Tumor Cell Targeting of Stabilized Liposome Conjugates

*Experimental studies using boronated DNA-binding agents*

BY

ERIKA BOHL KULLBERG

ACTA UNIVERSITATIS UPSALIENSIS
UPPSALA 2003
Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbecklaboratoriet, Uppsala, Friday, June 6, 2003 at 13:15 for the degree of Doctor of Medical Sciences. The examination will be conducted in English.

Abstract

To further develop cancer therapy, targeted delivery of cell killing agents directly to tumor cells is an interesting approach. This thesis describes the development of PEG-stabilized liposome conjugates targeting either epidermal growth factor receptor (EGFR) using its natural ligand EGF, or human epidermal growth factor receptor 2 (HER-2) using the antibody trastuzumab. Both receptors are known to be overexpressed on a variety of tumors. The liposomes were loaded with the boronated compounds water soluble boronated acridine (WSA) or water soluble boronated phenantridine (WSP), compounds primarily developed for boron neutron capture therapy, BNCT.

The liposome conjugates bound specifically to their receptors in cell culture. Because the WSA conjugates exhibited the most favorable boron uptake this compound was chosen for further study. The WSA-loaded liposome conjugates was internalized, an important characteristic for BNCT, and had a long retention inside the cells. The cellular localization of WSA, studied using fluorescence was found to be mainly cytoplasmic.

To increase the boron uptake studies comparing different incubation methods was performed. It was shown for both EGF and trastuzumab targeted liposomes the uptake could be increased over 10 times by changing from incubation in monolayer culture to incubation in cell suspension in roller flasks. With this treatment the boron concentrations reached after 24 h incubation time was 90 ppm for EGF-liposomes and 132 ppm for trastuzumab-liposomes, levels that are clinically interesting.

To study the cell-killing efficacy of the liposome-conjugates an experimental BNCT study was performed using EGF-liposome-WSA on cultured glioma cells. About half the number of thermal neutron was needed to inactivate 90% of the cells if the cells had been incubated with EGF-liposome-WSA compared to control cells. When comparing the survival to dose it was shown that to inactivate 90% of the cells 2.9 Gy was needed for EGF-liposome-WSA and neutrons compared to 5.6 Gy with $^{137}$Cs gamma.

The biodistribution of EGF-liposomes was also studied in mice. It was compared to EGF and it was found that the addition of a PEG-stabilized liposome to EGF significantly reduced EGF uptake in liver and kidneys, the circulation time in blood was prolonged as well. The reduced liver uptake might be due to inability of the 100 nm liposomes to pass the sinusoidal fenestrations of the liver and bind to the EGFR-rich hepatocytes. The reduced liver uptake potenitates the use of EGF-liposome conjugates for systemic injection.

Keywords: Liposome, EGF, HER-2, Targeting, boron

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ISSN 0282-7476
ISBN 91-554-5647-2
urn:nbn:se:uu:diva-3435 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-3435)
“You've got to work hard, you've got to work hard. If you want anything at all”

_Depeche mode_
This thesis is based on the following papers, which will be referred to in the text by their roman numerals I-VI


Reprints were made with kind permission from American Chemical Society (I), International Journal of Oncology (II) and Kluwer Academic/ Pleunum Publishers (III)
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<td>55</td>
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>AR</td>
<td>Amphiregulin</td>
</tr>
<tr>
<td>BMRR</td>
<td>Brookhaven medical research reactor</td>
</tr>
<tr>
<td>BNCT</td>
<td>Boron neutron capture therapy</td>
</tr>
<tr>
<td>BNL</td>
<td>Brookhaven national laboratory</td>
</tr>
<tr>
<td>BOPP</td>
<td>Boronated protoporphyrine</td>
</tr>
<tr>
<td>BPA</td>
<td>Borophenylalanine</td>
</tr>
<tr>
<td>BSH</td>
<td>Sulphhydryl borane</td>
</tr>
<tr>
<td>CAT</td>
<td>Chloramine-T</td>
</tr>
<tr>
<td>CBA</td>
<td>Carboranylalanine</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
</tr>
<tr>
<td>CPA</td>
<td>Carboranylpropylamine</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>DSPC</td>
<td>Distearoyl phosphatidylcholine</td>
</tr>
<tr>
<td>DSPE</td>
<td>Distearoyl phosphatidylethanolamine</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ETA</td>
<td>Pseudomonas exotoxin A</td>
</tr>
<tr>
<td>Fab</td>
<td>Monovalent antibody fragment</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma multiforme</td>
</tr>
<tr>
<td>GM₁&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Monosialoganglioside</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>Heparin binding EGF</td>
</tr>
<tr>
<td>HER-2-4</td>
<td>Human EGFR 2-4</td>
</tr>
<tr>
<td>HRG</td>
<td>Herugin</td>
</tr>
<tr>
<td>HTR</td>
<td>Hitachi training reactor</td>
</tr>
<tr>
<td>ICP-AES</td>
<td>Inductively coupled plasma-atomic emission spectrometry</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively coupled plasma-mass spectrometry</td>
</tr>
<tr>
<td>ID</td>
<td>Injected dose</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoproteins</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MPS</td>
<td>Mononuclear phagocyte system</td>
</tr>
<tr>
<td>NRG</td>
<td>Neuregulin</td>
</tr>
<tr>
<td>PDT</td>
<td>Photodynamic therapy</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>PPM</td>
<td>Part per million (µg/g)</td>
</tr>
<tr>
<td>RID</td>
<td>Radioimmunodiagnostic</td>
</tr>
<tr>
<td>RIT</td>
<td>Radioimmunotherapy</td>
</tr>
<tr>
<td>ScFv</td>
<td>Single-chain antibody fragment</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming growth factor alpha</td>
</tr>
<tr>
<td>WSA</td>
<td>Water soluble acridine</td>
</tr>
<tr>
<td>WSP</td>
<td>Water soluble phenantridine</td>
</tr>
</tbody>
</table>
1. Introduction and background

Cancer therapy today is efficient in treating solid tumors in places reachable for surgery or radiotherapy, the major treatment modalities today for primary tumors and large metastases. Chemotherapy is also effective in treatment of residual and spread disease in some tumor types, for example lymphomas. However, these treatment modalities cannot cure a large number of patients due to location of the tumor, the presence of disseminated cells or recurrence of a drug resistant disease. Targeted therapy might be of help when other curative treatments fail. Tumor therapy seeking out the disseminated cells in the bloodstream and lymphatic vessels and finding the residual cells after surgery is an appealing approach gaining interest.

Figure 1. The principle of tumor targeting with different carrier molecules. The targeting agent binds to targets on the cell surface and the toxic substances can execute their actions. A) An antibody with a toxic agent. B) A polymer chain conjugated to a targeting agent and loaded with toxic agents. C) A targeted liposome loaded with toxic agents.
1.1 Tumor targeting

Cells have on their surface specific molecules designed to regulate several processes such as differentiation and growth. These surface molecules can be overexpressed on tumor cells and are therefore referred to as tumor associated antigens. Tumors are also known to overexpress receptors for, for example, growth hormones, vitamins and lipids. These overexpressed structures can be targeted and used for therapy by an antibody or a receptor ligand to which a toxic substance of a radionuclide has been coupled.

**Figure 2.** A tumor cell with some of the more frequently targeted structures and examples of their targeting agents.

Antibodies, most frequently monoclonal antibodies, mAbs, are used to target tumor specific structures in several ways. In radioimmunotherapy, RIT, the radionuclide attached to the antibody is chosen to deliver local radiation energy in order to kill the targeted cells efficiently. The most frequently used radionuclides for therapy are $\beta$-emitters like $^{131}\text{I}$ and $^{90}\text{Y}$. In radioimmunodiagnostics, RID the same targeting principle is applied but the radionuclides are chosen to emit X-ray and gamma suitable for external detection, for example $^{111}\text{In}$ and $^{99m}\text{Tc}$. So far, successful therapy has been accomplished with haematopoetic tumors, such as non-Hodgkin's lymphoma. Antibodies targeting the tumor antigens CD-19, CD-20, CD-22 or CD-37 have been used with $^{131}\text{I}$ or $^{90}\text{Y}$ and have shown good specificity and therapeutic results (1, 2). A humanized antibody, rituximab, directed
towards CD-20 have shown good treatment effects (60% response) not just as a radiolabeled antibody, but also in itself. A non-humanized version of this antibody, ibritumomab, has shown response rates of 80% when labeled with $^{90}$Y (1, 2).

Tumor cells can also be eradicated using antibodies conjugated to toxic substances, such as ricin, genistein and pseudomonas exotoxin A (ETA). Antibodies can be used for immunotherapy by themselves or conjugated to a superantigen to evoke a more powerful immunoreaction towards the targeted tumor structure.

The use of antibodies has been hampered by the fact that most mAbs are derived from mouse and can therefore evoke an immune response towards the injected murine antibody thereby disabling further injections. By changing the non-binding parts of an antibody to human parts, a humanized chimeric antibody is created, being much more tolerated for repeated dosing. In some cases when a smaller targeting agent is needed, only the binding part of the antibody, the Fab fragment can be used. The smallest parts of the antibody, the variable regions, so called single-chain fragments, ScFv, can also be used for targeting.

If the targeted structure in question has a natural ligand, then this ligand, or a derivative of it, can be used for targeting. For the overexpressed epidermal growth factor receptor, EGFR, the ligand EGF can be used for targeting. More about this receptor and its ligand can be read below. The vitamin folate receptor is often overexpressed on various types of tumors, such as ovarian, colorectal and endometrial carcinomas (3), and the folic acid or folate has been used for targeted delivery of both radionuclides and drug carriers (3). Neuroendocrine tumors often overexpress the somatostatin receptor, and a somatostatin analogue, octreotide, has been used for both imaging and therapy of this kind of tumors (4). Many types of tumors also overexpress receptors for low density lipoproteins, LDLs. This has awakened the interest for use of LDLs as delivery vehicles for chemotherapeutics. Experiments have been performed to load anthracyclins into LDL particles with promising results regarding stability and toxicity (5, 6).

There are several ways to deliver the toxic agents with targeted therapy. The simplest are radiolabeled antibodies and ligands. To increase the amount of radionuclides or toxic agents, carriers can be attached to the targeting agent. Possible carriers are polymers such as dextranes, liposomes, and chelates. There are advantages and disadvantages for the use of large carrier molecules, the main advantage being the fact that more toxic agents can be loaded. Compared to a small peptide or ligand molecule, the larger constructs have a completely different circulation pattern and usually longer circulation times. This can be beneficial, giving
the construct more time to find disseminated tumor cells, but a large size might also give limited passage through capillary walls and therefore hamper the possibilities to target the tumor cells in normally vascularized tissue. Smaller molecules, on the other hand, can have a too quick passage through the body and be excreted before finding the tumor cells to exert their toxicity (4).

1.2 Liposomes

Liposomes are phospholipid bilayer spheres composed of lipophilic double membranes with an aqueous core. Liposomes have been proposed as drug-delivery vehicles since the mid 1970’s (7). Hydrophilic drugs can be loaded in the aqueous core and lipophilic drugs in the double membrane. The early liposome in vivo experiments, using large (>200 nm), often multilamellar liposomes (8), had problems with rapid removal from the bloodstream by cells of the mononuclear phagocyte system MPS (9). To circumvent this problem, sterically stabilized liposomes were constructed and examined during the late 1980’s, where a polymeric coat was used to shield the liposomes from opsonization and recognition by the cells of the MPS (9, 10). Two main formulations of stabilized liposomes were proposed: liposomes with monosialoganglioside, GM₁ (9) and liposomes with polyethylene glycol, PEG (10, 11).

Liposomes have been shown to gather in sites with increased capillary blood-flow and leaky vasculature, such as inflammations (12, 13) and tumors (14-16). This tumor-homing effect is used for all commercially available liposome formulations today.

1.2.1 Commercially available liposome formulations

Some of the most potent drugs for cancer therapy are the anthracyclins: doxorubicin and daunorubicin. Unfortunately their use is constrained by highly problematic systemic toxicities. For this reason the most studied drug delivery systems are designed to enhance or preserve the toxicity of anthracyclins against tumor cells but reduce the side effects for normal tissues, such as cardiotoxicity and bone marrow damage. Current active loading methods make it possible to load 10⁴ anthracyclin molecules into the aqueous core of each liposome.
Figure 3. The structure of a stabilized liposome. A) The polar headgroup of the phospholipid. B) The lipophilic tails of the phospholipid. C) The polymer brush (PEG) stabilizing the liposome and making it less prone to uptake by the immune system. D) Lipophilic drugs that can be loaded in the lipid bilayer. E) Hydrophilic drugs that can be loaded in the aqueous core.

The drug Myocet™ (Elan Pharmaceuticals) consists of doxorubicin enclosed in moderately sized liposomes (190 nm). Myocet™ gives limited prolonged circulation compared to free drug but reduces the toxicity due to altered biodistribution of doxorubicin. In trials where Myocet™ has been tested against free doxorubicin in metastatic breast cancer, the liposomal drug exhibited less cardiotoxicity and neutropenia (17). However, it remains a controversy whether Myocet™ is equally effective as the free drug (18).

For the drug DaunoXome® (Gilead Sciences) small liposomes (45 nm) loaded with daunorubicin are used. These liposomes have proven to extend circulation times due to their small size and rigid bilayer. The drug has been shown to be active against Kaposis sarcoma (19).

To increase the stability and circulation time the liposomes can be, as described above, coated with a layer of polyethylene glycol. This is the case for the doxorubicin loaded liposome formulation known as Doxil®/Caelyx® (Alza corporation). The liposomes are small (100 nm), rigid and coated with approximately 5 mol% PEG. They have been shown
to have a long circulation time, which increases accumulation in tumor tissue. Doxil®/Caelyx® has proved to decrease side effects of doxorubicin, such as nausea and hair loss (alopecia) significantly, but it also induces some new toxicities, the most noticeable being the palmar-plantar erythrodysesthesia known as hand-foot syndrome. This syndrome is due to the fact that the liposomes get stuck in the small capillaries of the palms and soles, giving rise to high local doxorubicin concentrations. This syndrome is the usual dose limiting toxicity of Doxil®/Caelyx®. The drug has been shown to be effective against a number of solid tumor types, such as Kaposi sarcoma (20, 21) and ovarian cancer (22, 23) and has also been tested for metastatic breast cancer with promising results (24, 25).

Table 1. Commercially available liposome formulations

<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
<th>Structure</th>
<th>Drug</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myocet™</td>
<td>Elan</td>
<td>liposome</td>
<td>Doxorubicin</td>
<td>Metastatic breast cancer</td>
</tr>
<tr>
<td>DaunoXome®</td>
<td>Gilead</td>
<td>liposome</td>
<td>Daunorubicin</td>
<td>Kaposi’s sarcoma (KS)</td>
</tr>
<tr>
<td>Doxil®/Caelyx®</td>
<td>Alza</td>
<td>PEG-liposome</td>
<td>Doxorubicin</td>
<td>KS, ovarian carcinoma</td>
</tr>
<tr>
<td>AmBisome</td>
<td>Gilead</td>
<td>liposome</td>
<td>Amphotericin B</td>
<td>Anti fungal</td>
</tr>
</tbody>
</table>

Not only drugs for tumor therapy have been developed. AmBisome (Gilead Sciences) is a liposomal formulation of amphotericin B proven very effective against fungal infections. Amphotericin B forms an ionic complex with the phospholipids in the bilayer and is not, compared to the formulations with anthracyclins described above, loaded in the aqueous core. AmBisome was designed as very rigid, small, unilamellar liposomes (<100 nm) with long circulation times (26). It has been shown that AmBisome liposomes preferentially bind to fungal cells and in some non-elucidated mechanism penetrate the fungal cell wall and can there execute its toxicities. The effectiveness of AmBisome relative to free Amphotericin B has been tested and showed to be slightly better for AmBisome for treatment of leukemia induced fungal infection (27). The side effect profile of AmBisome was significantly better than that of Amphotericin B (27).

In conclusion it can be said that the results of the today available commercial formulations using liposomes are not dramatically better compared to the free drugs, but since the toxicities seem to be lower in all
cases a higher dose might be given, thereby potentiating the use of liposome formulations.

1.2.2 Tumor targeting liposomes
To increase the tumor-specificity of liposomes a targeting agent can be attached. There have been several strategies for attachment of tumor targeting agents, but the prevailing and most successful is to attach the targeting agent to the distal end of a PEG molecule on the outside of the liposome. This has proved to increase the targeting ability compared to if the targeting agent is attached to the lipid head group (28). There have been several conjugation-chemical approaches to achieve this (29-32).

Several studies, both in vitro and in vivo, have been performed using targeted liposomes; so far none have performed clinical studies though. Among the most studied tumor targets with liposomes are the folate receptor, Human EGF receptor 2 (HER-2), CD-19 and the transferrin receptor.

A number of studies with folate-targeted liposomes loaded with anthracyclines (33, 34) has been performed showing that the folate receptor is suitable for liposome delivery with high specificity and internalization abilities. Several other therapies using liposomes targeting the folate receptor have been suggested, such as antisense delivery (35) and photodynamic therapy (PDT) (36). Liposomes targeting the folate receptor loaded with boronated compounds have also been studied with promising results (37-39).

The HER-2 has been studied as a target since early 1990’s. Suzuki et al (40) studied doxorubicin loaded liposomes with antibodies targeting either the p185 residue or the p125 residue, and it was shown that targeting p185 was superior. All further studies have targeted this epitope on HER-2. Goren et al. (41) showed 1996 that the uptake in cell culture was 16 times better for HER-2 targeted liposomes than non-targeted. Kirpotin et al (42) constructed immunoliposomes with Fab-fragments targeting HER-2, and they showed good binding and proven endocytosis in vitro. They also showed that the number of targeting molecules on each liposome could be optimized for increased uptake. For binding 40 Fab/liposomes was optimal, but for internalization a plateau was reached at 10-15 Fab/liposome (42). Park and co-workers (43-45) have studied HER-2 targeting extensively and have shown therapeutic efficacy in several animal studies. They have tested liposomes with both Fab-fragments and single chain fragments, ScFv, against the p185 epitope, and both conjugates showed equal effect. Immunoliposomes loaded with doxorubicin have been shown to be better
in animal studies than free doxorubicin, non-targeted liposomal doxorubicin or HER-2 antibody treatment (trastuzumab).

Lopez de Menezes et al (46-48) have targeted the tumor antigen CD-19 successfully on B-lymphoma cells both in vitro and in vivo. They have also performed ex-vivo experiments targeting CD-19 positive B-cells of multiple myeloma patient blood. The in vivo studies in mice showed that doxorubicin in immunoliposomes targeting CD-19 gave much better results than free doxorubicin or doxorubicin in non-targeted liposomes. As a test of the specificity, liposomes with a non-idiotype antibody was used with very limited uptake.

The transferrin receptor has been studied using transferrin-conjugated liposomes. Sarti et al. (49) showed that transferrin liposomes interacted specifically with cultured cells and that they were internalized via receptor mediated endocytosis. Inuma et al. (50) developed cisplatin loaded transferrin liposomes that proved to increase the cisplatin levels of disseminated tumor cells in ascites significantly. It was also shown by electron microscopy that gold labeled transferrin liposomes were located on the plasma membrane of cultured cells or in endosomes in the process of endocytosis.

1.3 The EGFR-family and its ligands

1.3.1 Receptors

The epidermal growth factor receptor, EGFR, is a 170 kDa transmembrane receptor present in many non-haematopoetic human tissues. It is composed of three major domains: a cysteine rich extracellular domain connected via a transmembrane lipophilic segment to an intracellular protein tyrosine kinase domain activated by ligand binding. The type 1 subclass of the tyrosine kinase receptor family does not only involve EGFR (ErbB-1), but also HER-2 (ErbB-2/Neu), HER-3 (ErbB-3) and HER-4 (ErbB-4). They all have the same basic structure as described above with high degrees of homology between the different receptors (51). The most highly related structure is the intracellular tyrosine kinase domain and the least related is the intracellular carboxylic terminal. The e-terminal region contains most of the tyrosines that, when autophosphorylated after activation, attract and bind specific substrates or adapter proteins involved in downstream signaling (51).

All EGF-receptors are involved in the mediation of proliferation and differentiation of normal cells and their importance in development has
been shown by the study of genetically modified mice (52). EGFR loss leads to embryonic or perinatal lethality with mice showing abnormalities in multiple organs. HER-2 knock-out mice died at mid-gestation due to malfunction in heart development. This phenotype is also shared by HER-4 knockouts. Mice that lack HER-3 develop a few days further but still have non-functional hearts and neural crest defects. These data show that the EGFR family plays critical roles in modulating specific aspects of vertebrate development. In the adult organism the receptors are also necessary. For example, mammary gland development is dependent on EGFR function and lactation is dependent on HER-2 and HER-4. Loss of HER-2 has been shown to delay the onset of puberty (52).

Table 2. The EGF-receptor family and its ligands

<table>
<thead>
<tr>
<th>Receptor</th>
<th>ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>EGF, AR, TGF-α, BTC, EPR, HB-EGF</td>
</tr>
<tr>
<td>HER-2</td>
<td></td>
</tr>
<tr>
<td>HER-3</td>
<td>HRG (NRG 1-2)</td>
</tr>
<tr>
<td>HER-4</td>
<td>BTC, EPR, HB-EGF, HRG (NRG1-4)</td>
</tr>
</tbody>
</table>

1.3.2 Ligands

EGF-family hormones are initially synthesized as membrane-anchored precursors that are subsequently cleaved to release soluble hormone. They are as mature hormones 50-60 amino acids long proteins and share a strong homology throughout 50 amino acids, in which the important feature is six characteristically spaced cysteines that form three intramolecular disulfide linkages. This defines a secondary structure comprising two sets of anti parallel β-sheet structures with little or no helical conformation (53, 54).

The family consists of epidermal growth factor (EGF), amphiregulin (AR), transforming growth factor alpha (TGF-α), betacellulin (BTC), epiregulin (EPR), and heparin binding EGF (HB-EGF) that bind EGFR; the three latter bind HER-4 as well. Neuregulins (NRG), of which the most well known is heregulin (HRG, NDF, NRG-1), bind to HER-3 and HER-4 (52-54). No natural ligand for HER-2 has yet been discovered. Increasing evidence suggests that it functions mainly as a co-receptor. If heregulin binds to HER-3/4 and the receptor heterodimerizes with HER-2, it has been shown that the ligand associates closely enough with HER-2 to be cross-linked (51). It has also been shown that HER-2 potentiates the other's signals when forming heterodimers (55).

EGF was first described by S. Cohen and was purified from mouse salivary gland. It was found to promote growth and development of
epidermal cells and was therefore named mouse epidermal growth factor, mEGF. It has 53 amino acids and a molecular weight of 6 kDa (56). It follows the general structure described above. H. Gregory described the human version later in the 1970s. It was found in human urine and inhibited gastric acid secretion. It was therefore named urogastrone. Human EGF and mEGF are cross-reactive and show 70% similarity of the amino acids and the three disulfide bonds are formed at the same relative positions (57).

1.3.3 Signaling

When a ligand binds its receptor dimerization takes place, either with another receptor of the same sort (homodimer) or with another receptor of the same receptor family (heterodimer). The dimerization starts a signal cascade that is dependent on the activating ligand, the receptor and the dimerization partner (54). A signal cascade starts with receptor phosphorylation of specific c-terminal sites that provide binding sites for adapter proteins such as Shc, Crk and Grb2, or kinases such as Src, Chk, and PI3K. All receptors can activate Shc or Grb2 to start the mitogen-activated protein (MAP) kinase pathway that results in DNA replication and proliferation. The HER-3 receptor is non-functional when homodimerized but very potent when dimerized with HER-2 (54). In fact HER-2 is the preferred heterodimerization partner for all EGFR-family receptors (55).

The receptor is down-regulated as a result of a response feedback loop. EGFR down-regulation of the receptor is mediated by internalization and degradation in lysosomes, a process known as endocytosis. (58). Tyrosine kinase activity greatly enhances this process by stabilizing receptor association with the endocytosis apparatus. (59). Eps15 is an example of an EGFR specific substrate that is involved in coated pit mediated internalization and is needed for endocytosis (60). This specificity for Eps15 of EGFR is probably why EGFR is the only EGFR-family receptor that is readily encycytesed when activated by its natural ligands (61).

1.3.4 In cancer development

EGFR is overexpressed in a variety of tumor tissues, for example in gliomas the EGF receptor gene has been shown to be amplified (62-64), as well as EGF-receptor mRNA (63). EGF-receptors have also been shown to be overexpressed in bladder carcinoma, where the expression was associated with the stage and grade of the tumor, indicating a poor prognosis (65). Also in breast cancer the overexpression of EGFR is
correlated with poor prognosis (51). Breast cancer cells have been shown to be potential targets for EGFR directed therapy, since EGF labeled with \(^{111}\)In has been shown to be selectively radiotoxic to breast cancer cells (66). EGFR overexpression has also been found in lung cancer (67) and in tumors of head and neck (68). Not only overexpression of the receptor has been found, the ligands EGF and TGF-alpha have proved to be overexpressed in gliomas as well. In a study by Ekstrand et al. all tested glioma tumors had mRNA expression of one or both of the ligands (69).

HER-2 receptor is known to be overexpressed in adenocarcinoma, especially in the breast (70) and in the ovary (71).

The EGFR family receptors have been shown to play many roles in the development of cancer, which might explain why they are overexpressed in tumors. Co-expression of HER receptors and ligands leads to receptor activation and stimulation of tumor cell proliferation and apoptotic resistance, thus providing a survival advantage. The HER-2 receptor signaling pathway has also been shown to impact neoangiogenes and tumor cell dissemination at several levels (70). In the breast cancer cells that overexpress HER-2, EGFR primarily forms heterodimers with HER-2, and the EGFR-HER-2 heterodimeres are impaired in EGF-induced endocytosis and downregulation. The impaired endocytosis leads to sustained signaling in response to EGF and subsequently stimulates the overproliferation and transformation of breast cancer cells (51, 72, 73).

Overexpression of HER-2 in breast carcinoma cells induces a mitogenic phenotype. Overexpression of HER-2 alone has been shown to be sufficient to increase cell migration and invasion. The EGFR-family receptors have also proved to mediate resistance to chemotherapy; expression and activation of HER-2 confers resistance to cisplatinum and tamoxifen but increases sensitivity to anthracyclins (70).

1.3.5 For cancer therapy

In therapy there is a number of ways that the activation pathway of the overexpressed EGF receptor system can be targeted. Antibodies can be used as antagonists of the ligands, peptides that inhibit receptor dimerization can be used, as well as drugs that block tyrosine kinase activity. Growth factors or antibodies conjugated to toxins or radionuclides can target the receptor structures (74).

Several EGFR-targeting monoclonal antibodies that have been able to inhibit proliferation of a variety of human cancer cell lines in culture and in xenograft models (75) have been developed. The most successful so far is mAb 225, which has been shown to target lung tumor and metastases in a phase I study. Unfortunately, an anti-mouse response was elicited, so a
chimeric version has been developed in order to be able to be injected repeatedly without evoking any immune response against the injected antibody. Since the tumor response after treatment with EGFR blocking antibodies is cytostatic rather than cytocidal, a tumoricidal moiety can be attached to the antibody. MAb 225 has been used as a delivery agent for toxins such as exotoxin A, ETA (75). Antibodies, most notably mAb 225, have also been shown to act as chemosensitizing agents in combination with chemotherapy (76).

The EGF molecule itself can be used as a targeting agent for cancer therapy. EGF-polylsines have been developed for oligonucleotide delivery and they have shown positive results in A549 cells (77). Several reports have used EGF as a targeting agent, multiple EGF-dextran conjugates have been developed for both radionuclide and boron delivery to tumors (78-80). Clinical trials have been undertaken studying the uptake of EGF-dextran in bladder carcinoma using bladder instillations (81). The use of EGF-chelates for radionuclide delivery has proved very promising (82) and clinical trials towards brain tumors are not far away.

There have been several trials performed using the EGFR tyrosine-kinase inhibitor ZD1839, Iressa, and it is shown to be effective towards various tumors with high selectivity for the EGFR. Its actions are mostly considered to be cytostatic, but cytocidal effects have been shown in combination with chemotherapeutics such as cisplatin and taxanes and in combination with radiotherapy as well (83).

Trastuzumab (Herceptin) is a humanized antibody against HER-2 that has proved to inhibit tumor growth in certain tumors (71, 84). The mechanisms behind this inhibition have been studied quite extensively and several hypotheses have been made. It has primarily been shown that trastuzumab arrests cells in the resting G1 phase of the cell cycle (85). Trastuzumab is also known to cause DNA strand breaks in HER-2 overexpressing cell lines BT-474 and SKBR-3. This might be the reason for synergy effects with chemotherapy, such as doxorubicin (86). The antibody is also known to mediate apoptosis for example in SKBR-3 cells (87).

Trastuzumab have been most frequently studied for therapy of HER-2 positive metastatic breast cancer, both as a single therapeutic agent (84) and in combination with chemotherapeutics (88). It was shown that the antibody inhibited tumor growth when used alone but had synergistic or additive effect when used in combination with the most common anticancer drugs (88). Unfortunately, the combination of trastuzumab and anthracyclins, though very potent towards tumor cells, proved to be too cardiotoxic (88). To prevent this toxic effect trials have been started studying the synergy of trastuzumab and Doxil®/Caelyx® (89).
1.4 BNCT

Boron neutron capture therapy, BNCT, is a binary therapy system which is very appealing. The tumor cells are loaded with high amounts of boron and thereafter irradiated with thermal neutrons (0.025 eV), resulting in the production of highly cell-toxic ionizing particles of short range with localization within the tumor cell.

Some atoms are known to have large cross sections for absorption of thermal or epithermal neutrons and $^{10}$B is one of them with a cross section of 3837 barn (1 barn = $10^{-28}$ m$^2$). The naturally occurring boron is a mixture of the two isotopes $^{10}$B (20%) and $^{11}$B (80%). $^{11}$B has a $10^6$ times smaller cross section for thermal neutrons. Therefore only $^{10}$B-enriched compounds are considerable in a therapeutic situation. Upon neutron capture $^{10}$B can undergo two reactions.

\[
^{10}\text{B} + ^1\text{n}_{\text{th}} \xrightarrow{(6.3\%)} ^7\text{Li}^{3+} \quad \text{(1.78 MeV) } R= 9.7 \text{ µm} \\
^{10}\text{B} + ^1\text{n}_{\text{th}} \xrightarrow{(93.7\%)} ^4\text{He}^{2+} \quad \text{(1.01 MeV) } R= 4.8 \text{ µm} \\
\]

\[
^{10}\text{B} + ^1\text{n}_{\text{th}} \xrightarrow{(6.3\%)} ^7\text{Li}^{3+} \quad \text{(1.47 MeV) } R= 8.0 \text{ µm} \\
^{10}\text{B} + ^1\text{n}_{\text{th}} \xrightarrow{(93.7\%)} ^4\text{He}^{2+} \quad \text{(0.84 MeV) } R= 4.2 \text{ µm} \\
\]

\[
^{10}\text{B} + ^1\text{n}_{\text{th}} \xrightarrow{(6.3\%)} \gamma \quad \text{(0.48 MeV)}
\]

Figure 4. Boron neutron capture reactions

In order to be effective, at least 10-30 µg $^{10}$B need to be delivered per gram tumor tissue corresponding to a concentration of 10-30 ppm. To obtain this concentration approximately $10^9$ $^{10}$B atoms need to be delivered to each tumor cell (90). The required dose is given by the dose limitations due to captures in naturally occurring elements in tissue. The most abundant are $^1$H and $^{14}$N with cross sections of 0.33 and 1.81 barns, respectively. The captures in $^1$H produce mainly gamma which gives a background dose to normal tissues in a therapeutic situation. $^{14}$N captures produces protons with a range of 10-11 µm, which can give dose limiting radiation effects in the irradiated area. A neutron fluence of about $10^{12}$-$10^{13}$ thermal neutrons/cm$^2$ is approximately the upper limit for normal tissue.

1.4.1 History

The use of $^{10}$B for medical purposes was depicted as early as 1936 by Locher (91). The first clinical trial of BNCT was initiated at Brookhaven
national laboratory (BNL) by Farr and Sweet in 1951. From 1959 to 1961 a series of patients received BNCT at the Brookhaven Medical Research Reactor (BMRR) of BNL (92). The malignancy chosen to study was glioblastoma multiforme, GBM, a localized brain malignancy with low survival. Despite aggressive efforts, no powerful treatment modality has emerged. Another group of patients was treated at the Massachusetts Institute of Technology (MIT) during 1959-1961 (93). These trials used four different boron compounds. Clinical results from these studies were disappointing and the last clinical BNCT trial for decades in the USA was performed in 1961 (92, 93). The disappointing results of all trials above were thought to depend on poor penetration of the thermal neutron beam and too little boron in the tumor; the tumor to blood ratios were less than one. Experiments using higher fluencies to ensure therapeutic levels at depth caused severe damage to the scalp in some patients; this might have been due to high boron concentrations in the blood (92, 93).

In Japan BNCT experiments were started in 1968. Dr Hatanaka, who had collaborated with Dr Sweet, began clinical trials at the Hitachi Training Reactor (HTR) using sulfhydryl borane Na\textsubscript{2}B\textsubscript{12}H\textsubscript{11}SH (BSH). Almost 150 patients with various forms of brain malignancies, mostly GBM, were treated. To ensure thermal neutrons in the tumor, Hatanaka et al irradiated their patients with open skulls. This was also done to prevent damage to the scalp, as seen in the US studies. Hatanaka showed some success with his studies including 9 patients with more than 10-year survival. Six of these were able to live a normal life without any signs of disease (94).

Hatanaka's results, though not undisputed, awakened the interest for BNCT in USA and Europe again. During the 1980's interest was focused on the compound borophenylalanine (BPA). It is a boronated amino acid that can be taken up in the cells by the naturally occurring amino acid transport system. One of the major problems with BPA is low solubility, which can be overcome by complexion with fructose (95).

A new higher energy neutron beam, with epithermal neutrons, was developed at BMRR. The epithermal neutrons are slowed down to thermal neutrons in the skull and the brain tissue. The use of BPA for clinical trials of GBM was approved by the FDA in 1994 and between 1994 and 1999 53 patients were treated. The results from the first 38 patients indicated no severe BNCT related toxicities. It was also shown that the time to progression was comparable to that after conventional treatment (96). In 1996 another study using BPA was initiated at MIT. In the MIT study 22 patients had been treated by 1999 and mixed results were obtained. Two patients exhibited a complete radiographic response, and 13 of 17 patients had measurable reduction in tumor volume for the first months after
irradiation, after which the disease either stabilized or increased. A number of acute side effects were noted, in particular effects due to increased intracranial pressure (97, 98). In Petten, the Netherlands, a study using BSH was started in 1997. After 26 treated patients it could be determined that no dose-limiting toxicities had been observed and no conclusions regarding the efficiency of the treatment compared to conventional treatment could be drawn (99, 100). Another study using BPA was initiated in 1999 in Finland, in which 18 patients without earlier radiation treatment have been treated. The one-year survival is estimated to be 61%. A different protocol accepting patients with previous radiotherapy has also started and no serious acute BNCT-related adverse effects has been encountered (101).

In 2001 the first BNCT study in Sweden was initiated at the Studsvik R2-0 reactor using an epithermal neutron beam, and for the first 17 patients no severe BNCT related acute toxicities have been observed. The compound used is BPA and high blood boron levels have been reached (102, 103). It is still too early to evaluate the efficacy of the treatment.

1.4.2 Compounds for BNCT

The compounds mainly used for clinical studies so far are the low molecular weight compounds BSH and BPA. In order to deliver more boron, carborane compounds were designed. A carborane is a boron cage containing 10 boron atoms. The first carborane compound constructed was an analog to BPA known as carboranylalanine, CBA. CBA was shown to improve boron uptake compared to BPA in vitro but not in vivo (104, 105), probably due to the lipophilicity of the carboranyl group (104).

Boron-containing nucleosides have also been of interest, as biosynthetically active tumor cells need building blocks for their DNA. Boron-containing moieties, either as a single dihydroxyboryl group or as a carboranyl moiety, have been inserted into pyrimidine nucleosides (106).

Other low molecular weight compounds have been of interest, such as boron containing porphyrines, of which the most studied is a boronated protoporphyrin known as BOPP (107). Since the protoporphyrin is a sensitizer for near infrared irradiation, the boronated protoporphyrin can be used for dual treatment, both BNCT and photodynamic therapy, PDT. BOPP was shown to augment boron uptake compared to BSH and BPA (107, 108) and bind selectively to glioma cells in vivo (109).

Proximity to DNA increases the lethality of the capture reaction (90) and therefore large interest has been put into the development of DNA-intercalating boron compounds based on the well known DNA-intercalating groups phenylphenantridine (110) and acridine (111).
compound based on naphtalemide has also been developed (112). Boron rich oligomeric phosphate diesters have also been shown to have DNA-binding properties (113, 114).

All compounds described so far are not tumor specific, or their tumor specificity is based on the increased metabolism of tumor cells. For tumor specific delivery, efforts have been made to develop boron-carrying antibodies or antibody fragments (114, 115). The problem with boron carrying antibodies is to obtain enough boron on each antibody, at least $10^3$ boron atoms per antibody is needed (115), and still retain immunoreactivity. If instead a carrier molecule with the possibility to deliver large amounts of boron is attached to the targeting molecule this problem might be circumvented. The most studied carrier molecules for tumor specific delivery of boron are starburst dendrimers (116), low density lipoproteins, LDL (117, 118), boronated dextrans (79, 80) or boron carrying liposomes.

1.4.3 BNCT and liposomes

Liposomes have been proposed as delivery agents for BNCT and have during the last decades been studied both with and without a targeting agent on the liposome.

Hawthorne and co-workers reported the use of liposomes as delivery agents for boron in 1992 (119). They showed that liposomes with mean diameters of 70 nm or less were capable of encapsulating high concentrations of water soluble ionic boron compounds, and that they were able to deliver this boron to subcutaneous tumors in mice. The boron concentrations reached over 15 ppm and the tumor to blood ration was over 3. Further studies (120) showed even better tumor uptake, 30-50 ppm and tumor /blood ratio of 5. They obtained similar results using liposomes where lipophilic boron compounds were encapsulated in the membrane (121). By using liposomes combining the two approaches, with both hydrophilic and lipophilic boron compounds, tumors in mice could be successfully targeted (122). The boron concentration reached 50 ppm and a tumor to blood ratio of 5-10 was obtained.

Metha et al (123) have studied BSH in liposomes with and without PEG. They were shown to have significant improvement in circulation time compared to free BSH after tail vein injection in mice. The circulation time proved to be the highest for the PEG-stabilized liposomes.

Moraes et al (124) developed liposomes loaded with the compound o-carboranylpropylamine (CPA). The results showed that the compound could be loaded into liposomes at a concentration of $10^4$ molecules/vesicle. Both PEG-stabilized and conventional liposomes were studied. It was
shown in cell culture that CPA toxicity decreased after liposome entrapment (CPA concentration 0.15 mM).

Table 3. Experiments with liposomes for boron delivery

<table>
<thead>
<tr>
<th>Liposome formulation</th>
<th>Boron compound</th>
<th>Target tissue</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-targeted</td>
<td>Ionic, water soluble compounds</td>
<td>Tumors in mice</td>
<td>(119, 120)</td>
</tr>
<tr>
<td>Non-targeted</td>
<td>Lipophilic boron compounds</td>
<td>Tumors in mice</td>
<td>(121)</td>
</tr>
<tr>
<td>Non-targeted w/wo* PEG</td>
<td>BSH</td>
<td>Mice without tumors</td>
<td>(123)</td>
</tr>
<tr>
<td>Non-targeted w/wo* PEG</td>
<td>CPA</td>
<td>Cultured glioma cells and lymphocytes</td>
<td>(124)</td>
</tr>
<tr>
<td>Anti CEA-liposomes</td>
<td>Ionic water soluble compounds</td>
<td>Pancreatic cancer cells in vitro and tumors in mice</td>
<td>(126,127)</td>
</tr>
<tr>
<td>Non targeted liposomes</td>
<td>Ionic water soluble compounds</td>
<td>Breast cancer cells in vitro</td>
<td>(125)</td>
</tr>
<tr>
<td>Folate-liposomes with PEG</td>
<td>Lipophilic boron compounds</td>
<td>KB cells in vitro</td>
<td>(39)</td>
</tr>
<tr>
<td>Folate-liposomes with PEG</td>
<td>Anionic boron compounds</td>
<td>KB cells in vitro</td>
<td>(37)</td>
</tr>
<tr>
<td>Folate-liposomes with PEG</td>
<td>Anionic boron compounds</td>
<td>Transplanted lung cancer in mice</td>
<td>(38)</td>
</tr>
</tbody>
</table>

* w/wo*, with or without

The experiments described above used liposomes without a targeting agent. Yanagie et al. (125-127) were the first to examine targeting liposome as a mean for boron delivery. They targeted the carcino-embryonic antigen, CEA, on pancreatic cancer cells and experiments in cell-culture showed that liposomes targeting CEA and enclosing $^{10}$B-compounds could, after neutron irradiation, inhibit cell-growth. The cell-growth was inhibited to approximately 30% after irradiation with $5 \times 10^{12}$ $\text{neq/cm}^2$. It was also shown, using the same conjugate, that pancreatic cancer tumor cells transplanted to mice could be growth inhibited after CEA-$^{10}$B-liposome targeting and neutron irradiation. The same group has shown that the growth of breast cancer cells could be inhibited by use of liposomes with $^{10}$B and neutron irradiation. No targeting agent was used in this study (125).

Recently Lee and co-workers in a series of publications studied the use of the folate-receptor for liposomal delivery of boron. Pan et al. (37) showed in a study that as much as 1500 $\mu$g boron /$10^9$ cells could be delivered. However, the uptake could not be blocked by adding an excess of folate. Using a different boron compound, specificity could be obtained,
and the boron concentration was still very high (around 500 µg /10^9 cells). In the next study (38) it was shown that boron could be delivered to implanted tumors in mice using folate receptor targeting liposomes. The biodistribution showed tumor to blood ratios up to 6 after 96 h. In another study a lipophilic boron compound was used and the uptake of folate-targeted liposomes in KB-cells was examined (39). It was shown that it was possible to deliver 587 µg boron/10^9 cells with high specificity. The corresponding uptake in cells not expressing the folate receptor was less than 10%.
2. Aims

The aim of this thesis is to develop liposome-ligand conjugates with specificity for EGFR or HER-2, and to study these conjugates in cell culture with regard to stability, uptake specificity and intracellular processing. Further, to evaluate the cell killing potency of the liposome conjugates in an in-vitro system after neutron activation. Finally, to evaluate the use of liposome conjugates for systemic injection by studying the biodistribution of EGF-liposomes compared to EGF in mice.
3. Materials, methods and techniques

Some of the most important materials and methods used in this thesis are presented here. For a more thorough description the reader is referred to the enclosed papers.

3.1 Liposomes

Liposomes are, as previously mentioned, phospholipid bilayer spheres. The ones used in this study were composed of disteaoryl phosphatidylcholine (DSPC), (57%), cholesterol (40%) and disteaoryl phosphatidylethanolamine-polyethylenglycol (DSPE-PEG), (3%). This giving rigid, stable and long circulating liposomes (128).

The liposomes were prepared by freeze-thawing and extrusion. The lipids were dissolved in chloroform and dried to a lipid film that was hydrated and heated to 60°C and thereafter frozen in liquid nitrogen. The freezing and heating was repeated seven times. The liposomes were then extruded ten times through a 100 nm filter to obtain liposomes of similar size. The size 100 nm was chosen because liposomes of that size have been shown to be the most stable in blood circulation (128).

Figure 5. Cryo-TEM picture of PEG stabilized liposomes loaded with WSA. The drug can be seen as the dark globular spots inside the liposomes. Bar is 100 nm. Photo kindly supplied by Markus Johnsson.
To get the boronated compounds into the liposomes, active loading techniques based on pH-gradients were used. Briefly, the liposomes have been prepared using a buffer with low pH (pH 4) and after extrusion the pH on the outside of the liposomes was raised to 7-8. The compounds for loading, in this case weak bases, were added to the solution and entered the core due to equilibrium. As a result of the lower pH on the inside, the compounds were protonated and thus trapped inside. This trapping of compound made the gradient behave like a pump, and approximately 98\% of added compound entered the liposome core. The molar loading ratios for the compound used in the papers herein were 0.2:1 and 0.1:1 (compound: liposome), giving approximately $10^4$ molecules per liposome. As much as $10^5$ molecules loaded per liposome have been performed during development of the production (129).

![Diagram of pH-gradient loading of drugs](image)

**Figure 6.** The principle for pH-gradient loading of drugs. Figure kindly provided by Markus Johnsson.

3.2 Boronated DNA-binding compounds

It has been shown that if the boron is located close to the DNA less amounts are needed (90). Therefore, DNA-binding boronated compounds have been developed. Two of them, water soluble boronated phenantridine, WSP, and water soluble boronated acridine, WSA, have been used in this thesis (110, 111) and were loaded into liposomes using the pH gradient loading technique described above (129).

WSP is composed of a phenylphenantridine chromophore coupled to a boron cage (10 boron atoms) and was used in paper II. The most commonly known phenylphenantridine is ethidium bromide, a widely used DNA stain in life science. The phenantridine ring system intercalates in the major groove, and the amino groups at position 3 and 8 are located inside the helix and interact horizontally with the sugar phosphate backbone. The phenyl group and the substituent at position 5 are externally positioned in
the minor groove. The substituent at position 5, where the boron cage is attached in WSP, has little or no effect on DNA binding (130, 131). The phenantridinium chromophore is fluorescent with an excitation around 546 nm.

WSA is composed of 9-aminoacridine, a boron cage with 10 boron atoms and spermidine tails that have been added to increase the water solubility and the affinity for DNA. The compound has been used in paper I-V. The acridine chromophore is known to interact with DNA in different ways. In one case the molecule is stacked between G-C base pairs with the 9-aminogroup pointing into the minor groove. In another case the drug molecule is intercalated asymmetrically between the bases of one strand only (130, 132). This depicts that chains attached to the 9-aminogroup (as the boron cage and spermidine tails in the case of WSA) do not interfere with the interaction (133). Boron-containing acridines were synthesized as early as 1967 (134), and the acridine moiety has also been proposed as an anti tumor agent labeled with, for example, $^{125}$I (135-137). The most well-known acridine compounds are the vital dye Acridine Orange and the drug Proflavine. WSA is fluorescent and was studied using excitation at 488 nm.

Figure 7. The chemical structures of the DNA binding compounds WSP and WSA.

WSP1 ($M_r = 599.7$)

WSA1 ($M_r = 746.1$)
As a comparison regarding DNA targeting we have also studied doxorubicin, a well-known anti-neoplastic agent that intercalates DNA. Doxorubicin has been extensively studied in liposomes. The commercially available liposome formulation Doxil®/Caelyx® uses doxorubicin as active agent. Doxorubicin was used in paper III and is also fluorescent with excitation 488 nm.

The toxicity of WSA and WSP were tested, using clonogenic survival in cell culture. WSP was shown to decrease survival significantly even at low (1 µg/ml) concentrations, while WSA was much better tolerated even up to 20 µg/ml. The toxicity was reduced for both compounds if they were enclosed in PEG-stabilized liposomes. For WSP the reduction in toxicity was striking: even at the concentration 20 µg/ml, only a slight reduction in survival could be seen (138).

3.3 Cellular models

The work in this thesis is largely based on studies of cultured tumor cells. The receptor specificity and intracellular processing of the conjugates can be studied in a cell-culture system, but more complex aspects, such as distribution and tumor selection, need to be investigated in studies using animal models. Tumor cells can be grown as monolayer culture on the bottom of culture dishes, as cell suspensions in culture medium in flasks, or as spheroids, three-dimensional cellular clusters. For this thesis three cell-lines have been used, all express the targeted receptors to a high extent.

A glioma cell line, U-343 MGa Cl2:6, has been used in paper I-III and V. The cell line is known to overexpress EGF-receptors and has approximately 10^5 receptors per cell (139). These cells have been used for monolayer culture, in pellet cell-suspension and in roller-flasks. These cells were also used for clonogenic survival after experimental BNCT in paper V.

A squamous carcinoma cell line, A-431, has been used in paper III and was chosen because of its large overexpression of EGF-receptors, approximately 10^6 receptors per cell (140). This cell line has been used for monolayer culture only.

A breast-cancer cell line, SK-BR-3, overexpressing the HER-2 receptor, the average number of receptors is 10^6, was used in paper IV. The cells were used in monolayer culture and in roller flasks.

All cell lines were grown in Ham’s F-10 medium, supplemented with 10% foetal calf serum, L-glutamine (2 mM) and PEST (penicillin 100 IU/ml and streptomycin 100 µg/ml) using humidified air at 37°C with 5% CO₂.
3.4 Boron determinations

In order to measure the boron concentration in samples the cells were digested in HNO\textsubscript{3} under heat and pressure (141). After digestion the samples were diluted in MilliQ water with 5% HNO\textsubscript{3} and then measured using either inductively coupled plasma-mass spectrometry (ICP-MS) or inductively coupled plasma-atomic emission spectrometry (ICP-AES).

In both procedures the liquid sample was injected in the inductively coupled plasma and the sample was atomized and ionized by the high temperature of the plasma (6000 K). Detection of which atoms were present was then performed with mass spectrometry or atomic emission spectrometry. The detection limits for boron of the instruments used are about 5 ppb and 50 ppb, respectively. ICP-MS was used for boron determinations in paper II-V and ICP-AES was used in paper V.

3.5 Neutron irradiation at the BNCT facility in Studsvik

A facility for BNCT has been built at the R2-0 research reactor at Studsvik. The filter/moderator system used for this study is the same that is currently employed for BNCT clinical trials (102, 103). The transport of neutrons generated by the reactor core, with a mean energy of 2 MeV across different materials (the filter), results in a final spectrum enhanced in the energy range between 0.4 eV and 10 keV. This is the range that is usually referred to as epithermal energy range. The slowing-down process (i.e. the shift of the neutron spectrum toward lower energies) is mainly driven by the combination of different layers of aluminum and Teflon, while the removal of the thermal neutron component, which cannot go deep into the tissue, is performed by a $^6$Li filter positioned just at the end of the beam. The cell sample irradiations of this study took place in a 20X 20X 15-cm\textsuperscript{3} PMMA phantom at a depth of 3 cm, where it had previously been found that the peak of the thermal neutron distribution produced is located.

3.6 Animals

Mice were used to study the biodistribution of EGF and EGF-liposomes in paper VI. Female NMRI mice were used, and they were housed in a controlled environment and fed ad libitum. Mice are known to have a large quantity of EGF receptors primarily in the liver (142), which makes systemic injection for tumor targeting with EGF less useful.
4. Preparation of liposome conjugates

The thesis is based on targeted stabilized liposome conjugates (summarized in table 4), and examines their construction and their behavior in vitro and in vivo. The most important aspects regarding the preparation and the optimizations made are presented in this chapter. For more thorough descriptions on the methods and materials used the reader is referred to the enclosed papers.

Table 4. The liposome conjugates used in paper I-VI

<table>
<thead>
<tr>
<th>Liposome conjugate</th>
<th>Conjugation method</th>
<th>Used in paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{125}$I-EGF-liposome</td>
<td>Maleimide-PEG-DSPE</td>
<td>I</td>
</tr>
<tr>
<td>$^{125}$I-EGF-liposome-WSA</td>
<td>—</td>
<td>I-III</td>
</tr>
<tr>
<td>$^{125}$I-EGF-liposome-WSP</td>
<td>—</td>
<td>II</td>
</tr>
<tr>
<td>$^{125}$I-EGF-liposome-DOX</td>
<td>—</td>
<td>III</td>
</tr>
<tr>
<td>EGF-3H-liposome-WSA</td>
<td>—</td>
<td>III</td>
</tr>
<tr>
<td>$^{125}$I-EGF-liposome-WSA</td>
<td>NHS-PEG-DSPE</td>
<td>V</td>
</tr>
<tr>
<td>$^{125}$I-EGF-liposome</td>
<td>—</td>
<td>VI</td>
</tr>
<tr>
<td>$^{125}$I-Trastuzumab-liposome-WSA</td>
<td>—</td>
<td>IV</td>
</tr>
<tr>
<td>Trastuzumab-3H-liposome-WSA</td>
<td>—</td>
<td>IV</td>
</tr>
</tbody>
</table>

4.1 Micelle-transfer procedure

Liposome conjugates were prepared using the micelle transfer (post-insertion) technique (figure 8) (31, 143, 144). Briefly, liposomes are prepared and loaded with the desired compound separately, and the targeting agent is added afterwards. The targeting agent is conjugated to the distal end of a PEG$_{3400}$-DSPE lipid. The ligand-PEG-DSPE lipids form micelles in solution that can be mixed with the liposomes. At a high enough temperature the ligand-PEG-DSPE lipids then transfer from the micelles into the liposome membrane.
Figure 8. Schematic drawing of the micelle-transfer method. EGF/trastuzumab is attached to DSPE-PEG-maleimide/NHS lipids in micelles. The EGF/trastuzumab lipids, in the form of micelles, are mixed with preformed liposomes, and the EGF/trastuzumab-PEG-DSPE molecules are thereby incorporated into the liposome membranes.

The use of this method is appealing in the sense that there is no need to change the whole conjugation procedure if a new targeting agent or a new load is desired. Another advantage is that the targeting agent is known to be situated in the outer membrane. Further, the fact that PEG3400 is used for the ligand conjugation and PEG2000 is used for stabilization in the liposome membrane gives an extra long spacer-arm for the ligand, making sure that it is extended for maximal contact with the receptors.

4.2 Radiolabeling of the targeting agent

EGF or Trastuzumab was labeled with $^{125}$I using the Chloramine-T (CAT) method. This is a direct labeling method in which the CAT oxidizes the iodine ion so it becomes positively charged. The iodine ion then undergoes electrophilic substitution with tyrosine residues. The reaction can take place under physiological pH when labeling with $^{125}$I and can therefore be performed with largely preserved biological activity of the labeled protein.
4.3 Optimization of micelle-transfer conditions for EGF-liposomes using maleimide-PEG-DSPE (paper I)

$^{125}\text{I-EGF}$ was modified with Traut’s reagent (2-iminothiolane) to get a free –SH group for conjugation. The modified $^{125}\text{I-EGF-SH}$ was conjugated to maleimide-PEG-DSPE for 24 h in room temperature. After purification, the EGF-lipid was transferred to the preformed liposomes as described above. The transfer conditions were optimized in paper I. The optimized parameters were temperature, time and initial PEG concentration in the preformed liposome (figure 9).

The times used for incorporation were 1, 4 and 24 h. For unloaded liposome no significant increase was apparent after 1 h, Therefore 1 h was considered optimal. For the WSA loaded liposomes longer incorporation time, 15 h, was used for the experiments in paper II since the low incorporation yield made us want to maximize the number of incorporated EGF-lipids. However, it was later concluded that the increase in yield with time was marginal for the WSA loaded liposomes as well, and the longer time only constituted an increased risk for EGF degradation. Therefore 1 h incorporation time was used for the experiments in paper III.

The temperatures studied were room temperature (RT), 37°C and 60°C. It was concluded that 60°C was by far the most efficient transfer temperature for all experiments, and it was shown that the degradation was not extensive enough to defend use of a lower temperature.

The initial PEG-concentration in the preformed liposomes was also studied: 0, 3 and 5 % PEG was used. No clear difference could be noted for the three concentrations, but the yield seemed to be a little lower for the highest concentration at short times. It is beneficial to have PEG initially in the liposomes for stability, therefore 3 % PEG was used in the preformed liposomes for all experiments. It was also found that after incorporation of the ligand-PEG-lipids the PEG concentration in the outer layer was approximately 5%, which is found to be optimal for stability (145).

It is interesting to note that the WSA-loaded liposomes exhibit an over all lower yield compared to unloaded liposomes. This phenomenon is shown for all EGF-targeted WSA-loaded liposomes. The incorporation yield in WSP and doxorubicin loaded liposomes (which have lower concentrations of loaded drug) is more similar to unloaded liposomes. For the WSA-loaded liposomes the final concentration was 10-15 EGF/liposome.

The conjugate was shown to be stable; at 37°C 79% of the EGF-associated radioactivity remained in the liposome fraction after one week and at 4°C 90% remained after 3 weeks.
Figure 9. Incorporation of 125I-EGF-PEG-DSPE lipid molecules into preformed liposomes. The percentage of added lipid incorporated in the liposome membrane was measured using the 125I-label on EGF. The amount of added EGF lipid was 2.7% to total liposomal lipid.

A: 0% PEG in the preformed liposomes, B: 3% PEG-DSPE to total lipid, C: 5% PEG-DSPE to total lipid, D: WSA loaded liposomes with 3% PEG-DSPE, to total lipid. Error bars represent maximal errors from double experiments. Scale on y-axis represents percentage incorporated EGF-lipid.

4.4 Optimization of conditions for trastuzumab-liposomes using NHS-PEG-DSPE (paper IV)

125I-trastuzumab solution was added to NHS-PEG-DSPE, and after hydration at 60°C for 5 min, to allow for the lipids to get into micelles, the mixture was kept at room temperature for 1 or 3 h conjugation. No increase in yield could be seen after 1 h. Therefore 1 h conjugation was considered sufficient. 125I-trastuzumab-PEG-DSPE micelles were purified and thereafter mixed with the preformed liposomes at 60°C, for 1, 4 or 24 h. No results were obtained from incorporation for 24 h at 60°C since the treatment caused the samples to stick to the column and no separation was possible. Therefore 4 h was considered to give the best yield. The empty liposomes gave higher yields than the WSA loaded for both 1 and 4 h as
was previously shown with EGF-liposomes. The final yield for trastuzumab-liposome-WSA was approximately 35 antibodies/liposome. The conjugate was stable. After two weeks in 37°C still 95% of the ligand-associated radioactivity remained in the liposome fraction.

4.5 Preparation of EGF-liposomes using NHS-PEG-DSPE (paper V)

The preparation procedure of the EGF-liposome conjugate used in paper V was slightly different from the one in paper I-III (optimized in paper I). As for the trastuzumab-liposome conjugates NHS-PEG-DSPE was used instead of maleimide-PEG-DSPE and no thiolation of EGF was necessary. The times and temperatures used for conjugation and incorporation of trastuzumab-lipids into liposomes were adapted for these EGF-liposomes as well. Since no modification of the protein was necessary one purification step could be removed, this together with the slightly higher conjugation and incorporation yields obtained made the over all yield about twice as good as for the procedure in paper I. The final result concentration was 20-30 EGF/liposome.

4.6 $^3$H-labeled liposome conjugates (paper III and IV)

To prepare radiolabeled liposomes, $^3$H-cholesteryl ether was used. This is a non-exchangeable, non-degradable lipid marker that is used to study, for example, the cellular uptake of liposomes (146, 147). The $^3$H-cholesterol was added to the lipid mixture before freeze-thawing and was thus incorporated in the lipid membrane. For the attachment of a ligand the methods described above was used.

4.7 Comments

We have been able to develop stabilized liposome conjugates for targeting of EGFR and HER-2. The conjugates showed good stability. The amount of targeting agents per liposome, 10-30 for the EGF-liposome conjugates and around 35 for trastuzumab-liposome conjugates, is assumed to be enough for a good cellular binding and uptake, at least when antibody-liposome conjugates have been studied (29, 42). The increase from 10 to 30 EGF per liposome did not yield any extra cellular uptake either (data not shown).
The clear difference in incorporation yield between WSA loaded and non-loaded liposomes is interesting. A similar study published by Ishida et al (144) reports less incorporation yield into Doxil®/Caelyx® liposomes but conclude that it is the increased PEG concentration in these liposomes (5% compared to their unloaded liposomes with 4% PEG) that hinders the lipids from transferring to the liposome membrane. We do not exclude the possibility that the PEG concentration in the preformed liposomes influences the amount of incorporation but believe that the liposome load also plays a large part, at least in this study.
5. Cell experiments

In the studies of uptake and intracellular processing of the conjugates monolayer cell cultures were used. Most of the experiments (summarized in table 5) have been performed analyzing the radioactive label on the targeting agent (\(^{125}\text{I}-\text{EGF}\) or \(^{125}\text{I}-\text{trastuzumab}\)), the radioactive label in the liposome membrane (\(^3\text{H}-\text{cholesterol}\)) and the boron from the liposome load (WSA or WSP).

Table 5. Performed in vitro experiments

<table>
<thead>
<tr>
<th>Liposome conjugate</th>
<th>Cell line</th>
<th>Experiment</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{125}\text{I}-\text{EGF-liposome})</td>
<td>U343</td>
<td>Displacement</td>
<td>I</td>
</tr>
<tr>
<td>(^{125}\text{I}-\text{EGF-liposome-WSA}, EGF-(^3\text{H})-liposome-WSA)</td>
<td>U343 A-431</td>
<td>Displacement, Time dependent uptake Retention Internalization studies Fluorescence studies</td>
<td>I, II II, III</td>
</tr>
<tr>
<td>(^{125}\text{I}-\text{EGF-liposome-WSP})</td>
<td>U343</td>
<td>Time dependent uptake Fluorescence studies</td>
<td>II</td>
</tr>
<tr>
<td>EGF-liposome-DOX</td>
<td>U343 A-431</td>
<td>Fluorescence studies</td>
<td>III</td>
</tr>
<tr>
<td>(^{125}\text{I}-\text{EGF-liposome-WSA})</td>
<td>U343</td>
<td>Uptake in cell-suspension Clonogenic survival</td>
<td>V</td>
</tr>
<tr>
<td>(^{125}\text{I}-\text{trastuzumab-liposome-WSA trastuzumab-}^3\text{H-liposome-WSA})</td>
<td>SKBR-3</td>
<td>Displacement Time dependent uptake Retention Internalization studies Fluorescence studies Uptake in cell-suspension</td>
<td>IV</td>
</tr>
</tbody>
</table>

5.1 Test of receptor specificity

Tests where the receptors have been blocked by an excess of native non-radiolabeled targeting agent have been studied for both EGF and trastuzumab conjugates. Displacement curves where increasing
concentrations of the blocking agent have been added show receptor specificity for both liposome conjugates (figure 10, A, B). The unspecific binding was shown to be approximately 10-20 % for EGF-liposomes and 30% for trastuzumab-liposomes.

**Figure 10.** Displacement of A) $^{125}$I-EGF-liposome-WSA and B) $^{125}$I-trastuzumab-liposome-WSA after 4 h incubation. The maximal uptake was set to 1.

### 5.2 Time-dependent uptake

The time dependent uptake has been studied with and without blocked receptors for both $^{125}$I and $^3$H labeled conjugates (figure 11 A-D). It was shown for both $^{125}$I-EGF-liposome and $^{125}$I-trastuzumab-liposome conjugates that the uptake of $^{125}$I reached a plateau after 8-24 h (figure 11 A and C). In the case of $^{125}$I-trastuzumab-liposomes the uptake even decreased after 24 h. When looking instead at the uptake of $^3$H-liposome conjugates the uptake is continuous throughout the incubation times (figure 12 B and D). The most probable explanation for this is that in the case of $^{125}$I, EGF or trastuzumab is degraded and excreted so that the measured radioactivity does not correspond to the total uptake. The liposome label, $^3$H-cholesterylether, instead is known to be a non-exchangeable, non-degradable marker (147), so this label should represent the true uptake of ligand-liposome conjugates.
Figure 11. Time dependent uptake of A) $^{125}$I-EGF-liposome-WSA. B) EGF-$^{3}$H-liposome-WSA. C) $^{125}$I-trastuzumab-liposome-WSA. D) trastuzumab-$^{3}$H-liposome-WSA. Maximal uptake was set to 1. Unspecific uptake was determined by addition of an excess of EGF (A, B) or trastuzumab (C, D) to block the receptors.

The time dependent uptake of boron is shown in table 6. It was shown that in monolayer culture that the uptake of boron increases with time to approximately 6 ppm after 24 h incubation for EGF-liposome-WSA. EGF-liposome-WSP only reached 2 ppm and thus did not show any increase from the uptake with non-targeted liposomes. The uptake of WSA and WSP *per se* were 6.6 and 10.5 ppm, respectively. The choice was to continue further studies with WSA instead of WSP. One possible explanation for the difference might be that only half the amount of boron could be loaded into the liposomes when using WSP as compared to WSA. With trastuzumab-liposome-WSA the monolayer culture uptake after 4 h incubation reached 12 ppm.
Table 6. Uptake of boron in monolayer cell culture (ppm).

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Cell line</th>
<th>1h</th>
<th>4h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>WSP</td>
<td>U343</td>
<td>10.49 +/- 1.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liposome-WSP</td>
<td>——</td>
<td>1.80 +/- 0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGF-liposome-WSP</td>
<td>——</td>
<td>2.21 +/- 0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WSA</td>
<td>——</td>
<td>6.61 +/- 1.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liposome-WSA</td>
<td>——</td>
<td>0.29 +/- 0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGF-liposome-WSA</td>
<td>——</td>
<td>1.0 +/- 0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.4 +/- 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.0 +/- 1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGF-liposome-WSA A-431</td>
<td>1 +/- 0.04</td>
<td>1.4 +/- 0.03</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>4.5 +/- 1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trastuzumab-liposome-WSA SKBR-3</td>
<td>12 +/- 0.4</td>
<td></td>
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</tbody>
</table>

5.3 Retention

The retention of the conjugates after 24h incubation was studied with regard to the targeting agent, the liposome, and the load. For both EGF and trastuzumab conjugates the retention of $^3$H and boron was very good (figure 12 A-C), corresponding to the retention of the liposome and WSA. For EGF-liposome-WSA approximately 80% of the boron remained in both cell lines after 48 h incubation. For trastuzumab-liposome-WSA 70% of the boron remained after 48 h. The $^3$H-liposome label remained almost as long for both conjugates. There was a different result when the retention of $^{125}$I-EGF and $^{125}$I-trastuzumab was studied. For $^{125}$I-EGF-liposome-WSA approximately 50% was rapidly excreted during the first 4 hours and after 48 h only 20-30% remained. For $^{125}$I-trastuzumab-liposome-WSA the ligand was also rapidly excreted and 18% remained after 48 h.
Figure 12. Retention of $^{125}$I-EGF-liposome-WSA, EGF-$^3$H-liposome-WSA and EGF-liposome-WSA(boron) in A) U343 MGa Cl2:6 cells and B) A-431 cells. Figure C) 1 shows $^{125}$I-trastuzumab-liposome-WSA, trastuzumab-$^3$H-liposome-WSA and trastuzumab-liposome-WSA(boron) retention in SKBR-3 cells. The retention was, in all cases, studied after 24 h incubation and the uptake at 24 h was set to 100%.
5.4 Membrane-bound and internalized conjugate

The internalization of the conjugates was studied using the acid-wash technique developed by Haigler (148). In short, the cells were incubated for the various times and thereafter washed to remove unbound conjugate. Glycine-HCl acid (pH 2.5) was added to the cell dishes for 6 min at 4°C and is thought to disrupt the binding of receptors and ligands on the cell surface, i.e., the membrane bound conjugate ends up in the acid fraction. After another wash with the acid, 1 M NaOH was added to detach the cells. The radioactivity in the base fraction consisted of the internalized conjugate.

It was shown for both conjugates that the majority was internalized fairly rapidly. For EGF-liposome-WSA there was a difference between the two cell-lines tested. In U-343 MGa Cl 2:6 over 90% of the conjugate was internalized even after short times. In A-431 the majority of the conjugate was internalized but only after longer times. This difference might be explained by the fact that A-431 cells recycle their EGF receptors.

Trastuzumab-liposome-WSA was also shown to be mostly internalized, about 70% of the total cell-associated radioactivity was internalized when both the label on the liposome and on the antibody were studied (figure 13).

5.5 Intracellular localization using fluorescence

Both boronated compounds WSA and WSP are, as mentioned earlier, fluorescent and this could be used for intracellular localization studies. When incubating U-343 MGa Cl2:6 cells with the compounds per se, a nuclear staining could be seen indicating that the compound in itself is DNA-binding (figure 14 A, B). When enclosing the compound in targeted liposomes the staining is mostly cytoplasmic (figure 14 C,D). This phenomenon was most pronounced for cells incubated with EGF-liposome-WSA, which showed no nuclear staining at all. Even after prolonged incubation time the WSA did not seem to reach the nucleus. The same experiments have also been performed with trastuzumab-liposome-WSA and no clear nuclear staining was apparent (figure 14 F).
Earlier publications have shown that doxorubicin loaded targeted liposomes have nuclear staining after cellular uptake (33, 48). Experiments incubating cells with EGF-liposome-DOX (figure 14 E) and trastuzumab-liposome-WSA in SKBR-3 cells. Filled bars: internalized, empty bars: membrane-bound.
liposomes-DOX were therefore performed. These experiments showed nuclear staining of doxorubicin. It can be concluded that the EGFR and HER-2 pathways are working if nuclear delivery is desired, but the release from the liposomes might be a problem. This problem of compound release has been addressed by several groups proposing different liposomal formulations to make the liposomes release their content more readily after internalization (149-151).

**Figure 14.** Fluorescent images of A) WSP, B) WSA, C) EGF-liposome-WSP, D) EGF-liposome-WSA, E) EGF-liposome-DOX, F) Trastuzumab-liposome-WSA. after 24 h incubation. WSA and WSP concentration 1µg/ml, and DOX concentration 2µg/ml.
5.5 Optimization of boron uptake

To test if the uptake of boron using EGF or trastuzumab-liposome-WSA could be increased from the monolayer incubation, incubation in cell suspension in centrifuge tubes and in roller flasks were tested (figure 15).

Incubating monolayer culture with EGF-liposome-WSA, the boron content in the cells reached 7 ppm after 24 h incubation. If instead the cells were incubated as loose pellets in a centrifuge tube on gentle shake, the boron concentration reached over 40 ppm after 24 h incubation. Moreover the background was significantly lower for the pellet incubation, approximately 10%, while for the monolayer incubation it was almost 50%.

Unfortunately, the cells did not seem to survive the rather harsh treatment of incubating the cells as loose pellets, most probably due to the poor circulation of incubation medium. Therefore cells were incubated in suspension in small siliconized flasks kept on constant roll to ensure maximum medium circulation. Using this method boron concentration of 55 ppm was reached after only 4 h incubation. After 24 h almost 90 ppm was reached, but the cells had then started forming spheroids. The background was low, 20%, using this incubation method as well.

The increase in uptake using the different incubation methods is probably due to an increase in conjugate concentration (in the incubation medium). There might also be a more favorable geometry for uptake by incubating the cells in suspension with more accessible receptors.

The fact that the background is lower is not easy to explain, but it can be noted that the background is at approximately the same quantitative level for all three incubation methods. Thus, it is not the background that has been lowered but the specific uptake that has increased.

For trastuzumab-liposome-WSA the same increase in uptake when changing from monolayer culture to incubation in roller-flasks was seen. In monolayer culture the uptake reached 12 ppm after 4 h incubation, with a background of 75%. In roller flasks experiments more promising results were obtained with an uptake increase to 132 ppm and a background of 25%.
Figure 15. Boron uptake in cells after different incubation methods. The conjugate EGF-liposome-WSA was added to cell dishes, cells in suspension forming a loose pellet, or cells in suspension in roller flasks. Trastuzumab-liposome-WSA was added to cell dishes or cells in suspension in roller flasks. To test the specificity of the uptake, samples where the cell receptors had been blocked by addition of excess EGF or trastuzumab were also used. Incubation time was 4 and 24 h.

5.6 Comments

It was shown that all studied conjugates bound specifically to their receptors, were internalized and had good intracellular retention. Internalization is especially important for BNCT since the short range of the particles makes the compound more toxic if the decay takes place within the cell than on the cellular membrane (90).

The specificity, studied by the displacement assay, shows unspecific binding ranging from 10 to 30%. Other groups have shown similar results. Gabizon et al. showed that addition of free folate in excess inhibited 86% of the binding of folate-PEG-liposome constructs (152). Lee et al. reported unspecific binding of about 30% using folate liposomes and folic acid block (34). Lopez de Menezes et al report 50% reduction in uptake after addition of free anti-CD19 antibody compared to the uptake of anti-CD19-liposomes (46). The unspecific uptake will have to be studied in an in vivo situation to determine the uptake in healthy tissue. In a binary therapy like BNCT unspecific uptake is of less problem, since it is only the irradiated areas that are exposed to the toxic effects.

The uptake of boron, reaching 90 ppm for EGF-liposome-WSA and 132 ppm for trastuzumab-liposome-WSA, is at levels that are of clinical
interest if $^{10}$B-enriched compounds were used. Other groups report uptake in cell culture of up to 1500 ppm. However, these high levels were associated with very high (1300 ppm) unspecific uptake (37).
6. BNCT experiments on cultured glioma cells

To study the efficacy of the EGF-liposome-WSA conjugate a BNCT experiment was conducted, using clonogenic survival of cultured U-343 MGa Cl2:6 cells. After incubation with EGF-liposome-WSA the cells were irradiated with neutrons at the Studsvik R2-0 reactor. It is important to note that the WSA used in this study was not $^{10}\text{B}$ enriched and therefore only 20% of the boron in the cells was capable of neutron capture. If $^{10}\text{B}$ enriched compounds had been used the efficacy would have been higher.

6.1 Experimental details

Glioma cells were incubated for 4 hours as a cell suspension in roller-flasks with either EGF-liposome-WSA, EGF-liposome-WSA with excess of free EGF, or culture medium. After incubation the medium was removed and the cells were thoroughly washed to remove any unbound conjugate. The cells were resuspended in a small volume of culture medium and kept on ice throughout the irradiation to hinder any repair mechanisms. The cells were irradiated with 0, 1, 2 or $3\times10^{12}$ thermal neutrons/cm$^2$. After neutron irradiation the cells were counted, diluted and plated for clonogenic survival assay.

The clonogenic survival assay is a powerful tool to determine the toxicity of a compound or a treatment. A number of cells are plated in a petri dish, where only the surviving cells will continue to divide. After a number of doubling times (approx. 10) the surviving cells will have formed colonies that, after staining, are visible for the naked eye. The count of the colonies compared with the number of seeded cells gives a value of the clonogenic ability. Normalization to the clonogenic ability of untreated cells gives the survival.
6.2 Dose calculations

The physical dose contributions by the thermal neutron beam and the contributions from the boron uptake were calculated using Monte Carlo simulations (more information can be obtained in paper V), (Table 7). The major dose contributors for the thermal neutron beam were the photon dose with 0.9148 Gy/10^{12} thermal neutrons/cm^{2}, the nitrogen dose with 0.232 Gy/10^{12} n_{th}/cm^{2} and the fast neutron dose with 0.082 Gy/10^{12} n_{th}/cm^{2}. The dose from the boron was with the uptake found in the study 0.823 Gy/10^{12} n_{th}/cm^{2}. The boron uptake was 55 ppm although only 20%, 11 ppm, was \textsuperscript{10}B. If \textsuperscript{10}B enriched compound had been used, the dose from the boron would have been 4.11 Gy/10^{12} n_{th}/cm^{2}, more than four times higher than the dose from the neutron beam alone.

Table 7 Fluence rates and physical doses

<table>
<thead>
<tr>
<th>Fluence rates</th>
<th>Dose (Gy/10^{12} n_{th}/cm^{2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermal neutrons ((n_{th})):</td>
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<tr>
<td>6.35\times 10^{9} cm^{-2}s^{-1}</td>
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</tr>
<tr>
<td>Fast neutrons:</td>
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<tr>
<td>1.89\times 10^{9} cm^{-2}s^{-1}</td>
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<tr>
<td>Fast neutron dose</td>
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<tr>
<td>Nitrogen dose</td>
<td>0.232</td>
</tr>
<tr>
<td>Photon dose</td>
<td>0.9148</td>
</tr>
<tr>
<td>Boron dose (11 ppm)</td>
<td>0.823</td>
</tr>
<tr>
<td>Boron dose (55 ppm)</td>
<td>4.11</td>
</tr>
</tbody>
</table>

6.3 Cell survival after neutron irradiation

The survival of the glioma cells after neutron irradiation as a function of neutron fluence is shown in figure 16. It was shown that, if the cells had been incubated with EGF-liposome-WSA, only half the neutron fluence was needed to reduce the survival to 10% compared to irradiation of untreated control cells. After incubation for 4 h with EGF-liposome-WSA, the boron levels in the cells reached 55 ppm. Cells with blocked EGF-receptors reached 11 ppm resulting in an unspecific binding of approx. 20%. This low binding confirms receptor specificity. The difference seen in survival of the liposomes with and without blocked receptors also confirm specificity and the difference reflects the amount boron taken up by the cells.
Figure 16. Clonogenic survival following thermal neutron irradiation of cells treated with EGF-liposome-WSA, EGF-liposome-WSA with pre-blocked receptors or untreated cells. The data sets were in all cases fitted to an exponential model, \( S = \exp(-\alpha F) \) where \( F \) is the neutron fluence and \( \alpha \) is a fitting parameter.

Considering the survival as a function of dose, the results look similar as can be seen in figure 17. The survival was still lowest for the cells treated with WSA-liposomes. Comparing with gamma irradiation it could be seen that for 10% survival 2.9 Gy was needed for targeted liposomes and 5.6 Gy for \(^{137}\)Cs gamma. 3.6 Gy was needed for neutron irradiation only. The slight difference between blocked and non-blocked cells should be due to differences in boron localization. The boron uptake with blocked receptors is probably due to both unspecific cell binding (as is shown in paper III) and liposome leakage. It can be assumed that the uptake via receptor mediated endocytosis, as in the case for EGF-liposome-WSA, should deliver the boron to a more favorable intracellular position.
6.4 Comments

It is possible to reduce survival of cultured glioma cells 2 orders of magnitude by incubation with EGF-liposome-WSA and subsequent neutron irradiation. There was also a clear difference in survival if an excess of EGF blocked the uptake.

Previous studies of cell survival after neutron activation of liposome delivered boron also show decreased cell survival. Yanagie et al (125, 127) have performed in vitro studies of liposome-encapsulated $^{10}$B. They show that the cell growth of pancreatic cancer cells and breast cancer cells could be lowered to approximately 30% after neutron irradiation with $5 \times 10^{12}$ neutrons/cm$^2$. The cell growth was assayed by $^3$H-TdR incorporation. We have managed, at $3 \times 10^{12}$ neutrons/cm$^2$, to inactivate 99% of the cells.

With a $^{10}$B uptake of 11 ppm and a neutron flux of $3 \times 10^{12}$ neutrons/cm$^2$ 1% of the cells still survive. This means that for a tumor with $10^7$ cells 1000 will survive and continue to grow. If $^{10}$B-enriched compounds could be used, the survival would be much lower, hopefully by several orders of magnitude.

Figure 17. Survival as a function of dose. The neutron irradiated data sets have been fitted to a purely exponential curve, $S = \exp(-\alpha D)$ and the gamma irradiated are fitted to a linear quadratic curve fit, $S = \exp(-\alpha D - \beta D^2)$, where $D$ is the dose in Gy and $\alpha$ and $\beta$ are fitting parameters.
7. Biodistribution of EGF and EGF-liposomes

As shown earlier the EGFR is a suitable target for tumor therapy. Unfortunately, the biodistribution of EGF has proved that it is a not very successful candidate for systemic injection because of its short circulation time, mainly due to its rapid uptake in liver and kidneys (153). It has been reported that after 2.5 min only 7% of $^{125}$I-EGF remain in the bloodstream in rats (154). The hepatocytes in the liver are known to have a large number of EGF-receptors (142) that effectively binds and degrades EGF.

In mice the sinusoidal fenestrations lining the vessels in the liver are approximately 100 nm (155). That means that a particle needs to be smaller than 100 nm to leave the vessels and reach the hepatocytes. If the EGF were to be coupled to a particle of this size or larger, maybe the binding to EGFRs of the hepatocytes might be circumvented. We wanted to investigate whether the attachment of a liposome to EGF would increase circulation time of EGF and decrease the binding to the liver due to its size. The liposomes with similar size, as the epithelial fenestrations of the liver, should reduce the hepatocytic uptake of EGF, while some non-parenchymal uptake of the EGF-liposomes would still be present due to the liposome uptake by Kupffer cells.

7.1 Experimental procedures

Mice were injected in the tail vein with $^{125}$I-EGF-liposome conjugate or $^{125}$I-EGF and after various times the animals were sacrificed. Blood, urine and organs were collected according to a standard protocol with 20 organs. The radioactivity of the organs was measured using a gamma counter after weight determinations.

To see if the EGFR-specific uptake of $^{125}$I-EGF and $^{125}$I-EGF-liposome could be blocked, an excess of EGF was injected into the mice 15 min prior to $^{125}$I-EGF or $^{125}$I-EGF-liposome injection. The mice were sacrificed 15 min after the second injection. Blood and organs were collected according to standard protocol, weighed, and thereafter the radioactivity was measured.
7.2 Circulation time

As is shown in figure 18 the circulation time of $^{125}$I-EGF increased if 100 nm PEG-stabilized liposome were attached. 15 minutes after injection of $^{125}$I-EGF 3.5% injected dose per g (ID/g) was still in the blood stream. One hour later 3% ID/g remained. If a liposome was attached, the blood level changed to 25% ID/g after 15 min and 14% ID/g after 1h. If the EGF-receptors were blocked by injection of an excess of EGF, the blood level of $^{125}$I-EGF after 15 min was higher than for non-blocked mice, probably as an effect of blocked EGF-receptors in the liver. No difference in blood level could be seen for $^{125}$I-EGF-liposomes after blocking. The circulation halftime of $^{125}$I-EGF-liposomes is longer than $^{125}$I-EGF but lower than results obtained in other studies with liposomes which often have halftimes of several hours (12, 16). This can be explained by the fact that in this study the targeting agent is followed, not the liposome. If the liposomes were radiolabeled we could expect longer halftimes.

Figure 18. Levels of $^{125}$I-EGF (open bars) and $^{125}$I-EGF-liposomes (filled bars) in blood expressed as % ID/g. Error bars represent maximal errors, 4 animals per time point.

7.3 Uptake in liver and kidneys

The uptake of $^{125}$I-EGF in liver decreased significantly when 100 nm PEG-stabilized liposomes were attached to EGF as is shown in figure 19 A. For $^{125}$I-EGF the uptake was 51% ID/g 15 min after injection, and 1h after injection it had decreased to 3% ID/g, indicating rapid degradation. For $^{125}$I-EGF-liposomes the uptake 15 min after injection was 9% ID/g, and the radioactivity was not excreted as rapidly as for $^{125}$I-EGF. When the receptors were blocked the uptake of $^{125}$I-EGF altered dramatically. From
the previously shown uptake of 51% ID/g after 15 min, the uptake decreased to 2% ID/g indicating blockage of EGFR specific uptake to hepatocytes in liver. This dramatic difference was not seen when $^{125}$I-EGF-liposomes were used. The uptake was somewhat lowered if the receptors were blocked, indicating low EGFR specific binding to liver for $^{125}$I-EGF-liposomes. However, we can not exclude that there is some hepatocyte specific uptake, even though the slight difference after blockage indicates that the liver uptake of $^{125}$I-EGF-liposomes is by non-parenchymal cells to a high extent.

Figure 19. Levels of $^{125}$I-EGF (open bars) and $^{125}$I-EGF-liposomes (filled bars) in liver and kidney expressed as % ID/g. Error bars represent maximal errors, 4 animals per time point.

In the kidney there were also vast differences in the uptake patterns of EGF and EGF-liposomes (figure 19 B). The uptake of $^{125}$I-EGF was at 51% ID/g 15 min after injection and after 1h this had decreased to 8% ID/g. If the receptors were blocked, the uptake doubled due to the higher blood levels
as a result of blocked organs, for example the liver. It was clear that EGF was excreted through the kidneys. For $^{125}$I-EGF-liposomes the kidney uptake was as low as 4% ID/g 15 min after injection and slowly decreasing. No difference could be seen with blocked EGF receptors. These results indicate that EGF-liposomes are not excreted via the kidneys. Probably the size of the liposome disables it from renal excretion.

7.4 Uptake in other organs

There was an uptake in submaxillary gland of both $^{125}$I-EGF and $^{125}$I-EGF liposomes (table 8, 9). The accumulation of $^{125}$I-EGF was more rapid, but the same levels were reached for both the peptide and the conjugate. The submaxillary gland, being the main site of EGF production in mice, expresses EGF receptors (156), and it was shown that the uptake of $^{125}$I-EGF could be blocked. Since the blocking experiment only was performed with 15 min incubation time, no difference after blocking could be seen for $^{125}$I-EGF-liposomes. The uptake of $^{125}$I-EGF-liposomes in submaxillary gland did not reach its highest value until after 4h.

In the spleen there was a higher uptake of the liposome conjugate than of $^{125}$I-EGF (table 8, 9). This is probably due to the fact that liposomes are cleared by MPS uptake and the spleen has an important role in the MPS. There was no difference in the uptake if EGF-receptors were blocked, indicating that the splenic uptake is not mediated by EGFR.

The uptake of $^{125}$I in thyroid reached approximately the same levels for both $^{125}$I-EGF and $^{125}$I-EGF-liposomes (table 8, 9). The uptake increased with time corresponding to the release of $^{125}$I and $^{125}$I-tyrosine from degraded $^{125}$I-EGF and $^{125}$I-EGF-liposomes.

Table 8. Uptake of $^{125}$I-EGF in selected organs (%ID/g)

<table>
<thead>
<tr>
<th>Time</th>
<th>Submax.</th>
<th>Spleen</th>
<th>Thyroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min</td>
<td>16 +/- 6</td>
<td>3.9 +/- 1.2</td>
<td>0.3 +/- 0.5</td>
</tr>
<tr>
<td>15 min + EGF</td>
<td>5.2 +/- 1.5</td>
<td>2.1 +/- 0.3</td>
<td>0.19 +/- 0.05</td>
</tr>
<tr>
<td>1 h</td>
<td>24 +/- 19</td>
<td>1.8 +/- 0.6</td>
<td>1.1 +/- 0.4</td>
</tr>
<tr>
<td>2 h</td>
<td>13 +/- 5</td>
<td>1.8 +/- 0.1</td>
<td>2.2 +/- 1.3</td>
</tr>
<tr>
<td>4 h</td>
<td>14 +/- 6</td>
<td>0.88 +/- 0.10</td>
<td>3.9 +/- 1.5</td>
</tr>
<tr>
<td>8 h</td>
<td>8.1 +/- 3.3</td>
<td>0.58 +/- 0.14</td>
<td>4.5 +/- 1.8</td>
</tr>
<tr>
<td>24 h</td>
<td>0.42 +/- 0.53</td>
<td>0.06 +/- 0.04</td>
<td>7.8 +/- 4.7</td>
</tr>
</tbody>
</table>

* The values are expressed as %ID
### Table 9. Uptake of $^{125}$I-EGF-liposomes in selected organs (%ID/g)

<table>
<thead>
<tr>
<th>Time</th>
<th>Submax.</th>
<th>Spleen</th>
<th>Thyroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min</td>
<td>0.58 +/- 0.42</td>
<td>9.8 +/- 7.1</td>
<td>0.03 +/- 0.02</td>
</tr>
<tr>
<td>15 min + EGF</td>
<td>0.63 +/- 0.18</td>
<td>8.1 +/- 3.2</td>
<td>0.03 +/- 0.01</td>
</tr>
<tr>
<td>1 h</td>
<td>4.7 +/- 0.9</td>
<td>11 +/- 5</td>
<td>0.24 +/- 0.09</td>
</tr>
<tr>
<td>2 h</td>
<td>7.3 +/- 1.4</td>
<td>6.5 +/- 4.6</td>
<td>0.59 +/- 0.05</td>
</tr>
<tr>
<td>4 h</td>
<td>16 +/- 7</td>
<td>6.3 +/- 4.9</td>
<td>1.7 +/- 0.9</td>
</tr>
<tr>
<td>8 h</td>
<td>5.9 +/- 3.6</td>
<td>2.3 +/- 1.0</td>
<td>4.8 +/- 3.2</td>
</tr>
<tr>
<td>24 h</td>
<td>0.46 +/- 0.21</td>
<td>1.2 +/- 0.6</td>
<td>5.6 +/- 1.4</td>
</tr>
</tbody>
</table>

*The values are expressed as %ID.

### 7.5 Comments

We were able to alter the biodistribution of $^{125}$I-EGF by conjugation to 100 nm PEG-stabilized liposomes. The most important differences in biodistribution are the reduced uptake in the liver and the kidneys when EGF is conjugated to liposomes, as well as the elevated blood levels over time. The circulation half-time of $^{125}$I-EGF was prolonged when attaching it to liposomes, since the blood-levels were much higher for $^{125}$I-EGF-liposomes. To decrease liver and kidney uptake and to increase the blood concentration of EGF-conjugate is of greatest importance for the use of EGF as a targeting agent in nuclide therapy or other targeting modalities utilizing EGF against cancer. The approach presented here will probably reduce liver and kidney toxicity, while giving time for the EGF conjugate to reach the tumor cells.
8. Summary and future work

8.1 Summary

This thesis has mainly dealt with the development of targeted liposome conjugates and their behavior in cell culture and in mice.

Liposome conjugates with high stability targeting EGFR and HER-2 have been constructed. They showed receptor specificity and internalize into the tumor cells after binding. The cellular retention of the liposome conjugates after 24 h incubation was also found to be good. Thus, the described liposome systems seems of interest of tumor therapy. There is potential to load the liposomes with various toxic agents such as toxins, radionuclides and nuclides for neutron activation.

The compounds WSA and WSP have previously been loaded into liposomes at high levels with low leakage (129). We have shown that the use of EGF and trastuzumab conjugated liposomes loaded with WSA results in high boron levels after incubation in a cell suspension culture. The boron levels reached in vitro are high enough to be of clinical interest. Further, by applying enriched $^{10}$B the situation will be even better. With EGF-liposome-WSA, the high boron levels were used to kill cultured glioma cells. After neutron irradiation survival was studied, using the clonogenic survival assay. Significant differences in survival were seen for cells incubated with conjugate and cells that were only neutron irradiated.

The in vivo studies, comparing the uptake of $^{125}$I-EGF and $^{125}$I-EGF-liposomes in selected organs in mice, showed that the uptake in liver and kidneys was significantly lower for $^{125}$I-EGF-liposomes and the circulation time in blood was longer. The fact that the $^{125}$I-EGF-liposome conjugates does not assemble in the liver to the same high extent as free $^{125}$I-EGF makes this conjugate promising for systemic injection.
8.2 Future work

The most interesting areas to continue the work with stabilized liposome ligand conjugates, in my point of view, are the following:

1. Further study of EGF-liposomes *in vivo*, using animals with xenografted tumors to study tumor specific delivery, will be performed to conclude whether EGF-liposomes are suitable for systemic drug delivery.

2. The high boron level reached in cell culture for trastuzumab-liposome-WSA conjugates, 132 ppm, makes this conjugate a promising candidate for experimental BNCT. It would also be very interesting to perform BNCT on tumor bearing animals, using both EGF-liposome and trastuzumab-liposome conjugates. If $^{10}$B enriched compounds could be used the therapeutical results would be dramatically improved when using WSA loaded liposomes.

3. Further development of the liposome conjugates with new DNA-binding compounds based on doxorubicin is in progress. Doxorubicin, which has been shown to be specifically delivered to the DNA after liposome targeting, is of specific interest for labeling with the Auger electron emitting nuclide $^{125}$I. Auger electron emitting $^{125}$I gives high cell damage (about one double strand break per decay), if the nuclide is situated within the DNA (157).

4. It would also be exciting to study whether the intact antibody of trastuzumab-liposomes has a negative impact on the tumor specific delivery. Previous studies by Park et al have shown that Fab- or ScFv-conjugated liposomes targeting HER-2 have at least as good characteristics as the antibody conjugated liposomes (158) *in vitro* and *in vivo*.

5. Targeted liposomes loaded with radiometals, for example $^{111}$In, would also be of interest to study. Radiometals are known to have good intracellular retention, and being able to deliver large amounts by using loaded liposomes would be of great value for imaging and possibly also therapy.
Acknowledgements

The work in this thesis have been conducted at the division of Biomedical Radiation Sciences (BMS), Uppsala University, with financial support mainly from Cancerfonden. I would like to thank everyone who has helped me along the way and a send a special thank you to:

Dr. Lars Gedda, my supervisor. For being the perfect supervisor, you have always taken part in my research with great interest and you have always found the time to talk to me no matter how trivial the issue.

Prof. Hans Lundqvist and Ass. Prof. Bo Sternerlöst, Co-supervisors, for always being supportive and helpful.

Prof. Jörgen Carlsson, Head of the BMS department and examiner. For creating a wonderful warm and supportive atmosphere. It has been a pure pleasure working at BMS. Thank you also for always taking such great interest in my work.

Quichun Wei, co-author, thank you for all help with the trastuzumab-work and for the animal experiments.

Marika Nestor, co-author, for working patiently and independently with all those cell experiments and for putting up with me as a supervisor.

Prof. Katarina Edwards, Nill Bergström, Dr. Markus Johnsson and Maria Arfvidsson, Co authors. For all help with liposomes, answering all my questions and swiftly supplying me with new liposome batches on short notice. Thank you for a lot of fun as well.

Prof. Stefan Sjöberg and his Ph.D. students, past and present, for supplying WSA and WSP.

Ass. Prof. Jacek Capala and Charlotta Persson. Thank you for all help with the experiments at Studsvik.
Dr Peter Frisk, Ulla Johansson, Ass. Prof Ulf Lindh and Dr Jean Pettersson. For helping me with a lot of ICP boron analyzes.

Dr Stefan Gunnarsson, for introducing me to the world of confocal microscopy.

Dr Anna Orlova and Ass. Prof. Vladimir Tolmachev. For instructions and help during my brief period as "labeling chemist" working with A3.

Veronika Asplund Eriksson, som håller ordning på allt och alla och också har varit en trevlig rumskamrat.

Maria Östh-Eklind för administrativ hjälp.

Åsa Liljegren Sundberg, för en massa kul hela tiden på BMS.

Gamla och nya doktorander, för en massa roliga saker som gör livet kul. Man får ALLTID minst ett skratt om dagen på BMS.

Mina föräldrar och min syster, tack för att ni alltid trott på mig, stöttat mig och pushat mig att göra mitt bästa.

Kullbergfamiljen, för att ni tagit mig till er och alltid orkat uppbåda intresse för hur det går med min "infekterade celler" m.m.

Mathias, min käre make… För att du är så bra och för att du får mig att känna mig som den roligaste, vackraste och smartaste kvinnan på jorden.
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