Chapter 1

General introduction
Antibody-based therapy has become one of the most important and successful therapeutic modalities to treat cancer. The first application was just 20 years ago, but the concepts are much older. Antibody-based therapy is actually one of the oldest treatments for cancer patients. Back in 1895, two French researchers described a serotherapy in which they treated patients with antisera derived from animals that were injected with cancer cells of the patient themselves\textsuperscript{1}. In some of the patients improvements were observed, however, severe immunological reactions occurred in most cases. The antisera they used consisted of a large variety of antibodies directed towards different antigens. Some of these antigens are expressed not only on malignant cells, but also on normal cells, which caused the immunological reactions. If a method could be developed to produce antibodies directed against one specific antigen, a possible treatment against cancer and many other diseases could be envisioned. This was the foundation of the ‘magic bullet’ hypothesis, proposed a decade later by Paul Ehrlich\textsuperscript{2}. His theory implied that if a compound could be made that selectively targets a disease, then a toxin for that organism could be delivered along with this selective agent.

**Antibody structure and function**

Natural human antibodies are large protein molecules produced by plasma cells, a specific type of white blood cells, and are used to identify and neutralize foreign organisms or molecular structures, called antigens. The structure of antibodies was discovered in the late 1960s\textsuperscript{3,4}. Antibodies consist of two identical heavy chains that are interconnected forming a Y-shape and two light chains that form extra legs to the top of the Y (Figure 1). Heavy and light chains both have a constant and a variable region. Heavy chains determine the class of the antibody, of which there are five types in mammals. The tail of the Y is indicated as the Fc part and the variable regions are the upper parts of the heavy and light chains that form the two upper legs (Fab-part) of the Y. The two light chains are identical and also consist

![Figure 1 - Schematic representation of a mAb.](image-url)
of a bottom constant region and a top variable region. Antigen binding occurs at the variable part. When the antibody is bound to the antigen by the Fab (Fragment, antigen binding) region on the top, the tail, called Fc (Fragment, crystallization) region makes it possible to initiate the further immune response that will remove the antigen from the system.

**Development of monoclonal antibodies**

From the early 1900s on, many researchers tried to find a method to develop antibodies directed against one specific antigen, i.e. monoclonal antibodies (mAbs). In 1951, Henri Kunkel investigated cells from multiple myeloma patients and discovered that the malignant plasma cells produce antibody molecules with one single specificity instead of several\(^5\). However, these cells produce antibodies at random, and researchers had no method to find out against which antigen the antibody was directed. In the late 1960s, researchers began to develop methods to isolate and propagate B-cells that only produce one specific antibody directed against one specific antigen\(^6\). The limitation of these methods was the restricted lifespan of the B-cells and the small amount of antibodies produced. In 1975, Köhler and Milstein succeeded in fusing a mouse B-cell, producing a specific and well-known antibody, with a mouse myeloma cell\(^7\). This method was called the ‘hybridoma technique’. By using this hybridoma technology they obtained an immortal cell line, which was capable of very high and pure production of one specific antibody.

This method to develop specific antibodies did not immediately lead to convincing clinical successes, since the antibodies were derived from mice. Application of murine mAbs met a few limitations: they are quickly removed from the blood, inflammatory reactions can occur, and human anti-mouse antibodies (HAMAs) can develop. When HAMAs are present, patients cannot receive multiple doses of the mAb, because of anaphylactic reactions that might occur\(^8\). By genetic engineering, chimeric antibodies were developed that are partially mouse and partially human. The DNA encoding the variable parts of the murine heavy and light chains are fused to the DNA encoding the constant regions of the human heavy and light chains, and these fusion constructs are brought into cultured cells for chimeric antibody expression. This engineering also allows the design of antibodies of a particular isotype such as IgG1 or IgG4. With this approach immune reactions directed against the Fc part of the mAb can be avoided, while the antigen binding part is not altered. As a next step it became possible to replace most of the murine heavy and light chains by the human heavy and light chains, with just the antigen binding parts of murine origin remaining. These mAbs are called “humanized”. Nowadays techniques are available to develop also fully human mAbs, and in some approaches the use of mice or other animals is no longer needed. These methods
also enable the engineering of a variety of antibody fragments, such as: diabodies, minibodies, affibodies and nanobodies. With the increase in mAb-development, rules for the nomenclature also have been set. All mAbs should have -mab as a suffix, and are preceded by the origin-determination: -o for mouse, -u for human, -xi for chimeric, -xizu for chimeric/humanized, and -zu for humanized.

**First mAbs and their mechanisms of action**

All of the current FDA-approved anti-cancer mAbs are immunoglobulins G (IgGs), with a variety of mechanisms of action, not always predictable and not always known for a new mAb. Often these mechanisms work in parallel, depending on the targeted molecule and the isotype of the mAb. Mechanisms of action include: (i) inhibition of cell signaling, (ii) induction of apoptosis, (iii) antibody-dependent cellular cytotoxicity (ADCC), and (iv) complement-dependent cytotoxicity (CDC). While the first two mechanisms are direct effects of the antibody, the next two are immune-mediated responses. In addition a mAb can serve as a carrier for a toxic agent. The delivery of a toxic agent is central part of this thesis and will be discussed later.

The different mechanisms of action are explained hereafter in more detail: (i) in the inhibition of cell signaling the antibody has an antagonistic effect by binding a membrane-bound receptor, often a growth factor receptor that shows and increased level of expression in tumor cells. This either prevents the growth factor from binding to its receptor or interferes with dimerization of the receptor. The result is a decreased signaling in the downstream pathway, leading to inhibition of unwanted cell proliferation and, ideally, cell death. The antibody could also target the growth factor itself, and as a result binding to the receptor cannot occur and cell growth is halted; (ii) antibodies that induce apoptosis have an agonistic effect, and binding to a receptor directly triggers apoptotic signaling in the tumor cells. The Fc-region of IgGs mediates immune-mediated responses: after binding to a target molecule, the Fc-regions engage effector cells of the immune system that subsequently kill the tumor cells; (iii) ADCC is induced by binding of the Fc-region of the mAb to the FceRIIIa (CD16) receptors on e.g. natural killer (NK) cells, after which NK cells are activated and release cytolytic granules that cause cell death; and (iv) CDC is induced when the Fc-region of the mAb binds to soluble complement factor C1q. This triggers the complement cascade that ultimately leads to cell death.

**Clinically approved mAbs**

The developments of the last years have led to 17 currently FDA- and European Medicines Agency (EMA)-approved mAbs (Table 1). Some of these are exploited as naked mAbs and some as antibody-conjugates.
<table>
<thead>
<tr>
<th>Name</th>
<th>Marketed by</th>
<th>Class</th>
<th>Target</th>
<th>First approved indication</th>
<th>Reported mechanisms of action</th>
<th>Approval year</th>
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<tbody>
<tr>
<td>Rituximab (Rituxan)</td>
<td>Biogen Idec./Genentech</td>
<td>Chimeric IgG1</td>
<td>CD20</td>
<td>Non-Hodgkin’s Lymphoma</td>
<td>ADCC, CDC, Induction of Apoptosis</td>
<td>1997</td>
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<td>Trastuzumab (Herceptin)</td>
<td>Genentech</td>
<td>Humanized IgG1</td>
<td>HER2</td>
<td>Breast Cancer</td>
<td>Signal Inhibition, ADCC</td>
<td>1998</td>
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<td>Alemtuzumab (Campath)</td>
<td>Sanofi-Aventis</td>
<td>Humanized IgG1</td>
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<td>CDC, Induction of Apoptosis</td>
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<td>Ibritumomab tiuxetan (Zevalin)</td>
<td>Biogen Idec.</td>
<td>Murine IgG1</td>
<td>CD20</td>
<td>Non-Hodgkin’s Lymphoma</td>
<td>Radioisotope Delivery (90Y)</td>
<td>2002</td>
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<td>Tositumomab (Bexxar)</td>
<td>GlaxoSmith-Kline</td>
<td>Murine IgG2a</td>
<td>CD20</td>
<td>Non-Hodgkin’s Lymphoma</td>
<td>Radioisotope Delivery (131I), ADCC, CDC, Induction of Apoptosis</td>
<td>2003</td>
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<td>Cetuximab (Erbitux)</td>
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<td>Chimeric IgG1</td>
<td>EGFR</td>
<td>Squamous Cell Carcinoma of the Head and Neck</td>
<td>Signal Inhibition, ADCC, CDC</td>
<td>2004</td>
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<td>Genentech</td>
<td>Humanized IgG1</td>
<td>VEGF</td>
<td>Colorectal Cancer</td>
<td>Signal Inhibition</td>
<td>2004</td>
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<td>Amgen</td>
<td>Human IgG2a</td>
<td>EGFR</td>
<td>Colorectal Cancer</td>
<td>Signal Inhibition, ADCC</td>
<td>2006</td>
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<td>Ofatumumab (Arzerra)</td>
<td>Genmab/GSK</td>
<td>Human IgG1</td>
<td>CD20</td>
<td>Chronic Lymphocytic Leukemia</td>
<td>ADCC, CDC</td>
<td>2009</td>
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<td>Denosumab (Xgeva)</td>
<td>Amgen</td>
<td>Human IgG2</td>
<td>RANKL</td>
<td>Bone Metastases</td>
<td>Signal Inhibition</td>
<td>2010</td>
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<td>Ipilimumab (Yervoy)</td>
<td>Bristol-Myers Squibb</td>
<td>Human IgG1</td>
<td>CTLA-4</td>
<td>Metastatic Melanoma</td>
<td>Signal Inhibition</td>
<td>2011</td>
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<td>Brentuximab vedotin (Ad cetris)</td>
<td>Seattle Genetics</td>
<td>Chimeric IgG1</td>
<td>CD30</td>
<td>Hodgkin Lymphoma</td>
<td>ADC</td>
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<td>Pertuzumab (Perjeta)</td>
<td>Genentech</td>
<td>Humanized IgG1</td>
<td>HER2</td>
<td>Breast Cancer</td>
<td>Signal Inhibition, ADCC</td>
<td>2012</td>
</tr>
<tr>
<td>Trastuzumab emtansine (Kadcyla)</td>
<td>Genentech</td>
<td>Humanized IgG1</td>
<td>HER2</td>
<td>Breast Cancer</td>
<td>ADCC, Signal Inhibition, ADCC</td>
<td>2013</td>
</tr>
<tr>
<td>Obinutzumab (Gazyva)</td>
<td>Genentech</td>
<td>Humanized IgG1; Glyco-engineered</td>
<td>CD20</td>
<td>Chronic Lymphocytic Leukemia</td>
<td>ADCC, CDC, Induction of Apoptosis</td>
<td>2013</td>
</tr>
<tr>
<td>Pembrolizumab (Keytruda)</td>
<td>Merck Sharp &amp; Dohme Corp</td>
<td>Humanized IgG4</td>
<td>PD-1R</td>
<td>Metastatic Melanoma</td>
<td>Signal Inhibition</td>
<td>2014</td>
</tr>
<tr>
<td>Nivolumab (Opdivo)</td>
<td>Bristol-Myers Squibb</td>
<td>Human IgG4</td>
<td>PD-1R</td>
<td>Metastatic Melanoma</td>
<td>Signal Inhibition</td>
<td>2014</td>
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</table>
Naked mAbs

In 1997, rituximab was the first mAb in oncology that was approved by the US Food and Drug Administration (FDA). It is directed against the CD20 antigen and used for patients with non-Hodgkin’s lymphoma (NHL). Hematologic malignancies are relatively easy to target, because the cells are in close connection with the blood stream and therefore directly accessible for mAb-therapy. CD20 is highly expressed on normal B cells and B-cell lymphoma cells and is not shed into the plasma. In vitro, no internalization after binding of rituximab is observed, but in vivo internalization cannot be excluded. Although also normal B cells are cleared by rituximab treatment, this seems to have no effect on the immune system, since no increase in the incidence of infections is observed in these patients. Upon antibody binding, B-cell proliferation is directly inhibited, nuclear DNA fragmentation is induced and this leads to cell death by apoptosis. After being a decade on the market, rituximab still makes 5 billion dollar sales per year.

One year after the approval of rituximab, a mAb targeting solid tumors was approved: trastuzumab. This humanized mAb is directed against human epidermal growth factor receptor 2 (HER2), which is expressed by many epithelial tissues and over-expressed in 20-30% of breast cancers. Trastuzumab binds to the extracellular domain IV of the HER2 receptor and blocks activation of the downstream signaling cascade. Other indicated effects are prevention of HER2-receptor dimerization, increased endocytotic destruction of the receptor, inhibited shedding of the extracellular domain, and activation of the immune system by ADCC. Trastuzumab is used for treatment of metastatic HER2-positive breast cancer and as adjuvant therapy for other HER2-positive breast tumors.

Alemtuzumab became approved in 2001, and is an anti-CD52 mAb used for patients with B-cell chronic lymphocytic leukemia. It exerts anti-tumor activity by ADCC. In 2012, alemtuzumab was withdrawn from the market and was relaunched in 2014 as a treatment for multiple sclerosis at a much higher price.

Cetuximab, approved in 2004, is directed against the epidermal growth factor receptor (EGFR = HER1) and was first used in advanced colorectal cancer. It is only effective for tumors that express EGFR and have a wild type KRAS gene. Like trastuzumab, cetuximab blocks the downstream signaling cascade after binding with high affinity to the receptor. KRAS is downstream in the signaling cascade, when mutated it causes uncontrolled cellular proliferation and as it acts downstream of EGFR it makes the tumor cell insensitive for cetuximab treatment. It also triggers ADCC and CDC. Currently, cetuximab is registered for the treatment of metastatic colorectal cancer (mCRC), metastatic non-small cell lung cancer (mNSCLC) and head-and-neck squamous cell carcinoma (HNSCC).
Bevacizumab, also approved in 2004, is a mAb directed against vascular endothelial growth factor (VEGF), a growth factor associated with angiogenesis and overexpressed in many solid tumors. Bevacizumab binds to VEGF, thereby causing inhibition of angiogenesis. When tumors grow larger, the cells become hypoxic and start releasing VEGF and other angiogenic factors. This makes bevacizumab suitable for use in many different solid tumor types. Currently it is approved for treatment of patients with mCRC, NSCLC, metastatic breast cancer, metastatic renal cell cancer, prostate cancer, and glioblastoma.\(^\text{14}\)

Panitumumab, approved in 2006, is an anti-EGFR mAb, like cetuximab, used in the treatment of mCRC and also requires wild type KRAS to be effective. In contrast to cetuximab, which is a chimeric IgG1 and therefore ideally suited for ADCC activity, panitumumab is a human IgG2 mAb, less active in ADCC. However, clinical data do not show differences in the efficacy of these two anti-EGFR mAbs in colorectal cancer.\(^\text{15}\) While there is a difference in HNSCC.

Ofatumumab, approved in 2009, targets CD20 like rituximab and is approved for use in treatment of chronic lymphocytic leukemia (CLL). The binding and immunological properties of ofatumumab differ from those of rituximab,\(^\text{16}\) therefore it might be a suitable mAb to treat rituximab-refractory patients. In 2012, a clinical study showed a modest efficacy of ofatumumab treatment in rituximab-refractory patients.\(^\text{17}\)

Denosumab, approved in 2010, targets less frequent occurring tumors. It is directed against the receptor activator of nuclear factor kappa-B ligand (RANKL), a surface molecule on osteoclasts, and is used for treatment of bone metastases from solid tumors and for unresectable giant cell bone tumors.\(^\text{18}\)

Ipilimumab, the first immune checkpoint mAb (elaborated in the part ‘Other next generation mAbs’) approved in 2011, is used for treatment of metastatic melanoma and is directed against cytotoxic T lymphocyte–associated antigen 4 (CTLA-4), a protein receptor that down-regulates the immune system.\(^\text{19}\)

Pertuzumab, approved in 2012, is also directed against HER2 and used to treat patients with metastatic breast cancer. Pertuzumab targets a different motif in HER2 than trastuzumab: it binds domain II of the extracellular domain of HER2, and is more effective in preventing receptor heterodimerization with EGFR, HER3, and HER4, and subsequent cell signaling. Preclinical studies showed synergy between trastuzumab and pertuzumab, providing possibilities for combination therapy.\(^\text{20}\) Therefore, a phase II clinical study was performed, which showed that the addition of pertuzumab to the standard trastuzumab and docetaxel combination treatment resulted in a significant clinical benefit.\(^\text{21}\)
**Conjugated mAbs**

Using mAbs as delivery vehicles of toxic agents is one of the most promising applications in antibody therapy. During the earliest mAb research this approach was tested because of the initial lack of successes with naked therapeutic murine mAbs.

Delivery of toxic payloads can be divided in three categories: radioimmunoconjugates, immunotoxins and antibody-drug conjugates (ADCs). The development of mAbs coupled to radioactive or toxic compounds makes the chemistry more complicated and demands a higher standard of safety measurements: (i) conjugates preferably have exactly the same pharmacokinetics as their non-conjugated counterparts; (ii) biological half-life and binding properties to the target should not be altered after conjugating an additional group to the mAb; (iii) *in vivo* stability of the chemical bond between the payload and the mAb is also of major importance, since it is undesirable that some of the toxic molecules are released from the conjugate before they reach the tumor site.

Radioimmunoconjugates are mAbs with a radionuclide or radionuclide-containing compound attached to it. When the mAb binds to the tumor cell, the radiation damage causes cell death of the targeted cell and possibly the surrounding cells, depending on the radiation energy of the radionuclide. For use in this therapeutic setting, the radionuclide needs to have a physical half-life long enough to allow the mAb to reach the tumor and have an α- or β- decay. The two radionuclides used in FDA-approved radioimmunoconjugates are $^{131}$I and $^{90}$Y, with half-lifes of 8.02 days and 2.66 days, respectively. $^{90}$Y emits high-energy β’s that have a mean range in tissue of 2.7 mm, and is therefore most suitable for large tumors; $^{131}$I emits β’s with lower energy, the mean tissue range is 0.8 mm, and is better suited for treatment of smaller tumors. The radionuclide is coupled directly to the tyrosines of the mAb ($^{131}$I) or via a chelate ($^{90}$Y). The two radioimmunoconjugates that made it to approval are ibritumomab tiuxetan ($^{90}$Y) in 2002 and tositumomab ($^{131}$I) in 2003 (see Table 1). Both antibodies are of murine origin with CD20 as the target. They are used for treatment of patients with NHL, which is attractive since these cells are sensitive for radiation. Nevertheless, unlabeled mAbs like rituximab and its successors are still favored in clinical use, since working with radiopharmaceuticals always requires extra precautions for the patient and physician. In recent years, new radioimmunoconjugates have not been approved for clinical use. Main reasons are the inadequate targeting of many tumor types for radioimmunotherapy and the previously mentioned extra precautions that need to be implemented for transportation and clinical use when working with radiopharmaceuticals.

Immunotoxins have a catalytic toxin as therapeutic entity. These toxins are enzymes of bacteriological origin or other types of pathogens. After endocytosis, the cleaved toxins cause termination of protein synthesis, which results in cell
death by apoptosis. A complication in the development of immunotoxins is that the toxins are of non-mammalian origin, which causes a reaction of the human immune system in patients. Neutralizing antibodies are produced against the toxin, limiting the number of treatments\(^23\). Also, most toxins have binding sites for surface molecules expressed by normal tissues, and these binding sites have to be modified before the toxins can be used therapeutically. In addition, therapy studies revealed that efficacy could be impeded by cellular resistance mechanisms\(^24\). While immunotoxins have a lot of potential, a large effort in optimization is required before they can be successfully brought to the clinic.

ADCs are currently the most thoroughly explored immunoconjugates. For the last 20 years researchers have tried to develop the perfect combination of a highly toxic payload, linked through a stable linker to a specifically and selectively targeting mAb. Early studies tested classical chemotherapeutic compounds, such as doxorubicin, coupled to a mAb, but these conjugates failed to show therapeutic efficacy\(^25\). These failures can be explained by the small amount and relatively low potency of the toxic compound that is released in the tumor. Compared to a single injection of the free toxic compound, the amount of toxic compound that is delivered to the tumor is much lower when coupled to a mAb\(^26\). Therefore, the ideal ADC allows the application of highly toxic compounds, much more toxic than current chemotherapeutics, that otherwise have a therapeutic window which is too narrow to allow their use as free drug\(^27\). This also requires stable conjugation to avoid systemic toxicity by release of the toxic compound in the circulation, but once internalized the drug should be released to be able to kill the tumor cell. Only then, the desired therapeutic effect will be observed.

The toxic compounds used for ADCs can be divided in two main categories: DNA-damaging agents and microtubule inhibitors. The most studied toxins are tubulin inhibitors derived from maytansine, maytansoids, and the auristatins, synthetic analogues derived from the natural occurring dolastatin 10, and the DNA-damaging agent calicheamicin\(^28\). The development of synthetic versions of these natural occurring toxins provided the possibility of adding specific chemical groups, which enables easy coupling to linkers while keeping toxicity in the nanomolar range.

Gemtuzumab-ozogamicin was in 2000 the first FDA approved ADC and used for the treatment of acute myeloid leukemia (AML) (Table 1). This IgG4 mAb is directed against CD33, a surface glycoprotein, and the cytotoxic antibiotic calicheamicin is linked to this mAb by a hydrazone (pH labile) linker. The IgG4-isotype was used because it does not induce ADCC activity and related cytokine release. Gemtuzumab-ozogamicin was used as single agent for patients over 60 with
CD33-positive AML in first relapse or those that are not considered candidates for cytotoxic chemotherapy. However, 10 years after its approval, gemtuzumab-ozogamicin was voluntarily withdrawn from the market due to the lack of clinical benefit and unacceptable systemic cytotoxicity. The poor results with this first approved ADC demonstrate the importance of getting more knowledge on the in vivo behavior of ADCs, e.g., in animal models.

A decade after the approval of gemtuzumab-ozogamicin, the second ADC was approved for clinical use: brentuximab vedotin (Table 1), directed against CD30 and used to treat patients with Hodgkin’s lymphoma. The drug used in this ADC is the auristatin derivative monomethyl auristatin E (MMAE), coupled to the mAb via a cleavable peptide (valine-citrulline) linker. Brentuximab vedotin was developed after early-phase patient data revealed only modest clinical activity of the naked mAb, originally named cAC10. In 2003, the ADC was developed under the name cAC10-vcMMAE/SGN-35, but the ADC was renamed brentuximab vedotin in 2008 when clinical studies were started.

The next approved ADC was ado-trastuzumab-emtansine in 2013 (Table 1). The ADC consists of trastuzumab and the maytansine derivate DM1, coupled via a thioether linker proclaimed to cause the release of the drug upon proteolytic degradation only. The thioether linked ADC gave the better therapeutic effect, since the release of the toxic compound in the circulation was minimized compared to other, less stable, hydrazone linkers. Ado-trastuzumab emtansine is approved for treatment of patients with metastatic breast cancer who previously received trastuzumab and a taxane, separately or in combination.

At the moment more than 30 ADC’s are in early- or late-stage clinical development. The ADCs in late stage clinical development all contain the previously mentioned toxic compounds (Table 2).

Researchers tried to learn from the withdrawal of gemtuzumab ozogamicin and developed a closely related ADC: inotuzumab ozogamicin, the same drug and linker, coupled to CD22, which is another surface glycoprotein that can be used to treat patients with AML/NHL. The naked CD22 antibody showed no clinical efficacy, but is readily internalized, which makes it ideal for use in an ADC, as was confirmed by preclinical and early clinical studies. Clinical phase III studies with izotuzumab ozogamicin in combination with rituximab are currently ongoing.

Pinatuzumab vedotin is also directed against CD22 and contains an auristatin toxic compound, like in the approved brentuximab vedotin. It is also a potential competitor of rituximab, the current standard of care for treatment of NHL in combination with chemotherapy, as was shown in preclinical in vivo efficacy studies.
Therefore, a phase II study was started that combines pinatuzumab vedotin with rituximab\textsuperscript{15}, to find a possible treatment for rituximab-refractory patients.

For treatment of solid tumors, glembatumumab vedotin and lorvotuzumab mertansine are currently in phase II studies. Glembatumumab vedotin contains a mAb directed against glycoprotein non-metastatic melanoma protein B (GPNMB), which is mainly expressed in breast cancers and melanomas. The toxic MMAE is conjugated to the mAb via a cleavable peptide linker, like in brentuximab vedotin. Clinical studies with glembatumumab vedotin for treatment of both advanced melanoma\textsuperscript{36} and breast cancer\textsuperscript{37} show promising results. Lorvotuzumab mertansine is composed of an anti-CD56 antibody, linked via a cleavable disulfide linker to the maytansinoid DM1. CD56 is the neuronal cell adhesion molecule and is expressed by various tumor types. Lorvotuzumab mertansine has shown efficacy in combination therapy in multiple myeloma patients\textsuperscript{38} and shows potential in small-cell lung cancer (SCLC) xenograft models\textsuperscript{39}. These combined results were promising enough to immediately start with the phase II clinical trial for treatment of SCLC\textsuperscript{35}.

Despite increasing popularity of ADCs, it is fair to state that the development of ADCs is still at its infancy. At this moment, most ADCs under clinical development contain maytansines or auristatins as the toxic payload (Table 2). It is not totally clear whether releasable, cleavable or non-cleavable linkers are best suited for appropriate drug delivery to the tumor. To get better understanding of optimal ADC design, \textit{in vivo} tracking of ADCs would be very informative.

**Table 2 - Clinical-stage ADC pipeline, adapted from Mullard\textsuperscript{15}**

<table>
<thead>
<tr>
<th>ADC</th>
<th>Lead</th>
<th>Lead indications</th>
<th>Target</th>
<th>Payload</th>
<th>Phase</th>
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<td>Inotuzumab ozogamicin (CMC-544)</td>
<td>Pfizer</td>
<td>Aggressive non-Hodgkin’s lymphoma; ALL</td>
<td>CD22</td>
<td>Calicheamicin</td>
<td>III</td>
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<td>RG-7596</td>
<td>Genentech</td>
<td>DLBCL and follicular non-Hodgkin’s lymphoma</td>
<td>CD79b</td>
<td>MMAE</td>
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<td>GPNMB</td>
<td>MMAE</td>
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<td>Sanofi</td>
<td>DLBCL; ALL</td>
<td>CD19</td>
<td>DM4</td>
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<td>Doxorubicin</td>
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<td></td>
<td>(also known as TROP2 or EGP1)</td>
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<tr>
<td>Labetuzumab- SN-38</td>
<td>Immunomedics</td>
<td>Cancer; colorectal cancer</td>
<td>CEA (also known as CD66e)</td>
<td>Irinotecan metabolite</td>
<td>I</td>
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<tr>
<td>IMGN-853</td>
<td>ImmunoGen</td>
<td>Ovarian tumor; solid tumor</td>
<td>Folate receptor 1</td>
<td>DM4</td>
<td>I</td>
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<tr>
<td>IMGN-529</td>
<td>ImmunoGen</td>
<td>B cell lymphoma; CLL; non-Hodgkin’s lymphoma</td>
<td>CD37</td>
<td>DM1</td>
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<tr>
<td>RG-7458</td>
<td>Genentech</td>
<td>Ovarian tumor</td>
<td>Mucin 16</td>
<td>MMAE</td>
<td>I</td>
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<tr>
<td>RG-7636</td>
<td>Genentech</td>
<td>Melanoma</td>
<td>Endothelin receptor ETB</td>
<td>MMAE</td>
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<tr>
<td>RG-7450</td>
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<td>Prostate cancer</td>
<td>STEAP1</td>
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<td>RG-7600</td>
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<td>Not disclosed</td>
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<tr>
<td>RG-7598</td>
<td>Genentech</td>
<td>Multiple myeloma</td>
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<td>ALL; aggressive non-Hodgkin’s lymphoma</td>
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<td>CD19</td>
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<td>Vorsetuzumab mafodotin</td>
<td>Seattle Genetics</td>
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<td>CD70</td>
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<td>Agensys</td>
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<td>SLC44A4</td>
<td>MMAE</td>
<td>I</td>
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<tr>
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<td>AGS-16</td>
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<td>Guanylyl cyclase C</td>
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<td>SAR-566658</td>
<td>Sano菲</td>
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<td>Mucin 1</td>
<td>DM4</td>
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<td>AMG-172</td>
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<td>CD70</td>
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<td>Bayer</td>
<td>Cancer; mesothelioma</td>
<td>Mesothelin</td>
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ALL, acute lymphoblastic leukemia; CEA, carcinoembryonic antigen; CLL, chronic lymphoblastic leukemia; DLBCL, diffuse large B cell lymphoma; GPNMB, glycoprotein NMB; MMAF, monomethyl auristatin F; NSCLC, non-small-cell lung cancer; PSMA, prostate-specific membrane antigen; STEAP1, six-transmembrane epithelial antigen of prostate 1; TACSTD2, tumour-associated calcium signal transducer 2.
Other next generation mAbs

While in the ADC-development the focus is on improving the linker/toxic compound chemistry, the mAb itself is also undergoing development, contributing to next generation mAbs. Glycoengineered mAbs are modified in their glycosylation pattern in the Fc-region to enhance ADCC and CDC, via improved binding to Fcγ receptors on immune effector cells. This creates a more potent naked mAb, compared to a normal IgG1, with ofatumumab as the first successful example of this strategy and obinutuzumab the second that was approved in 2013 (Table 1).

A new and exciting development is the clinical application of mAbs that inhibit immune-checkpoints. When an immune response is elicited, also a negative feedback mechanism is activated to dampen the response, and these mechanisms are indicated as immune-checkpoints. These mechanisms are protective, but hamper effective anti-tumor immune responses. In fact tumor cells can exploit these mechanisms to counteract immune responses. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1) have proven to be important immune-checkpoint proteins and promising therapeutic targets for mAbs. The previously mentioned ipilimumab is directed against CTLA-4 and nivolumab and pembrolizumab are directed against PD-1. Nivolumab and pembrolizumab, approved in 2014 (Table 1), reactivate T cells by blocking APC (antigen presenting cell) inhibition of T cells or by blocking tumor cells, which often inactivate T cells in the microenvironment via their overexpressed PD-1 ligands (e.g. PD-L1). An advantage of these immune-checkpoint inhibiting mAbs is that they can be beneficial in many types of tumors, hematological and solid.

Multi-specific antibodies are able to recognize more than one target molecule, by exploiting single heavy chain-light chain pairs from antibodies with distinct specificities. The different pairs can be linked to form two arms with different specificity within a single IgG molecule. MEHD7945A, currently in phase II clinical trials, is directed against EGFR as well as HER3, which are closely related receptors, and shows promising results in the treatment of multidrug resistant tumors.

Besides intact mAbs and antibody fragments, nanobodies included, advances in genetic engineering have led to the development of several non-immunoglobulin-like binding molecules. They have antibody-like binding properties, but completely lack the antibody scaffold; and the novel structures are designed as derivatives of naturally occurring substrates or proteins. Anticalins, affibodies, adnectins, designed ankyrin repeat proteins (DARPins) and avimers are among the most developed non-Ig-based protein scaffolds. Claimed advantages of these ligands are their small size, max. 200 amino acids, and the lack of immunogenicity. Adnectin CT-322 and DARPin AGN150998/MP0112 are currently in clinical phase II development.
Nuclear imaging in mAb development and applications

The continuous progress in the development of mAbs and mAb conjugates demands for accurate, preferably quantitative, methods to monitor the in vivo behavior of these potentially toxic molecules. Therefore, not only mAbs have been in development for therapeutic exploitation the last decades, but many researchers have put as much effort in the development of diagnostic mAbs as well.

Most commonly used diagnostic mAbs for imaging purposes consist of a mAb carrying a radioactive isotope for use in single photon emission computed tomography (SPECT) or positron emission tomography (PET). The quality of gamma cameras as well as PET-scanners in terms of sensitivity and resolution has improved over the last years, causing an increase in mAb-imaging. However, PET imaging has a higher sensitivity and more accurate quantification than SPECT.

Immuno-PET, imaging of mAbs labeled with positron emitters, can provide whole body images, thereby revealing important information both about the tumor and possible metastases. Also the level of antigen expression in tumor and other tissues can be determined, indicating the tumor-specificity of the mAb: crucial knowledge for therapeutic potential of the mAb. Immuno-PET can also be used to optimize mAb dose scheduling and to track changes in tumor biology over time, including assessments of the antigen expression before and after therapy. Furthermore, immuno-PET provides the possibility to select patients for therapy, thereby increasing therapeutic efficacy and decreasing adverse events.

The radionuclides that can be used for immuno-PET, need to have a physical half-life that matches the biological half-life of the mAb. Since almost all approved mAbs and mAbs in clinical development are intact mAbs with a half-life of several days, $^{124}$I ($t_{1/2}=100.3$ hours) and $^{89}$Zr ($t_{1/2}=78.4$ hours) are attractive candidate radionuclides. For both isotopes labeling procedures have been developed that ensure safety-controlled production for human use all over the world. Conjugation with $^{124}$I is relatively easy, only an oxidizing agent is necessary to enable the direct labeling reaction of $^{124}$I to the tyrosine moiety of the mAb. Labeling mAbs with $^{89}$Zr requires premodification of the mAb, which implies the introduction of an extra group - a chelator - to be able to attach the metal radionuclide in a stable way. The first method used for clinical immuno-PET was published in 2003 and required a two steps conjugation. The same group published an improved version in 2010 with a single step conjugation.

An important reason to choose between $^{124}$I or $^{89}$Zr is the nature of the mAb, whether it is internalizing or not. Iodine is released from the cells after internalization, while the zirconium is residualizing and remains in the cell after internalization. The latter makes $^{89}$Zr the radionuclide of choice for many immuno-PET applications.

Many successful preclinical and clinical studies with $^{124}$I- and $^{89}$Zr labeled mAbs have been performed and are described in detail in comprehensive reviews.
**Near-infrared imaging in mAb applications**

Although immuno-PET has many advantages for whole body imaging, resolution is limited and real-time imaging is impossible. Photoinmunodetection, in which mAbs are labeled with fluorescent dyes, might have a complementary clinical potential to immuno-PET. It allows high-resolution, real-time, dynamic imaging of superficial tissue layers at the cellular level, without radiation burden to the patient. Therefore, it might be ideal for confirmation of mAb binding to subsets of cells. In addition, it might be a powerful clinical tool for the delineation and characterization of early-stage or residual disease, for example of cancer during surgery or in a screening setting. A limitation of optical imaging in comparison to PET imaging is the difficulty of deep tissue imaging and of quantification.

Fluorescent dyes in the near-infrared (NIR) region are the most suitable for use in imaging. The light emitted after excitation is in the region of 650-900 nm. At this wavelength tissue absorption and scattering is low, tissue penetration is in the range of 1 to 2 centimeters and tissue auto-fluorescence in not interfering with dye signal. The challenge is to develop a fluorescent probe that maintains its fluorescent properties after conjugation to mAbs. Studies with ICG, the only FDA-approved NIR dye, showed promising results when used as a single agent, however, when the molecule was modified for coupling to a mAb a serious loss of fluorescence was observed. Different fluorescent dyes have been tested for coupling abilities and the dye with the highest potential to be used in the clinical setting is IRDye800CW (Licor Bioscience). IRDye800CW can be functionalized with either an NHS or a maleimide reactive group, ideal for conjugation to mAbs, as illustrated in several preclinical studies with IRDye800CW-mAb conjugates.

The first clinical trials with IRDye800CW labeled mAbs – bevacizumab and cetuximab – are currently ongoing (Clinicaltrials.gov, NCT01987375, NCT02113202, NCT01508572, NCT01972373, NCT02129933).

Even more attractive seems the use of dual-labeled mAbs, since combining PET and optical imaging techniques would provide the ideal probe: the location and size of the tumor and metastases can be determined with immuno-PET, followed by a complete tumor resection aided by imaging of the NIR-labeled mAb during surgery. For these dual-labeled conjugates with IRDye800CW, FDA-approved mAbs trastuzumab and bevacizumab as well as new mAbs were used. Also different PET radionuclides were used. Besides the expected $^{89}$Zr, also $^{64}$Cu ($t_{1/2} = 12.7$ hours) was investigated. All studies show tumor specific uptake of the conjugate with both imaging modalities, confirming the potential of IRDye800CW. However, clinical translation of dual-labeled conjugates still has some hurdles to take. Clinical use of $^{64}$Cu with intact mAbs seems less desirable, since the half-life of $^{64}$Cu does not match the biological half-life of intact mAbs in humans. Moreover,
stable coupling of $^{64}$Cu to mAbs is challenging, and sequestration of $^{64}$Cu in the liver might occur. In addition, the number of conjugated dye and chelator groups per mAb was different for every study performed, and this might affect antigen binding and pharmacokinetics. When clear protocols would be available for the reproducible production of stable dual-labeled mAbs, clinical use of PET/NIR molecules will most probably be the next translational step.

**OUTLINE OF THIS THESIS**

As outlined above in Chapter 1, mAbs have shown to be very promising candidates in cancer therapy as naked single agents, but mostly when used as carrier for tumor-specific delivery of potent toxic compounds. The latter requires a reproducible synthesis protocol that is relatively straightforward and GMP compliant. Moreover, labeling of the mAbs and mAb-conjugates facilitates product development and their clinical applications. The more toxic the naked mAb or the toxic compound to be delivered by the mAb are, the more important it becomes that tumor targeting is “precise”; precise at the level of the individual patient and precise at the level of a high uptake in the tumor and a low uptake in the normal tissues. In this thesis the development of tools to allow the quantitative tracking of mAbs in vivo by PET or optical imaging is described, which might be of help in steering efforts in the development and (pre)clinical application of precision medicine with mAbs and other biologicals.

In Chapter 2, we focus on the use of radioactive labels in the development and in vivo validation of novel antibody drug conjugates (ADCs). ADCs comprise a class of drugs receiving a lot of attention from pharmaceutical companies involved in the development of anti-cancer drugs (see General introduction). We introduce a new family of highly potent drugs, tubulysins, to be coupled to mAbs as novel ADCs. For efficient ADC development, tubulysins are labeled with $^{131}$I, while the mAb under investigation in this study (trastuzumab) is labeled with $^{89}$Zr. By using this approach of “dual-radiolabeling” we monitor the direct conjugation of tubulysin derivatives to trastuzumab, to get full control over drug-antibody ratio (DAR), and we characterize the tubulysin-trastuzumab conjugates for their pharmacokinetics and tumor targeting properties. Finally, we describe the in vivo efficacy of these ADCs, which have been developed by guidance of radiolabeling technology.

As outlined in the introduction section, $^{89}$Zr labeling of mAbs allows the quantitative in vivo characterization of mAbs and other biologicals using PET imaging. PET imaging is particularly well suited for whole body imaging, but is not qualified to show tumor targeting at the cellular level. For the latter, labeling of mAbs with
fluorescent dyes in combination with optical imaging, is better suited as it allows high resolution and real time imaging. Irrespective the use of PET radionuclides or fluorescent dyes for mAb tracking, to be acceptable for pharmaceutical companies and regulatory authorities, the procedures for coupling to mAbs should we well controlled and inert, not affecting the biological properties of the mAb.

In Chapter 3, we use \(^{89}\)Zr labeling as an internal reference standard to prove that labeling of the mAbs cetuximab and bevacizumab with the NIR dye IRDye800CW can be performed in a controlled an inert way, without affecting binding and biodistribution characteristics of the mAbs. To this end, different equivalents of IRDye800CW are coupled to the mAbs.

To allow broad scale preclinical and clinical application of single- as well as of dual-modal fluorescence and PET imaging, Chapter 4 presents a detailed protocol for the inert coupling of IRDye800CW and/or \(^{89}\)Zr to mAbs in a current good manufacturing practice-compliant (cGMP) way. The previous study (Chapter 3) provided the necessary information to design a user and product friendly protocol: to avoid unnecessary radiation exposure to the product and personnel, first the chelator desferal (Df) is conjugated to the mAb, second the NIR-dye IRDye800CW is coupled, and finally the conjugate is labeled with \(^{89}\)Zr.

While imaging of mAbs labeled with NIR dyes showed promising results, quantification of the fluorescent signal is more challenging. In Chapter 5, we evaluate a new method to quantify the uptake of IRDye800CW-cetuximab in organs and tumors. Also here we use dual-labeling with \(^{89}\)Zr and standard gamma ray quantification method as reference and for validation purposes.

In Chapter 6 a summary of the results is given as well as a general discussion and future perspectives on the topics. In Chapter 7 a summary in Dutch is provided.
REFERENCES

3. Porter RR. The structure of antibodies. The basic pattern of the principal class of molecules that neutralize antigens (foreign substances in the body) is four cross-linked chains. This pattern is modified so that antibodies can fit different antigens. Sci Am 1967;217:81-7 passim.


