A chemical biology approach for targeting of ligand-drug conjugates

Hoogendoorn, S.

1

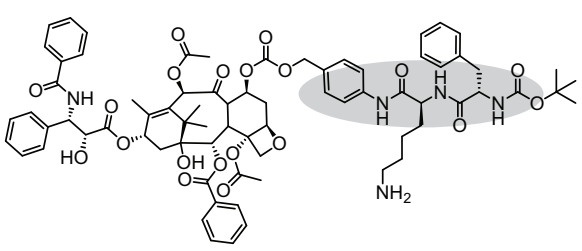
General introduction

Many complex biological processes are regulated by cell signaling, where a secreted ligand travels short or long distances through an organism or tissue until it is recognized by a cell surface receptor. These receptors, proteins residing in the cellular membrane, function as a communication channel between the extra- and intracellular space, translating the binding of a ligand into a variety of signaling cascades which ultimately lead to a cellular response. Receptor expression is cell-type specific so that the ligands that bind them exert a localized effect. This principle is essential for maintaining normal tissue homeostatis and dysregulation of cellular communication lies at the basis of a variety of diseases, such as cancer or autoimmune diseases. In his pioneering work in the early 20th century, Paul Ehrlich proposed the presence of a ligand-receptor interaction and the possibilities that this provides for selective targeting of chemotherapeutics.[1] With his studies on the cellular localization of colored dyes he laid the groundwork for the realisation that the chemical properties of a compound, in combination with the cell on which it acts, determine the effects. He recognized the potential of organic chemistry as a means to create those molecules that interact with a specific cellular target as indicated by his statement "wir müssen zielen lernen, chemisch zielen lernen" ("we have to learn how to aim, aim chemically").[2,3] More than a hundred years later, the search for Ehrlich's "magic bullet" has not ceased and the area of drug targeting approaches is still very much alive. In this chapter, current strategies for the targeted delivery of imaging or therapeutic agents that employ ligand-receptor interactions will be highlighted. In line with the projects discussed in the later chapters of this thesis, the focus will be on those examples that make use of synthetic approaches to reach this goal.

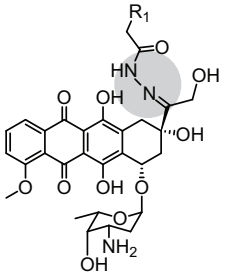
General principles of receptor-mediated targeted delivery

When discussing targeted delivery, it is important to distinguish which kind of targeting is envisaged, systemic, intracellular or both. In the ideal case, the ligand-drug conjugate enters the circulation, and accumulates at the site where its action is needed by being captured by the surface receptors. For this to happen, the affinity of the ligand and the expression levels of the receptor should be sufficiently high. Ideally, the receptor is only expressed on the diseased cells, such as tumor cells, because even when overexpressed, the lower levels on the more abundant healthy cells will compete for binding and can thus act as a sink. Non-specific interaction with surrounding cells should preferably be very low.

Once arrived at the site of action, multiple options for further, intracellular, delivery exist, depending on the nature of the ligand and the design of the ligand-drug conjugate. Ligands are generally classified as agonists, which activate receptors, or antagonists, which block the receptor and thereby prevent activation. Over the years it has become clear that this classification does not cover all possibilities, with ligands being able to induce full or partial effects that can even be opposite to that generated by an endogenous agonist.[4] Moreover, besides the orthosteric binding site where the endogenous ligand binds, there are often other, allosteric, sites where ligand binding can occur. Some receptors contain multiple ligand recognition domains, leading to enhanced affinity for multivalent ligands, examples of which will be discussed in more detail later on. Binding of a ligand, typically an agonist, to the receptor might result in receptor-ligand internalization. Depending on the type of targeted therapy, this might be an advantage or a disadvantage. The two-step antibody-directed enzyme prodrug therapy (ADEPT) is designed such that an enzyme, required to release the active drug, is delivered to the binding site so that the drug can then freely diffuse into the tissue.[5]



1
Boc-Phe-Lys-PABC-7-Paclitaxel



2
Doxorubicin-hydrazone linker-R1

Figure 1.1: Strategies for controlled release in the lysosomes of paclitaxel or doxorubicin by employing Cathepsin B-cleavable (Phe-Lys) or acid labile (hydrazone) linkers. PABC: self-immolative *p*-aminobenzylcarbamate linker. Linker regions are indicated in grey.

In most cases drugs or drug formulations need to be delivered intracellularly by receptor-mediated endocytosis to exert their effect. For some therapies, such as enzyme replacement therapy for lysosomal storage disorders, the endolysosomal pathway is the endpoint of the delivery. In many other cases, the drug needs to be released from the endolysosomes

to reach its intracellular target, requiring alternative strategies such as synthetic cleavable linkers between the ligand and the drug. The use of a cathepsin B cleavable dipeptide linker (**1**, Figure 1.1) has been used to release the cytotoxic anticancer drugs paclitaxel (acting on microtubules), doxorubicin (DOX) (a DNA intercalator), and mitomycin C (a DNA crosslinker) from the lysosomes.[6,7] Making use of the increasingly acidic pH of the endolysosomes is another means of controlled drug release as shown by the introduction of an acid labile hydrazone linkage onto DOX, which was released in the lysosomes (**2**, Figure 1.1).[8,9] In case of targeted delivery for diagnostic purposes, such as a fluorescent dye or a radiotracer, it will depend on the final goal whether an antagonist or an agonist is the preferred ligand. Antagonists are the ligands of choice to visualize a receptor population on the cell membrane without inducing a direct effect, whereas if receptor-ligand internalization and trafficking are the subjects of interest, agonists are preferred.

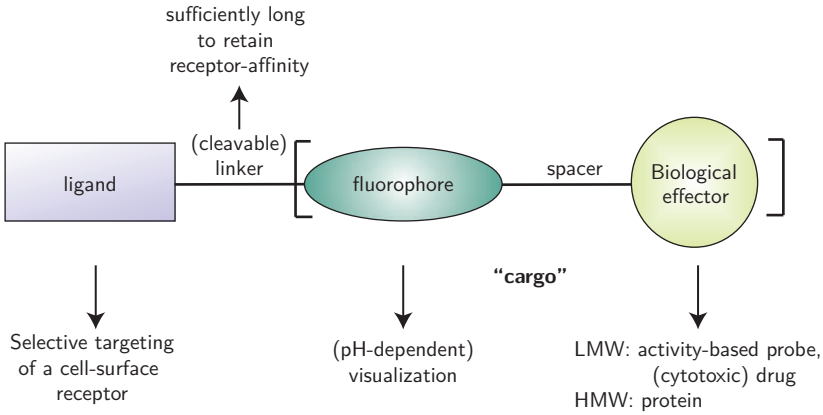


Figure 1.2: General structure of constructs for targeted drug delivery.

The general structure of the (bio)molecules employed for targeted delivery thus consists of the receptor-binding ligand, a (cleavable) linker and 'cargo' (Figure 1.2). The cargo could involve a therapeutic agent such as a drug or a protein and alternatively it could be a reporter group, such as a fluorescent dye, to visualize and study the delivery process and/or an activity-based probe to assess the activity of an intracellular enzyme. This general structure forms the basis of the synthesized constructs that are presented in this thesis. In Chapters 2 and 5 fluorescent DCG-04, an activity-based probe for lysosomal cathepsins was introduced as cargo, in Chapter 4 the protein Hsp70, and in Chapters 7-9 fluorescent dyes for visualization and carboranes as potential drugs. The ligands that are found in the literature can be divided in two main categories: antibodies (or fragments thereof) directed to a specific cell receptor or antigen, or non-antibody receptor ligands, spanning a wide variety of synthetic and endogenous ligands. Even though the antibody category has its distinct advantages in the sense of affinity and selectivity, the synthetic ligands hold a lot of promise because of the relatively easy access to large amounts of defined material which can be chemically tuned to contain the properties of interest (see Chapters 2, 4-8).

In the following sections selected literature examples of synthetic ligands that are used as targeting devices are discussed, based on the receptor type that they bind.

G protein-coupled receptors

The large family of G protein-coupled receptors (GPCRs) is one of the most druggable and many synthetic small molecules or peptides have been developed that are able to act on these receptors.[10,11] Endogenous ligands for GPCRs span a wide range of biomolecules, from small compounds such as neurotransmitters to large protein hormones. Although the structural features of individual GPCRs vary considerably, they are all characterized by a seven-transmembrane domain (Figure 1.3). A heterotrimeric G-protein is bound to the C-terminus which dissociates upon activation of the receptor. Depending on the nature of the α-subunit of the G-protein (G_s, G_i,G_q or $G_{12/13}$) distinct intracellular signaling cascades are set in motion.[12] An elaborate desensitization/resensitization mechanism is used to prevent overstimulation of the system. G protein-coupled receptor kinases (GRKs) phosphorylate the receptor,[13] enabling the binding of β-arrestins. Arrestin binding prevents the binding of G-proteins, thereby desensitizing the receptor for further stimulation. In a next step, the receptor is sequestered from the cell membrane in clathrin-coated vesicles. This can either lead to resensitization, in which the receptor is dephosphorylated and recycled back to the surface in an active form, or to downregulation of the receptor from the cell membrane by degradation in the endolysosomal pathway.[14] The fate of the ligand upon endocytosis varies. If the receptor-ligand complex is stable, the ligand might recycle back to the membrane together with the receptor where it is released into the extracellular space.

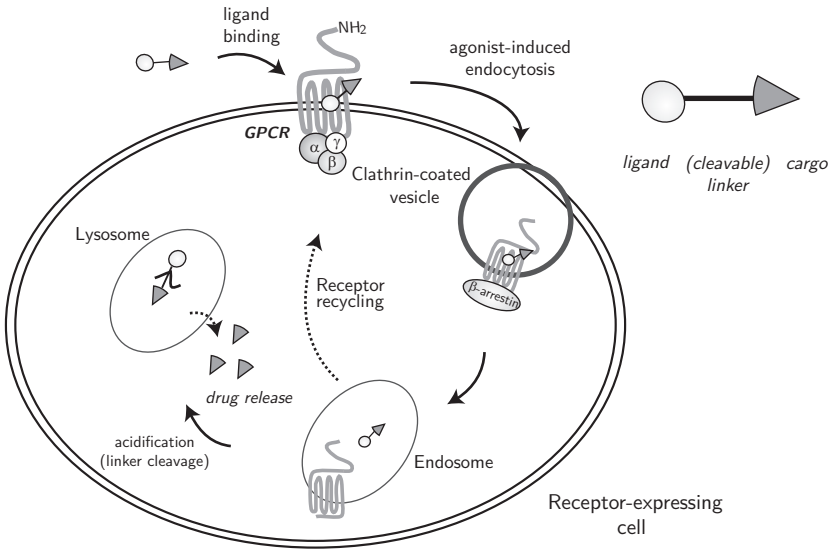
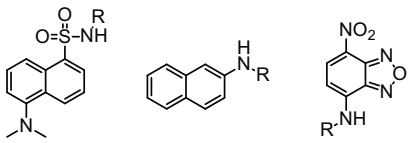


Figure 1.3: Graphic representation of agonist-induced internalization of GPCRs and a possible trafficking route of the ligand-drug conjugate.

Alternatively, the ligand dissociates from the receptor in the endolysosomal pathway. It is then either trafficked further to the lysosomes, or it diffuses out of the vesicles into the cytoplasm (Figure 1.3).

The discovery of allosteric binding sites, topographically distant from the orthosteric site, created novel opportunities for the design and development of structurally diverse ligands.[15–17] The glycoprotein hormone receptors, follicle-stimulating hormone receptor (FSHR), luteinizing hormone receptor (LH/hCGR) and thyroid-stimulating hormone receptor (TSHR), for example have a large N-terminal hormone binding domain and an allosteric binding site in the seven-transmembrane domain.[18] Many low molecular weight ligands that bind to the allosteric sites of these receptors have been developed[19–22] to interfere with their biological activity. There are, however, only limited examples of small-molecule GPCR ligands that are used for targeting purposes. One of the reasons for this is that structural alterations, required to introduce a chemical ligation handle needed for attachment of a label or a drug, can have a large influence on the affinity, potency and selectivity of a small molecule. For this approach to work, detailed structure-activity relationship (SAR) studies are often needed to arrive at the most optimal structure that still complies with these criteria.[23] Based on a low molecular weight dihydropyridine (DHP) agonist of the FSHR,[24,25] a SAR study was conducted to find the optimal length and positioning of a ligation handle-containing PEG-spacer (see Chapter 6). The optimal structure obtained from this was employed in the synthesis of DHP-analogues containing a fluorescent dye, with the aim to develop a diagnostic tool to visualize receptor binding, internalization and trafficking (see Chapter 7).



3 dansyl | **4** naphtylamine | **5** NBD

Figure 1.4: Structures of small fluorophores dansyl, naphtylamine and NBD.

Fluorescent ligands for several GPCRs have been reported in the literature, sometimes with unexpected changes in the pharmacological profile from agonist to antagonist.[26,27] Most examples make use of relatively small fluorophores such as dansyl **3**, naphthylamine **4** or NBD **5** (7-nitrobenz-2-oxa-1,3-diazol-4-yl) (Figure 1.4) to diminish the influence of the dye on the binding properties of the ligand. A drawback hereof is that these dyes consequently have short emission and excitation wavelengths, limiting their use in a cell- or tissue-based system because of high levels of autofluorescence (Figure 1.4).[26,28] Some recent studies have successfully employed red-emitting dyes in the synthesis of low molecular weight fluorescent ligands to visualize receptors. Holtke *et al*. synthesized a near-infrared Cy5.5-conjugate of the nonpeptidyl selective endothelin A receptor antagonist PD156707 (**6**, Figure 1.5) which was shown to bind to the receptor with similar affinity as the lead compound and could be used to visualize the receptor on different cell types.[29] By introduction of a conjugation handle onto the core of SR144528, a cannabinoid CB_2 receptor ligand, Bai *et al*. could introduce a near-infrared dye. No thorough studies were performed on the phar-

macological properties of the resulting fluorescent ligand NIR-mbc94 (**7**, Figure 1.5), but it was shown that receptor expressing cells were much more fluorescent than wildtype cells and that this fluorescence could be competed with non-labeled SR144528.[30] In a follow-up study, NIR-mbc94 was shown to be indeed a selective ligand for the CB_2 receptor albeit with a much lower binding affinity than the lead SR144528 (K_i =260 nM vs 1 nM). Endogenous levels of receptor could be fluorescently labeled and quantified using this fluorescent ligand, showing its potential for use in a high-throughput screen for novel CB_2 receptor ligands.[31] The potential of fluorescent ligands when used together with advanced confocal fluorescence microscopy techniques, such as fluorescence correlation spectroscopy (FCS), has been shown with fluorescently labeled adenosine A1 and A3 receptor ligands. In these studies, the ligand-receptor interactions could be thoroughly studied which led to the identification of two distinct agonist-occupied receptor populations, based on their motility in the membranes of living cells.[32,33]

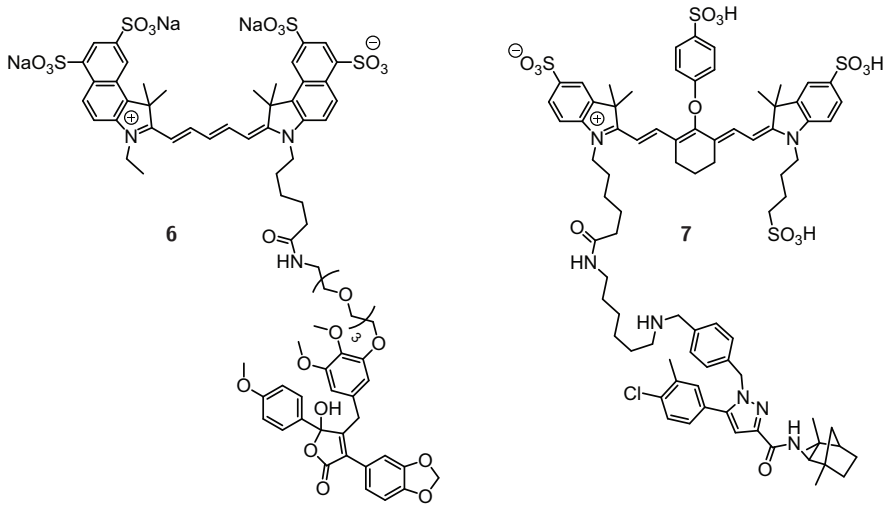
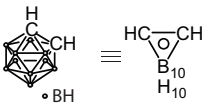


Figure 1.5: Near-infrared fluorescent GPCR ligands Cy5.5-PD156707 **6** and NIR-mbc94 **7**.

GPCRs that have endogenous peptide or protein ligands have been more often explored for targeted delivery, because of the ease of access to a synthetic peptide (fragment) by solid-phase peptide synthesis (SPPS). Besides, SPPS enables peptide modification by extension of its N- or C- terminus or by introduction of an unnatural amino acid. The Y receptor family is regulated by abundantly present endogenous neuropeptide Y (NPY) ligands.[34] Shorter fragments of the endogenous peptides or peptide-like structures resembling the C-terminus have been synthesized and were shown to be antagonist or agonists for the different subtypes of Y receptors.[35] The scope of the use of NPY ligands has expanded by the discovery of the presence of the Y_1-receptor subtype on breast and metastatic tumors.[36] Radiolabeled Y_1-selective peptides were subsequently used *in vitro* as well as *in vivo* as a diagonistic tool to visualize the overexpressed receptor in breast tumor tissue and metastases sites.[37] This result prompted the researchers to investigate the use of NPY ligands

to deliver a cytotoxic agent to the tumor site. In an approach analogous to that described in Chapter 8, the agent of choice was an *o*-carborane moiety. Carboranes are boron-rich lipophilic polyhedral structures with potential application in a binary anti-cancer therapy named boron-neutron capture therapy (BNCT).[38] Both individual components (carboranes and thermal neutrons) are relatively non-toxic in themselves, whereas a combination of both leads to a nuclear reaction and release of high energy particles that are detrimental for the cells which are exposed to it. A prerequisite for effective therapy is a high concentration of boron preferentially accumulated in tumor cells as opposed to healthy cells. Intracellular delivery would allow for the most efficient approach, since the trajectory of energy release by the formed α-particles is estimated to coincide with the average diameter of a cell.[39]



8

Figure 1.6: Different depictions of *o*-carborane

Attachment of *o*-carborane (**8**, Figure 1.6) to a Y_1R selective [F^7, P^{34}]-NPY derivative via the ϵ-amino group of lysine-4 resulted in a slight loss in binding affinity and diminished activity compared to [F^7, P^{34}]-NPY. Affinity and activity were still in the nanomolar range, however, and the ligand was shown to be selective for the Y_1R over the Y_2R subtypes. This peptide was also capable of inducing receptor-internalization of only Y_1R as seen by microscopy studies on HEK293 cells transiently expressing human Y_1R conjugated to enhanced yellow fluorescent protein (EYFP). It remains elusive what the fate of the carborane-containing ligand was in this system, since it could not be visualized.[40]

The overexpression of peptide hormone receptors such as the somatostatin receptor and the gonadotropin-releasing hormone (GnRH) receptor in various tumor tissues has also been established.[41] The activities of toxic conjugates of a peptide-based GnRH receptor agonist with doxorubicin or an analogue hereof, 2-pyrrolino-DOX, were compared with that of the cytotoxic drug alone. Targeted analogues were shown to be much less toxic *in vivo* while having significantly more antitumor activity on tumors that did express the receptor.[42,43] The doxorubicin containing analogue (AN-152) is currently being evaluated in clinical trials.[44] The somatostatin receptor (sst) is present on many neuroendocrine tumors.[45] Synthetic analogues with enhanced plasma stability and affinity towards sst compared to endogenous somatostatin peptides have been described. These analogues, octreotates, consist of the pharmacophoric part of somatostatin and contain a threonine at the C-terminus (**9**, Figure 1.7). Various radiolabeled octreotate analogues have been reported that find use as radiotracers or in radionuclide therapy.[46,47] Conjugation of a cyanine dye to octreotate yielded a fluorescent analogue (**10**, Figure 1.7) that was shown to be internalized in sst-expressing cells and accumulated specifically in primary human neuroendocrine tumor cells. This example shows the potential of synthetic ligands for tumor visualization.[48] The use of octreotate as a tumor targeting entity for BNCT has also been explored. Mier *et al*. have attached *closo*-borane mercaptoundecahydrododecaborate (BSH) to maleimide-conjugated Tyr^3-octreotate, but no biological studies were conducted.[49] In a more recent

study by Betzel and co-workers, one or two *o*-carboranes were attached to octreotate via linkers of different size. *In vitro* radioligand binding studies showed these analogues to be potent binders of the various sst-subtypes with enhanced selectivity for the sst_2-subtype compared to the reference peptide somatostatin 28 (SRIF-28). Binding was shown to be dependent on spacer length, with increased binding at longer length, and on the number of carboranes attached, with one (**11**, Figure 1.7) being favored over two (**12**, Figure 1.7). Although these compounds show potential for use as targeting agents for BNCT, no specificity or internalization data is currently available.[50]

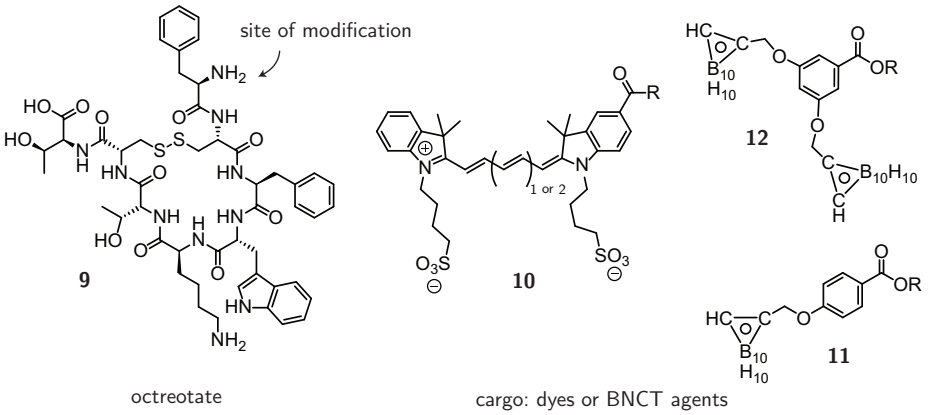


Figure 1.7: Synthetic somatostatin analogue (octreotate) used to target fluorescent dyes or carboranes for BNCT to sst-expressing tissues.

Lectin receptors

Proteins that are able to recognize and bind carbohydrates such as glycan chains present on glycoproteins, are collectively named lectins. These can be soluble or membrane-bound, and the latter category encompasses an increasing variety of lectin receptors.[51] With the advancements in carbohydrate synthesis, such as solid-phase methods, more complex oligosaccharide structures have become within reach. Although the lectin binding properties of these oligosaccharides have often been investigated, little work has been done on the use of synthetic carbohydrates as targeting entities.[52] In analogy with the work discussed above and in Chapter 8, some work on carbohydrate-carborane compounds as targeted BNCT-agents has been performed. An additional advantage of the use of carbohydrates in this context is their hydrophilicity, thereby somewhat compensating the hydrophobic nature of the carborane cage. Orlova *et al*. conjugated the disaccharide lactose to *o*-carboranylacetic acid with the aim of targeting lactose-binding lectins on melanoma cells. No biological evaluation of the compounds was conducted however, and in a follow-up study it was found that *o*-carborane at the α-position of a carbonyl results in deboronation to the corresponding *nido* compound when dissolved in water or methanol.[53] Tietze *et al*. have published several papers on the subject of glycoside modified carboranes for BNCT.[54,55] In their studies, carboranes were introduced by the reaction of decaborane with a terminal or in-

ternal alkyne on either *O*- or *C*-glycosides. *In vitro* studies on C6 rat glioma cells and B-16 melanoma cells showed dose-dependent uptake of a maltoside-carborane construct resulting in much higher intracellular boron levels than obtained with currently approved BNCT agents. *In vivo* studies in rat showed accumulation in brain tumor tissue, but similar concentrations were found in the blood, resulting in unwanted side-effects.[54] It remains elusive whether these constructs are taken up by cells in a receptor-dependent fashion and if so, which receptor is responsible for the uptake.

Cells of the immune system, especially dendritic cells (DCs) and macrophages, express different C-type lectin receptors which are characterized by their calcium dependency in ligand binding and conserved carbohydrate recognition domains (CRD). An important member is the macrophage mannose receptor (MR, CD206) which recognizes oligosaccharides terminating in mannose, fucose or N-acetylglucosamine. The receptor contains eight CRDs (Figure 1.8), which have been re-named C-type lectin-like domains (CTLDs) because most of the domains lack ligand binding activity.[56,57] Both CTLD4 and CTLD5 have been implied as the domains involved in ligand binding.[58] Having two ligand binding domains within one polypeptide chain allows for a stronger interaction with multivalent ligands and possibly ligand specificity, although the MR is also able to capture antigens with single mannose residues.[59,60] Besides the MR, dendritic cells express other mannose-binding C-type lectins, such as dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN, CD209) (Figure 1.8).

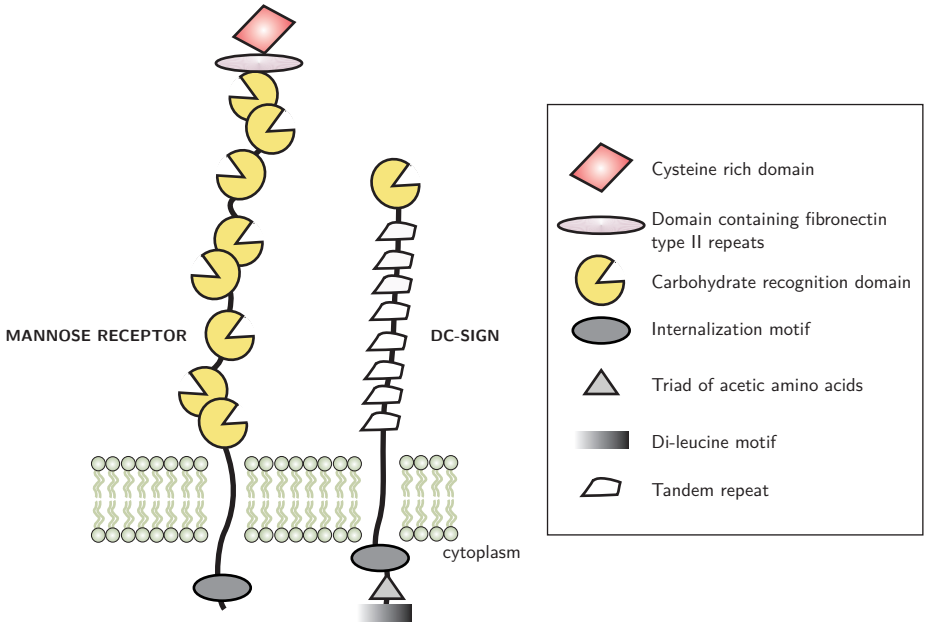
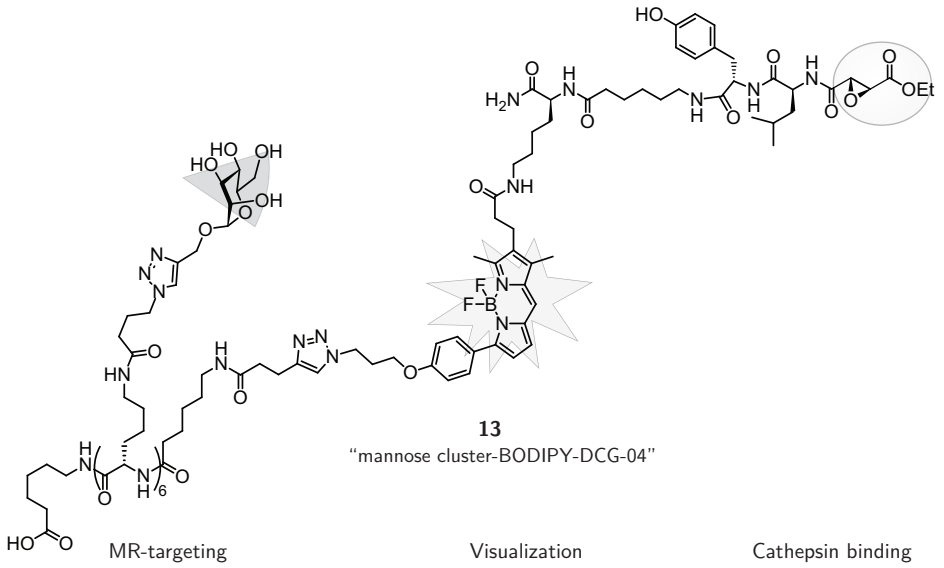


Figure 1.8: Schematic representation of the mannose receptor and DC-SIGN.

DC-SIGN belongs to type II C-type lectins, with an intracellular N-terminus, whereas the MR is a type I receptor, with an extracellular N-terminus. DC-SIGN contains only one CRD, but has been shown to multimerize in order to bind multivalent ligands with high affinity.[59,61] Furthermore, structural examination of DC-SIGN bound to either a high-mannose *N*-linked oligosaccharide or a complex-type *N*-linked glycan showed an unexpected interaction between the receptor and internal mannose residues.[62] Both receptors share an intracellular motif that allows internalization of the receptor in clathrin-coated pits. Targeting of either receptor might thus result in the selective internalization of ligands into the endolysosomal pathway of professional antigen-presenting cells.

Kikkeri *et al*. used carbohydrate modified quantum dots which showed enhanced uptake in liver cells when used *in vivo* compared to unmodified quantum dots. This finding suggests that receptor-mediated uptake, via the mannose receptor when using mannose or the asialoglycoprotein receptor when using D-galactose conjugated to quantum dots, plays a role in their endocytosis.[63] In a study by Hillaert *et al*., six propargyl mannoses were clicked onto an azide-modified peptide scaffold in order to obtain a multivalent 'mannose cluster'. This cluster was conjugated to the fluorescent activity-based probe (ABP) BODIPY-DCG-04 with the aim to deliver the ABP to the lysosomes of DCs and macrophages. It was indeed shown that incorporation of the cluster led to temperature-dependent internalization of the compound **13** (Figure 1.9), indicating a receptor-mediated process. Uptake and subsequent cathepsin labeling by the ABP could be blocked by addition of mannan, a natural occurring oligomannoside, to the medium of DCs, further establishing the receptor dependency. In Chapter 2 an extension of this work is described. Instead of a regular BODIPY dye, a pH-activatable BODIPY dye was incorporated in the mannose cluster-BODIPY-DCG-04 construct.



13
"mannose cluster-BODIPY-DCG-04"

Figure 1.9: Targeted fluorescent activity-based probe for cathepsins.

The pH at which the BODIPY dye becomes fluorescent is dependent on the alkyl-substituents on the *meso*-aniline group and is thus synthetically tunable (see also Chapter 3). With the use of multiple constructs incorporating BODIPY dyes with differing pH-dependencies, live-cell imaging experiments could be performed to study the trafficking of these compounds in the endolysosomal pathway.[64]

Targeting of larger cargo, such as proteins, to lysosomes of specific cell-types lies at the basis of therapeutic intervention in lysosomal storage disorders. Enzyme replacement therapy (ERT) relies on the uptake of recombinant lysosomal enzyme into the endolysosomal pathway of disease-affected cells, to restore normal lysosomal function. In case of Gaucher disease, β-glucocerebrosidase (GBA1) is the enzyme of interest which needs to be targeted to mannose-binding lectins on macrophage (Gaucher) cells. Current ERTs for Gaucher use different approaches for glycan remodeling in order to expose core mannose or high mannose glycan chains on the recombinant GBA1. In Chapter 4 an alternative approach is investigated to target a protein to dendritic cells. Recombinant heat shock protein 70 (Hsp70) containing a C-terminal 'sortase sequence' (LPETGG) was conjugated to GGG-BODIPY-mannose cluster by employing the bacterial enzyme Sortase A. Uptake of the resulting Hsp70-BDP-MC in DCs was studied by confocal microscopy and gel analysis, showing that a relatively small synthetic entity such as the mannose cluster can function as a targeting device for a large protein.

In Chapter 5 the mannose cluster is modified to contain mannose-6-phosphate residues instead of mannoses, with the aim of targeting the mannose-6-phosphate receptor (MPR). The main role of the MPRs is to route newly synthesized lysosomal proteins from the *trans*-Golgi network to the lysosomes. Although mainly localized intracellulary, a fraction of the receptor population is continously recycling between the cell membrane and the intracellular environment. Zhu *et al*. have used synthetic M6P-containing oligosaccharides to target recombinant acid α-glucosidase to M6PR-expressing muscle cells affected by Pompe disease.[65,66] Dephosphorylation of M6P by serum phosphatases, yielding mannose, results in enhanced uptake by MR-expressing liver cells and less or no selective delivery to the intended target site. More stable M6P analogues that do have receptor affinity but are not a substrate for phosphatases are therefore interesting alternatives. Several isosteric and non-isosteric analogues of M6P have been synthesized and tested for their receptor binding affinities.[67] Isosteric mannose-6-phosphonate (M6Pn) was shown to have similar affinity for the CI-M6PR as M6P and would be a suitable alternative as targeting entity.[68] As an example hereof, Barragan *et al*. have shown that vesicles containing M6Pn coupled to a steroid are targeted to MCF-7 breast cancer cells. Although promising, conclusive experiments for the involvement of the CI-M6PR in their uptake have not been conducted as yet.[69]

Folate receptor

The best studied example of a small molecule used to target a cell membrane receptor is likely that of folic acid targeting the folate receptor. Folic acid is the oxidized form of folate, an essential vitamin for mammals. Capturing of exogenous folate by cells is thus of great importance. Two transporters for folate have been identified, the "reduced-folate carrier" system, a low affinity carrier which is present on virtually all cells, and the folate receptor (FR), a high affinity binder of folic acid and highly overexpressed on various tumor cells.[70] The low affinity of the reduced-folate carrier system for folic acid, combined with the favorable expression pattern of the FR makes folic acid a highly suited organic molecule for tumor targeting.[71] Furthermore, folic acid retains its high affinity for the FR when conjugated through its γ-carboxyl group, is stable, readily available and cheap.[72] Leamon and Low demonstrated for the first time that covalent attachment of folic acid to macromolecules such as serum albumin or horseradish peroxidase results in their uptake in living cells via the folate receptor.[73,74] Since then many examples have appeared in the literature of folic acid-conjugates for various application such as radiotherapy, contrast agents and immuno- or chemotherapy.[70,75,76] A near-infrared dye was conjugated to folic acid, resulting in a tumor-targeted imaging agent that was shown to accumulate in ovarian cancer tissue *in vivo*.[77]

FR-mediated endocytosis occurs in caveolae, in a clathrin-independent fashion.[78] The resulting endosomes have been shown to be recycling and because folic acid retains its high receptor-affinity inside endosomes, the amount of folate-conjugated drug which is released inside the cell is limited. The pH of the FR-containing endosomes remains relatively high (average $\sim$6.5) and folate-drug conjugates that rely on an acid-cleavable linker system for drug release have been shown to be ineffective.[76,79] A better strategy is to make use of the reducing properties of the endosome by introduction of a self-immolative disulfide linker between folic acid and a drug of choice.[80] The applicability of this approach was shown by Yang *et al*. who introduced a FRET pair (BODIPY-FL/rhodamine) onto folic acid via an S-S linkage (**14**, Figure 1.10). Upon cleavage of the disulfide linker in the endosomes, the two dyes are dissociated resulting in loss of the FRET signal and recovery of the green fluorescent signal.[81] Conjugation of the maytansinoid DM1, a highly cytotoxic drug, to folic acid via an S-S-containing linker led to selective targeting of and cytotoxicity in FR-expressing cells.[82] The many successful *in vitro* and *in vivo* examples of folate receptor-mediated targeting open up the way for clinical trials to further investigate the potential of folate conjugates as targeted immuno- or chemo-therapeutic agents.

Conclusion

The examples highlighted in this chapter show that small-molecule synthetic ligands hold a lot of promise as targeting entities. Organic chemistry allows for a thorough evaluation of possible sites of ligand modification and linker systems and for the development of new classes of ligands for unexplored receptors. Furthermore, synthetic dyes have greatly advanced the imaging applications and synthetic approaches to cytotoxic molecules such as carboranes for BNCT are ever expanding. To arrive at clinically relevant targeting entities,

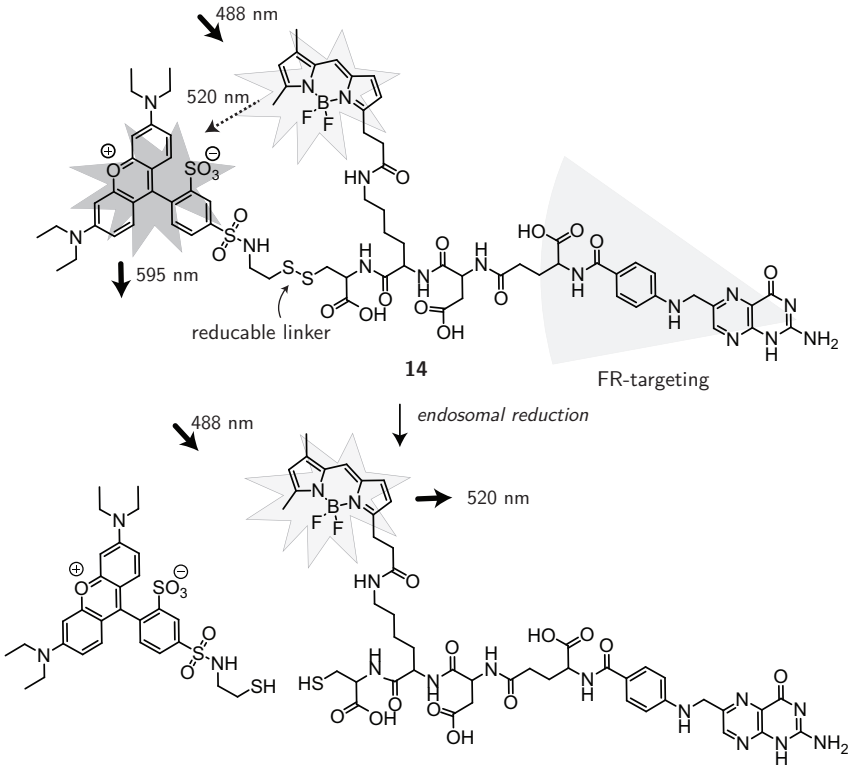


Figure 1.10: Endosomal uptake via the folate receptor was studied using a FRET dye pair linked to folic acid via an S-S linker. Irradiation with 488 nm light resulted in a red (595 nm) FRET signal. Upon cleavage of the disulfide bridge under endosomal reducing conditions, the two dyes were dissociated and fluorescence emission changed to green (520 nm).

either as diagnostics or therapies, however, *in vitro* binding assays to assess the ligand-receptor interaction are insufficient. Biochemical experiments addressing receptor pharmacology, and the behaviour of the ligand-cargo constructs in a more complex setting such as cells, tissue or *in vivo* are essential. When chemistry and biology are highly integrated much can be gained, as shown by the few examples that have resulted in well-defined receptor-ligand pairs that can be used for efficient targeting. In that way, a variety of 'magic bullets', each with its own target can be accomplished.

Aim and outline of this thesis

The research described in this thesis aims at the development of constructs containing a receptor-targeting entity covalently bound to biologically active cargo. Chemical and biological approaches are combined to arrive at the ultimate goal: selective delivery of the cargo to a cell-type, tissue or organelle of interest. In all cases, a fluorescent dye is incorporated to visualize the construct by fluorescence microscopy or in-gel fluorescence scanning. In Chapters 2, 3 and 8, pH-dependent BODIPY dyes are investigated with the aim to selectively visualize intracellular acidic compartments. Targeted receptors that will be

discussed include mannose-binding lectins (Chapters 2 and 4), the mannose-6-phosphate receptor (Chapter 5) and the follicle-stimulating hormone receptor (Chapters 6-9). With the exception of the work described in Chapter 9, where recombinant FSH is used as the targeting entity, the ligands that are employed are synthetic and (relatively) low molecular weight in nature. A variety of cargo is introduced, ranging from an activity-based probe (Chapters 2 and 5), carboranes for BNCT (Chapter 8) to the protein Hsp70 (Chapter 4). In the concluding Chapter 10 the findings are summarized and alternative and future strategies towards targeted delivery of these receptors are discussed. Broader applications of some of the synthesized entities, such as pH-dependent dyes and functionalized carboranes, are presented here as well.

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