 amino acids linker. This finding offers an important insight...
into the influence of the linker on the properties of the affibody drug conjugates. In paper III, we investigated the influence of different cytotoxic payloads, as part of an affibody-drug conjugate targeting HER2, on binding properties, cytotoxicity, biodistribution, and anti-tumor effect. The combination of a potent cytotoxic effect in vitro, and a high tumor uptake in vivo, resulted in a superior anti-tumor effect for ZHER2-ABD-mcMMAF at lower doses compared to the previously investigated ZHER2-ABD-mcDM1. Importantly, it maintained a favorable toxicity profile with lower liver uptake compared to ZHER2-ABD-mcDM1. The affibody-drug conjugate ZHER2-ABD-mcMMAF holds great promise as a valuable agent for HER2-targeted cancer therapy. In paper IV, we generated a series of HER2-targeted affibody-drug conjugates fused with different PAS or XTEN polypeptides. We evaluated the ability of the XTEN and PAS polypeptides to extend the plasma half-life, and their influence on tumor uptake, and tissue biodistribution. We compared our new constructs with the previously developed construct, ZHER2-ABD-mcDM1, where an albumin binding domain was used for half-life extension. It was found that the ABD-fused affibody-drug conjugate demonstrated superior tumor uptake and tumor-to-normal-organ ratios compared to the PASylated and XTENylated affibody-drug conjugates. It is possible that ABD is better also for other cancer-targeting strategies where a high tumor uptake while maintaining comparable accumulation in normal tissues is desired. In paper V, we compared the binding properties and cytotoxic potential of a monovalent and a bivalent HER3-targeting affibody-drug conjugate. The biodistribution and therapeutic potential of the bivalent drug construct were evaluated. We found that the bivalent ZHER3-ABD-ZHER3-mcDM1 is a highly potent drug conjugate with favorable biodistribution and anti-tumor efficacy. These results suggest that ZHER3-ABD-ZHER3-mcDM1 holds promise for future clinical development as a potential therapeutic option for patients with HER3 over-expressing cancer.

In summary, the potential for modification and optimization through the design of diverse components within HER2 and HER3-targeting affibody-drug conjugates significantly enhances therapeutic effectiveness, thereby encouraging prospective advancements in the development of targeted drug conjugates.
Sammanfattning

Cancer är en stor folkhälsoutmaning och den näst vanligaste dödsorsaken i världen, med miljontals nya fall som diagnostiseras varje år. Traditionella cancerbehandlingar som kirurgi, strålbehandling och kemoterapi är många gånger effektiva, men kan också orsaka skador på friska celler, vilket leder till biverkningar. Riktad terapi är ett mer exakt och fokuserat tillvägagångssätt för cancerbehandling, där syftet är att rikta in sig på cancercellerna samtidigt som de normala cellerna lämnas opåverkade. Det är särskilt effektivt vid cancer där specifika molekylära mål är kända, såsom undergruppen av bröstcancerpatienter med HER2-överuttryck eller i undergruppen patienter med pankreascancer med HER3-överuttryck.

Antikroppsläkemedelskonjugat (ADC) är en viktig typ av tumörriktad terapi, med tolv läkemedel godkända för klinisk användning av FDA. De utnyttjar den höga specificiteten hos monoklonala antikroppar och är konjugerade med mycket cytotoxiska läkemedel för att öka ackumuleringen av läkemedlen i tumören, för mycket specifikt och effektivt dödande. Traditionella ADC:er är dock kanske inte det optimala formatet för riktad leverans av cellgifter. De begränsas av sina relativt höga molekylvikter, vilket resulterar i relativt låg penetration av solida tumörer.


Sammanfattningsvis visar avhandlingen att modifiering och optimering av olika komponenter inom HER2- och HER3-inriktade affibody-läkemedelskonjugat kan förbättra den terapeutiska effektiviteten avsevärt, och resultaten uppmuntrar därigenom till ytterligare utveckling av riktade läkemedelskonjugat.

Translated by Torbjörn Gräslund with the help of Google translate
List of appended papers

This thesis is based on the following published articles and manuscripts. Full versions of the papers are appended at the end of the thesis.


* These authors contributed equally to this article.

All papers are reprinted with permission from the copyright holders.
Respondent’s contribution to appended papers

I. Contributed to design, and performed the experiments together with co-authors. Responsible for the production, conjugation, and characterization of the drug conjugates. Analyzed the data and wrote the manuscript partly with co-authors.

II. Contributed to planning, designing, and performing the experiments together with co-authors. Responsible for cloning, production, conjugation and characterization of the drug conjugates. Analyzed the data and wrote the manuscript with co-authors.

III. Performed the experiments together with co-authors. Responsible for in vitro characterization of the drug conjugates. Analyzed the data and wrote the related experimental section and reviewed the manuscript.

IV. Contributed to planning, designing, and performing the experiments together with co-authors. Responsible for cloning, production, conjugation and characterization of the drug conjugates. Analyzed the data and wrote the manuscript with co-authors.

V. Contributed to planning, designing, and performing the experiments together with co-authors. Responsible for cloning, production, conjugation and characterization of the drug conjugates. Analyzed the data and wrote the manuscript with co-authors.
Published work not included in the thesis

Public defense of dissertation

This thesis will be defended on Friday 22nd September 2023, at 10:15, Oskar Kleins Auditorium, Roslagstullsbacken 21, AlbaNova University Center, Stockholm, for the degree of “Teknologie doktor” (Doctor of Philosophy, Ph.D.) in Biotechnology.

Respondent:
Jie Zhang, M.Sc. in Clinical Pharmacy
Department of Protein Science, School of Engineering Sciences in Chemistry, Biotechnology and Health
KTH Royal Institute of Technology, Stockholm, Sweden

Faculty opponent:
Prof. Marika Nestor
Department of Immunology, Genetics and Pathology
Uppsala University, Uppsala, Sweden

Evaluation committee:
Assoc. Prof Thuy Tran
Department of Oncology-Pathology
Karolinska Institute, Stockholm, Sweden

Kristina Viktorsson, Ph.D.
Department of Oncology-Pathology
Karolinska Institute, Stockholm, Sweden

Assoc. Prof. Mats Martinell
Department of Industrial Biotechnology, School of Engineering Sciences in Chemistry, Biotechnology and Health
KTH Royal Institute of Technology, Stockholm, Sweden

Chairman:
Prof. Amelie Eriksson-Karlström
Department of Protein Science, School of Engineering Sciences in Chemistry, Biotechnology and Health
KTH Royal Institute of Technology, Stockholm, Sweden
Respondent’s main supervisor:
Prof. Torbjörn Gräslund
Department of Protein Science, School of Engineering Sciences in Chemistry, Biotechnology and Health
KTH Royal Institute of Technology, Stockholm, Sweden

Respondent’s co-supervisor:
Prof. Per-Åke Nygren
Department of Protein Science, School of Engineering Sciences in Chemistry, Biotechnology and Health
KTH Royal Institute of Technology, Stockholm, Sweden
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABD</td>
<td>Albumin-binding domain</td>
</tr>
<tr>
<td>ADA</td>
<td>Adenosine deaminase</td>
</tr>
<tr>
<td>ADAPT</td>
<td>ABD-Derived affinity protein</td>
</tr>
<tr>
<td>ADC</td>
<td>Antibody drug conjugate</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>AffiDC</td>
<td>Affibody drug conjugate</td>
</tr>
<tr>
<td>ALCL</td>
<td>Anaplastic large cell lymphoma</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>CDC</td>
<td>Complement-dependent cytotoxicity</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte associated molecule-4</td>
</tr>
<tr>
<td>DAR</td>
<td>Drug to antibody ratio</td>
</tr>
<tr>
<td>DARPin</td>
<td>Designed ankyrin repeat protein</td>
</tr>
<tr>
<td>DTT</td>
<td>DL-Dithiothreitol</td>
</tr>
<tr>
<td>ECD</td>
<td>Extracellular domain</td>
</tr>
<tr>
<td>E.coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ESP</td>
<td>Engineered alternative scaffold protein</td>
</tr>
<tr>
<td>Fc</td>
<td>Crystallizable fragment</td>
</tr>
<tr>
<td>FcRn</td>
<td>Neonatal Fc receptor</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. Food and drug administration</td>
</tr>
<tr>
<td>FGE</td>
<td>Formylglycine-generating enzyme</td>
</tr>
<tr>
<td>GHD</td>
<td>Growth hormone deficiency</td>
</tr>
<tr>
<td>GRAS</td>
<td>Generally recognized as safe</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HER3</td>
<td>Human epidermal growth factor receptor 3</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>IAA</td>
<td>Iodoacetamide</td>
</tr>
<tr>
<td>IC50</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenously</td>
</tr>
<tr>
<td>$K_a$</td>
<td>Association rate constant</td>
</tr>
<tr>
<td>$K_d$</td>
<td>Dissociation rate constant</td>
</tr>
<tr>
<td>$K_D$</td>
<td>Equilibrium dissociation constant</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>mc</td>
<td>Maleimidocaproyl</td>
</tr>
<tr>
<td>MSA</td>
<td>Mouse serum albumin</td>
</tr>
</tbody>
</table>
NK cell  Natural killer cell
NSCLC  Non-small cell lung cancer
PE  *Pseudomonas aeruginosa* exotoxin A
PEG  Polyethylene glycol
p.i.  Post injection
RP-HPLC  Reversed-phase high-performance liquid chromatography
SC  Subcutaneously
SCID  Severe combined immunodeficiency disease
SMCC  Succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate
SPR  Surface plasmon resonance
TCEP  Tris (2-carboxyethyl) phosphine
T-DM1  Trastuzumab emtansine
vc  Valine-citrulline
Z  Affibody molecule
ZTaq  Thermus aquaticus DNA polymerase-targeted affibody
# Table of contents

## Chapter 1 Targeted Cancer Therapy .................................................1

1.1 HER Family ..............................................................2

1.1.1 HER2 ..............................................................3

1.1.2 HER3 ..............................................................4

1.2 Other targets for cancer therapy ...........................................5

## Chapter 2 Antibody and Affinity Proteins ........................................7

2.1 Monoclonal Antibody .........................................................7

2.2 Antibody Fragments .........................................................8

2.3 Engineered Alternative Scaffold Proteins ..........................10

2.3.1 Affibody Molecules .................................................11

2.3.2 ABD-Derived Affinity Proteins .................................13

2.3.3 Designed Ankyrin Repeat Proteins .............................13

2.4 Half-life Extension of Protein ...........................................15

2.4.1 Attachment to Polymers ............................................15

2.4.2 Albumin Binding .....................................................19

2.4.3 Fc Fusion ............................................................21

## Chapter 3 Antibody Drug Conjugates and Targeted Drug Conjugates .........23

3.1 Antibody Drug Conjugates ................................................23

3.1.1 Linker Moiety .......................................................26

3.1.2 Cytotoxic Payload ..................................................33

3.1.3 Lessons Learned from ADCs in the Clinic and Clinical Trials ....37

3.2 Targeted Drug Conjugates .................................................39

## Chapter 4 Present Investigation ....................................................42

4.1 *Paper I* - Affibody-Derived Drug Conjugates Targeting HER2: Effect
doing Drug Load on Cytotoxicity and Biodistribution ..................43

4.2 *Paper II* - Effect of Inter-Domain Linker Composition on Biodistribution
of ABD-Fused Affibody-Drug Conjugates Targeting HER2 ..........50

4.3 *Paper III* - Comparison of HER2-Targeted Affibody Conjugates Loaded
with Auristatin- and Maytansine-Derived Drugs .......................56
4.4  *Paper IV* - A Comparison of *in vivo* Half-life Extension of Affibody-Drug Conjugates by PASylation, XTENylation and Albumin Binding .................................................................64

4.5  *Paper V* - Pre-clinical Evaluation of Drug Conjugates Based on Affibody Molecules Targeting HER3-Expressing Tumors .........................71

4.6  Concluding Remarks and Future Perspectives ........................................76

**Acknowledgement** ..................................................................................79
**References** ..............................................................................................82
Chapter 1

Targeted Cancer Therapy

Cancer is becoming the leading cause of death in the world, killing nearly 10 million people in 2020 [1]. Cancer is often a serious disease in which abnormal cells in the body grow uncontrollably and may spread into normal tissues. Cancer can occur essentially anywhere in the body and can affect people of all ages, even though it becomes more common with increasing age. There are over 100 different types of cancers, with breast, prostate, and lung cancers being the most common types. The cause of malignant transformation is not completely understood for all cancers, but certain risk factors, such as exposure to chemicals or radiation, an unhealthy lifestyle, and some chronic infections may increase the risk of disease development [2]. Early diagnosis and effective treatment increase the patient’s chances of survival. There are numerous types of treatments, which are utilized depending on the type and stage of cancer. Common treatments include surgery, chemotherapy, radiation therapy, immunotherapy, and targeted therapy.

Targeted therapy refers to a type of cancer treatment that selectively targets specific small molecules or proteins that often play an important role in cancer development and progression. Unlike traditional chemotherapy, which can affect both normal and cancer cells, targeted therapy is designed to specifically kill cancer cells while minimizing damage to healthy tissues. Targeted therapy forms a basis for precision medicine, a medical approach that takes into account individual differences in genes, environment, and lifestyle [3]. This approach can provide a more personalized and effective option for cancer treatment.

The discovery and development of targeted therapies is based on a deep understanding of the genetic and molecular changes in tumor cells. By identifying specific proteins or pathways that are crucial for the growth and spread of cancer cells, researchers can
develop small molecules or proteins, such as monoclonal antibodies, that target those specific molecules [4]. Monoclonal antibodies usually aim to recognize and bind to specific targets on the cancer cell’s surface. They act as a drug through different mechanisms, for example by triggering the immune system to act on the tumor. Targeted therapies have been shown efficacious in the treatment of many cancer types, including breast cancer, lung cancer, and melanoma [5].

1.1. The HER Family

The Human Epidermal Growth Factor Receptor (HER) family is a target for cancer therapy. Also known as the ErbB family, it includes four transmembrane receptor tyrosine kinases HER1 (EGFR), HER2, HER3, and HER4. All four receptors consist of an extracellular domain (ECD), an intracellular domain (ICD), and a transmembrane domain (TM) [6]. These receptors are crucial components of cell signaling pathways that regulate cell growth, proliferation, differentiation, and survival [7].

EGFR, HER3, and HER4 are activated by ligands, and HER2 is constantly in an active state. After activation, the receptors come together to form dimers, which are then phosphorylated on specific tyrosine residues in the cytoplasmic tails. The phosphorylated ICDs may then activate intracellular signaling pathways. These pathways ultimately regulate different cellular processes [8]. The ErbB family is involved in the activation of many intracellular signaling pathways, including the mitogen-activated protein kinase (MAPK), phosphatidylinositol-3 kinase (PI-3K), phospholipase C (PLC-γ), and the signal transducer and activator of transcription (STAT) pathways (Fig.1) [9].
Figure 1. HER family signaling pathways. The figure is drawn with reference to \[10\]. HER2 has no binding ligand, but the other three have activating ligands. One of the preferred partners for HER2 dimerization is HER3, which has an impaired tyrosine kinase domain. When they form dimers, it leads to the activation of downstream signaling pathways that regulate cell proliferation, differentiation, and survival.

Therefore, targeting the HER family and its downstream signaling pathway has emerged as a promising approach for cancer treatment.

1.1.1. HER2

HER2 has been identified as a key regulator in the progression of different types of tumors especially breast cancer \[9\]. In normal cells, HER2 induces signals for cell growth and division, but in some types of cancer, HER2 overexpression can lead to uncontrolled abnormal growth of tumor cells. In addition to breast cancer, HER2 overexpression is related to other types of cancer, such as gastric cancer \[11\] and ovarian cancer \[12\].

HER2 is unique among family members in that it has no natural ligand and its activation is based on the homodimerization or heterodimerization with another HER receptor \[13,14\]. HER2-positive cancers can be treated with targeted therapies that selectively block the function of the HER2 receptor, such as trastuzumab and pertuzumab. Trastuzumab (Herceptin®) is a monoclonal antibody approved by the FDA to specifically target HER2-positive breast cancer. Depending on the stage and type of
breast cancer, trastuzumab can be given alone or in combination with other treatments, like surgery, chemotherapy, or hormone therapy [15]. Pertuzumab (Perjeta®) is another monoclonal antibody that inhibits the dimerization of HER2 with other HER family members. It binds to a different part of the receptor than trastuzumab, enhancing the blockade of signaling if given together with trastuzumab. Thus, it is often used in combination with trastuzumab and other therapies as part of the standard treatment for HER2-positive breast cancer [16].

1.1.2. HER3

HER3 also known as ErbB3, is another receptor in the HER family. HER3 is overexpressed in a variety of cancer types, particularly breast, ovarian, and lung cancer [17]. When HER3 is activated by binding to a ligand, such as heregulin (HRG, neuregulin-1), it triggers downstream signaling pathways that promote tumor cell growth and survival. HER3 has impaired kinase activity, and compared to HER2, it has a low expression level in tumors. For these reasons much effort in the development of targeted therapies towards the HER family has been directed towards HER2, and the therapeutic potential in the development of targeted therapies toward HER3 may have been underestimated.

However, HER3 can form heterodimers with other receptors, such as the HER2-HER3 heterodimer, which is highly oncogenic [19]. HER2 then activates HER3 by phosphorylation of the intracellular domain, thereby leading to the activation of downstream signaling pathways, including the PI-3K/AKT pathway that promotes cell proliferation [17].

There are several HER3-targeting antibodies in development and clinical trials. Patritumab is a monoclonal antibody against HER3 that is currently in clinical trials for the treatment of colorectal and lung cancers [19,20]. It competitively blocks the binding of neuregulin to HER3, thereby disrupting cancer cell proliferation and survival. In the phase II study, the combination of patritumab and erlotinib did not show a significant improvement among the patients with advanced NSCLS [21]. Seribantumab (MM-121) is another monoclonal antibody targeting HER3 that prevents the binding of neuregulin and heterodimerization [22]. It was investigated in a phase I study in combination with paclitaxel in gynecological and breast cancers [23]. Similarly to patritumab, a phase II trial of seribantumab in combination with docetaxel for patients with HRG-positive NSCLC was terminated as it failed to improve progression-free survival [24].
Although no HER3-targeted treatment has been approved by the FDA, many HER3-targeting monoclonal antibodies have shown preclinical efficacy [25]. Promising clinical studies support the development of HER3-targeted therapy for cancer treatment, despite the failures of patritumab and seribantumab.

1.2. Other Targets for Cancer Therapy

A variety of molecules and small proteins have been identified that are involved in signal transduction for cell growth and division. These molecules and proteins would be ideal targets for cancer therapy. Below are a couple of examples of other targets for which targeted therapy is under development or is used clinically.

EGFR (Epidermal growth factor receptor) also belongs to the HER family and is involved in the regulation of cell proliferation and differentiation. It is highly expressed in many tumors such as breast and lung cancers [26,27]. Targeting EGFR has been a promising therapeutic strategy for cancer treatment. Cetuximab (Erbitux®) and panitumumab (Vectibix®) targeting EGFR are two monoclonal antibody drugs that have been approved for clinical treatment of colorectal cancer [28].

VEGF (Vascular endothelial growth factor) is a protein that plays an important role in angiogenesis. Its overexpression promotes the growth and spread of tumors. Bevacizumab (Avastin™) is an anti-VEGF-A mAb that prevents the VEGF-A ligand from binding to its receptors for the treatment of metastatic colorectal cancer and certain eye disease [29,30].

PD-1 (Programmed cell death protein 1) and PD-L1 (Programmed death-ligand 1) are checkpoint proteins, and can be targets for cancer-targeted therapy. PD-1 is a protein expressed on the surface of T cells, and PD-L1 is a protein that is sometimes expressed on the surface of cancer cells. When PD-L1 binds to PD-1, it sends the signal to the T cell which suppresses the immune response against the cancer cells. As a result, the cancer cell will evade the immune system and continue to grow uncontrollably. Immune checkpoint inhibitors that target PD-1 or PD-L1 are able to block the signaling pathway and activate an immune cell response against the cancer cells. Anti-PD-1 antibodies such as pembrolizumab (Keytruda®) and nivolumab (Opdivo®) are approved by the FDA for the treatment of NSCLC (Non-small cell lung cancer) [31,32]. Anti-PD-L1 antibodies such as durvalumab (Imfinzi®) and atezolizumab (Tecentriq®) have been approved in combination with chemotherapy for the treatment of extensive-stage small cell lung cancer [33].
CTLA-4 (Cytotoxic T Lymphocyte Antigen-4) is another checkpoint protein present on the surface of T cells. When it binds to its ligand CD80 (B7-1) or CD86 (B7-2), T cell activation will be inhibited [34]. Monoclonal antibodies targeting CTLA-4, such as ipilimumab (Yervoy®) can promote T cell activation and enhance anti-tumor immunity. It has been approved by the FDA for the treatment of late-stage melanoma and renal cell carcinoma [35,36]. In addition to ipilimumab, another anti-CTLA-4 antibody, tremelimumab, has been developed for cancer therapy. The regimen tremelimumab with durvalumab and chemotherapy was approved by the FDA in 2022 for metastatic NSCLC [37].

TIGIT (T cell immunoreceptor with immunoglobulin and ITIM domain) has emerged as a promising immune checkpoint for cancer therapy. It is a membrane receptor belonging to the TIGIT family expressed on various immune cells, including T cells and NK (Natural killer) cells. TIGIT can deliver an inhibitory signal to cells by binding to its ligand CD155, and suppressing the activation of immune cells [38]. After the interaction between TIGIT and CD155, dendritic cells (DCs) expressing CD155 may acquire tolerogenic properties. Tolerogenic DCs are characterized by a reduction in antigen presentation, low expression of co-stimulatory molecules, and decreased production of proinflammatory cytokines (e.g., IL-12) while promoting the secretion of anti-inflammatory cytokines (e.g., IL-10). These changes collectively lead to impaired activation of T cells. Tiragolumab is an anti-TIGIT mAb that was approved in 2021 by the FDA for the treatment of metastatic NSCLC in combination with the PD-L1 inhibitor Atezolizumab [39].

Overall, a growing number of novel targets, including checkpoint proteins, play an important role in targeted cancer therapy and their potential in combination with other treatments has been explored to improve cancer patient outcomes [40].
Chapter 2
Antibodies and Affinity Proteins

2.1. Monoclonal Antibodies

Antibodies (Ab), also called immunoglobulins (Ig), are large proteins produced by the immune system to recognize foreign substances, such as bacteria and viruses\(^1\). These foreign objects are called antigens. Antibodies play a crucial role in defending humans against infections and diseases by identifying antigens. There are five isotypes of antibodies, including IgG, IgM, IgA, IgD, and IgE, each of which has different functions and characteristics. The different classes of antibody molecules are distinguished by their heavy chain. IgG or immunoglobulin G will be the focus of this section of the thesis. They are produced by plasma cells and are the most abundant antibodies in the human blood and tissues.

The full-length IgG molecule (150 kDa) is Y-shaped and consists of two heavy chains (50 kDa for each chain) and two light chains (25 kDa for each chain), which are linked by disulfide bonds (Fig. 2)\(^2\). Light chains are composed of one variable domain (V\(_L\)) and one constant domain (C\(_L\)) and the heavy chains are comprised of one variable domain (V\(_H\)) and three constant domains (C\(_{H1}\), C\(_{H2}\), C\(_{H3}\)). The V part of the Y-like antibody molecule contains one variable region and one constant region from each heavy and light chain, called the antigen-binding fragment (Fab fragment), which is responsible for specifically binding to the antigen on the surface of foreign cells. The trunk of the Y-shape is formed by the constant domains of the heavy chains, also referred to as the crystallizable region (Fc region). The function is to interact with the Fc receptor on the immune cell surface and regulate the biological activity of the immune system.
Complementarity-determining regions (CDRs) are located in the variable domains of IgG that are responsible for recognizing and binding to the antigens \[^{[43]}\]. Three CDRs (CDR1-3) are unique in amino acid sequences and can selectively bind different epitopes of many different antigens. The Fc region does not bind to antigens, but interacts with other immune cells, such as natural killer cells (NK cells), macrophages, and B cells, as well as activating the complement system \[^{[44]}\]. Another function of the Fc region is to interact with the neonatal Fc receptor (FcRn) to transport the antibody over epithelial layers and also to maintain the IgG concentration in circulation. The details of the interaction between FcRn and the Fc region will be discussed in the section “Half-life extension of proteins”.

IgG molecules can be used for a variety of applications in research, diagnostics, and therapy. It can be used as a probe to quantify proteins, nucleic acids, or small molecules. For certain infectious disease diagnoses and cancer screening, antibodies are able to detect specific molecules. Two important applications of antibodies are to make therapeutic agents for the treatment of cancer and autoimmune disorders.

### 2.2. Antibody Fragments

Antibody fragments are antibody-derived proteins that lack specific regions of a full-length antibody, and most retain the function to bind specific antigens. Most fragments are relatively small proteins as they are generated by the removal or modification of the heavy or light chains of the antibody. Compared with full-length antibodies, they have different properties such as higher tissue penetration, a rapid clearance rate, and typically lower immunogenicity \[^{[45]}\]. There are many types of antibody fragments, including the Fab, Fab’, F(ab’)\(_2\), and scFv.

A Fab (antigen-binding fragment) consists of a variable domain and a constant domain from both the heavy and light chains. Since it does not have any Fc region, the molecular weight is approximately 50 kDa. One therapeutic drug consisting of a Fab is ranibizumab (Lucentis\(^{®}\)), which targets VEGF-A and is used for the treatment of macular degeneration \[^{[46]}\]. The Fab’ fragment has a molecular weight of about 55 kDa and contains the Fab region and part of the Fc region. It is similar to the Fab fragment, but there is a free sulfhydryl (-SH) group. In contrast, F(ab’)\(_2\) is a divalent molecule with a molecular weight of 110 kDa generated by two Fab moieties linked by a disulfide bond. An scFv, or single-chain variable fragment, is derived from the variable regions of the heavy and light chains, which are linked by a peptide. Similar to Fab and its fragments, based on two variable regions, this allows the recombinant protein to fold and form a cohesive unit that selectively recognizes and binds to the antigen with high
affinity. Due to their small size with a molecular weight of 25 kDa, scFvs have better tissue permeability compared to full-length antibodies and larger fragments.

A particular type of antibody fragment is the so-called nanobodies, also known as VHH domains [47]. They are derived from heavy-chain only antibodies, a type of antibodies that are present in camelids and sharks. The heavy-chain-only antibodies lack light chains and the nanobodies are derived from the V\textsubscript{H} domain. It is the smallest antibody-derived fragment with a molecular weight of only 15 kDa. Nanobodies have shown promise in many different applications such as \textit{in vivo} imaging and the treatment of cancer and viral infections [48,49,50].

Engineered antibody variants such as diabodies, triabodies, and bispecific antibodies (Fig.2), are modified from antibodies aimed to have specific properties. They can be used in lots of applications, including therapeutics and disease diagnosis. A diabody is composed of two variable regions from two antibodies connected by two peptide linkers. They are one-third the size of the full-length antibody and typically bivalent molecules with two antigen-binding sites that identify different targets. Alternatively, if the two variable regions are from the same antibody, it can create a monospecific diabody with two binding sites for the same antigen. A triabody is a type of tri-specific antibody that is typically engineered to bind three different antigens simultaneously. Triabodies have been investigated for potential therapeutic applications, especially in cancer treatment. The bispecific antibody is the antibody that has been engineered with two distinct binding sites, each with specificity for a unique target antigen. There are a variety of formats for bispecific antibodies, including IgG-like bispecific antibodies, BiTEs (bispecific T-cell engagers), and DARTs\textsuperscript{®} (dual-affinity retargeting) proteins. An IgG-like bispecific antibody has a similar structure compared to a full-length antibody, with two different antigen-binding domains. A BiTE is comprised of two different single-chain variable fragments linked by a single peptide chain. They are investigated for cancer treatment where one scFv binds to a T cell, and the other binds to the tumor cell, which causes the T cell to produce cytotoxic activity to attack the tumor cell [51]. A DART molecule has a similar structure to a diabody, but it contains an additional interchain disulfide bridge for stability.
2.3. Engineered Alternative Scaffold Proteins

Antibodies have been highly effective in targeted tumor therapy, leading to FDA approval and ongoing clinical trials of numerous antibodies. However, antibodies are not always the perfect choice for each application. Firstly, the antibody has a molecular weight of 150 kDa, resulting in poor penetration into tumors, compared to smaller proteins \[^{52}\]. Secondly, antibody production is relatively costly and requires mammalian cells to produce. However, it should be noted that it becomes less of a problem, due to the development of production and purification methods that are more and more efficient. Thirdly, many antibodies are originally of murine origin, which sometimes leads to immunological reactions. However, nowadays there are methods to create fully human-derived antibodies, either by \textit{in vitro} selection from fully human antibody fragment libraries or by immunizing mice with fully human IgG-loci in the genome \[^{53}\]. Problems with immunogenicity may be less of a problem in the future.

Engineered alternative scaffold proteins (EAPs) are types of versatile binding frameworks, which can be generated to have high specificity and affinity for desired antigens. The EAPs usually have desirable characteristics, e.g., a small size, a robust fold, and are easy to produce. A small size sometimes enables the chemical synthesis of the engineered alternative scaffolds, which also allows for the introduction of unnatural amino acids and other building blocks in the structure \[^{54}\]. Unnatural amino acids may for example be used for drug conjugation and labeling with imaging probes. Furthermore, many alternative scaffolds have high thermal stability, which enables
storage at room temperature for a longer time without experiencing loss of activity \(^{[55]}\). In addition, engineered alternative scaffold proteins can often be easily produced with high yields in bacterial hosts such as *Escherichia coli* (*E. coli*). Usually, the affinity protein is generated by display technologies, including cell surface display, phage display, or yeast display selections. The process can be summarized in two steps; a library of variants is first created by random mutagenesis, followed by the isolation of suitable binders by biopanning from the library.

Several engineered alternative scaffold proteins have been developed for future use as cancer therapeutics, such as affibody molecules, ABD-derived affinity proteins (ADAPTs), and designed ankyrin repeat proteins (DARPins) (Fig.3).

![Figure 3](image.png)

**Figure 3.** Representative structures of engineered alternative scaffolds. Affibody (PDB: 2KZJ), ABD (PDB: 1GJS), and a bispecific DARPin (PDB: 6FPA). The 3D structures are visualized by Pymol Molecular Graphics System, Version 2.4.2 based on the deposited coordinates in the Protein Data Bank.

### 2.3.1. Affibody Molecules

Affibody molecules are a class of small (7 kDa), high-specific binding proteins, based on a scaffold of 58 amino acids. The scaffold is also referred to as the Z domain, derived from the B domain in the IgG-binding region of staphylococcal protein A \(^{[56]}\). Affibody molecules typically have a three-helix bundle structure in an antiparallel orientation. They often have excellent biophysical properties, such as a high melting point, rapid refolding, and high solubility. Usually, new affibody candidates are selected from a library, where 13 amino acids in helices 1 and 2 have been randomized \(^{[57]}\) (Fig.4). A variety of successful binders toward biomedically relevant targets have been developed, such as HER2, HER3, FcRn, and EGFR \(^{[58,59,60,61]}\).
Figure 4. Representative structure of an affibody molecule binding to HER2 (PDB: 2KZJ) with the 13 randomized positions in helix 1 and 2 marked. The 3D structure was visualized with the Pymol Molecular Graphics System, Version 2.4.2.

The first candidate of a HER2-targeting affibody molecule (ZHER2:4) was discovered with an affinity (K_D value) of 50 nM [58]. It was further affinity matured to the affibody molecule ZHER2:342 with an affinity of 22 pM [62]. Further engineering led to ZHER2:2891 with improved hydrophilicity and thermal stability. It was created by the substitution of an additional 11 amino acids in the framework compared to ZHER2:342. It has a high affinity to HER2 with a K_D value of 76 pM [63]. Since the size of an affibody molecule is smaller than an antibody, and below the kidney filtration cut-off, it is rapidly cleared from circulation. This is an advantage for some applications and has enabled the HER2-targeting affibody molecule ZHER2:342 to be tested in phase II clinical trials for in vivo imaging of HER2 status in breast tumors [64]. Furthermore, the affibody molecule is a cysteine-free scaffold that allows for the introduction of cysteines at a desired position in the construct for specific attachment of e.g., drugs and chelating groups. An attractive approach is to use a highly potent payload to generate affibody-drug conjugates, which exhibit a cytotoxic effect on HER2-high expression cell lines and has been shown to inhibit tumor growth in vivo [65].

Affibody molecules have also been developed against the HER3 receptor, which by themselves have shown potential as a therapeutic agent in a preclinical model of pancreatic cancer [59]. However, the development of drugs targeting HER3 poses a challenge due to its high level of endogenous expression, especially in the liver. In addition, the expression level of HER3 is lower in most tumor cells compared to HER2, with a maximum of 50,000 receptors/cell [66]. An affibody molecule ZHER3:08698 has
been engineered with a high affinity of 50 pM against HER3 [67]. It was converted to an affibody-drug conjugate, which was found to be cytotoxic to HER3-positive cell lines [68]. The application of affibody-drug conjugates will be discussed in Chapter 3.1.

In conclusion, affibody molecules are a group of promising affinity scaffold proteins that have been investigated for a variety of applications, including cancer therapy and \textit{in vivo} imaging.

\textbf{2.3.2. ABD-Derived Affinity Proteins}

ABD-Derived affinity protein (ADAPT) is another type of affinity protein that is derived from the albumin-binding domain (ABD) of protein G [69]. The ADAPTs usually fold into three helices that are arranged in an anti-parallel manner. They are small proteins with a molecular weight of 5 kDa consisting of 46 amino acids. They have been engineered to specifically bind to a variety of targets, for example, HER2, HER3, and TNF-\(\alpha\) [70,71,72].

ADAPTs have been investigated in preclinical models and in a clinical trial of radionuclide molecular \textit{in vivo} imaging. A clinical trial (NCT03991260) revealed that \textsuperscript{99m}Tc-labeled ADAPT6 can provide high-contrast imaging of HER2 expression in breast cancer. A combination of rapid target accumulation and rapid clearance of unbound agents is advantageous and highly beneficial for such applications. The first ADAPT variant targeting HER2, ADAPT6, was generated with an affinity of 0.5 nM for radionuclide imaging. \textsuperscript{111}In/\textsuperscript{68}Ga-DOTA-(HE)\textsubscript{3}-ADAPT6 showed specific binding to HER2-expression cells [73]. An ADAPT-drug conjugate ADAPT6-ABD-mcDM1 was developed for targeting cancer tumors. It included an albumin binding domain for half-life extension (see Chapter 2.4). The resulting drug conjugate retained a high affinity towards HER2 and showed a potent cytotoxic effect [74].

Taken together, ADAPTs have shown potential in a wide range of applications, particularly as drug conjugates, and as diagnostic probes for \textit{in vivo} tumor imaging in the clinical trial.

\textbf{2.3.3. Designed Ankyrin Repeat Proteins}

Designed ankyrin repeat proteins (DARPins) are another group of small (14 - 17 kDa), engineered proteins that are developed for a variety of biotechnological and medical applications. The DARPin scaffold is composed of 4 to 6 ankyrin repeat domains with 33 amino acids each. Each domain has two antiparallel \(\alpha\)-helices linked by a loop,
which then form a β-turn \[^{75}\]. Like other scaffold proteins, DARPins have excellent properties for biomedical applications, including high thermal stability (melting temperature up to 90°C), rapid refolding, and ease of engineering and production \[^{76}\]. The DARPin molecules can be selected with phage display, yeast display, and ribosome display from large combinatorial libraries. DARPins have been generated against several targets with picomolar affinities, such as HER2 (90 pM) \[^{77}\], EpCAM (68 pM) \[^{78}\], and VEGF-A (1-4 pM) \[^{79}\].

Furthermore, since DARPins typically have short plasma half-lives due to their small size, strategies to achieve a longer \textit{in vivo} half-life for therapeutic applications have been developed. For example, DARPins have been modified by the addition of a unique cysteine residue for PEG conjugation or with a peptide chain for ABD conjugation. Abicipar pegol is a DARPin-based VEGF-A antagonist with a PEG chain for half-life extension. It has been shown to be in circulation for more than 13 days in humans and also maintains a high affinity for VEGF-A \[^{80}\]. Recently, FDA declined approval of abicipar pegol for the treatment of neovascular AMD (age-related macular degeneration) due to an unfavorable risk/benefit ratio. Another approach is to create a multivalent or multispecific DARPin construct, incorporating a module binding to albumin. MP0250, a multispecific DARPin drug targeting HGF/VEGF has been tested in clinical trials with a potentially extended half-life of up to 11 days \[^{81}\].

The small size of DARPins results in rapid clearance from the blood and normal tissues by the kidneys. This allows for high-contrast \textit{in vivo} imaging. A DARPin-based probe, a \(^{99m}\)Tc labeled HER2-targeting molecule \(^{99m}\)Tc-(HE)\(_3\)-G3, could for example distinguish between HER2-positive and HER2-negative breast cancers in a phase I clinical trial, and demonstrated a suitability for further clinical development \[^{82}\]. Another HER2-specific DARPin was expressed as a fusion protein with a truncated version of the highly toxic Exotoxin A from \textit{Pseudomonas aeruginosa}, DARPin-LoPE. It showed significant cytotoxicity against the HER2-positive cell line SKOV3 and potently inhibited the growth of HER2-expressing human ovarian cancer xenografts in mice \[^{83}\].

In conclusion, engineered alternative scaffold proteins appear to be attractive options for cancer therapeutic and \textit{in vivo} imaging applications. Undoubtedly, these non-IgG scaffold proteins open new avenues for the biopharmaceutical market. In particular as protein-based radiotracers, and drug-conjugates, as well as many other applications and formats.
2.4. Half-life Extension of Protein

Over the past few decades, a large number of therapeutic proteins and peptides have been developed for the biopharmaceutical market. One of the biggest challenges limiting the efficacy of these polypeptides is their rapid elimination in circulation due to their short plasma half-life. Scaffold proteins are usually small with a molecular weight below the renal filtration cutoff of approx. 70 kDa, which leads to rapid elimination through the kidneys.

Pharmacokinetics (PK) is the study that involves the quantitative analysis of how drugs are absorbed, distributed, metabolized, and eliminated in the body. Absorption means the process by which a therapeutic drug moves from administration to systematic circulation in the bloodstream. Drug half-life ($T_{1/2}$) is the time for the drug concentration to decrease by half, which represents the rate of drug elimination. Currently, the majority of protein-based drugs on the market are given through intravenous (IV) or subcutaneous (SC) injections at frequent intervals, which may cause patient discomfort. Therefore, it is desirable to prolong the half-life of the therapeutic protein drug to minimize the injection frequency.

With the exception of monoclonal antibodies, which have weeks of plasma half-life, most proteins are eliminated from circulation in 1-2 days. The small size and hydrophobicity of protein molecules are significant factors affecting half-life, as it is related to renal filtration and hepatic metabolism. Size, molecular weight, and surface charge determine the rate of elimination. Many studies have shown different technologies for half-life extension, that can enhance the half-life by about 2-100 fold and thus improve the pharmacokinetic parameters for a variety of proteins [84]. Over the past years, several strategies have been developed to increase the therapeutic half-life of therapeutic proteins and peptides by attaching a polypeptide chain or synthetic polymer to increase the size, such as PEG, PAS, and XTEN. Other strategies which increase the size and also allow for FcRn-mediated recycling include an attachment to e.g., albumin and Fc.

2.4.1. Attachment to Polymers

PEGylation is a popular technology where polyethylene glycol (PEG) molecules are attached to a protein-based drug to modify its properties. PEGylation involves the covalent attachment of one or more PEG chains to the molecule of interest through a chemical reaction, such as amine coupling or thiol coupling. PEG molecules are hydrophilic and uncharged polymers that are non-biodegradable, but they are generally...
considered to be non-toxic and non-immunogenic \cite{85}. Each ethylene oxide unit of a PEG molecule can bind two or three \( \text{H}_2\text{O} \) molecules, resulting in a large complex and a more hydrophilic product \cite{86}. In this way, PEGylation can increase the solubility of the protein and prolong its circulating half-life in the body, possibly making it a more effective drug \cite{87} (Fig 5).

Typically, PEGs of 1-5 kDa are used for conjugation to antibodies, while PEGs of 20-50 kDa are used for conjugation to smaller peptides. PEGylation has been in development for the clinical market for more than 25 years and was the first successful technology for improving the pharmacokinetics of therapeutic agents \cite{88}. PEGylation is now an established strategy for protein half-life extension which has been approved by FDA with over 30 PEGylated drugs \cite{89}. One example of a PEGylated drug is Adagen\textsuperscript{®}. The active protein is ADA (adenosine deaminase), which was first studied by Davis and colleagues in the 1970s. Adagen\textsuperscript{®} was the first PEGylated protein approved by the FDA for clinical use in 1990 for the treatment of patients with severe combined immunodeficiency disease (SCID). ADA itself has a very short half-life of only a few minutes. Conjugating PEG to ADA can extend its half-life to 48-72 h. The findings by Davies and colleagues \cite{90} suggested that PEGylation can confer proteins with an extended half-life and a significantly reduced immunogenicity.

Currently, PEGylation is used in several drugs, including drugs for the treatment of cancer, chronic kidney disease, and gastrointestinal disorders. Most commonly, the PEGylated drugs have a larger PEGs chain, exceeding 40 kDa, compared to the first-generation drugs \cite{91}. However, PEGylation has shown some disadvantages during drug development, for example, it requires chemical coupling and modification of the proteins and sometimes gives a poor bioavailability of the PEGylated proteins due to waxy solutions.

Based on the successful pharmaceutical development of PEGylation, novel half-life extension approaches have been investigated as alternatives, for example, attachment of polypeptide chains. Similar to PEGylation, the new strategies also increase the hydrodynamic radius aiming to avoid kidney filtration. In general, polypeptides have the characteristics of biodegradability, non-immunogenicity, non-toxicity, high safety, and easy synthesis \cite{92}.
Antibodies and Affinity Proteins

\[\text{Figure 5.} \text{ Schematic illustration of the half-life extension strategy by attachment to polymers. The figure is drawn with reference to }^{[93]} \text{. Therapeutic proteins with a hydrodynamic radius of less than 70 kDa have rapid renal clearance, while therapeutic proteins bound to PEG or PAS/XTEN polymers can avoid rapid renal clearance.}\]

PASylation is one of these approaches that use an unstructured polypeptide sequence, which is composed of 100-600 amino acids with sequences with a random arrangement of three amino acids, proline, alanine, and serine (PAS) \(^{[94]}\). It is an uncharged, hydrophilic polymer with similar properties to PEG. PAS polypeptides can be expressed in \textit{E. coli} as it does not require glycosylation. It is usually recombinantly expressed as a fusion to the therapeutic protein, a strategy that avoids chemical coupling or other complicated modifications. Moreover, PAS is biodegradable and thus can be quickly eliminated from organs in the body while showing stability in serum \(^{[94]}\). Regarding the tunability of the protein half-life, the different sequence length of PAS is associated with increased plasma half-life.

Currently, growth hormone deficiency (GHD) is a disease caused by insufficient growth hormone in the human body, especially in children. The treatment of GHD requires daily injections for life, which may affect patient compliance. One example using a PAS polypeptide was its fusion with human growth hormone (hGH). PASylated-hGH was shown to have a 94-fold longer half-life of 4.42 hours via i.v. injection compared to less than 3 minutes of unfused hGH \(^{[94]}\). In the study, it was also found that PASylation did not affect hGHS affinity for its receptor. XL-Protein GmbH is a biotech company in Germany that focuses on the development of PASylated therapeutic proteins, including alternative scaffold proteins, cytokines, antibody
fragments, peptides, imaging tracers, and bispecific proteins. Another PASylated fusion drug, leptin-PAS, which is undergoing a phase II clinical trial for the treatment of obesity, showed a 21-fold increase in hydrodynamic volume and increased half-life from 26 minutes to 20 hours. Also, in this case, PASylation did not affect the binding activity of leptin [95].

XTEN is another class of unstructured hydrophilic, biodegradable, non-immunogenic polypeptides, composed of six natural amino acids, Ala, Asp, Pro, Gly, Thr, and Ser. They can also be expressed in *E. coli* cells. Unlike PAS polymers, XTEN is a random arrangement of the six amino acids without repetitive sequences. It typically has 864 residues with a molecular weight of 79.2 kDa, while a group of shorter polypeptides has been selected from the full-length XTEN, including XTEN144, XTEN288, and XTEN576 [96]. Longer XTEN polypeptides have a larger size and are expected to show a slower kidney clearance. XTENylation has been used to extend the half-life of a wide range of proteins and peptide-based therapeutics by increasing the hydrodynamic volumes. Numerous *in vitro* and *in vivo* studies have shown that XTEN does not accumulate in healthy tissues and biodegrades rapidly. The findings indicate that XTEN has the potential to be a safer alternative than PEG for extending the half-life of therapeutic proteins.

Teduglutide (GLP-2G) is a recombinant human glucagon-like peptide 2 variant that exhibits resistance to proteolytic degradation and is used for the treatment of gastrointestinal disease. It has an extended half-life of 3-5 hours compared to the native GLP2, which has a short half-life of 7 minutes due to fast proteolytic degradation [97]. The XTENylated fusion protein GLP-2G-XTEN has been shown to have an increased half-life in the circulation of mice up to 34 hours [98]. Another promising development for XTENylation is the treatment of diabetes. Type-2 diabetes is the most common diabetes in the world and the symptoms include high blood sugar, weight loss, and frequent urination. Exenatide (Byetta®) is a GLP1 receptor agonist approved for the treatment of Type-2 diabetes but requires subcutaneous injections twice a day due to its fast clearance. Exenatide has a short half-life of about 2.4 hours, and the XTENylated fusion protein Exenatide-XTEN864 has an extended half-life of 128 hours, which has the potential to be developed as a once-monthly injection to improve patient compliance [99]. Moreover, a study demonstrated the potential of using XTENylated imaging probes. Annexin A5 (AnxA5) is a protein used in the detection of apoptosis *in vitro* and *in vivo*, but its plasma half-life is below 7 minutes, resulting in a low concentration in the target tissue when used as an imaging agent. Therefore, a new imaging agent, XTEN-AnxA5, consisting of human AnxA5 and the XTEN288 polypeptide was generated. It showed an improved accumulation in tumor tissues compared to AnxA5.
itself. The results suggested that the XTENylated fusion protein increases the blood half-life to approximately 1 hour and encourages the development of other XTENylated imaging probes in the future \[^{100}\].

This chapter summarizes strategies for extending the half-life of protein drugs by the attachment to polymers. PEGylation, PASylation, and XTENylation extend the hydrodynamic radius with good stability and solubility.

### 2.4.2. Albumin Binding

Human serum albumin (HSA) is the most abundant protein in plasma with a molecular weight of approximately 67 kDa and a very long half-life of 19 days. It is negatively charged which helps to further delay its filtration through the kidney thus extending its half-life \[^{101}\]. The neonatal Fc receptor (FcRn) is a protein that has been shown to play an important role in modulating the serum level of IgG and serum albumin by binding in a pH-dependent manner \[^{102,103}\]. Generally, FcRn can bind to IgG and HSA in endosomes at acidic pH (<6.5), followed by recycling to the cell surface and release at neutral pH in the blood (>7). In this way, IgG and albumin rely on FcRn recycling to avoid lysosomal degradation, thereby extending the half-life (Fig 6).

**Figure 6.** FcRn-mediated recycling. After internalization, serum albumin and IgG bind to FcRn in the acidic endosome, FcRn bound proteins are recycled and released at physiologic pH, while other, unbound proteins, are degraded in the lysosome.
Consequently, a strategy to improve the \textit{in vivo} half-life of proteins and peptides is to attach them to albumin by a non-covalent or a covalent bond. Non-covalent attachment can be achieved through specific binding to albumin, while covalent attachment is achieved through conjugation or by expression of the protein from a gene fusion with albumin \cite{104}.

There are different ways to achieve non-covalent binding to albumin. One way is to take advantage of the ability of albumin to act as a transporter \textit{in vivo}, reversibly binding to a wide range of endogenous and exogenous ligands, including fatty acids, penicillin, and warfarin to increase their half-life and bioavailability \cite{105}. One drug utilizing non-covalent albumin binding is Insulin detemir (Levemir\textsuperscript{®}) which is a conjugate of insulin and fatty acid and is used for the treatment of diabetes. Upon entering the bloodstream, the fatty acid binds to albumin forming a non-covalent interaction which in turn leads to a longer residence time in circulation. This has resulted in an increased half-life from 5 minutes to 6 hours \cite{106}.

Moreover, some proteins can bind to albumin specifically, which also leads to an increased half-life. One example is the albumin binding domain (ABD) from streptococcal protein G, a small (46 amino acids, 5 kDa) protein domain with a three-helix fold \cite{69}. This ABD has for example been expressed as a fusion protein with the affibody molecule $Z_{HER2:342}$ to prolong the plasma half-life. It was shown that the fusion protein maintained a high affinity to HSA. The half-life was greatly extended compared to the half-life of the affibody molecule alone and a 25-fold reduction in renal uptake was recorded \cite{107}. Another affibody variant targeting HER2, $Z_{HER2:2891}$, has also been expressed as a fusion protein with the ABD from protein G. The results showed an increase in the half-life from minutes to 14 hours in mice \cite{65}.

Some drugs include covalently attached HSA. HSA is in those drugs attached chemically by a linker, or, if the drug is a polypeptide, is expressed recombinantly as a fusion protein with HSA. An example of chemical linking is a conjugate consisting of doxorubicin and albumin for the treatment of cancer. It is becoming a potential clinical candidate due to the high stability of the molecule and favorable efficacy compared to doxorubicin itself \cite{108}. Fusion of the genes encoding the drug and albumin provides a simple and direct process to link them together covalently. Such fusion proteins have been recombinantly produced in a variety of expression hosts, such as the yeast \textit{Saccharomyces cerevisiae} and mammalian cells (CHO, HEK293) \cite{109}. Human GLP-1 has a half-life of only 1-2 minutes, whereas the albumin-fused GLP-1, albiglutide\textsuperscript{®} showed a plasma half-life of 11 hours in mice and 3 days in monkeys. It has been developed into a once-weekly injection for the treatment of type 2 diabetes \cite{110}.
Albumin fusion also allows for the construction of bivalent and bispecific fusions, where drugs are fused to the N and C terminus of albumin \([111]\). MM-111 (Merrimack) is a bispecific antibody-derived drug candidate consisting of a human scFv targeting HER2 and a human scFv targeting HER3 linked by modified HSA. This novel trimeric complex showed an effective inhibition for HER2-expressing tumor cell proliferation and is in a clinical trial for the treatment of advanced gastric cancer \([112,113]\).

Evidence suggested that human albumin is a very good molecule for plasma half-life extension of therapeutic drugs based on its biochemical and biophysical properties.

### 2.4.3. Fc Fusion

Hundreds of IgG-based drugs have been approved for clinical use or are in clinical development for a wide range of diseases, such as cancer, infectious disease, and cardiovascular disease \([114,115]\). Most IgG-based half-life extending proteins utilize Fc domain-coupled fusions and the relationship to FcRn for achieving pharmacokinetic properties and functions. One of the advantages of manufacturing a drug as an Fc-fusion is the improved half-life mediated by the interaction with FcRn, which may reduce the frequency of dosing (Fig 7). Apart from the extension of the plasma half-life, fusion to Fc also often results in the improvement of other properties of the drug, such as increased solubility and stability \([116]\).

![Figure 7. FcRn-mediated recycling of therapeutic proteins. Therapeutic proteins attached to the Fc region and albumin, serum albumin, and Fc bind to FcRn in the acidic endosome, endosomal recycling FcRn bound therapeutic proteins are recycled and released at physiologic pH, while the unbound proteins are degraded in the lysosome.](image-url)
FcRn is a transmembrane glycoprotein that is expressed in numerous cell types, including endothelial cells, epithelial cells, and immune cells \[^{117}\]. It consists of a heavy chain (α chain) and a light chain (β2-microglobulin) that are linked by non-covalent association. The heavy chain is composed of three extracellular domains (α1-3), a transmembrane domain, and a cytoplasmic domain \[^{118}\]. In humans, the natural ligands of FcRn are IgG and HSA.

The ability of FcRn to bind to its ligand in a pH-dependent manner is crucial for many of its functions, such as recycling and transcytosis. These functions allow the ligand to be protected from degradation in the lysosomes and allow transport through epithelial cell layers. Recombinant expression of a protein drug as a fusion to Fc may enable an easier manufacturing process since it can be purified with Protein A-based affinity chromatography \[^{119}\]. Furthermore, it sometimes increases the yield \[^{120}\].

Orencia® (Abatacept), is an Fc-fused drug consisting of the ECD of human CTLA-4 and the Fc domain of IgG1 with a half-life of 13 days in humans \[^{121}\]. It was approved in 2011 for the treatment of rheumatoid arthritis \[^{122}\]. Engineered Fc- domains with increased affinity for FcRn have also been developed. One drug including an engineered Fc is ACT-F. It has a plasma half-life of 45 days in cynomolgus monkeys, and the Fc variant has been engineered to increase the affinity for FcRn from 1200 nM to 97 nM. Compared to the same drug with wild-type Fc its plasma half-life was increased \[^{123}\].

Overall, protein half-life extension strategies have the potential to improve therapeutic efficacy and also reduce the dosing frequency in patients. However, these approaches should be carefully optimized to evaluate the benefits of extended half-life as well as potential drawbacks such as toxicity, immunogenicity, and potential hampering of biological activity.
Chapter 3

Antibody Drug Conjugates and Targeted Drug Conjugates

3.1. Antibody Drug Conjugates

Monoclonal antibodies are well-suited for targeted cancer therapy. However, as monotherapy, for example, the mAb trastuzumab, has shown only modest anti-tumor efficacy against breast cancer [124]. Antibody-drug conjugates (ADCs) are a type of biopharmaceutical drug aimed at cancer-targeted therapy with potent cytotoxic action. It consists of three parts, a monoclonal antibody (mAb), a linker connecting the antibody and the drug, and a cytotoxic payload (Fig 8). The monoclonal antibody recognizes and binds to a specific protein or tumor antigen on the surface of the cancer cell, enabling the ADC to selectively target the tumor cells. Once it is internalized by endocytosis, the cytotoxic payload is released directly into the cancer cell to exert anti-cancer effects while minimizing side effects on healthy cells.

Figure 8. The general structure of an ADC. The antibody-drug conjugate consists of three components: antibody, linker, and cytotoxic payload.

Compared with traditional chemotherapy, ADCs offer a number of advantages, such as improved specificity, increased efficacy, and reduced toxicity. In addition, this approach can increase the concentration of the payload in cancer cells to improve efficacy. As of 2023, more than 100 ADCs are in clinical and preclinical development.
The FDA has approved twelve ADCs for the treatment of a variety of cancers. Two of them are trastuzumab emtansine (T-DM1, Kadcyla®) for HER2-positive breast cancer, and brentuximab vedotin (Adcetris®) for Hodgkin’s lymphoma, and certain types of non-Hodgkin’s lymphoma[^125].

Since the tumor cells have high HER2 expression levels in 25-30% of all breast cancers, HER2 was recognized a long time ago as a potential target for ADC cancer therapy[^126]. Trastuzumab emtansine (Kadcyla®) was the second FDA-approved ADC (not counting gemtuzumab ozogamicin) for cancer treatment. It was approved in 2013 for certain types of breast cancer. It is composed of two different parts, where one is the drug trastuzumab (Herceptin®), which is a mAb against HER2-positive breast cancer cells, and the other is emtansine, which is a chemotherapeutic drug. T-DM1 works by attaching itself to HER2-positive breast cancer cells, after which it is internalized and the chemotherapeutic drug is released into the cells (Fig 9). In the case of T-DM1, a stable linker called succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) is used to link emtansine to trastuzumab. It is designed to maintain stability in the bloodstream and release the payload in the tumor cells after internalization. After degradation of the mAb part of T-DM1 in the lysosomes the active payload is lysine-MCC-DM1, which is released from the lysosome. Lysine-MCC-DM1 cannot pass the cell membrane, and there is, therefore, no bystander effect by T-DM1[^127].

The payload emtansine, also known as DM1, is a potent cytotoxic agent that targets and disrupts the polymerization of microtubules. T-DM1 has a drug-to-antibody ratio (DAR) of 3.5, which means that each antibody molecule is linked to an average of 3.5 cytotoxic payload molecules. DAR is a critical characteristic of ADCs that can affect their potency. The higher the DAR, the more drug each antibody can deliver to the tumor cell, which might lead to a more efficacious drug. Therefore, DAR is an essential parameter that needs to be considered during development and optimization[^128]. The combination of trastuzumab and emtansine makes T-DM1 an effective treatment for HER2-positive breast cancer. In one of the clinical trials, T-DM1 has an improved median overall survival of 5.8 months compared with a combination treatment of lapatinib and capecitabine[^129]. There are adverse effects associated with T-DM1 treatment. T-DM1 is usually given by intravenous injection and, commonly, patients experience side effects, such as fatigue, nausea, vomiting, and low blood cell counts[^130].
**Figure 9.** General mechanism of T-DM1. T-DM1 binds to and forms a complex with HER2 on the cancer cell, followed by endocytosis. The internalized complex undergoes lysosomal degradation and releases DM1 into the cytoplasm, resulting in the inhibition of tubulin polymerization and leading to tumor cell death. In the case of T-DM1 the cytotoxic effect is not induced in bystander cells because of the impermeability of the plasma membrane of T-DM1.

Brentuximab vedotin (Adcetris) is an ADC approved in 2011 to treat certain types of Hodgkin lymphoma and a type of non-Hodgkin lymphoma called systemic anaplastic large cell lymphoma (ALCL). It consists of the anti-CD30 chimeric monoclonal antibody brentuximab (cAC10) and monomethyl auristatin E (MMAE) linked by a cathepsin cleavable linker (VC). Brentuximab vedotin specifically targets CD30 which is present on the surface of the tumor cells. The payload MMAE is delivered by brentuximab, where it disrupts the polymerization of the microtubules, which inhibits cancer cell growth, resulting in cell death [131]. In a clinical trial conducted in 2010, it was found that among patients with Hodgkin lymphoma, 34% achieved complete remission after treatment with Adcetris. The trial also showed that tumor regression was observed in 94% of the patients. In the case of ALCL, 87% of the patients had at least a 50% reduction in tumor size and 97% had some degree of tumor shrinkage [132].

As mentioned earlier, an ADC consists of three components, so there are lots of important factors that need to be considered when designing an ADC. For example, an ideal antibody for ADC generation should have a high affinity for its tumor antigen, be efficiently internalized, have low immunogenicity, and have a long half-life in
circulation. In addition, the target antigen should ideally be highly expressed in tumor cells and lowly expressed in normal tissues. The linker should have high stability in circulation but should release the cytotoxic payload efficiently after internalization. The payloads are the key effector of the ADCs. They should be highly potent agents with high stability and solubility.

The use of various linker strategies and payloads has created new perspectives for improving the therapeutic index of ADCs and enhancing drug delivery to tumors while minimizing drug exposure to healthy tissues \[133\]. In the following chapter, I will discuss strategies for linker optimization and cytotoxic warhead selection.

### 3.1.1. Linker Moiety

The linker is a crucial component that joins the monoclonal antibody and the payload in an ADC. By optimizing the linker, it is possible to enhance the stability of the conjugate, extend the plasma half-life, and facilitate target internalization. According to its cytotoxic drug release mechanism, ADC linkers can be divided into two categories, cleavable and non-cleavable. Many of the ADCs that are currently undergoing clinical evaluation use cleavable linkers. These linkers should be stable in the bloodstream and release the drug within the target-expressing cells. The release occurs mostly in the endosomal compartments and is triggered in various ways, such as enzymatic cleavage, reducing environment, or acidic pH. ADCs with cleavable linkers typically release drugs that can also have a bystander effect on neighboring antigen-negative cells due to the high membrane permeability of the released drug. In addition to an increase in the killing of cells in the tumor vicinity, it may result in systemic toxicity and a reduced therapeutic window \[134\].

A variety of strategies have been investigated with the aim of improving the solubility of the drugs and also increasing the DAR in ADCs, as well as overcoming resistance mechanisms mediated by proteins such as MDR1 that transport the chemotherapeutic payload out of the cells \[133\]. Several approaches have utilized cleavable linkers to selectively release the payload in the cytoplasm, optimizing the linker to allow passage through the cell membrane to induce the bystander effect, or using a charged linker that is unable to cross the membrane thereby limiting the bystander effect, depending on whether the bystander effect is desired or not. Cleavable and non-cleavable linkers have both been evaluated in the development of clinically approved ADCs and in ADCs that are undergoing clinical trials \[135\].
Non-cleavable linkers are designed so that the payload is still attached to the antibody after internalization, i.e., there are no drug release mechanisms. After internalization the ADC is transported to the lysosomes where the antibody part is degraded, leaving the drug connected to the linker and the amino acid in the mAb to which it was connected.

### 3.1.1.1. Cleavable Linkers

Effective cleavable linkers are critical for the success of ADCs. They should be stable in the bloodstream for an extended time while also enabling efficient payload release in the tumor cells. The cleavable linkers can be divided into two main types, chemically labile linkers, and enzyme cleavable linkers.

#### 3.1.1.1.1. Chemically Labile Linkers

Chemically labile linkers include acid-sensitive linkers and disulfide linkers, and they are widely used in ADC development. These linkers are designed to be cleaved when the environment becomes more acidic or when there is an increased level of glutathione (Fig 10).

Hydrazone linkers are a class of acid-cleavable linkers that remain stable in the blood circulation at neutral pH, but can selectively release the drug in an acidic cellular compartment, such as lysosome (pH 4.8) and endosome (pH 6) [136]. The first clinically approved ADC was gemtuzumab ozogamicin (Mylotarg®) for the treatment of CD33-positive acute myeloid leukemia (AML). It is composed of a synthetic derivative of calicheamicin and an anti-CD33 monoclonal antibody IgG4k (hP67.6) which are linked by a hydrazone linker [137]. After its initial FDA approval, Mylotarg® was withdrawn from the market by the FDA due to its toxicity, which was partially attributed to poor stability in plasma [138]. However, in 2017, Mylotarg® was again approved by the FDA.
Antibody Drug Conjugates and Targeted Drug Conjugates

![Chemically cleavable linkers](image)

**Figure 10.** Chemically cleavable linkers. (A). Hydrazone linker. The cleavage of this linker occurs in acidic environments, such as endosomes and lysosomes. (B). Disulfide linker. This disulfide bridge is reduced by intracellular reducing agents, such as glutathione, to release the drug.

A disulfide linker (-S-S-) is a covalent bond involving two sulphydryl (SH) groups from cysteine residues or other SH molecules through an oxidation reaction. It is based on the reduction difference between the intracellular environment and the environment in plasma [139]. After the internalization of a disulfide bond coupled ADC, the payload can be released in the lysosome either through disulfide exchange or by the action of a reducing agent like glutathione. Mirvetuximab soravtansine (ELAHERE®) has been recently approved by the FDA for the treatment of epithelial ovarian cancer and consists of an anti-FRα mAb conjugated to DM4 via a glutathione cleavable linker [140].

### 3.1.1.1.2. Enzyme Cleavable Linkers

The use of enzyme-cleavable linkers in the development of ADCs has become increasingly popular and emerged as a promising strategy due to their superior plasma stability and controlled release mechanism. Enzyme cleavable linkers utilize the presence of hydrolytic enzymes that have the ability to recognize specific peptide sequences or carbohydrate patterns, which enables the degradation of the linker. An important aspect during the development of enzyme cleavable linkers was to take advantage of the difference in enzyme composition between the bloodstream and endosomes/lysosomes, to achieve cleavage and release of the payload only in the intracellular compartments. These release mechanisms ensure that the cytotoxic payloads are released in the tumor cells, resulting in increased efficacy and reduced toxicity compared to other cleavable linkers [141].
The most widely used enzyme-cleavable linkers are the protease-cleaved linkers. Cleavage is achieved by intracellular proteases, for example by cathepsin B. Proteases such as cathepsin B may be present at higher levels in tumor cells than in healthy cells [142]. A number of peptide linkers have been developed, including the valine-citrulline (Val-Cit) dipeptide linker and the phenylalanine-lysine (Phe-Lys) dipeptide linker. The cathepsin cleavage is usually combined with a self-immolating part of the linker, such as the para-amino benzyloxycarbonyl (PABC), where cathepsin cleavage induces 1,6-elimination of PABC with subsequent release of the active drug [143] (Fig 11). Until now, five of the approved ADCs utilize enzyme cleavable linkers: brentuximab vedotin (Adcetris®), polatuzumab vedotin-piiq (Polivy®), enfortumab vedotin (Padcev®), trastuzumab deruxtecan (Enhertu®), and tisotumab vedotin-tftv (Tivdak®).

**Figure 11.** Enzyme cleavable linkers. (A). Peptide-based linkers can be cleaved by cathepsin B and the PABC group allows the release of the free drug. (B). Glucuronide linkers are cleaved by β-glucuronidase, which is responsible for catalyzing the hydrolysis of bonds in β-glucuronic acid to release the payload.
Non-peptide cleavable linkers (β-Glucuronide linkers) are another type of enzyme-cleavable linker used in ADCs. These linkers contain a hydrophilic sugar group, and are designed to be cleaved by β-glucuronidase, an enzyme that is present in lysosomes and is overexpressed in many tumor cells \[144\]. When the ADC enters the tumor cell, the β-glucuronide linker is cleaved, resulting in the release of the sugar moiety and triggering self-immolation of the PAB group, followed by the release of the free cytotoxic drug. Examples are an anti-CD30 and an anti-CD70 monoclonal antibody conjugated to a minor groove binder (MGB) through a β-glucuronide linker. Both drug candidates were found to efficiently deliver the cytotoxic payload to the cancer cells to induce a strong cytotoxic effect \[145\].

One of the initial cleavable ADC linkers contained an acid-labile hydrazone, which was designed for a specific cleavage in the tumor cells. However, this type of linker was found to release the payload prematurely, resulting in damage to normal tissues. As a result, new ADC linkers were developed including more stable disulfide-cleavable linkers and enzyme-cleavable linkers.

### 3.1.1.2. Non-Cleavable Linkers

Another group of ADCs utilizes non-cleavable linkers (Fig. 12). As mentioned previously, non-cleavable linkers have significantly improved plasma stability compared to cleavable linkers \[146\]. The use of a non-cleavable linker in an ADC requires that the monoclonal antibody is degraded in the lysosome after its internalization in order to release the active payload. This limits the “bystander” effect. An example is T-DM1, an ADC using a non-cleavable linker, where the drug cannot cross the cell membrane after degradation of the mAb part in the lysosome because of a positively charged lysine residue that is still attached to the drug after lysosomal degradation \[147\]. Another example of ADCs with non-cleavable linkers is Belantamab mafodotin-blmf for the treatment of relapsed multiple myeloma using a maleimidocaproyl (mc) linker for the attachment of MMAF (mcMMAF) \[148\].

It is important to consider the differences between the parent drug and ADC metabolites when designing ADCs. An example is MMAE, a cytotoxic payload that is most effective in its native form and much less cytotoxic when attached to a non-cleavable linker \[149\]. However, MMAF, another cytotoxic payload used in ADCs, has been shown to maintain its potency when attached to an alkyl chain \[150\]. Therefore, the linker type should be chosen to be suitable for the particular drug used.
3.1.1.3. Attachment Sites

The design and performance of ADCs are influenced significantly by the attachment sites on the antibody, which could be largely attributed to the chemical groups present on the linkers \[^{[151]}\]. For the ADCs approved for clinical use, the most common conjugation methods use lysines or reduced cysteines in the mAb for drug attachment. These natural amino acids are present in varying numbers in antibodies, and they can be modified to create both heterogeneous and homogeneous ADCs \[^{[152]}\]. Heterogeneous ADCs mean in this context that different antibody molecules have different numbers and spatial positioning of the drugs, resulting in decreased stability and efficacy. In contrast, the creation of homogeneous ADCs uses specific conjugation sites in the antibody to achieve control of drug attachment. This usually leads to a more stable ADC with better efficacy \[^{[153]}\].

Lysines are typically more abundant and have a less uniform distribution in antibodies compared to cysteines. Lysine residues have free amino groups and form relatively stable amide bonds with the linker when creating an ADC \[^{[154]}\] (Fig 13). An example of a heterogeneous ADC is T-DM1, where the drug DM1 is conjugated to lysine residues on trastuzumab. The final product is a mixture of molecules with different DAR.

**Figure 12.** Non-cleavable linkers. Non-cleavable linkers are stable inside the cell and require the monoclonal antibody to be degraded in the lysosome after the internalization of the ADC in order to release the active payload. (A). Maleimide cyclohexane linker. (B). Maleimidocaproyl linker.
Drug attachment to lysines is a non-specific reaction and there appears to be little preference for different lysines in the antibody. Moreover, it has been found that the conjugation sites can greatly affect the stability and pharmacokinetic properties of an ADC \cite{155}. It is therefore important to give the sites of drug attachment consideration when constructing ADCs.

**Figure 13.** Some examples of drug conjugation sites. (A). Conjugation of the drugs to lysine residues. (B). Conjugation of the drugs to cysteine residues. (C). Conjugation using an enzyme-based reaction to a SMARTag.

Cysteines are connected by disulfide bonds in an antibody. For IgG, there are four interchain cysteines that can be selectively reduced to create eight attachment sites to be used for drug attachment. In reality, it is difficult to achieve a homogenous ADC product when addressing cysteines for drug attachment and the DAR may range from 0 to 8. A study on the antibody-drug conjugate cAC10-Val-Cit-MMAE, using different DAR ratios to determine the effect of drug loading on therapeutic potential, has shown that when the DAR is above 4, the resulting ADC had reduced tolerability, increased plasma clearance rate, and lower efficacy \cite{156}. Tris (2-carboxyethyl) phosphine (TCEP) and DL-Dithiothretiol (DTT) are chemical reagents commonly used to reduce disulfide bridges in proteins, which can provide a thiol moiety for the attachment of drugs. The
free thiol groups may react with maleimide or halogenoalkane groups in the linker drug \cite{157}. A recent strategy for the creation of ADCs utilizes additionally inserted cysteine residues that can be specifically addressed with linker drugs. It has been developed by Genentech \cite{154}. The strategy identified cysteine substitutions on both the light and heavy chains which did not affect the antibody folding or antigen binding. One example of the application of this strategy is the design of an ADC which consist of MMAE attached to an antibody targeting MUC16, an ovarian cancer antigen. The ADC has shown promising results, where it is tolerated better compared to the conventional ADC where the drugs are attached by cysteine sulphydryl groups while maintaining a similar efficacy.

Another way to achieve site-specific conjugation is to use a tag consisting of genetically encoded amino acids that are incorporated into the sequence of the mAb. These tags can be specifically recognized by suitable enzymes, such as sortases or formyl glycine-generating enzymes (FGE). Sortases are enzymes that can attach polyglycine-tagged payloads to a target antibody by a specific recognition sequence (LPETG) that can be inserted into the heavy or light chains \cite{158}. The FGE-tag technology is termed SMARTag (Specific Modifiable Aldehyde Recombinant Tag) and functions in a similar way. FGE recognizes a specific amino acid motif (Cys-X-Pro-X-Arg) that can be site-specifically inserted into an antibody. FGE converts cysteine to formylglycine. The resulting aldehyde of formylglycine can then be used as a site for the covalent attachment of aldehyde-specific warheads \cite{159}.

Overall, considerable efforts have been dedicated to developing and optimizing sites in mAbs to provide handles for site-specific drug conjugation, which can lead to more homogeneous and stable conjugates with improved pharmacokinetic properties.

3.1.2. Cytotoxic Payload

When selecting the payload for an ADC, a number of factors need to be considered, including solubility, conjugation stability, and potency \cite{160}. These properties are essential to ensure the effectiveness of the ADC in delivering the cytotoxic effect to the target cells. High solubility in water is important to prevent aggregation. Many potential payloads have low solubility, which can, to some extent, be increased by using a linker containing hydrophilic sections such as PEG. This can allow for generating ADCs with increased DAR \cite{161}. Conjugation stability is also an essential factor for the ADC as it affects efficacy and safety. The drug must be stably attached to the mAb while in the bloodstream, preventing premature release which may lead to side effects. A novel strategy using pH-sensitive prodrugs has been introduced. This approach leads to the
prodrugs can release the cytotoxic agent in the acidic tumor cells. The resulting doxorubicin prodrug showed that it is less cytotoxic than doxorubicin in neutral pH conditions and has similar cytotoxicity under acidic conditions [162]. The ideal payload should also be potent enough and preferably more cytotoxic to tumor cells than to normal cells.

Several types of payloads are used in ADCs, including microtubule polymerization inhibitors, DNA-damaging drugs, and, more recently, topoisomerase inhibitors. These categories of payloads affect critical cellular processes necessary for cell survival.

3.1.2.1. Microtubule Polymerization Inhibitors

A popular class of payloads is the microtubule polymerization inhibitors. Most commonly they are either maytansine derivatives such as DM1 and DM4 or auristatin derivates such as MMAE and MMAF. By inhibiting the polymerization of microtubules, these payloads prevent cells from dividing and growing, eventually leading to death. It is possible that such drugs are more cytotoxic to fast-dividing cells than to slow-dividing cells since they prevent the formation of the spindle during mitosis, a key process in cell division. Since cancer cells divide faster than most normal cells it can be speculated that they will be more cytotoxic to cancer cells than most normal cells. However, there are some fast-dividing cells in the body such as the hematopoietic stem cells in the bone marrow. Because of this, a common adverse effect of T-DM1 is low blood count [163].

A group of microtubule polymerization inhibitors is the auristatins. MMAE and MMAF are two such drugs, which are synthetic derivatives of dolastatin 10, a natural anti-mitotic agent isolated from *Dollabella auricularia* [164]. Both MMAE and MMAF are peptide-like compounds and have good stability in plasma and are resistant to degradation by proteases. The difference between MMAE and MMAF is that MMAF has a negatively charged phenylalanine at the C-terminus, while MMAE is uncharged, which makes it more difficult to cross cell membranes (Fig 14). As a free drug, MMAE exhibits greater membrane permeability and a lower IC₅₀ compared to MMAF. However, MMAF is a more hydrophilic payload and has a reduced propensity to aggregate, resulting in lower systemic toxicity [165]. Currently, there are five auristatin-based ADCs approved by the FDA for clinical use, including Adcetris®, Polivy®, Padcev®, Blenrep®, and Tivdak®. Adcetris® is created by combining brentuximab, which is a monoclonal antibody that targets CD30 with MMAE using a cleavable linker. It is used in the treatment of relapsed Hodgkin lymphoma [132]. Blenrep® is a humanized...
monoclonal antibody against the BCMA (B-cell maturation antigen) conjugated with MMAF for the treatment of relapsed multiple myeloma \cite{166}.

**Figure 14.** Structures of (A) MMAE and (B) MMAF. The purple area contains the differences between the payloads.

Another popular type of microtubule polymerization inhibitor is the maytansinoids, for example, DM1 and DM4. They are from maytansine, a natural, highly anti-mitotic product, originally derived from the African shrub *Maytenus ovatus* \cite{167} (Fig 15). Maytansine and its analogs act as inhibitors of microtubule assembly by binding to the tubulin protein at the same binding site as vinblastine. This leads to the disruption of microtubule formation, which is necessary for various cellular processes such as cell division and intracellular transport. The maytansinoids have a potent cytotoxic effect, and they are stable in plasma. Until now, several ADCs including maytansinoids have undergone clinical evaluation and some have been approved by the FDA for clinical use. For example, T-DM1, an FDA-approved ADC consists of the maytansinoid DM1 conjugated to the anti-HER2 monoclonal antibody trastuzumab. It has demonstrated remarkable effectiveness against metastatic breast cancer \cite{167}. ELAHERE®, another FDA-approved ADC consists of the anti-FOLR1 (folate receptor 1) humanized monoclonal antibody M9346A conjugated to DM4 for the treatment of FRα-positive platinum-resistant ovarian cancer \cite{140}. IMGN901 (Lorvotuzumab mertansine) consists of the CD56-targeting antibody lorvotuzumab and the maytansinoid DM1 for the treatment of CD56-positive small-cell lung cancer and ovarian cancer \cite{168}.
3.1.2.2. DNA Damaging Payloads

Over 60% of the ADCs currently undergoing clinical trials use microtubule polymerization inhibitors as their cytotoxic payloads \[^{169}\]. However, other types of payloads are also in clinical trials, particularly those involving DNA-damaging agents.

Calicheamicins are a class of highly potent DNA-damaging payloads produced by the bacterium *Micromonospora echinospora*. They can bind to the minor groove of DNA and induce specific double-stranded breaks \[^{170}\]. Two ADCs (Mylotarg® and Besponsa®) are approved by the FDA for clinical use and have calicheamicin as payload. Mylotarg® is a monoclonal antibody against CD33 linked to calicheamicin. It is used for the treatment of acute myeloid leukemia \[^{171}\]. Besponsa® is a CD22-directed ADC that combines an anti-CD22 humanized mAb with calicheamicin. It is used to treat relapsed B-cell precursor acute lymphoblastic leukemia \[^{172}\].

Pyrrolobenzodiazepines (PBD) are another group of highly potent DNA-damaging agents isolated from the bacteria *Streptomyces* \[^{169}\]. PBDs can insert into the DNA double helix, causing structural changes that lead to DNA damage. Despite their significant antitumor efficacy, PBD exhibit cardiotoxicity, which has discontinued clinical use. However, ongoing research suggests that PBD can be employed in antibody-drug conjugate for cancer therapy. PBD dimers typically have picomolar IC\(_{50}\) in a variety of cell types \[^{173}\]. Zynlonta® is an FDA-approved ADC that is composed of an antibody targeting CD19 with a PBD payload. It is used for the treatment of adult relapsed large B-cell lymphoma \[^{174}\].
3.1.2.3 Topoisomerase Inhibitors

Camptothecin (CPT) analogs are a class of potent antitumor agent that inhibits topoisomerase I. The source of Camptothecin is the Chinese ornamental tree *Camptotheca acuminata*. In initial clinical trials, CPT exhibited promising anticancer activity, particularly against breast, ovarian, colon, lung, and stomach cancers[^43^]. However, its therapeutic use faced challenges due to low solubility and reported adverse effects. Currently, two CPT analogues have been granted FDA approval and are presently employed as anticancer agents in cancer treatment: topotecan and irinotecan[^175^]. There are two FDA-approved ADCs utilize camptothecin analogs as cytotoxic payloads. Enhertu® consists of the HER2 mAb trastuzumab and DXd (a DX-8951 derivative) payload[^176^]. Trodelvy® is a conjugate of a Trop-2-directed antibody and an SN-38 drug. It is approved for clinical use in the treatment of triple-negative breast cancer[^177^].

3.1.3. Lessons Learned from ADCs in the Clinic and Clinical Trials

Antibody-drug conjugates (ADCs) represent a relatively new category of antitumor drugs that aims to combine the specificity of monoclonal antibodies with the cytotoxic effect of chemotherapy. Since the first ADC was evaluated in a clinical trial in the 1990s, numerous subsequent studies have shown significant potential for the treatment of various types of cancers. Until now, twelve ADC drugs have been approved by the FDA for clinical use[^178^]. Furthermore, there are currently more than 100 ADC candidates in different stages of clinical trials[^146^]. An overview of the FDA-approved ADCs including target, linker, payload, DAR, and approval year is shown in Table 1.
<table>
<thead>
<tr>
<th>Trade Name</th>
<th>General Name</th>
<th>Target</th>
<th>Linker</th>
<th>Payload</th>
<th>DAR</th>
<th>Approval Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mylotarg</td>
<td>Gemtuzumab ozogamicin</td>
<td>CD33</td>
<td>Hydrazine</td>
<td>Calicheamicin</td>
<td>2-3</td>
<td>2017; 2000</td>
</tr>
<tr>
<td>Adcetris</td>
<td>Brentuximab vedotin</td>
<td>CD30</td>
<td>mc-VC-PABC</td>
<td>MMAE</td>
<td>4</td>
<td>2011</td>
</tr>
<tr>
<td>Kadcyla</td>
<td>Trastuzumab emtansine</td>
<td>HER2</td>
<td>Non cleavable SMCC</td>
<td>DM1</td>
<td>3.5</td>
<td>2013</td>
</tr>
<tr>
<td>Besponsa</td>
<td>Inotuzumab ozogamicin</td>
<td>CD22</td>
<td>Hydrazine</td>
<td>Calicheamicin</td>
<td>6</td>
<td>2017</td>
</tr>
<tr>
<td>Polivy</td>
<td>Polatuzumab vedotin-piiq</td>
<td>CD79b</td>
<td>mc-VC-PABC</td>
<td>MMAE</td>
<td>3.5</td>
<td>2019</td>
</tr>
<tr>
<td>Padcev</td>
<td>Enfortumab vedotin</td>
<td>Nectin-4</td>
<td>mc-VC-PABC</td>
<td>MMAE</td>
<td>3.8</td>
<td>2019</td>
</tr>
<tr>
<td>Enhertu</td>
<td>Trastuzumab deruxtecan</td>
<td>HER2</td>
<td>Tetrapeptide</td>
<td>DXd</td>
<td>8</td>
<td>2019</td>
</tr>
<tr>
<td>Trodelvy</td>
<td>Sacituzumab govitecan</td>
<td>Trop-2</td>
<td>CL2A</td>
<td>SN-38</td>
<td>7.6</td>
<td>2020</td>
</tr>
<tr>
<td>Blenrep</td>
<td>Belantamab mafodotin-blmf</td>
<td>BCMA</td>
<td>mc</td>
<td>MMAF</td>
<td>4</td>
<td>2020</td>
</tr>
<tr>
<td>Zynlonta</td>
<td>Loncastuximab tesirine-lpyl</td>
<td>CD19</td>
<td>Dipeptide</td>
<td>PBD dimer</td>
<td>2.3</td>
<td>2021</td>
</tr>
<tr>
<td>Tivdak</td>
<td>Tisotumab vedotin-tftv</td>
<td>Tissue factor</td>
<td>mc-VC-PABC</td>
<td>MMAE</td>
<td>4</td>
<td>2021</td>
</tr>
<tr>
<td>ELAHHERE</td>
<td>Mirvetuximab soravtansine</td>
<td>FRα</td>
<td>sulfo-SPDB</td>
<td>DM4</td>
<td>N/A</td>
<td>2022</td>
</tr>
</tbody>
</table>
Despite progress in the development and use of ADCs, there are still numerous challenges that need to be addressed. These challenges include pharmacokinetics, side effects, tumor targeting, payload release, and drug resistance [133].

Following administration, three major forms of ADC may exist in the bloodstream, including the fully intact ADC, the unconjugated antibody, and the free cytotoxic drug [179]. In a typical pharmacokinetic profile for an ADC, the concentrations decrease over time due to the internalization and degradation of the ADC. These factors must be considered for an optimal ADC selection [180]. Factors that impact the clearance of antibodies include the interaction with the neonatal Fc receptor (FcRn). Through binding to ADCs in the endosome of cells that have taken up the ADC, FcRn facilitates the export of ADCs to the extracellular compartment [181]. In addition, the metabolism of the free cytotoxic payload is another factor affecting the characteristics of ADCs.

The most frequent severe side effects detected in the FDA-approved ADCs are hematotoxicity, hepatotoxicity, and gastrointestinal reactions, which may be related to the premature release of payloads into the circulation and the undesirable uptake in healthy cells [182]. It is necessary to optimize the next generations of ADC to decrease the side effects.

In comparison to conventional chemotherapy drugs, ADCs have a larger molecular weight, which can affect the ability to penetrate tumors. Therefore, it is crucial to consider the permeability of the drug when designing an ADC. Moreover, the “bystander effect” of some ADCs can kill surrounding tumor cells that do not have an expression of the target antigen. Avoiding the effect in some conditions is an important aspect to consider when designing an ADC.

Drug resistance is another challenge for an ADC. Although the underlying mechanism of drug resistance in ADCs is not well defined, several possible mechanisms have been identified based on preclinical models, including downregulation of the targeted antigens, over-expression of drug transporter proteins, dysfunction of ADC trafficking pathways, and modifications of the targeted receptor [183].

3.2. Targeted Drug Conjugates

ADCs are composed of a monoclonal antibody, a linker, and a cytotoxic payload drug. Therefore, any modification of these three components will impact the function and efficacy of the ADC. For example, different antibodies that target the same antigen can exhibit distinct binding affinity and induce an effect with varying efficacy. Furthermore,
bispecific antibodies may give more possibilities for the development of ADCs. These designs have the potential to enhance internalization and improve tumor selectivity, thus increasing the efficacy of ADC therapy. For example, BL-B01D1 is a novel bispecific ADC that comprises a bispecific antibody targeting EGFR and HER3, linked to a topoisomerase I inhibitor payload through a cleavable linker. Remarkably, BL-B01D1 has shown promising effectiveness in patients with extensively treated metastatic or locally advanced solid tumors [184]. Another strategy for developing ADCs is to change the conventional monoclonal antibody structure and use a small molecular weight fragment to couple to the payload to enhance the ability to penetrate tumor tissues and deliver the payload. For example, using alternative scaffolds for drug delivery may result in the development of novel cancer therapy options, that may have properties that in some cases are better than the conventional ADCs.

Compared with traditional ADCs, the alternative scaffold conjugates often have a smaller size, allowing for better penetration of solid tumors. In addition, many can be easily expressed in bacterial systems which may lower the production cost. Alternative scaffold proteins generally exhibit greater thermal stability and chemical stability and usually have a faster and easier refolding process, which may facilitate the use of a broader range of chemical conjugation reactions. Moreover, some alternative scaffold lacks cysteine residues, which enables the introduction of unique cysteines for site-specific conjugation.

Due to the novelty and ongoing development of alternative scaffold conjugates, there are currently a limited number of studies on them. Therefore, it is necessary to evaluate potential alternative scaffold conjugates for further development of cancer treatments. Affibody-drug conjugates appear to be a good format for the creation of alternative scaffold conjugates (Fig 16). For example, \( Z_{\text{HER2:2891}-\text{ABD-mcDM1}} \) is an affibody-drug conjugate comprising the payload DM1 conjugated to an affibody molecule targeting HER2 [185]. A unique cysteine was placed at the C-terminus for the site-specific conjugation of mcDM1. The molecular weight is only 14 kDa, which is much lower than the 150 kDa of a traditional ADC. However, one of the challenges of these small scaffold conjugates is the fast clearance from plasma. Therefore, in this construct, an ABD was included to extend the half-life in the circulation.
Another example is the DARPin-based conjugate MSA-Ec1-MMAF which targets the epidermal cell adhesion molecule (EpCAM) and contains the cytotoxic payload MMAF, linked to the DARPin through a thioether bond formed between a cysteine residue and a maleimide group. In this construct, an unnatural amino acid, azidohomoalanine, was introduced at the N-terminus. Dibenzocyclooctyne-modified MSA was then conjugated to the azide group to prolong the in vivo half-life. It shows a 22-fold extended retention time in the circulation of around 17 hours compared to the conjugate without the MSA domain [186].

A third example of an alternative scaffold conjugate is ADAPT6-ABD-mcDM1. It is an ADAPT-based drug conjugate. This construct included an ADAPT6 molecule that targets HER2, an ABD to extend the half-life in circulation, and the potent cytotoxic payload DM1. The ADAPT-based conjugate demonstrated a remarkable ability to selectively bind to HER2-overexpressing cells. It exhibited potent cytotoxicity against cells overexpressing HER2 with IC50 values ranging from 5 to 80 nM [74].
Chapter 4

Present Investigation

Over the past decade, antibodies have emerged as a prominent class of molecules for targeted cancer therapy, either as single agents or as conjugates with a payload. Alternative scaffold proteins have become complementary agents to antibodies in recent years. This thesis aims to develop and evaluate one class of alternative scaffold proteins, the affibody molecules, with conjugated cytotoxic drug payloads, for targeted cancer therapy in vitro and animal models. The studies focused on affibody molecules targeting HER2 and HER3, with the aim of optimizing and evaluating different conjugate designs. In paper I, the effect of drug load on cytotoxicity and biodistribution of affibody-derived drug conjugate targeting HER2 was investigated. In paper II, the biodistribution of different inter-domain linker compositions of an ABD-fused affibody-drug conjugate targeting HER2 was investigated. In paper III, the anti-tumor efficacy of HER2-targeted affibody conjugates loaded with auristatin- and maytansine-derived drugs was compared using mice bearing SKOV3 (HER2-expressing cell line) xenografts. In paper IV, different strategies for half-life extension by PASylation, XTENylation, and albumin binding, of affibody-drug conjugates targeting HER2 were investigated. In paper V, the effect of the molecular design of HER3-targeting affibody-drug conjugates on the BxPC3 pancreatic human tumor model was investigated in vivo.
Affibody molecules have shown significant potential as vehicles for the delivery of cytotoxic payloads for targeted cancer therapy due to the possibility of generating high-affinity binders, a simple manufacturing process, and the ability to optimize the loading and spatial orientation of the drug molecules. The affibody-based drug conjugates comprise three key components, including an affibody, a linker, and a cytotoxic payload.

In general, the drug-to-affinity protein ratio (DAR) affects the efficacy of the drug. For antibodies, a low DAR value often leads to insufficient delivery of the cytotoxic payload to the tumor cells with low levels of the target antigen, while a high DAR value may positively affect the toxicity but lead to accelerated hepatic clearance \[^{187}\]. In addition, a high DAR is associated with changes in the binding properties and physical instability of the conjugates, which could impact their effectiveness \textit{in vivo} \[^{188}\].

An opportunity for affibody-drug conjugates is that they allow for site-specific attachment of the drugs and easy control of the drug loading. The affibody molecules are cysteine-free structures, thus a desired number of cysteine residues can be selectively engineered at desired positions, followed by site-specific conjugation of the drug using thiol-directed chemistry. Cytotoxic payloads, such as DM1, exhibit a hydrophobic character, which can contribute to its uptake in the liver. Previous studies have demonstrated that incorporating a hydrophilic linker consisting of the amino acid sequence Glu-Glu-Glu adjacent to a C-terminal cysteine residue, where DM1 was attached, could decrease the hydrophobicity of the conjugates and reduce its uptake by the liver \[^{189}\].

In this study, I have investigated the effects of increasing the ratio of a cytotoxic drug to affibody from one to three in a HER2-targeted affibody-drug conjugate. The anti-HER2 affibody molecule ($Z_{\text{HER2:2891}}$) was recombinantly expressed as a fusion protein with an albumin-binding domain (ABD\textsubscript{035}) to extend the plasma half-life. Additionally, one or three cysteine residues adjacent to Glu-Glu-Glu were introduced at the C-terminus of the conjugates, to which the cytotoxic payload DM1 was conjugated through a non-cleavable maleimidocaproyl linker (mc). The affibody-drug conjugates are illustrated in Fig 17. The \textit{in vivo} biodistribution of the resulting drug conjugates $Z_{\text{HER2-ABD-mcDM1}}$ and $Z_{\text{HER2-ABD-mcDM13}}$ were determined in mice bearing HER2-overexpressing SKOV3 xenografts.
Different HER2-targeting affibody molecules were generated and expressed in *E.coli* BL21 Star. The proteins were produced in the cytoplasm and were purified by HSA-affinity chromatography, which interacted with the ABD in the fusion proteins. A reducing agent was added to reduce potentially oxidized cysteines in the C-termini of the constructs, followed by conjugation of the payload mcDM1 or alkylation of the cysteines to generate the non-toxic control. Due to the ability of efficient refolding of the fusion proteins, the conjugates were further purified by RP-HPLC under denaturing conditions. The conjugates were characterized biochemically by SDS-PAGE, SEC, and RP-HPLC (Fig 18). The RP-HPLC showed that the cytotoxic payload mcDM1 increased the hydrophobicity of the conjugates, since they were eluted at a higher acetonitrile concentration (later in the chromatogram) compared to the constructs with DAR=1 and DAR=0.
To investigate the influence of mcDM1 drug load on the affinity properties, the binding affinity of the affibody-drug conjugates to HER2 and serum albumins (HSA and MSA) was determined by a Biacore biosensor (Table 2). $Z_{\text{HER2}}$-ABD-mcDM1$_3$ had a weaker affinity for both HER2 and serum albumins than $Z_{\text{HER2}}$-ABD-mcDM1 and $Z_{\text{HER2}}$-ABD-AA$_3$. The reason for this loss in affinity cannot presently be explained.

Table 2. The affinity constants for affibody-drug conjugates.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Ligand</th>
<th>$K_a$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)</th>
<th>$K_d$ (s$^{-1}$)</th>
<th>$K_D$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Z_{\text{HER2}}$-ABD-mcDM1$_3$</td>
<td>HER2</td>
<td>$3.1 \times 10^5$</td>
<td>$2.1 \times 10^{-4}$</td>
<td>$6.9 \times 10^{-10}$</td>
</tr>
<tr>
<td></td>
<td>HSA</td>
<td>$3.0 \times 10^5$</td>
<td>$6.0 \times 10^{-5}$</td>
<td>$2.0 \times 10^{-10}$</td>
</tr>
<tr>
<td></td>
<td>MSA</td>
<td>$5.2 \times 10^5$</td>
<td>$3.0 \times 10^{-3}$</td>
<td>$5.8 \times 10^{-9}$</td>
</tr>
<tr>
<td>$Z_{\text{HER2}}$-ABD-mcDM1</td>
<td>HER2</td>
<td>$1.5 \times 10^6$</td>
<td>$5.7 \times 10^{-5}$</td>
<td>$3.8 \times 10^{-11}$</td>
</tr>
<tr>
<td></td>
<td>HSA</td>
<td>$2.4 \times 10^6$</td>
<td>$2.2 \times 10^{-3}$</td>
<td>$9.4 \times 10^{-10}$</td>
</tr>
<tr>
<td></td>
<td>MSA</td>
<td>$1.0 \times 10^6$</td>
<td>$3.0 \times 10^{-4}$</td>
<td>$3.0 \times 10^{-10}$</td>
</tr>
<tr>
<td>$Z_{\text{HER2}}$-ABD-AA$_3$</td>
<td>HER2</td>
<td>$1.0 \times 10^6$</td>
<td>$7.7 \times 10^{-5}$</td>
<td>$5.2 \times 10^{-11}$</td>
</tr>
<tr>
<td></td>
<td>HSA</td>
<td>$2.7 \times 10^6$</td>
<td>$2.7 \times 10^{-3}$</td>
<td>$1.0 \times 10^{-9}$</td>
</tr>
</tbody>
</table>

An in vitro cytotoxicity analysis was performed for the affibody-drug conjugates and the corresponding non-toxic control in cell lines with different HER2 expression levels (Fig 19). For all three high HER2-expressing cell lines (SKOV3, SKBR3, AU565), both affibody-drug conjugates showed a strong dose-dependent cytotoxic effect. For the SKBR3 and AU565 cell lines, there was no obvious difference in the IC$_{50}$ value for
the high and low DAR construct. However, \( Z_{\text{HER2}}^{\text{-ABD-mcDM13}} \) showed a stronger cytotoxic effect with an \( IC_{50} \) value of 12.4 nM compared to \( Z_{\text{HER2}}^{\text{-ABD-mcDM1}} \) for the SKOV3 cell line. For the cell lines with low HER2 expression, essentially no cytotoxic effect was observed. It is evident that the cytotoxicity is related to the interaction between the affibody molecules and HER2, and that the cytotoxicity is cell-line dependent.

**Figure 19.** Cytotoxicity analysis of the affibody-drug conjugates. The viability of the cell lines was determined and plotted against the concentration of AffiDCs. Each data point is the average of quadruplicate experiments, and the error bars correspond to 1 SD.

To analyze the drug conjugates further they were radiolabeled with 99m-technetium. The binding specificity of the affibody-drug conjugates was tested on the HER2-overexpressing cell lines SKOV3 and BT474. Cells incubated with radiolabeled affibody-drug conjugates were blocked by pre-incubated non-radiolabeled affibody-drug conjugates (Fig 20A). The blocking results strongly suggested that the interaction of the affibody-drug conjugates and HER2-overexpressing cells was HER2-dependent. The assay also revealed a notably elevated level of non-specific binding for \( Z_{\text{HER2}}^{\text{-ABD-mcDM13}} \) to the cells, in comparison with the other conjugates. The increased lipophilicity of multiple mcDM1 drugs is a possible reason for the higher level of non-specific binding.

In an internalization experiment, the cell-related radioactivity and internalized fraction were measured at different time points over 24 hours for the radiolabeled drug conjugates (Fig 20B). A fast cellular association rate was found for all three affibody-
drug conjugates for SKOV3 cells. For the BT474 cells, the association rates for the three conjugates were slower and increased continuously for 24 hours.

**Figure 20.** (A) *In vitro* binding specificity of radiolabeled affibody-drug conjugates on the SKOV3 and BT474 cell lines. Non-blocked indicates that the cells were only incubated with radiolabeled conjugates, while blocked indicates that the cells were pre-incubated with a non-radiolabeled conjugate to block available HER2 receptors prior to incubation with the radiolabeled drug conjugates. Each bar represents the average of three individual experiments, and the error bars correspond to 1 SD. The asterisks (*) correspond to significant differences ($p < 0.05$). (B) The cellular processing of radiolabeled affibody-drug conjugates by SKOV3 and BT474 cells over 24 hours. Each time point is the average of three individual measurements, and the error bars in the figure correspond to 1 SD.

The binding affinity of the radiolabeled conjugates to HER2-expressing SKOV3 cells was evaluated in real-time using a LigandTracer instrument (Fig 21). From the interaction map data, it was found that $Z_{\text{HER2-ABD-mcDM1}}$ and $Z_{\text{HER2-ABD-mcDM1}}$ interacted through a single process, while $Z_{\text{HER2-ABD-AA3}}$ interacted through two processes. The impact of mcDM1 drug load on the binding properties was consistent with the SPR results, i.e., the high-DAR drug conjugate had a weaker affinity to the cells compared to the low-DAR drug conjugate.
Figure 21. Real-time binding results and interaction map analysis of radiolabeled affibody-drug conjugates on SKOV3 cells. (A) Z\textsubscript{HER2}-ABD-AA\textsubscript{3}. (B) Z\textsubscript{HER2}-ABD-mcDM1\textsubscript{3}. (C) Z\textsubscript{HER2}-ABD-mcDM1.

Moreover, an \textit{in vivo} biodistribution experiment was carried out to investigate the uptake of the AffiDCs in mice bearing HER2-overexpressing SKOV3 xenografts (Fig 22). By comparing the blood retention of Z\textsubscript{HER2}-ABD-mcDM1\textsubscript{3} and Z\textsubscript{HER2}-ABD-mcDM1 at 4 hours and 24 hours p.i., it was observed that Z\textsubscript{HER2}-ABD-mcDM1\textsubscript{3} had more rapid clearance than Z\textsubscript{HER2}-ABD-mcDM1. These findings are consistent with the conclusions from a study on ADCs, which demonstrated that an increased DAR correlated with higher hydrophobicity, leading to more rapid elimination from the bloodstream \cite{190}. Z\textsubscript{HER2}-ABD-mcDM1\textsubscript{3} exhibited higher liver uptake compared to Z\textsubscript{HER2}-ABD-mcDM1, indicating increased hepatic clearance. A previous study has reported that for radiolabeled targeting proteins and peptides, the presence of a hydrophobic moiety or a positively charged region can promote liver uptake \cite{191}. The tumor uptake increased over time. However, a notable difference was observed in which the tumor uptake of Z\textsubscript{HER2}-ABD-mcDM1\textsubscript{3} was considerably lower than Z\textsubscript{HER2}-ABD-mcDM1 at both time points. At 24 hours, the tumor uptake for Z\textsubscript{HER2}-ABD-mcDM1\textsubscript{3} had reached 6.8 %ID/g, while it was 3.3 %ID/g for Z\textsubscript{HER2}-ABD-mcDM1.
However, since $Z_{HER2}$-ABD-mcDM1$_3$ contained three times as many DM1 molecules, the delivery of the drug to the tumor was 1.45-fold higher than for $Z_{HER2}$-ABD-mcDM1.

![Biodistribution of radiolabeled affibody-drug conjugates in mice bearing human SKOV3 xenografts](image)

Figure 22. Biodistribution of radiolabeled affibody-drug conjugates in mice bearing human SKOV3 xenografts. The radioactivity is presented as the percent of injected dose per gram (%ID/g) and presented as the average values from 3 or 4 animals ± 1 SD.

In conclusion, increasing the DAR of HER2-targeting AffiDC from one to three resulted in a 1.45-fold increase in the delivery of cytotoxic mcDM1 to tumors. However, this increase was accompanied by a non-specific uptake in the liver. Therefore, further studies are necessary to investigate the molecular design of high-DAR AffiDCs to achieve efficient drug delivery to the tumors while minimizing the uptake in normal tissues.
4.2. Paper II – Effect of Inter-Domain Linker Composition on Biodistribution of ABD-Fused Affibody-Drug Conjugates Targeting HER2

In preclinical studies, affibody-based targeted drug conjugates, fused with an albumin binding domain (ABD) to extend their half-life, have shown potent anti-tumor activity \[^{185}\]. The use of these conjugates resulted in effective treatment and prolonged survival of mice bearing SKOV3, HER2-expressing xenografts. The payload mcDM1 increased the hydrophobicity of the conjugate, resulting in higher liver uptake. However, this effect could be partially mitigated by introducing a hydrophilic linker consisting of three glutamates between the ABD and mcDM1, which reduced the hepatic uptake of the anti-HER2 AffiDCs \[^{189}\]. This finding is significant as it reduces the risk of liver toxicity, a frequent reason for drug trial failures and withdrawal from the market \[^{192}\].

Moreover, we hypothesized that further optimization of the molecular design of the AffiDCs could enhance the cytotoxic effect while reducing systemic toxicity. This study aimed to investigate the effect of length and composition of the inter-domain linker between HER2-targeted affibody molecule (Z\(_{HER2}\)) and the ABD on the \textit{in vitro} functionality and \textit{in vivo} biodistribution of the AffiDCs containing a microtubule polymerization inhibitor maytansine derivative (mcDM1).

The inter-domain linker originally comprised a sequence of four glycines and one serine (GGGGS/G\(_4\)S), which is a version of the flexible (G\(_4\)S\(_n\))-linkers that are commonly used in biotechnology \[^{193}\]. The flexibility of the linker is a desired characteristic since we believe that the construct binds to its receptor \textit{in vivo} while binding to albumin. Since both albumin and the receptor are large molecules, and the AffiDC is small, a rigid linker may make it difficult to bind both proteins at the same time. Flexible linkers should comprise small and hydrophilic amino acids, a property satisfied by both serine and glycine \[^{194}\]. The length of the linker is another crucial consideration when designing a protein construct. A short linker may lead to inadequate separation between the structural domains, which can result in incorrect folding \[^{195}\] or impaired biological functions \[^{196}\]. However, long linkers may potentially form secondary structures that could also affect biological functions. It is worth noting that linkers connecting different domains in naturally occurring proteins typically are 3 to 15 residues long \[^{194}\].

In this study, linkers containing repeated sequences of three glycines and one serine (G\(_3\)S) or three serines and one glycine (S\(_3\)G) were evaluated. Affibody-drug conjugates with three different linkers, Z\(_{HER2}\)-(G\(_3\)S)\(_3\)-ABD-mcDM1, Z\(_{HER2}\)-(S\(_3\)G)\(_3\)-ABD-mcDM1, and Z\(_{HER2}\)-G\(_4\)S-ABD-mcDM1 (Fig 23) were constructed, and their properties were investigated including their \textit{in vivo} biodistribution.
The binding of the constructs to HER2, HSA, and MSA was analyzed with a real-time biosensor (Biacore instrument) to investigate if the composition of the linker affected their binding capacity (Table 3). The dissociation rates were similar for the three drug conjugates. The affinity to HER2 of $Z_{HER2}$-($G_3S$)$_3$-$ABD$-$mcDM1$ and $Z_{HER2}$-($S_3G$)$_3$-$ABD$-$mcDM1$ were weaker than $Z_{HER2}$-$G_4S$-$ABD$-$mcDM1$ due to a slower association rate. The weaker affinity of the constructs with long linkers may be the result of inter-domain interaction that induces steric hindrance and affect their ability to bind to HER2. However, it is noteworthy that despite the observed changes in linker length and composition, both $Z_{HER2}$-($G_3S$)$_3$-$ABD$-$mcDM1$ and $Z_{HER2}$-($S_3G$)$_3$-$ABD$-$mcDM1$ retained low nanomolar affinity to HER2, which may be sufficient for effective targeting of HER2-positive tumors. For $Z_{HER2}$-($G_3S$)$_3$-$ABD$-$mcDM1$ and $Z_{HER2}$-($S_3G$)$_3$-$ABD$-$mcDM1$, the affinity was weaker to both HSA and MSA compared with $Z_{HER2}$-$G_4S$-$ABD$-$mcDM1$ and the non-toxic control constructs due to slower on-rates.
Table 3. Affinity constants for AffiDCs.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Ligand</th>
<th>$K_a$ (M⁻¹·s⁻¹)</th>
<th>$K_d$ (s⁻¹)</th>
<th>$K_D$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Z_{HER2}$-$(G_3S)_3$-ABD-mcDM1</td>
<td>HER2</td>
<td>$9.7 \times 10^4$</td>
<td>$2.2 \times 10^{-4}$</td>
<td>$2.3 \times 10^{-9}$</td>
</tr>
<tr>
<td></td>
<td>HSA</td>
<td>$1.1 \times 10^5$</td>
<td>$1.6 \times 10^{-4}$</td>
<td>$1.4 \times 10^{-9}$</td>
</tr>
<tr>
<td></td>
<td>MSA</td>
<td>$4.5 \times 10^5$</td>
<td>$3.1 \times 10^{-3}$</td>
<td>$6.8 \times 10^{-9}$</td>
</tr>
<tr>
<td>$Z_{HER2}$-$(S_3G)_3$-ABD-mcDM1</td>
<td>HER2</td>
<td>$7.1 \times 10^4$</td>
<td>$2.5 \times 10^{-4}$</td>
<td>$3.5 \times 10^{-9}$</td>
</tr>
<tr>
<td></td>
<td>HSA</td>
<td>$8.5 \times 10^4$</td>
<td>$1.5 \times 10^{-4}$</td>
<td>$1.8 \times 10^{-9}$</td>
</tr>
<tr>
<td></td>
<td>MSA</td>
<td>$3.1 \times 10^5$</td>
<td>$3.3 \times 10^{-3}$</td>
<td>$1.1 \times 10^{-8}$</td>
</tr>
<tr>
<td>$Z_{HER2}$-$(G_4S)_3$-ABD-AA</td>
<td>HER2</td>
<td>$1.2 \times 10^6$</td>
<td>$2.6 \times 10^{-4}$</td>
<td>$2.2 \times 10^{-10}$</td>
</tr>
<tr>
<td></td>
<td>HSA</td>
<td>$7.9 \times 10^5$</td>
<td>$1.9 \times 10^{-4}$</td>
<td>$2.5 \times 10^{-10}$</td>
</tr>
<tr>
<td></td>
<td>MSA</td>
<td>$6.3 \times 10^6$</td>
<td>$4.5 \times 10^{-3}$</td>
<td>$7.1 \times 10^{-10}$</td>
</tr>
<tr>
<td>$Z_{HER2}$-$(S_3G)_3$-ABD-AA</td>
<td>HER2</td>
<td>$8.8 \times 10^5$</td>
<td>$6.6 \times 10^{-4}$</td>
<td>$7.5 \times 10^{-10}$</td>
</tr>
<tr>
<td></td>
<td>HSA</td>
<td>$9.8 \times 10^5$</td>
<td>$1.9 \times 10^{-4}$</td>
<td>$1.9 \times 10^{-10}$</td>
</tr>
<tr>
<td></td>
<td>MSA</td>
<td>$4.5 \times 10^6$</td>
<td>$3.3 \times 10^{-3}$</td>
<td>$7.4 \times 10^{-10}$</td>
</tr>
<tr>
<td>$Z_{HER2}$-G₄S-ABD-mcDM1</td>
<td>HER2</td>
<td>$7.5 \times 10^5$</td>
<td>$1.9 \times 10^{-4}$</td>
<td>$2.5 \times 10^{-10}$</td>
</tr>
<tr>
<td></td>
<td>HSA</td>
<td>$8.0 \times 10^5$</td>
<td>$2.1 \times 10^{-4}$</td>
<td>$2.6 \times 10^{-10}$</td>
</tr>
<tr>
<td></td>
<td>MSA</td>
<td>$3.8 \times 10^6$</td>
<td>$2.8 \times 10^{-3}$</td>
<td>$7.4 \times 10^{-10}$</td>
</tr>
</tbody>
</table>

Several cell lines with different HER2 expression levels were cultured with the conjugates to analyze their cytotoxicity (Fig 24). For HER2 high-expressing cell lines, including SKOV3, SKBR3, and AU565, the three conjugates showed a dose-dependent cytotoxic effect. The IC₅₀ values for the AU565 cell line were similar for all drug conjugates, and ranged from 1.2 to 2.4 nM. For the SKBR3 cells, the cytotoxic effect of the AffiDCs with the $(G_3S)_3$ and $(S_3G)_3$ linkers were slightly weaker than for the construct with the $G_4S$ linker, which correlated with their difference in affinity to HER2. In addition, for the SKOV3 cells dose-dependent curves were observed for the three drug conjugates, but they had a flatter shape. For SKOV3, the IC₅₀ values for $Z_{HER2}$-$(G_3S)_3$-ABD-mcDM1 was 48 nM, whereas for $Z_{HER2}$-$(S_3G)_3$-ABD-mcDM1 and $Z_{HER2}$-G₄S-ABD-mcDM1 they were 150 nM and 180 nM, respectively. The weaker cytotoxic effect on SKOV3 compared to AU565 and SKBR3 shows that it is more resistant to DM1 poisoning, a phenomenon that has also been observed in an earlier study [189]. SKOV3 can be regarded as the most difficult model of HER2 expressing cancer cell lines among the cell lines studied in this study.
**Figure 24.** The cytotoxicity of the affibody-drug conjugates. The Y-axis of the graph displays the relative viability of different cell lines, while the X-axis shows the concentration of the proteins. Each data was derived from four replicate experiments, and the average is displayed with error bars that correspond to 1 SD.

Further *in vitro* and *in vivo* experiments were carried out on constructs that were labeled with 99m-technetium. The binding specificity and rate of internalization showed no significant differences among the three antibody-drug conjugates (Fig 25A and B). A LigandTracer instrument was used to investigate the binding kinetics of the radiolabeled AffiDCs to HER2-expressing cells, and the data were used to plot an interaction map (Fig 25C). In the interaction map, two processes were detected for all three conjugates, suggesting two binding interactions with different affinities. The conjugates with (G₃S)₃ and (S₃G)₃ linkers exhibited slightly lower affinities compared to the conjugate with the G₄S linker. This result was consistent with the differences in affinities to HER2 found in the SPR analysis.
Figure 25. Cell binding specificity, rate of internalization, and interaction map of radiolabeled AffiDCs. (A) The HER2-expressing cell lines SKOV3, BT474, and SKBR3 were incubated with radiolabeled affibody-drug conjugates. HER2 receptors on the cells were blocked or not blocked by the same, non-radiolabeled, conjugates. The star sign (*) corresponds to significant differences (p < 0.05). (B) Cellular processing of the constructs in SKOV3 and BT474 cells. The data were normalized and 100% was set as the value obtained at 24 hours. The values are presented as the average of three samples ± 1 SD. (C) Interaction map of the interactions between the radiolabeled constructs and SKOV3 cells. The data are from duplicate experiments.

Next, the properties of the affibody drug conjugates were studied in mice. All three conjugates showed prolonged blood retention, with no significant differences at 4 and 24 hours. At 48 hours, the conjugates with the (G3S)3 and (S3G)3 linkers had a significantly increased blood retention compared to the construct with the G4S linker. The calculated plasma half-life ($T_{1/2}$) is displayed in Fig 26A. The (S3G)3-linked conjugate showed a slightly shorter half-life ($T_{1/2} = 9.2$ h) compared to the (G3S)3-linked conjugate ($T_{1/2} = 10.3$ h) and the G4S-linked conjugate ($T_{1/2} = 10.7$ h). The hepatic uptake was investigated and is plotted as the activity in the liver as a function of time (Fig 26B). The AUC was found to be noticeably smaller for the construct with the (G3S)3 linker (1.27-fold) and the construct with the (S3G)3 linker (1.25-fold) compared to the construct with the G4S linker. At 4 h, the conjugate with the (G3S)3
linker had the lowest liver uptake among the three conjugates. At 24 h and 48 h, there was no significant difference in liver activity for the three conjugates.

The aim of targeted therapy is to enhance the anti-tumor effect while reducing systemic toxicity. It can be achieved through molecular design to minimize the uptake of normal organs. In this study, a direct comparison of conjugates with different inter-domain linkers showed that the use of \((G_3S)_3\) and \((S_3G)_3\) linkers resulted in a significant reduction in liver uptake compared to the \(G_4S\) linker.

\[ \text{Figure 26. (A) Blood kinetics and (B) liver uptake. AUC refers to the area under curve. Each data is the average of four animals.} \]

In conclusion, the target binding and the biodistribution properties of the mcDM1-conjugated and ABD-fused affibody molecules are affected by the length and composition of the inter-domain linker. Specifically, the constructs with linkers with 12 amino acid residues showed lower affinity to HER2 and a weaker cytotoxic effect \textit{in vitro} compared to the construct with a 5 amino acid linker. However, the longer linker leads to lower hepatic uptake by approximately 1.2-fold compared to the \(G_4S\) linker, which may reduce potential hepatotoxicity.
4.3. Paper III – Comparison of HER2-Targeted Affibody Conjugates Loaded with Auristatin- and Maytansine-Derived Drugs

We have recently investigated a conjugate comprising an affibody molecule that targets HER2, fused with an albumin-binding domain (ABD) for extended half-life, and loaded with the cytotoxic maytansine derivative DM1. In this study, we compared AffiDCs loaded with different drugs. The original DM1-loaded AffiDC was compared with AffiDCs loaded with the auristatin MMAE and MMAF (Fig 27). The aim was to investigate the impact of the cytotoxic payloads on binding affinity, in vitro cytotoxicity, in vivo biodistribution, and anti-tumor effect.

Two major classes of cytotoxic payloads used in drug conjugates are the maytansinoids and the auristatins, both of which act by inhibiting tubulin polymerization. The maytansine derivate DM1 has been conjugated to the anti-HER2 monoclonal antibody trastuzumab creating an ADC known as trastuzumab emtansine. It is approved by the FDA for clinical use in the treatment of breast cancer [197]. MMAE, a derivative of dolastatin 10, is a type of payload that has been utilized in a variety of ADCs. For example, brentuximab vedotin, consists of an anti-CD30 mAb conjugated to MMAE. It has been approved for the treatment of Hodgkin lymphoma and systemic anaplastic large-cell lymphoma [198]. Monomethyl auristatin F (MMAF) is another derivative of dolastatin 10 that contains one extra charged phenylalanine compared to MMAE [141]. Belantamab mafodotin is an example of an ADC that targets CD38 and is conjugated with MMAF. This ADC was approved by the FDA in 2020 for first-line treatment of multiple myeloma [199].

**Figure 27.** Chemical structure of the cytotoxic payloads and schematic representation of the affibody-drug conjugates.
Affibody-drug conjugates loaded with different cytotoxic payloads were generated and analyzed by SDS-PAGE (Fig 28A). The results showed high purity and the expected molecular weight. The drug conjugates were further analyzed by size-exclusion chromatography under native conditions. The results indicated that the conjugates were present as monomers and no degradation products were observed (Fig 28B). Moreover, RP-HPLC was used to analyze all drug conjugates, and the results showed that they were eluted as single peaks showing high purity (Fig 28C). The affibody-drug conjugates loaded with MMAE or MMAF were eluted slightly later compared to the drug conjugate loaded with DM1, suggesting that these conjugates are more hydrophobic.

Figure 28. Characterization of the AffiDCs. (A) SDS-PAGE analysis of the conjugates, lane M1 and M2 are marker proteins with molecular weights indicated to the left and right, respectively. (B) Size-exclusion chromatography analysis. (C) RP-HPLC analysis.

Further characterization of the affibody-drug conjugates involved determining their affinities to the targets, HER2, HSA, and MSA using a biosensor (Table 4). All three drug conjugates exhibited strong affinities to HER2, with equilibrium dissociation constants (K_D) ranging from 0.33 to 0.46 nM. The binding affinities for HSA ranged from 0.088 to 0.24 nM. The affinities to MSA were weaker than the affinities to HSA for all affibody-drug conjugates.
Table 4. Equilibrium dissociation constants (K_D) of the AffiDCs. Each value is the average of three replicate measurements ± 1 SD.

<table>
<thead>
<tr>
<th>Drug conjugate</th>
<th>HER2 (nM)</th>
<th>HSA (nM)</th>
<th>MSA (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZHER2-ABD-mcMMAE</td>
<td>0.38 ± 0.01</td>
<td>0.088 ±0.01</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>ZHER2-ABD-mcMMAF</td>
<td>0.46 ± 0.01</td>
<td>0.24 ± 0.14</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>ZHER2-ABD-mcDM1</td>
<td>0.33 ± 0.01</td>
<td>0.091 ± 0.005</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>ZTag-ABD-mcMMAF</td>
<td>NB*</td>
<td>1.92±0.02</td>
<td>5.85± 0.05</td>
</tr>
</tbody>
</table>

*NB: No binding was detected.

To compare the potency of the affibody-drug conjugates loaded with the auristatin derivatives or DM1, cell lines with different HER2 expression levels were incubated with different concentrations of the drug conjugates, and the cell viability was measured (Fig 29, Table 5).

The BT474 cell line, which has high HER2 expression, was found to be the most resistant to the cytotoxic effects of the drug conjugates. Notably, only ZHER2-ABD-mcMMAF showed a cytotoxic effect with an IC_{50} value of 0.01 nM and a cell survival rate of 40% to 50%. At present, the resistance mechanism of the BT474 cells to tubulin polymerization inhibitor is unknown. However, it was evident that the cell line exhibited greater sensitivity to MMAF compared to the other two drugs. ZHER2-ABD-mcMMAE and ZHER2-ABD-mcDM1 did not have any effect on the BT474 cell line. Significant differences were observed between the three drug conjugates in SKOV3 cells. ZHER2-ABD-mcMMAF was the most potent construct with an IC_{50} value of 12 nM, whereas ZHER2-ABD-mcDM1 was 40-fold less potent than ZHER2-ABD-mcMMAF. Furthermore, no effect of ZHER2-ABD-mcMMAE on SKOV3 cells was observed. For SKBR3 cells, the ZHER2-ABD-mcMMAF drug conjugate was the most potent. For AU565 cells, ZHER2-ABD-mcMMAF was also found to be the most potent construct, with an IC_{50} value 10 times lower than ZHER2-ABD-mcDM1 and 100 times lower than ZHER2-ABD-mcMMAE.
The cytotoxicity of the affibody-drug conjugates was investigated in HER2-expressing cell lines. The X-axis corresponds to the concentrations of the affibody-drug conjugates, and the Y-axis depicts the relative cell viability. Each data point is the mean of four measurements with error bars corresponding to 1 SD.

The IC\textsubscript{50} values of the three affibody-drug conjugates.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Z\textsubscript{HER2-ABD-mcDM1} (nM)</th>
<th>Z\textsubscript{HER2-ABD-mcMMAE} (nM)</th>
<th>Z\textsubscript{HER2-ABD-mcMMAF} (nM)</th>
<th>Z\textsubscript{Taq-ABD-mcMMAF} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKOV3</td>
<td>412</td>
<td>ND</td>
<td>12</td>
<td>190</td>
</tr>
<tr>
<td>SKBR3</td>
<td>1.4</td>
<td>8.2</td>
<td>0.2</td>
<td>170</td>
</tr>
<tr>
<td>AU565</td>
<td>2.5</td>
<td>24</td>
<td>0.2</td>
<td>180</td>
</tr>
<tr>
<td>BT474</td>
<td>ND</td>
<td>ND</td>
<td>0.01</td>
<td>110</td>
</tr>
<tr>
<td>A549</td>
<td>ND</td>
<td>ND</td>
<td>215</td>
<td>ND</td>
</tr>
</tbody>
</table>

Next, the drug conjugates were labeled with 99m-technetium for further analysis. The SKOV3 and BT474 cell lines were incubated with radiolabeled drug conjugates to investigate their rates of internalization. The rates of association were measured over a 24-hour period (Fig 30). The three affibody-drug conjugates rapidly associated with SKOV3 cells, reached a plateau within 4-6 hours, and were effectively internalized. The association rates with BT474 cells were slower, and the rate of internalization was slower resulting in lower internalized fraction after 24 hours.
Figure 30. Determination of the association rates and internalization of the AffiDCs in SKOV3 and BT474 cells. (A) Z\textsubscript{HER2}-ABD-mcMMAE. (B) Z\textsubscript{HER2}-ABD-mcMMAF. (C) Z\textsubscript{HER2}-ABD-mcDM1. Each data point represents the average of three replicate experiments, and the error bars correspond to 1 SD.

To investigate the tumor targeting ability and compare the uptake of the three radiolabeled drug conjugates, female BALB/c nu/nu mice bearing SKOV3 xenografts were intravenously injected. The radioactivity uptake in different organs, tissues, and tumors was quantified at 4, 24, and 48 hours (Fig 31). At 4 hours post-injection, the blood retention of Z\textsubscript{HER2}-ABD-mcMMAE was significantly higher than Z\textsubscript{HER2}-ABD-mcMMAF and Z\textsubscript{HER2}-ABD-mcDM1. The liver uptake of Z\textsubscript{HER2}-ABD-mcMMAF was significantly lower at 4 hours that the other two. The AUC for liver uptake of Z\textsubscript{HER2}-ABD-mcMMAE and Z\textsubscript{HER2}-ABD-mcDM1 were similar, whereas Z\textsubscript{HER2}-ABD-mcMMAF exhibited a 1.1-fold lower uptake. In contrast, the tumor uptake increased over time for all affibody-drug conjugates, except for Z\textsubscript{HER2}-ABD-mcMMAE which
showed a tendency to decrease from 24 to 48 hours. It was observed that both $Z_{HER2}$-ABD-mcMMAF and $Z_{HER2}$-ABD-mcDM1 exhibited similar uptake in the tumors at all measured time points. At 4 h post-injection, $Z_{HER2}$-ABD-mcMMAE had a significantly higher tumor uptake compared to the other two conjugates.

**Figure 31.** (A) Blood kinetics. (B) Liver uptake. (C) Tumor uptake of the radiolabeled AffiDCs at 4, 24, and 48 hours. Each data point represents the average of the measured radioactivity from four animals, and the error bars indicate 1 SD.

The anti-tumor activity of $Z_{HER2}$-ABD-mcMMAF in female BALB/c nu/nu mice bearing SKOV3 xenografts was compared to $Z_{HER2}$-ABD-mcDM1, which has earlier shown remarkable anti-tumor efficacy in our previous studies [185]. A lower dose was administered in this experiment compared to the previous studies to be able to observe differences between the two drug conjugates. It was therefore expected that a weak effect on the tumors should be observed for animals receiving $Z_{HER2}$-ABD-mcDM1. The survival curves and the therapeutic effects are displayed in Fig 32. The mice in the $Z_{Taq}$-ABD-mcMMAF and PBS groups exhibited exponential tumor growth. The median survival had not been reached in the $Z_{HER2}$-ABD-mcMMAF group by the end of the study (90 days), while it was 56 days for the group receiving $Z_{HER2}$-ABD-mcDM1 (Fig 32A). In the $Z_{HER2}$-ABD-mcMMAF group, 50% of the mice achieved complete remission without any detectable tumor at the end of the study. Another 20% of the mice-maintained remission but had visible tumors, while the remaining 30% exhibited a partial response. Specifically, 10% of the mice showed delayed tumor growth, and
Present Investigation

20 % experienced tumor ulceration without further growth. Among the $Z_{HER2}$-ABD-mcDM1 group, 20 % of the mice achieved complete remission, while 30 % experienced a partial response. However, 50 % of the mice did not respond to the treatment at all (Fig 32B). There was no notable difference in animal weight between the groups, which suggests that the treatment was well-tolerated (Fig 32C).

**Figure 32.** In vivo therapy study. (A) Survival of mice bearing SKOV3 xenografts treated with 2.9 mg/kg of the affibody-drug conjugates loaded with MMAF or DM1, non-targeting control $Z_{Taq}$-ABD-mcMMAF, or PBS. (B) Therapy outcome of the different treatment groups (n=8-10). The effects were classified as follows: no response, which referred to exponential tumor growth; partial response, which referred to delayed tumor growth or tumor ulceration without growth; sustained remission, which involved the presence of macroscopic tumors by the end of the study; and complete remission, which indicated the absence of tumors by the end of the study. A chi-square test was utilized to determine the difference between the groups, and the significance levels are denoted by asterisks. Specifically, * represents $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.0001$. (C) Mean animal weights of the different groups during the treatment. Each data point is the average of 8-10 mice ± 1 SD.

In conclusion, this study compared the properties of affibody-drug conjugates loaded with the different payloads MMAF, MMAE, and DM1. $Z_{HER2}$-ABD-mcMMAF exhibited the most potent cytotoxic effect on HER2-positive cell lines. In the therapy study, the MMAF-containing conjugate achieved complete tumor regression in 50% of
mice bearing SKOV3 tumors at a dose of 2.9 mg/kg. In contrast, ZHER2-ABD-mcDM1 only provided a moderate anti-tumor effect at this relatively low dose. Therefore, the combination of potent in vitro cytotoxicity, minimal in vivo uptake in normal tissues, and effective tumor delivery resulted in a superior anti-tumor effect of ZHER2-ABD-mcMMAF. Moreover, the therapy was well tolerated, and no adverse effects were observed.
4.4. Paper IV – A Comparison of \textit{in vivo} Half-life Extension of Affibody-Drug Conjugates by PASylation, XTENylation and Albumin Binding

Affibody molecules are small engineered alternative scaffold proteins with a three-helix fold with a molecular weight of 6.5 kDa. The small size of affibody molecules may enable rapid penetration of solid tumors and may also lead to long tumor retention when strong affinity binders are used \cite{200}. Nonetheless, due to their small size which is less than the kidney filtration cut-off size, affibody molecules are eliminated quickly through renal filtration and thus have a short plasma half-life in circulation.

In this study, we investigated \textit{in vivo} half-life extension strategies for HER2-specific affibody-drug conjugates (AffiDCs). The properties of the AffiDCs were investigated in terms of binding affinity, \textit{in vivo} cytotoxicity, and tissue biodistribution. In a previous study, our group found that the affibody-drug conjugate $Z_{\text{HER2}}$-ABD-mcDM1, where the ABD was used for half-life extension, has a significant anti-tumor effect and led to increased survival in mice with implanted tumors \cite{201}. Here, we started with the same drug conjugate and generated versions where we removed the ABD and instead added unstructured polypeptide chains with different lengths. The polypeptides were PAS300 (300 amino acids), PAS600 (600 amino acids), XTEN288 (288 amino acids), or XTEN576 (576 amino acids). These unstructured polypeptides increased the size and can be used to prolong the half-life in circulation. They may lead to better tumor uptake.

The affibody constructs are represented in Figures 33A and B. A DNA sequence encoding the amino acids MHEHEHE was added to the N-terminus of the genes encoding $Z_{\text{HER2:2891}}$-Cys-XTEN/PAS and $Z_{\text{HER2:2891}}$-ABD-Glu$_3$Cys. The cysteines were used to conjugate the cytotoxic drug mcDM1.
Figure 33. Design of Z\textsubscript{HER2}-mcDM1-XTEN288, Z\textsubscript{HER2}-mcDM1-XTEN576, Z\textsubscript{HER2}-mcDM1-PAS300, Z\textsubscript{HER2}-mcDM1-PAS600, and Z\textsubscript{HER2}-ABD-mcDM1. (A) Schematic representation of the affibody-PAS and the affibody-XTEN constructs. (B) Schematic representation of the affibody-ABD construct.

The fusion proteins were produced in \textit{E}.\textit{coli}, and were purified, followed by DM1 conjugation to the cysteine in each construct. An SDS-PAGE analysis showed high purity of all five conjugates (Figure 34A). It is interesting to note that the PASylated and XTENylated drug conjugates migrate more slowly than what would be expected based on their molecular weight. The calculated molecular weights of Z\textsubscript{HER2}-mcDM1-XTEN288 and Z\textsubscript{HER2}-mcDM1-PAS300 are 35 kDa and 33.7 kDa, respectively, but they migrated as they had a molecular weight of 60 kDa and 130 kDa. A possible contribution to this behavior is that a protein fused with a hydrophilic polypeptide chain moves towards the anode with slower mobility, because it has decreased binding to SDS. Since PAS polypeptides are uncharged, and XTEN polypeptides are negatively charged, the PASylated protein migrated more slowly than the XTENylated protein \[^{202}\]. The approximate sizes of fusion proteins were determined by analytic SEC (Figure 34C), and the results showed a slightly larger size of the constructs with XTEN576 and PAS600 proteins compared to the constructs with XTEN288 and PAS300. The RP-
HPLC results showed that the drug conjugates were pure, and PAS/XTENylated conjugates were more hydrophilic than the conjugate with ABD (Figure 34B).

**Figure 34.** Characterization of HER2-targeting affibody fusion proteins. (A) SDS-PAGE analysis of purified drug conjugates under reducing conditions. The molecular weights of marker proteins are indicated to the left of the gel. (B) RP-HPLC analysis from 20% to 60% acetonitrile in water with 0.1% TFA during 40 min. (C) Analysis by SEC under native conditions. The numbers above the panel indicate the molecular weights of standard proteins in kDa.

The binding kinetics of the affibody drug conjugates to HER2 were determined by SPR on a Biacore T200 (Figure 35). The association rates ($k_a$) of the PASylated conjugates were similar compared with $Z_{\text{HER2}}$-ABD-mcDM1 and ranged from $2.6 \times 10^5$ to $3.7 \times 10^5$ 1/Ms. However, the XTENylated conjugates have a slower association rate ($6.1 \times 10^4$ 1/Ms and $9.1 \times 10^4$ 1/Ms, respectively). The dissociation rates ($k_d$) of the five conjugates, were found to be similar. The equilibrium dissociation constants ($K_D$) for the PASylated conjugates were similar to the $K_D$ value of the conjugate with ABD, and stronger than the $K_D$ values for the XTENylated conjugates.
Figure 35. Biosensor analysis. Dilution series of the five constructs were injected over a chip with immobilized HER2. All concentration series were injected in duplicates and each panel is an overlay of both series for each conjugate. The concentrations are indicated to the right.

Next, the cytotoxic effect on cancer cell lines was investigated. On HER2-positive cells, all conjugates showed a dose-dependent cytotoxic effect (Figure 36). For the SKOV3 cell line, the IC<sub>50</sub> values were very similar among all conjugates, ranging from 26 to 43 nM. Z<sub>HER2</sub>-ABD-mcDM1 and Z<sub>HER2</sub>-mcDM1-PAS600 showed a slightly better cytotoxic effect on the AU565 cell compared to the other three. For the SKBR3 cell line, the IC<sub>50</sub> value of Z<sub>HER2</sub>-ABD-mcDM1 was 0.7 nM, which was lower than for the other four conjugates (ranging from 4.1 to 9.6 nM). It was interesting to note that the curves of the XTEN/PAS-conjugates had a different appearance, with a shallower slope. In conclusion, the results indicated that there is no large difference in cytotoxic effect between the five conjugates. No cytotoxic effect was observed on the HER2-negative MCF7 cell line in our study. In the A549 cell line, with low HER2 expression, only the highest concentration of Z<sub>HER2</sub>-mcDM1-PAS600 showed a cytotoxic effect.
Figure 36. Cytotoxicity of the affibody-drug conjugates. Cells were incubated with dilution series of the different constructs for 72 hours before the determination of cell viability. Each experiment was performed in quadruplicate, and the mean is displayed with error bars corresponding to 1 SD.

To be able to study the conjugates in vivo they were radiolabeled with 99m-techtentium. The five $^{99m}\text{Tc}$-labeled affibody-drug conjugates were injected intravenously in CD-1 mice and the activity in organs and tissues was measured at 4, 24, and 48 hours after injection to determine the biodistribution (Figures 37).

All conjugates had prolonged retention in the blood (Figure 37A). $^{99m}\text{Tc}$ZHER2-mcDM1-PAS300 and $^{99m}\text{Tc}$ZHER2-mcDM1-XTEN288 had the shortest half-life (7.3 and 7.6 hours, respectively), followed by $^{99m}\text{Tc}$ZHER2-mcDM1-XTEN576 (8.7 hours) and $^{99m}\text{Tc}$ZHER2-ABD-mcDM1 (9.0 hours). The longest half-life of 11.6 hours was observed for $^{99m}\text{Tc}$ZHER2-mcDM1-PAS600.

For all conjugates, the uptake in the liver was relatively low at all studied time points and the area under the curve (AUC) values for the XTENylated and PASylated conjugates was lower or similar to the AUC value for $^{99m}\text{Tc}$ZHER2-ABD-mcDM1 (Figure 37B).

The kidneys had the highest uptake of activity for all conjugates, which indicated predominant renal excretion. At 4 and 24 h p.i. the conjugates with the larger molecular weight, $^{99m}\text{Tc}$ZHER2-mcDM1-XTEN576 and $^{99m}\text{Tc}$ZHER2-mcDM1-PAS600, had lower uptake in the kidneys compared to $^{99m}\text{Tc}$ZHER2-mcDM1-XTEN288, $^{99m}\text{Tc}$ZHER2-mcDM1-PAS300 and $^{99m}\text{Tc}$ZHER2-ABD-mcDM1, resulting in an
approximately two-fold smaller AUC value (Figure 37C). The AUC values for \([^{99m}Tc\]Tc-ZHER\(_2\)-mcDM1-XTEN288\) and \([^{99m}Tc\]Tc-ZHER\(_2\)-mcDM1-PAS300\) were comparable with the AUC value for \([^{99m}Tc\]Tc-ZHER\(_2\)-ABD-mcDM1\).

**Figure 37.** Comparison of (A) blood kinetics, (B) liver uptake, and (C) kidney uptake of \([^{99m}Tc\]Tc-labeled affibody-drug conjugates at 4, 24, and 48 h post-injection (p.i.) in CD-1 mice (n = 4). The uptake is shown as %ID/g ± 1 SD. The half-life in blood was calculated using a one-phase decay function in GraphPad Prism version 9.4.1. AUC refers to the area under the curve and is given in parenthesis after the names of the conjugates in panels B and C.

To study the specificity of tumor targeting, the tumor uptake of \([^{99m}Tc\]Tc-labeled affibody-drug conjugates in BALB/c nu/nu mice bearing HER2-expressing SKOV3 xenografts was compared to the tumor uptake in mice bearing HER2-negative Ramos xenografts. At 48 h after injection, the uptake of the XTENylated and PASylated conjugates in the SKOV3 xenografts was significantly (\(p < 0.001\)) higher than in the Ramos xenografts (Figure 38A).

The comparison of uptake in SKOV3 xenografts among the five conjugates 48 h p.i. showed that \([^{99m}Tc\]Tc-ZHER\(_2\)-ABD-mcDM1\) provided a significantly (\(p < 0.001\)) higher tumor uptake (8.8 ± 1.2 %ID/g) than the other conjugates (Figure 38B). The uptake of \([^{99m}Tc\]Tc-ZHER\(_2\)-mcDM1-PAS600\) was significantly (\(p < 0.05\)) higher than that of \([^{99m}Tc\]Tc-ZHER\(_2\)-mcDM1-PAS300\) (5.6 ± 1.1 %ID/g vs. 3.3 ± 0.3 %ID/g). There were no significant (\(p > 0.05\)) differences between the uptake of \([^{99m}Tc\]Tc-ZHER\(_2\)-mcDM1-XTEN288, \([^{99m}Tc\]Tc-ZHER\(_2\)-mcDM1-XTEN576\) and \([^{99m}Tc\]Tc-ZHER\(_2\)-mcDM1-PAS300\) in the SKOV3 xenografts.
Figure 38. Tumor targeting of $[^{99m}\text{Tc}]$Tc-labeled affibody-drug conjugates at 48 h p.i. in BALB/c nu/nu mice bearing HER2-expressing SKOV3 xenografts (n = 4). (A) Targeting specificity comparison with HER2-negative Ramos xenografts (n = 4). (B) Side-by-side uptake comparison of the five conjugates. Tumor uptake is shown as %ID/g ± 1 SD. Asterisks correspond to significant differences, where * corresponds to $p < 0.05$, *** corresponds to $p < 0.001$, and **** corresponds to $p < 0.0001$; ns – not significant.

Our findings describe the properties of affibody drug conjugates which have had their half-lives extended by different strategies. The results show how the ABD or PASylation or XTENylation influence the properties and biodistribution and further, provide significant insights into the optimization of affibody-derived drug conjugates. In conclusion, PASylation and XTENylation of an affibody-based drug conjugate appears to be less efficient strategies for tumor-targeted drug delivery compared to utilizing fusion with ABD. XTENylation and PASylation provided significantly lower values of tumor uptake with similar or higher accumulation in normal organs and tissues, resulting in decreased tumor-to-organ ratios.
4.5. Paper V – Pre-clinical Evaluation of Drug Conjugates Based on Affibody Molecules Targeting HER3-Expressing Tumors

The HER family comprises several receptor tyrosine kinases, namely HER1/EGFR, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4. After activation, these receptors may form homo- or heterodimers with other members of the HER family. Among these receptors, HER3 has presented a challenge as a therapeutic target. It is typically expressed at a relatively low level of up to 50,000 receptors per cell \[^{66}\], in contrast to HER2, which can reach up to 2 million receptors per cell \[^{203}\]. Clinical trials with HER3-targeting drugs have yielded disappointing results. As a result, there is a need for the development of approaches that can potentially enhance the efficacy of HER3-targeting drugs.

In a previous study, we have developed and investigated the properties of the affibody-drug conjugate \(Z_{\text{HER3}:08698}\)-ABD-mcDM1 \[^{68}\]. Similar to earlier studies in this thesis we used an albumin binding domain (ABD) for half-life extension. We coupled the potent cytotoxic drug DM1 via a non-cleavable maleimidocaproyl (mc) linker to the protein. In this study, we have conducted a comparative analysis of the cytotoxic effect of a monovalent and bivalent affibody-drug conjugate targeting HER3 on the HER3-expressing cell lines BxPC3 and DU145. We also assessed the therapeutic potential of the bivalent conjugate by treating mice, bearing tumors derived from the BxPC3 cell line. This experiment aimed to determine the effectiveness of the bivalent HER3-targeting affibody-drug conjugate \textit{in vivo} and its potential as a treatment strategy for HER3-overexpressing tumors.

The composition of three affibody constructs, \(Z_{\text{HER3}}\)-ABD-\(Z_{\text{HER3}}\)-mcDM1, \(Z_{\text{HER3}}\)-ABD-mcDM1, and \(Z_{\text{HER3}}\)-ABD-\(Z_{\text{HER3}}\)-AA, is shown in Figure 39A. The purity of the constructs was determined by SDS-PAGE (Fig 39B) and it migrated at the expected molecular weights. To further validate the purity, RP-HPLC was performed, and the results demonstrated a purity exceeding 95% for all three constructs (Fig 39C). From Figure 39C, it is evident that \(Z_{\text{HER3}}\)-ABD-\(Z_{\text{HER3}}\)-mcDM1 was eluted later compared to \(Z_{\text{HER3}}\)-ABD-\(Z_{\text{HER3}}\)-AA, indicating an increase in hydrophobicity resulting from DM1 conjugation. Moreover, \(Z_{\text{HER3}}\)-ABD-mcDM1 was eluted later than \(Z_{\text{HER3}}\)-ABD-\(Z_{\text{HER3}}\)-mcDM1, suggesting that the increase in hydrophobicity by DM1 was more pronounced for the smaller protein. As shown in Figure 39D, the mono/multimeric state of the constructs was investigated by SEC analysis. The chromatograms showed a single peak for all three constructs, of essentially the correct molecular weight, suggesting that they are in a monomeric state.
Figure 39. Schematic overview of the anti-HER3 affibody constructs. (A) The composition of the three affibody constructs, which contain one or two HER3 affibody domains and an ABD. (B) SDS-PAGE shows the molecular weight of the constructs. (C) RP-HPLC analysis. (D) Analytical SEC analysis of the constructs.

SPR binding analysis was used to determine the affinity between the constructs and the extracellular domains of HER3 and murine ErbB3. As shown in Figure 40, the affinities of the three affibody constructs were similar, with $K_D$ values ranging from 3 to 4 nM. However, due to a rapid dissociation rate, the affinity of $Z_{HER3}$-ABD-$Z_{HER3}$-mcDM1 and $Z_{HER3}$-ABD-mcDM1 to mErbB3 was 10 times lower than the affinity to HER3. Similarly, for $Z_{HER3}$-ABD-$Z_{HER3}$-AA, the affinity to mErbB3 was 4 times lower than its affinity to HER3.
Figure 40. Sensorgrams from SPR binding analysis. The three affibody constructs were immobilized and were used to capture HER3 and mErbB3, which were injected with two-fold dilution concentrations from 6 to 100 nM.

The properties of the constructs were further investigated *in vitro*, by determining their cytotoxic effect on the BxPC3, DU145, and SKOV3 (HER3-) cell lines (Fig 41). A dose-dependent cytotoxic effect of Z\_HER3-ABD-Z\_HER3-mcDM1 and Z\_HER3-ABD-mcDM1 was observed on the BxPC3 cell line. The IC\(_{50}\) value of Z\_HER3-ABD-Z\_HER3-mcDM1 was 3 nM, while Z\_HER3-ABD-mcDM1 exhibited an IC\(_{50}\) value of 14 nM. In the case of DU145 and SKOV3 cells, a cytotoxic effect was observed only at the highest concentration of 1500 nM for Z\_HER3-ABD-Z\_HER3-mcDM1 and Z\_HER3-ABD-mcDM1. Furthermore, there was no cytotoxic effect for the control Z\_HER3-ABD-Z\_HER3-AA on any of the three cell lines.
Figure 41. Cytotoxic effect of the anti-HER3 affibody constructs. The cell lines were cultured and treated with varying concentrations of the constructs. The cell viability is represented as a percentage of the viability of cells that were incubated without any construct, which was set to 100%. Each data point in the graphs represents the average of four independent experiments, and the error bars correspond to 1 SD.

From previous in vivo therapy study results, where $Z_{\text{HER3}}$-ABD-$Z_{\text{HER3}}$ without any conjugated drug was evaluated, we hypothesized that the cytostatic effect of $Z_{\text{HER3}}$-ABD-$Z_{\text{HER3}}$ could be enhanced by incorporating a cytotoxic DM1 molecule into the construct. The in vivo anti-tumor effect of $Z_{\text{HER3}}$-ABD-$Z_{\text{HER3}}$-mcDM1 was evaluated in mice bearing HER3-overexpressing BxPC3 xenografts with a combination treatment of the previously assessed construct $Z_{\text{HER3}}$-ABD-$Z_{\text{HER3}}$ and $Z_{\text{HER3}}$-ABD-$Z_{\text{HER3}}$-mcDM1. Three injections were given per week, of which two were $Z_{\text{HER3}}$-ABD-$Z_{\text{HER3}}$ and one was $Z_{\text{HER3}}$-ABD-$Z_{\text{HER3}}$-mcDM1. The control groups were treated with $Z_{\text{HER3}}$-ABD-$Z_{\text{HER3}}$ as monotherapy (three injections per week), or not treated (PBS). The average tumor volume growth curves of the three groups are shown in Figure 42A. The curves were drawn until 38% of the mice were euthanized. The combination treatment significantly decreased the tumor volume compared to both control groups. The combination group had a significantly longer median survival of 90 days than the monotherapy group of 68 days and the PBS group of 49 days (Fig 42B).
**Figure 42.** (A) Tumor growth is plotted as a function of time for BxPC3 xenografted mice. (B) Survival of the mice bearing BxPC3 xenografts.

In conclusion, despite the relatively low HER3 expression level on the cancer cells compared to HER2, it proved to be sufficient for induction of an anti-tumor effect *in vivo* in the combination treatment group, with significantly longer survival of the mice compared to the group receiving only $Z_{HER3}$-ABD-$Z_{HER3}$ or the vehicle control group (PBS).
4.6. Concluding Remarks and Future Perspectives

Cancer is characterized by the uncontrolled growth and spread of abnormal cells in the human body. Cancer is often a serious disease and can occur in different parts of the body and often leads to serious health complications and even death. Early diagnosis, a better understanding of the molecular mechanisms of the cancer cells, and improvements in the treatment have contributed to better patient outcomes and increased survival for many types of cancer. Conventional chemotherapy has been a cornerstone of cancer treatment for a long time. However, chemotherapeutic drugs target not only cancer cells but also normal cells, which may result in side effects. More recently, targeted therapy has emerged as a prominent and promising new generation of cancer treatment. Targeted therapy focuses on specific components of cancer cells, such as proteins or genes, which play crucial roles in the growth and spread of cancers. In my thesis, I focused on a small, engineered scaffold protein, affibody molecules, for targeted delivery of cytotoxic payloads to cancer cells.

Affibody molecules have a small size of 6.5 kDa, can often be generated with high affinity to their target, and usually have high stability. These features provide a reliable framework for the development of therapeutic and diagnostic applications. Affibody-drug conjugates (AffiDCs) are a class of targeted drug candidates that combine the specificity of affibody molecules with the cytotoxic effect of drug payloads.

In paper I, we investigated the influence of the drug-to-affibody ratio (DAR) on the pharmacokinetic profile of affibody-drug conjugates targeting HER2. We found that increasing the DAR from one to three resulted in a 1.45-fold increase in the delivery of cytotoxic mcDM1 to tumors. However, this increase was accompanied by increased non-specific uptake in the liver. The DAR value of affibody-drug conjugates is a critical parameter that influences the potency and efficacy of the AffiDCs as a targeted cancer therapy. A high DAR indicates that a large number of drug molecules are attached to each affibody, which can result in an increased drug payload delivered to the cancer cells. However, a high DAR value may lead to reduced stability and increased potential for non-specific tissue uptake. Therefore, further studies are necessary to investigate the molecular design of high-DAR AffiDCs to achieve efficient drug delivery to the tumors while minimizing the uptake in normal tissues. Achieving an optimal DAR is an important consideration in the development of AffiDCs to balance efficacy, stability, and safety.

The selection of the linker and payload in affibody-drug conjugates is also an essential step in designing an effective and safe targeted cancer therapy. In paper II and III, we
investigated the effect of the different inter-domain linkers and different cytotoxic payloads as part of affibody-drug conjugates targeting HER2. Paper II showed that the target binding and the biodistribution properties of the affibody-drug conjugates are affected by the length and composition of the inter-domain linker. The constructs with a 12 amino acid linker showed lower affinity to HER2 and a weaker cytotoxic effect \textit{in vitro} compared to the construct with a 5 amino acid linker. However, the longer linker leads to lower hepatic uptake which may reduce potential hepatotoxicity. These findings offer a valuable understanding of the molecular design in the development of targeted drug conjugate with decreased liver uptake. Paper III concluded that the developed affibody-drug conjugate $Z_{\text{HER2}}$-ABD-mcMMAF shows significant potential for HER2-targeted cancer therapy compared to the previously most efficient affibody-drug conjugate, $Z_{\text{HER2}}$-ABD-mcDM1. It also maintained a favorable toxicity profile with lower hepatic uptake.

Different strategies for half-life extension were described in paper IV. We generated a series of HER2-targeting affibody-drug conjugates fused with different lengths of PAS or XTEN polypeptides, and compared the constructs with the previously developed ABD-fused conjugate. It was found that the different strategies for half-life extension influence the properties and biodistribution of the drug conjugates. In conclusion, the ABD-fused affibody-drug conjugate appears to be more efficient than PASylated and XTENylated affibody-drug conjugates. It provides higher tumor uptake with similar normal tissue accumulation. Ongoing studies gave us significant insights into the optimization of targeted drug conjugates for further development and clinical applications.

In paper V, the binding properties and cytotoxic potential of monovalent and bivalent HER3-targeting affibody-drug conjugates were compared. We evaluated the biodistribution and therapeutic potential of the bivalent drug construct. The results suggested that the bivalent $Z_{\text{HER3}}$-ABD-$Z_{\text{HER3}}$-mcDM1 is a highly potent drug conjugate with a favorable biodistribution profile and anti-tumor efficacy compared to the monovalent conjugate. Despite that HER3 has a relatively low expression level in most cancer cells compared to HER2, it could be seen that the combination treatment including the bivalent $Z_{\text{HER3}}$-ABD-$Z_{\text{HER3}}$-mcDM1 and the non-toxic version, $Z_{\text{HER3}}$-ABD-$Z_{\text{HER3}}$, was sufficient for an anti-tumor effect and significantly prolonged survival of the tumor-bearing mice. These results suggest that $Z_{\text{HER3}}$-ABD-$Z_{\text{HER3}}$-mcDM1 holds promise for future clinical development as a potential therapeutic option.

So far, affibody-drug conjugates in targeted cancer therapy have shown significant promise for clinical development. Ongoing research is focused on developing and
optimizing drug conjugates for targeting other receptors for targeting other cancer types. The use of affibody molecules as targeting agents opens up new possibilities for personalized and precise cancer treatment. Consideration of the molecular design, drug payload selection, and half-life extension strategies is necessary for future clinical development.
Acknowledgements

During these four years at KTH, I received a lot of support and encouragement from many people, I am deeply grateful for the help from all of you!

First, I would like to thank all the foundations for financing my research projects and financing conference participation.

**Torbjörn**, my main supervisor, thank you for offering me the opportunity to be a Ph.D. student in your group. Your consistent support and guidance make you a perfect supervisor. Thank you for your endless patience on my projects, definitely my thesis, over the years. Whenever I have problems, you can always give me a lot of suggestions and discussions that help me solve the problems. I will always remember your insights, humor, and broccoli lunch!

**Per-Åke**, my co-supervisor and lab desk mate, thank you for sharing your thoughts and knowledge and giving me lots of suggestions on different projects, especially the CEA project. Your passion and seriousness for science have inspired me a lot. You can always ask interesting questions in all academic seminars. I am so surprised that you can remember all details of the experiments and give me very useful tips!

**PIs**: **Amelie**, thank you for being a committee member for my half-time seminar and giving me fantastic advice on my research projects, especially the dibromomaleimide project. **John**, thank you for being a committee member for my half-time seminar, and thanks for the inspiring suggestions. **Stefan**, you are a great leader. You always make Plan 3 well organized, for example, the safety round, cleaning day, and AlbaNova updates. **Johan N**, thank you for holding the seminar and giving me feedback on my presentations. You always give me impressive advice on my projects. **Johan R**, thanks for keeping introducing new biotechnology to plan 3. I am grateful for the experience of the literature seminar. **Sophia**, I am grateful for your research on the ADAPT project which gave me lots of background knowledge about scaffold proteins. **My**, your singing gave me a deep impression. **Cristina**, thanks for expanding biotechnology to broader interesting fields.

**Uppsala group**: **Anna**, **Vladimir**, **Anzhelika**, **Tianqi**, and **Sara**, thanks for your wonderful work and great collaboration! You have always been there to help and discuss with me. Your rigorous attitude, exceptional insights, and sense of
responsibility have left a lasting impression on me. It has been a pleasure working with you all.

Torbjörn group: For everyone from our group, thank you for the help and friendship! Haozhong, Wen, Shengze, and Hao, thank you for spending time to help me gain new skills and experience. You were always able to discuss with me and help me solve the problem when I had strange results in my experiments. Ruonan, Linda, and Javad thank you for the time we spent together in the lab and lunchroom. Thank you for always being there to chat and laugh!

HPA group: Hanna T, Lanlan, Malin W, and Gabriella, thanks for your help on the diabody project. You give me the best advice on HEK expression and purification. I am very appreciative of your suggestions.

Emma L, Maryam, thank you for taking care of the dish room and ordering packages. Your help has made the experiments much easier.

Kristina J, Christina, thanks for your administration of my Ph.D. study and comments on my eISP.

Lauren, thank you for taking the time to review my thesis and give me useful feedback.

Kristina W, thank you for the help with the CD and your view on the PNA project.

Hanna L, Magdalena, thanks for your feedback on the Monday seminar.

Jonas, I feel very lucky to have you as a colleague, and you are exceptionally kind to everyone. Whenever I have a problem, you are the first person that comes to my mind, and you always provide me with assistance. I am grateful to spend breaks talking about everything with you. Thank you so much for the great memories! Kim, you are a very warm person, and you always offer help to anyone facing challenges. I greatly admire your passion for experiments. I remember you wished me a Happy New Year in Chinese. Thanks for bringing all the laughs! Malin, Marit, and Emma, thanks for being responsible for the SPR, thank you so much for your help. I always enjoy chatting with you all during breaks and lunchtime. Moira, you use a notebook to record in every meeting, your passion for research is admirable. You’re always the first one to find out I cut my hair! Charles, thanks for the help on the HER3 project, you always give me the best advice for lab work. Anna, my neighbor, I am delighted that we were attending the PEGS in Spain. Thank you for the advice on the project and restaurant selection.
Ábel, Jacob, thanks for your experience on HPLC, and thanks for always helping me. I appreciate sharing lunchtime with you all. Aman, Andy, thank you for your wonderful defense, which inspired me a lot. Aman, thanks for your suggestions on my diabody project, and thanks for your SEC column. Siri, Cornelia, thank you for taking care of the cytoflex, and always giving me help with the selection. Luke, you give me a lot of help on the selection, thanks for your suggestions and also the protocol! Hannes, I can always hear your laughter in every corner of the lab, and I am grateful for the joy you bring us. Sanne, our refrigerator was leaking, and thank you for helping us face this unfortunate situation together on a Monday. Danilo, I am very glad to chat with you during the after-party. Welcome to visit China next time. Savinna, thank you for being responsible for the Tidaholm, you are so nice to everyone, and your passion for experiments has inspired me. Gustav, Max, I am so impressed by your songwriting and musical talent. Max, your plants and cats bring freshness to our office. Camilla, Charlotte, Mona W, and Num, it was a pleasure talking with you at lunchtime. I will remember this unforgettable memory.

Office members: Moira, Siri, Jonas, Max, Aman, and Anna-Luisa, thank you for the happy time we spent during the last four years. I am really enjoyable to be the officemate to all of you.

A big thanks to everyone at Plan 3, old and new friends, thank you for making the best place to work!

Last but not least, I would like to thank my family and friends. Qinda, thanks for your continuous support and love. Additionally, I want to thank my parents for their encouragement in my study. Qingyang, Qichen, Wanhong, Wanyu, Yang, Liuzhen, Cong, and Zhiwei, thanks for the unforgettable and happy time we spent together in Sweden.
References


(22) Schoeberl, B.; Faber, A. C.; Li, D.; Liang, M. C.; Crosby, K.; Onsum, M.; Burenkova, O.; Pace, E.; Walton, Z.; Nie, L.; et al. An ErbB3 antibody, MM-121, is active in cancers with ligand-dependent activation. *Cancer Res* 2010, 70 (6), 2485-2494.


(30) Fogli, S.; Del Re, M.; Rofi, E.; Posarelli, C.; Figus, M.; Danesi, R. Clinical pharmacology of intravitreal anti-VEGF drugs. *Eye (Lond)* **2018**, **32**(6), 1010-1020. DOI: [10.1038/s41433-018-0021-7](https://doi.org/10.1038/s41433-018-0021-7) From NLM.


(35) Cameron, F.; Whiteside, G.; Perry, C. Ipilimumab: first global approval. *Drugs* 2011, 71 (8), 1093-1104. DOI: 10.2165/11594010-000000000-00000 From NLM.


(47) Hassanzadeh-Ghassabeh, G.; Devoogdt, N.; De Pauw, P.; Vincke, C.; Muyldermans, S. Nanobodies and their potential applications. *Nanomedicine (Lond)* 2013, 8 (6), 1013-1026. DOI: 10.2217/nmm.13.86 From NLM.


5, 14. DOI: 10.1186/2046-1682-5-14  From NLM.
(90) Dozier, J. K.; Distefano, M. D. Site-Specific PEGylation of Therapeutic Proteins. International Journal of Molecular Sciences 2015, 16 (10), 25831-25864.
(98) Alters, S. E.; McLaughlin, B.; Spink, B.; Lachinyan, T.; Wang, C. W.; Podust, V;


(103) Roopenian, D. C.; Akilesh, S. FcRn: the neonatal Fc receptor comes of age. *Nat Rev Immunol* 2007, 7 (9), 715-725. DOI: 10.1038/nri2155 From NLM.

(104) Sleep, D.; Cameron, J.; Evans, L. R. Albumin as a versatile platform for drug half-life extension. *Biochimica et Biophysica Acta (BBA) - General Subjects* 2013, 1830 (12), 5526-5534. DOI: https://doi.org/10.1016/j.bbagen.2013.04.023.


(121) Lutt, J. R. Efficacy, safety, and tolerability of abatacept in the management of rheumatoid arthritis. *Open Access Rheumatol* 2009, 1, 17-35. DOI: 10.2147/oarrs.s4536 From NLM.


- 93 -


(163) Kowalczyk, L.; Bartsch, R.; Singer, C. F.; Farr, A. Adverse Events of
Trastuzumab Emtansine (T-DM1) in the Treatment of HER2-Positive Breast Cancer Patients. *Breast Care (Basel)* 2017, 12 (6), 401-408. DOI: 10.1159/000480492 From NLM.


(172) BESPONSA Prescribing Information. New York, N. P. I.


(174) ADC Therapeutics and Sobi Announce European Commission Approval of ZYNLONTA® (loncastuximab tesirine) for the Treatment of Relapsed or Refractory Diffuse Large B-cell Lymphoma, DECEMBER 21, 2022.

(175) Li, F.; Jiang, T.; Li, Q.; Ling, X. Camptothecin (CPT) and its derivatives are known to target topoisomerase I (Top1) as their mechanism of action: did we miss something in CPT analogue molecular targets for treating human disease such as cancer?
Am J Cancer Res 2017, 7 (12), 2350-2394. From NLM.


(184) Ma, Y.; Zhao, Y.; Fang, W.; Zhao, H.; Huang, Y.; Yang, Y.; Chen, L.; Hou, X.; Zou, W.; Ding, M.; et al. BL-B01D1, a first-in-class EGFRxHER3 bispecific antibody-drug conjugate (ADC), in patients with locally advanced or metastatic solid tumor: Results from a first-in-human phase 1 study. Journal of Clinical Oncology 2023, 41


