Tumour Targeting Using Radiolabelled EGF Conjugates

Preclinical Studies

BY

ÅSA LILJEGREN SUNDBERG
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Abstract

Tumour targeted radiotherapy is an appealing approach for treatment of disseminated tumour cells. A targeting agent that specifically binds to a structure on tumour cells is then used to transport therapeutically relevant radionuclides. The epidermal growth factor receptor, EGFR, is overexpressed on tumour cells in several malignancies, e.g. highly malignant gliomas. In this thesis, three types of radiolabelled EGF-conjugates, aimed for targeting to EGFR-expressing tumour cells, were developed and studied: EGF-dextran labelled with $^{131}$I, EGF labelled with $^{211}$At, and two EGF-chelates, DTPA-EGF and Bz-DTPA-EGF, labelled with the radioactive metals $^{111}$In and $^{177}$Lu.

The targeting properties of radiodinated EGF-dextran were first studied in cultured glioma cells. Radioidine coupled to the dextran part of EGF-dextran was retained in cells appreciably longer than radioidine coupled to EGF. This can give about 100 times increased radiation dose to tumour cells.

Targeting with $^{211}$At-EGF was investigated in combination with the tyrosine kinase inhibitor gefitinib (Iressa™, ZD1839). The uptake of $^{211}$At-EGF in EGFR-expressing tumour cells increased with increasing gefitinib concentrations. This was the case for both gefitinib-resistant and gefitinib-sensitive cell lines. The effect of the combined treatment on cell survival, however, differed between the cell lines in an unexpected way. In gefitinib resistant cells, combined treatment decreased cell survival approximately 3.5 times relative to $^{211}$At-EGF treatment alone. In gefitinib sensitive cells, however, combined treatment increased the cell survival (i.e. a protective effect).

The EGF-chelates studied $^{([111]}$In)DTPA-EGF, $^{([131]}$In)Bz-DTPA-EGF and $^{([177]}$Lu)Bz-DTPA-EGF all bound specifically with high affinity ($K_1 > 2$ nM) to EGFR on cultured glioma cells. They were internalised after binding, and the cellular retention of radionuclides was high (60% remained after 45 h). A biodistribution study in mice showed that liver and kidneys accumulated a majority of the radioactivity. The EGF-chelates bound EGFR specifically also in vivo. A tumour-to-blood ratio of 25 was achieved in a preliminary study.

Keywords: targeting, tumour, EGF, radionuclide, gefitinib, Iressa, ZD1839, glioma, therapy, diagnostics

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To my beloved Father

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


In 2002, the respondent changed last name from Liljegren to Sundberg.

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Front cover: Uptake of EGF-Texas Red in gefitinib treated U343 glioma cells. Cell tracker (green) was used to stain cell cytoplasm, and Hoechst 33342 (blue) was used to stain cell nuclei.
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### Abbreviations

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<tr>
<td>AR</td>
<td>Amphiregulin</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>BTC</td>
<td>Betacellulin</td>
</tr>
<tr>
<td>Bz-DTPA</td>
<td>Benzyl-diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>CAT</td>
<td>Chloramine-T</td>
</tr>
<tr>
<td>CDAP</td>
<td>1-cyano-4-dimethylamino pyridinium tetra-fluoroborate</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DOTA</td>
<td>Tetraazacyclododecanetetraacetic acid</td>
</tr>
<tr>
<td>DOTATOC</td>
<td>DOTA-D-Phe¹-Tyr³-octreotide</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethylenetriaminepentaacetic acid</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>EGF-TR</td>
<td>EGF-Texas Red</td>
</tr>
<tr>
<td>EPR</td>
<td>Epiregulin</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast performance liquid chromatography</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma multiforme</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine 5’-diphosphate</td>
</tr>
<tr>
<td>GMP</td>
<td>Good manufacturing practice</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine 5’-triphosphate</td>
</tr>
<tr>
<td>HAMA</td>
<td>Human anti-mouse antibody</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>Heparin binding EGF</td>
</tr>
<tr>
<td>HER1-4</td>
<td>Human EGFR 1-4</td>
</tr>
<tr>
<td>ID</td>
<td>Injected dose</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol(1,4,5)phosphate</td>
</tr>
<tr>
<td>Kᵣ</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>LET</td>
<td>Linear energy transfer</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>NRG</td>
<td>Neuregulin</td>
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<tr>
<td>NRG-1</td>
<td>Heregulin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3’-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol-3,4-biphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol-3,4,5-triphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLCγ</td>
<td>Phospholipase C-gamma</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine binding</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase with homology to tensin</td>
</tr>
<tr>
<td>RBE</td>
<td>Relative biological effect</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single photon emission computed tomography</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming growth factor alpha</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine kinase inhibitor</td>
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<td>WHO</td>
<td>World Health Organisation</td>
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Introduction

The major therapy modalities used for treating cancer today are surgery, external beam radiation, chemotherapy and hormonal therapy. Surgery and external radiation are used for solid primary tumours and large metastases, while chemotherapy is used for residual and spread disease. Chemotherapy can be efficient against certain malignancies, e.g. lymphomas, but problems arise when disseminated tumours cannot be treated effectively by chemotherapy. This is the case for example with patients with melanomas, highly malignant brain tumours and disseminated adenocarcinomas (e.g. breast and prostate carcinomas) where current therapy methods are mainly palliative.

An appealing concept for treating disseminated tumour cells is to use targeted therapy. In such therapy, a targeting agent that specifically binds to a structure exclusive for tumour cells is used to transport cytotoxic agents, like radionuclides or toxins. Unfortunately, there is a limited number of structures that are exclusively found on tumour cells. It is, however, fairly common that tumour cells express a much higher quantity of certain cell surface receptors than normal tissues do. For example, the epidermal growth factor receptor, EGFR, is overexpressed in several malignancies, e.g. lung, breast and bladder carcinomas, and high grade gliomas [121].

In this thesis, EGFR targeting was investigated, using the ligand EGF as targeting agent. Three types of EGF-based targeting conjugates were labelled with various radionuclides and studied in cultured cells and animals. Special attention was given to targeting glioma cells, since therapy of gliomas seems to be one of the applications best suited for EGFR targeting. The reason for this is that e.g. hepatocytes and epithelial cells naturally express EGFR [33, 52, 113, 158], which makes it inappropriate to use systemic administration of radiolabelled EGFR targeting agents. When targeting high grade glioma cells, this may be circumvented by using a locoregional, intracranial administration of the targeting agent.
Background

Gliomas

Every year, about 1300 people in Sweden are diagnosed with some form of tumour in the central nervous system. This corresponds to about 3% of the total incidence of new cancer patients. Brain tumours can originate from different types of brain cells. The most common primary brain tumours in adults, and unfortunately also the most aggressive ones, originate from supportive cells in the brain called glia cells, and these tumours are hence called gliomas. Gliomas can be divided into several subgroups, based on histological classifications (Table 1) [70]. One subgroup contains the astrocytic tumours. These are further classified into four grades by the World Health Organisation (WHO), with grade 1 being the least malignant form of tumour and grade 4 the most malignant and severe form [70, 73].

Table 1: Tumours of glial origin

- Astrocytic tumours
  - Pilocytic astrocytoma (WHO grade 1)
  - Diffuse astrocytoma (WHO grade 2)
  - Anaplastic astrocytoma (WHO grade 3)
  - Glioblastoma multiforme (WHO grade 4)
- Oligodendroglial tumours
  - Oligodendroglioma
  - Anaplastic oligodendroglioma
  - Mixed oligoastrocytoma
  - Anaplastic mixed oligoastrocytoma
- Ependymal tumours
  - Ependymoma

Gliomas of WHO grade 1 are called pilocytic astrocytomas. They are most often seen in children and are not considered malignant [73]. Gliomas of WHO grade 2, diffuse astrocytomas, account for about 35% of the human
astrocytic tumours, and they generally affect people between the ages of 20 and 40 years [70, 125]. These tumours are considered potentially malignant because of their infiltrative growth into normal brain tissue. Moreover, diffuse astrocytomas can progress into grade 3 or 4 astrocytomas [72]. Genetic alterations in the p53 gene [150], as well as overexpression of platelet derived growth factors (PDGF) and PDGF receptors [155], have been linked to the formation of diffuse astrocytomas [68, 125].

Anaplastic astrocytomas (WHO grade 3) can be diagnosed de novo, but are often detected in patients with a previous diffuse astrocytoma [68, 125]. These tumours have increased numbers of abnormal cells and cell nuclei, increased growth of blood vessels, and increased proliferation [68, 73]. The survival of patients with astrocytomas of WHO grade 3 is reduced compared to the lower grades. The most malignant of the astrocytic tumours are the glioblastomas (WHO grade 4). They account for about 50–60% of all astrocytic tumours [35]. A main characteristic of the glioblastomas, (also called glioblastoma multiformes, GBMs) is their extreme migrational potential [11, 45]. They appear to invade normal brain along the white matter tracts, around nerve cells, and along perivascular spaces [11]. To date, gliomas of WHO grade 4 are considered incurable.

One usually distinguishes between primary GBM, which occurs de novo, and secondary GBM that arises in patients with a previous lower-grade astrocytoma [10, 71, 72]. They often have different genetic alterations and may thereby have different pathogenesis [71, 72]. Secondary GBMs often have the same genetic alterations found in lower-grade astrocytomas, and typically develop in younger patients. Primary GBMs, on the other hand, are typically characterised by genetic alterations of the PTEN gene and amplification of the epidermal growth factor receptor (EGFR) locus, and they are most often detected in older patients [68, 71, 72].

Gliomas seldom metastasise outside the nervous system, but because of their infiltrative and migrational character these tumours are very difficult to cure [45, 73]. If possible, patients are usually treated with neurosurgery to remove the bulky part of the tumour, and the area around the tumour cavity is then irradiated by external beam irradiation [79]. Despite this, tumour recurrences are very common, and the median survival time for patients with GBM is usually less then 1.5 years [4, 79, 91, 130]. Chemotherapy is sometimes given to these patients, but most compounds used do not pass the blood-brain barrier effectively enough to inhibit growth of glioma cells [13, 45, 69].
The EGFR family

Receptors
The epidermal growth factor receptor (EGFR) family consists of transmembrane cell surface receptors that are involved in a wide range of cellular processes, including differentiation, proliferation, cell migration and apoptosis [159]. They are expressed in a variety of tissues of epithelial and mesenchymal origin [158]. Analyses of genetically modified mice have also shown that the receptors play a critical role in embryonic development [104, 158].

The receptor family consists of four members: EGFR (HER1, ErbB-1), HER2 (ErbB-2), HER3 (ErbB-3) and HER4 (ErbB-4). All receptors have the same basic structure with an extracellular ligand binding domain, a lipophilic transmembrane domain, an internal tyrosine kinase domain and finally a carboxy terminal domain where autophosphorylation occurs and signalling pathways are initiated. The degree of homology between the receptors differs between the different domains, with the tyrosine kinase domain having the highest sequence identities and the C-terminal region having the lowest [41].

The EGFR (HER1, ErbB-1) is a 170 kDa glycoprotein with 1186 amino acids that is expressed in several tissues, e.g in hepatocytes and epithelial cells [33, 52, 113]. The extracellular domain of EGFR consists of four subdomains, L1, CR1, L2 and CR2. The two ligand-binding L-subdomains form a β-barrel structure that recognizes and binds the ligand, with support from the two cysteine-rich CR-subdomains [60]. The hydrophobic transmembrane domain consists of 23 amino acids that form an α-helix [65, 153].

The intracellular tyrosine kinase domain has a high degree of homology between the different receptors of the EGFR family [41]. An exception is the HER3 receptor that has an impaired tyrosine kinase function [53]. The catalytic function of the tyrosine kinase involves transferring the gamma phosphate of ATP to the hydroxyl group of tyrosines in different proteins [63]. The ATP-binding pocket is hence used as target for anti-cancer drugs that inhibit the tyrosine kinase function [99].

Ligands
Receptors of the EGFR family are activated by binding of their respective ligands to the extracellular domain of the receptors. (An overview of the receptors and their ligands can be seen in figure 1.)
At least seven ligands are known to bind EGFR (HER1): the epidermal growth factor (EGF), transforming growth factor α (TGF-α), amphiregulin (AR), betacellulin (BTC), epiregulin (EPR), epigen and heparin binding EGF (HB-EGF) [56]. Neuregulins (NRG), of which the most well known is heregulin (NRG-1), bind both the HER3 and HER4 receptor. In addition to the neuregulins, HER4 has three other ligands: betacellulin (BTC), EPR and HB-EGF [116]. HER2 is considered an orphan receptor, since no natural ligand to the receptor has yet been identified.

The structures of the different ligands are related, with a consensus sequence of approximately 40 amino acids containing six cysteine residues spaced at defined intervals. These cysteins form three intramolecular disulfide bonds that restrain the molecule in a three-loop secondary structure [23, 121].

The ligand to EGFR, the epidermal growth factor, EGF, is a relatively small and compact protein of 6 kDa, consisting of 53 amino acids in a single polypeptide chain. The structure of the protein is stable, mainly due to many β-structures and three disulfide bonds. The three disulfide bonds are necessary for the biological function of the protein [17, 23]. Neither the N nor the C-terminal of the protein is believed to be involved in the receptor binding, as they are both distant from the supposed receptor binding site [17, 23].
Binding of ligand to a receptor triggers receptor dimerisation, which is essential for the subsequent generation of an intracellular signal. The receptor dimers can consist either of two identical receptors (homo-dimerisation) or of two different receptors (heterodimerisation). It has been shown that HER2 is the preferred dimerisation partner for all the other receptors in the EGFR family [49], but all possible homo- and heterodimeric receptor complexes between members of the EGFR family have been identified in different systems [51, 104, 159].

When receptors have dimerised, they are clustered together in clathrin coated pits on the cell surface, and are then internalised into the cell in endocytic vesicles (see figure 2). The endocytic vesicles fuse with endosomes and begin their passage through the endosomal compartments [137, 156]. Proteolysis of the receptor-ligand complex may begin in late endosomes, which contain functionally active enzymes [141]. Complete degradation of the complex is, however, thought to occur in mature lysosomes, where it is rapidly degraded to low molecular weight peptides [137]. Recycling of mainly non-occupied receptors, but also receptor-ligand complexes, can occur from all endosomal compartments, but recycling from late compartments is slower than from early ones [137, 151].

**Figure 2** Endocytosis and intracellular trafficking of EGFR.
Signalling

The function of EGFR receptors and ligands is to regulate proliferation, migration and survival of cells. This is accomplished by triggering a complex system of signalling pathways, which ultimately leads to DNA transcription and cellular response. When a ligand has bound a receptor and receptor dimerisation has occurred, the tyrosine kinase domains can auto- or transphosphorylate tyrosines in the c-terminal domain of the receptor. This provides sites for direct interaction with a number of adaptor proteins containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains [12, 104]. Which sites are phosphorylated and hence, which signalling molecules are engaged, is determined by the identity of the ligand as well as by the dimerisation partner [103].

The first signalling pathway shown to be downstream of the EGFR was the Ras and Shc-mitogen-activated protein kinase, or MAPK pathway. This pathway is an invariable target of all EGFR family ligands, and ultimately promotes cell proliferation. The pathway is activated by receptor interaction of the adaptor protein Grb2 in complex with the SOS guanine nucleotide exchange factor. SOS stimulates the exchange of GTP for GDP on the small G-protein Ras, and the activated Ras in its turn activates the serine/threonine kinase Raf-1. Through a series of intermediate kinases, Raf-1 activation leads to phosphorylation, activation and nuclear translocation of Erk-1 and Erk-2, which catalyse the phosphorylation of nuclear transcription factors [63, 65, 124].

**Figure 3** EGFR signalling including the PLCγ pathway, the PI3K pathway and the MAPK pathway.
In addition to the MAPK pathway, the PLCγ pathway and the phosphatidylinositol 3-kinase (PI3K) pathway are able to transduce EGFR mediated signalling. PLCγ activates second messenger systems that are required for cell motility [27], while the PI3K pathway is thought to be a mediator of the antiapoptotic effects following EGFR activation. In the PLCγ pathway, PLCγ associates with EGFR and then hydrolyses PIP2. This yields the second messengers DAG and IP3, which in their turn activate protein kinase C (PKC) and mobilise calcium stores from the endoplasmic reticulum. The PI3K pathway is mainly activated through the HER3 receptor, but activation in response to EGFR ligands occurs through formation of EGFR-HER3 heterodimers [65]. Activated PI3K, consisting of the two subunits p85 and p110, converts the plasma membrane lipid PIP2 to PIP3, which binds the protein Ser/Thr kinase Akt. Akt is then phosphorylated by the phosphoinositide-dependent kinase 1 (PDK1), ultimately resulting in cell survival response [18]. A schematic outline of the different signalling pathways can be seen in figure 3. The complexity of the signalling is, however, further increased by cross-talk between pathways.

Expression in tumours

The receptors of the EGFR family are, through their signalling, regulators of cell proliferation, motility and survival, and their genes are hence altered or amplified during tumour development in several malignancies. Most often, the gene is amplified, leading to overexpression of receptors in tumour cells [25]. Errors in the regulation of receptor production at translational/posttranscriptional levels may also result in receptor overexpression.

Overexpression and also structural alterations of EGFR are frequent in human malignancies. EGFR overexpression is moreover often accompanied by a high expression of ligand (especially TGF-α), resulting in frequent activation of EGFR signalling [121]. EGFR overexpression has been detected in, for example, gliomas [25, 127] and cancers of epithelial origin, such as those in lung [121], breast [149], bladder [16, 96, 97] and head and neck [30, 95]. About 50% of the glioblastoma multiforme tumours have overexpression of EGFR, most often because of amplification of the EGFR gene [25, 43, 127]. In gliomas [127], as well as in breast cancers [143] and bladder carcinomas [96], overexpression of EGFR is associated with advanced disease and poor prognosis. A mutated form of the EGFR, EGFRvIII, with a 267-aa deletion in the extracellular domain, has been found in glioblastomas [93, 127] and in carcinomas of breast, ovary [93] and lung [102]. EGFRvIII is believed to be constitutively phosphorylated, and therefore mediates an unattenuated signalling, although it cannot bind any known ligand [61].
Overexpression of HER2 is found in adenocarcinomas, especially in breast and ovary [15, 42]. About 15-30% of the invasive ductal breast cancers contain multiple copies of the HER2 gene, which has been linked to increased incidence of metastases and poor prognosis [42, 119]. Usually HER2 is relatively homogeneously expressed in the tumour tissue and the expression is retained in metastases [154].

HER3 is overexpressed in oral squamous cell cancer, where it correlates with lymph node involvement and decreased patient survival, but there is no evidence for gene amplification [128]. A reduced expression of HER4, relative to normal tissue, has been detected in breast and prostate tumours. This has been correlated with a differentiated phenotype [67].

Tumour targeting

A major problem in tumour therapy is to control disseminated tumour cells that remain after surgery and external radiotherapy. Chemotherapy can be efficient in treating spread or residual disease in some malignancies, e.g. lymphomas and testicular cancers. Chemotherapeutic agents, however, have the disadvantage that they are toxic not only to tumour cells but also to normal dividing cells, which may cause severe side effects. When chemotherapy cannot be used effectively against disseminated cells, as for example in GBM patients, there are few therapeutic alternatives, and the prognosis for these patients is often poor [130].

An ideal therapy in these cases would be a tumour specific targeted therapy, where tumour cell proliferation is selectively inhibited, without causing too severe damage to normal tissues. The intention of such a targeted treatment can either be to direct a cytotoxic agent specifically to the tumour cells, for example by using radiolabelled antibodies, or to stop tumour growth by inhibiting the function of molecules driving the tumour cells to proliferate. In both cases, a tumour specific target is needed.

Since receptors of the EGFR family, especially EGFR and HER2, are involved in the development of tumours and also overexpressed on the tumour cell surface in several malignancies, they can be considered as targets for such a specific tumour targeting treatment [86]. A major problem with this is, however, that normal cells also express receptors, although most often not to the same extent as in the tumours. For EGFR, there is, for example, quite a high receptor expression in hepatocytes, which makes it inappropriate to use systemic administration of EGFR directed targeting agents carrying a cytotoxic agent. For treatment of malignant gliomas, this problem may be circumvented if an intracranical locoregional administration is used. The targeting agent can then be administered into the operation cavity after the bulky tumour mass has been removed. The targeting agent
will hopefully diffuse through the brain tissue to reach disseminated tumour cells.

**Inhibiting growth factor receptor function**

In recent years a number of promising new anticancer drugs have been developed which target the function of growth factor receptors. The receptor function can be inhibited, either by blocking ligand binding to the extracellular part of the receptor, most often using antibodies, or by using small tyrosine kinase inhibitors that bind the intracellular part of the receptor. In both cases the aim is to block growth receptor signalling, and thereby inhibit tumour cell proliferation.

The antibodies generated to block ligand binding, and thereby also block receptor activation, are in most cases partially or fully humanised in order to limit HAMA (human anti-mouse antibody) immune responses. Cetuximab (C225) is a chimeric mAb directed against the extracellular domain of EGFR [5]. It has been used in several early clinical trials (phase I and II) for various cancers, including colorectal, and head and neck tumours, and both responses [36] and failures [94] have been reported. Another EGFR binding mAb is ABX-EGF. This is a fully humanised antibody with high affinity ($K_d = 0.5 \text{ pM}$) for EGFR. ABX-EGF is currently undergoing phase II trials in patients with renal, colorectal, prostate and non-small-cell lung cancers [114].

The most extensively studied mAb directed against receptors of the EGFR family is trastuzumab (Herceptin). It binds with high affinity ($K_d = 0.1 \text{ nM}$) to the HER2 receptor and is used against breast cancer cells overexpressing HER2. It acts by inducing antibody-dependent cell mediated cytotoxicity, as well as inhibiting receptor signalling. In a multicenter phase III trial, in which patients with metastatic HER2-expressing breast cancer were randomised to treatment with chemotherapy, with or without trastuzumab, the median survival was increased for those treated with trastuzumab [133].

In the last couple of years, inhibition of growth receptor function has developed from blocking receptor activation with mAb, towards the design of specific tyrosine kinase inhibitors (TKI). These compounds are small molecules that directly inhibit receptor tyrosine kinase activity by binding to the ATP-binding site on the intracellular part of the receptor. They thereby inhibit further signalling from growth factor receptors and consequently prevent tumour cell proliferation. One of the most studied tyrosine kinase inhibitors is the AstraZeneca developed drug gefitinib (Iressa, ZD1839). This quinazolone-based compound (the chemical structure is shown in figure 4), first described in 1997 [157], is a reversible ATP-competitive inhibitor of the EGFR tyrosine kinase. Gefitinib is an orally active drug with an $IC_{50}$ of 23 nM, and it was the first compound introduced in clinical studies as an
EGFR-targeted TKI. Several clinical studies with gefitinib have been performed, which have demonstrated promising clinical anti-tumour activity and an acceptable tolerability profile [7, 55, 140]. Gefitinib is currently in Phase III trials in advanced non-small-cell lung cancer and breast cancer [6].

![Chemical structures of the tyrosine kinase inhibitors gefitinib (Iressa, ZD1839), Tarceva (OSI-774), CI-1033, and EKB-569.](image)

Another EGFR-specific tyrosine kinase inhibitor is Tarceva (OSI-774). It behaves in a similar way as gefitinib, since it acts as a reversible ATP-competitive EGFR-TKI too. Tarceva is, however, even more specific for EGFR than gefitinib is, and has an IC₅₀ of 2 nM [92]. Since these drugs work by blockade of ATP, which is present at mM levels in cells, they have been administered using multiple-dosing schedules (e.g. daily for many days) in order to achieve and maintain the required plasma levels. In addition to the reversible TKIs, a number of irreversible TKIs against EGFR have been developed. These agents, e.g. CI-1033 and EKB-569, bind covalently to specific cystein residues in the ATP-binding pocket of EGFR [14]. CI-1033 acts against all members of the EGFR family, whereas EKB-569 only binds EGFR (HER1). In Phase I trials, both the irreversible TKIs seem to be generally well tolerated by patients [39, 140]. The chemical structures of the tyrosine kinase inhibitors are shown in figure 4.

**Targeting agents**

Anti-cancer drugs like gefitinib, which only affect the function of growth factor receptors, have one disadvantage: they are cytostatic rather than cytocidal. Even though it has been shown that TKIs can induce apoptosis in
a fraction of the treated cells [46], a majority of the tumour cells will probably remain alive after treatment.

Another approach of tumour targeting may then be of interest, namely to direct a cytotoxic agent specifically to tumour cells by using a tumour-targeting agent. A variety of targeting agents have been used for this purpose, and most often antibodies are used. Targeting with radiolabelled antibodies have been successful when treating haematopoietic tumours such as non-Hodgkin’s lymphoma [28, 66], but treatment of solid tumours have shown less progress [9, 22, 47, 146]. The main problem with antibodies in this case seems to be their inability to penetrate tissue fast enough, because of their large size [85, 89, 160].

Radiolabelled antibodies used for locoregional treatment of residual glioma cells in the brain have been shown to increase survival, but no curative effect has yet been achieved [3, 32, 58, 107, 117]. It has, however, not been elucidated whether the increased survival was due to a specific targeting, where antibodies diffused through brain tissue to reach the disseminated cells, or if it was due to non-specific irradiation by long range radiation from the radionuclides used. Targeting agents smaller than antibodies nevertheless seem preferable when targeting disseminated glioma cells [59, 89].

Possible alternatives to antibodies as targeting agents are, for example, antibody fragments, affibodies and small peptides.

There are some properties that are desirable for a good targeting agent:

- Good ability to diffuse in tissue
- Good water solubility
- High specificity for the target molecule
- High affinity of binding to the target molecule

Peptides and small proteins may have the potential to fulfil most of these requirements [152]. Radiolabelled analogues of the short peptide somatostatin have, for example, demonstrated encouraging results in the treatment of solid neuroendocrine tumours in several clinical trials [77, 108, 109, 148]. Radiolabelled somatostatin analogues have also been used for targeting gliomas [89]. The problem in that case is, however, that there is not any frequent expression of somatostatin receptors in high grade gliomas [24].

When targeting overexpressed growth factor receptors of the EGFR family, an option is to use receptor ligands as targeting agents. They are significantly smaller than antibodies (about 6 kDa rather than 150 kDa) which should favour their penetrating properties. They are, furthermore, quite stable molecules, in terms of insensitivity to pH, temperature and ion strength [17].
If a growth factor ligand is used as targeting agent, it will be internalised and processed by the tumour cells. The intracellular retention of the targeting agent, or more importantly the cytotoxic agent coupled to it, is therefore of importance, since the natural cellular processing of receptor-ligand complexes is fast. For example, $^{125}$I, coupled to EGF via direct labelling, is excreted as low molecular weight radiocatabolites from cells in culture within a few hours [132]. If the retention of radionuclides is not increased, there will be little time for radionuclides to damage the DNA of a targeted tumour cell. Furthermore, there is a risk that there will be problems with unspecific irradiation of normal tissues from radiocatabolites.

Radionuclides
When choosing a cytotoxic agent for coupling to a targeting agent, there are several alternatives. Toxins like ricin A, diphtheria toxin and pseudomonas exotoxin can be coupled to different targeting agents, either via chemical coupling or recombinant techniques, a strategy that has been tried for treatment of different malignancies [26, 82, 111, 122]. Cytotoxic agents like doxorubicin can also be used, if targeting liposomes are applied as transporting device [110]. Using drugs as cytotoxic agents, however, has disadvantages. The targeting agents may need to be internalised into tumour cells to have a cytotoxic effect, and all cells in e.g. a metastasis need to be targeted to get a curative effect. There is also a risk of tumour cells developing resistance against drugs.

By using radioactive nuclides as cytotoxic agents, these problems can, to a large extent, be avoided. Depending on the range of a radionuclide’s emitted particles, a radionuclide coupled to a targeting agent can irradiate not only the targeted tumour cell but also untargeted neighbouring tumour cells, an effect called the cross-fire effect. There is also no evidence of cells developing resistance against effects of radiation.

There are three types of radionuclides that can be considered for therapeutic purposes in radionuclide tumour targeting: alpha-emitters, beta-emitters and auger electron-emitters. Examples of such radionuclides are listed in table 2. The optimal radionuclide type for tumour targeting is very dependent on the targeting agent used, the characteristics of the target, the malignancy to be treated, size and morphology of tumours or metastases, administration route etcetera.

Generally, alpha-emitters seem to be the best alternative when single cells or small tumour clusters are to be treated. Since alpha particles have high LET (linear energy transfer) and high RBE (relative biological effectiveness) they are potent in killing cells, and their range is quite short (usually 50-100 µm) [145]. Alpha-emitters considered for radionuclide tumour targeting purposes are primarily $^{211}$At, $^{212}$Bi and $^{213}$Bi [64, 161]. The major obstacles concerning the use of these alpha-emitters are their poor availability at a
reasonable cost and their short physical half lives (7.2 h, 1.0 h and 45.6 min respectively) [64, 161]. To have an optimal effect of a targeted radionuclide therapy, the physical half lives of the radionuclides should be matched with the biologic half life of the targeting agent. The great benefit of alpha-emitters is that only a small number of decays are needed to kill a targeted cell [64].

Beta-emitters are suitable for targeting large tumour cell clusters (about 0.5 cm in diameter) [100], and might be advantageous to alpha-emitters in the sense that, due to the cross-fire effect, not all cells in a tumour need to be targeted in order to be killed [64]. This may overcome problems of heterogeneous expression of the target in tumour tissue, and poor tumour penetration of a targeting agent. Unfortunately, it also increases the non-specific toxicity to normal tissue. Beta-emitters, such as $^{90}$Y and $^{131}$I are frequently used in clinics. Beta particles emitted from $^{90}$Y are more energetic, with a mean path length of about 5 mm, compared to those emitted from $^{131}$I that have a 0.9 mm mean range [84]. On the other hand, $^{131}$I also emits gamma radiation that contributes to the whole body radiation dose. In recent years, there has been a growing interest in using the low energy beta-emitter $^{177}$Lu for radionuclide tumour targeting [138]. Lutetium-177 has a mean range of 0.28 mm in tissue which should limit the toxicity to normal tissue. Another advantage is that $^{177}$Lu also emits a small amount of gamma radiation with energy suitable for scintigraphic imaging [78].

When targeting single cells, the optimal path range of the radiation utilised should be comparable to the size of the targeted cell, so that a large fraction of the total radiation energy is deposited within the targeted cell. Auger-emitters emit low energy electrons that have a very short path length [101]. They should therefore be useful for killing single tumour cells [8, 84]. An auger emitter commonly considered is $^{125}$I, which emits electrons with a range of 0.06-17 µm [8]. This is too short to reach the DNA of a tumour cell if the radionuclide is not situated inside the cell. Preferably, auger-emitters should be associated with the DNA to have an optimal effect [8, 101]. Another auger-emitting radionuclide is $^{111}$In, often used for scintigraphic imaging using SPECT.

**Table 2: Potential radionuclides for targeted radiotherapy**

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Emission of therapeutic interest</th>
<th>Half life</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{90}$Y</td>
<td>High energy beta</td>
<td>2.7 days</td>
<td>Large tumour cell clusters</td>
</tr>
<tr>
<td>$^{111}$In</td>
<td>Auger cascade</td>
<td>2.8 days</td>
<td>Single tumour cells</td>
</tr>
<tr>
<td>$^{125}$I</td>
<td>Auger cascade</td>
<td>60 days</td>
<td>Single tumour cells</td>
</tr>
<tr>
<td>$^{131}$I</td>
<td>Medium energy beta</td>
<td>8 days</td>
<td>Medium tumour cell clusters</td>
</tr>
<tr>
<td>$^{177}$Lu</td>
<td>Low energy beta</td>
<td>6.7 days</td>
<td>Small tumour cell cluster</td>
</tr>
<tr>
<td>$^{211}$At</td>
<td>Alpha</td>
<td>7.2 h</td>
<td>Single tumour cells and small clusters</td>
</tr>
<tr>
<td>$^{212}$Bi</td>
<td>Alpha</td>
<td>1 h</td>
<td>Single tumour cells and small clusters</td>
</tr>
<tr>
<td>$^{212}$Bi</td>
<td>Alpha</td>
<td>0.76 h</td>
<td>Single tumour cells and small clusters</td>
</tr>
</tbody>
</table>
Since cancer patients very seldom have a spread disease with tumour cell clusters of a uniform size, the optimal solution when choosing radionuclide for targeted therapy might be to have a mixture of radionuclides that emit particles of different ranges. It has been shown that combined treatment of tumour-bearing rats with $^{90}$Y and $^{177}$Lu labelled somatostatin analogues, resulted in better survival than when rats were treated with only one of these radionuclides [37].
Aim

The aim of this thesis was to develop and preclinically characterise potential EGF-based targeting agents for targeted radiotherapy of EGFR-expressing tumours, especially malignant gliomas. Special attention was directed towards improving the intracellular retention of the targeting radionuclides. Furthermore, the potential to combine targeted radiotherapy of EGFR with inhibition of the EGFR signalling, using the tyrosine kinase inhibitor gefitinib, was to be evaluated.

Three different types of EGF-conjugates were studied: EGF-dextran labelled with radioactive iodine, EGF labelled with the alpha-emitter $^{211}$At, and EGF-chelates labelled with the radioactive metals $^{111}$In or $^{177}$Lu.
EGF-dextran (paper I and II)

Since radiohalogens like $^{125}$I, $^{131}$I and $^{211}$At are rapidly excreted from tumour cells when EGF is used as targeting agent [2, 20, 105, 131], efforts have been made to increase their intracellular retention. One way to increase the retention is to couple EGF to dextran. Dextran is a polysaccharide compound built up as a chain of glucose units. The glucose units are linked by $\alpha$ 1-6 bonds, with about 5% branching, mostly as a result of $\alpha$ 1-3 linkages. Due to the stability and excellent water solubility of dextran, it has been used for clinical purposes, e.g. as a blood expander. Dextrans exist naturally in a wide range of molecular weights, but can be separated into quite narrow average molecular weights.

Conjugate

The first EGF-dextran conjugate developed was conjugated using the CDAP-method [2, 74]. This is a “random coupling” since EGF can be conjugated to any of the glucose units of dextran [21]. The hydroxyl groups in dextran are activated by CDAP to form cyanate esters that can react with primary amino groups of EGF. Zhao et al then developed a more defined “end-end coupled” EGF-dextran conjugate [162]. This coupling took advantage of the fact that there is only one reducing end in dextran. It is situated at the C1 end, where an aldehyde is formed when the ring structure is open. This aldehyde was coupled by reductive amination to the N-terminal amino group of EGF. In this end-end-coupled conjugate, dextran can also be
used as carrier of radionuclides, by using the cyanylating agent CDAP to couple tyrosines to dextran. Radioactive iodine can then be coupled to the tyrosine residues via direct iodination, using chloramine-T as oxidising agent.

In both these conjugates, mouse EGF was used instead of human EGF. The reason for this is that mouse EGF has only one reactive amino group at physiological pH, the amino terminus, whereas human EGF in addition to that has two lysine residues that introduce two extra amino groups [17]. Consequently, if human EGF is used for coupling, dextran can be conjugated to any of the three reactive amino groups. With mouse EGF, it is reasonable to assume that the conjugates formed are homogenous. Mouse and human EGF have about the same affinity to human EGFR, but differ somewhat in amino acid composition [17, 44, 50].

In papers I and II, end-end coupled EGF-dextran conjugates were used. EGF was conjugated to dextran with a molecular weight of 13 or 9 kDa, using cyanoborohydride (NaCNBH₃) as reductive agent. The conjugate was then purified from unreacted EGF and dextran, using gel filtration and preparative gel electrophoresis. Tyrosines were coupled to dextran, using the CDAP-reaction, to allow radio-iodination of both the EGF part and the dextran part of the conjugate.

Radiolabelling of EGF-dextran conjugates with ¹²⁵I was performed using the chloramine-T method. Chloramine-T oxidises ¹²⁵I, which results in incorporation of ¹²⁵I into tyrosine residues in e.g. a target protein. In paper I, EGF-dextran was labelled with ¹²⁵I on the EGF part, on the dextran part, or on both parts of the conjugate. Labelling on both parts ([¹²⁵I]-EGF-dextran-[¹²⁵I]), was done as described above. The conjugate labelled only on the EGF part ([¹²⁵I]-EGF-dextran) was produced and labelled as described above, but since this conjugate should not be labelled on the dextran part, no tyrosines were conjugated to the dextran chain. The CDAP-reaction was therefore omitted. To label the conjugate on the dextran chain only (EGF-dextran-[¹²⁵I]), tyrosines were first labelled with ¹²⁵I and then conjugated to the dextran chain, using the CDAP-reaction.

### Binding and cellular processing

During the cell experiments, the human glioma cell line U343MGaCl₂:6 was used (from now on denoted U343). It expresses approximately 5×10⁵ EGFR/cell. The experiments were performed on cells in monolayer cultures. Unless otherwise stated, cell experiments were performed at 37°C.

In order to investigate the binding specificity of EGF-dextran, displacement tests were performed. Cells were then incubated on ice with radiolabelled conjugate and excess amounts of unlabelled EGF. The EGF-dextran conjugate was shown to bind specifically to EGFR, since binding of 18
radiolabelled conjugate could be displaced by increasing concentrations of unlabelled EGF (figure 6). At the highest EGF-concentration, approximately 10% of the EGF-dextran binding remained unblocked.

![Figure 6](image)

**Figure 6** Displacement of $^{125}\text{I}$-EGF-dextran-$^{125}\text{I}$ and $^{125}\text{I}$-EGF after 2 h incubation. The binding values are presented in arbitrary units.

In paper I, we studied the possibility of using dextran as a carrier of radionuclides, to improve their intracellular retention. Retention tests were performed with EGF-dextran conjugates labelled with $^{125}\text{I}$ on different parts: $^{125}\text{I}$-EGF-dextran-$^{125}\text{I}$, $^{125}\text{I}$-EGF-dextran and EGF-dextran-$^{125}\text{I}$. Comparisons were made with $^{125}\text{I}$-EGF. The radiolabelled compounds were incubated with cultured U343 cells for different times (0.5-48 h). The cells were then washed, and the retention of radioactivity was measured for up to 24 h.

It was shown that radioiodine coupled to the EGF part of EGF-dextran had an increased retention relative to radioiodinated EGF. The retention was, however, further increased if radioiodine was coupled to the dextran part. When the radionuclides were coupled only to dextran, 70-80% of the radioactivity was still cell associated after 24 h (figure 7). Radioactive compounds excreted from the cells were also separated on small gel filtration columns, to determine the approximate range of the molecular weights of the radiocatabolites. The results showed that radioactive compounds excreted from cells generally were of low molecular weight (less than 5 kDa) if radioiodine was coupled to EGF, and of high molecular weight (more than 5 kDa) if radioiodine was coupled to dextran (see table 3). We could hence draw the conclusions that the EGF-part of EGF-dextran is degraded in cells, and low molecular weight catabolites are quickly excreted. Dextran, on the other hand, seems not to be degraded to the same extent, but
instead accumulated in cells and only slowly released from the cells, mainly as high molecular weight compounds.

![Graph showing cell associated retention of radioiodine](image)

**Figure 7** Cell associated retention of radioiodine, after interrupted incubation with $^{125}\text{I}$-EGF, $^{125}\text{I}$-EGF-dextran, $^{125}\text{I}$-EGF-dextran-$^{125}\text{I}$ and EGF-dextran-$^{125}\text{I}$. The incubation was interrupted at the time of maximal binding for each conjugate.

**Table 3: Molecular weight distribution of excreted radioactivity**

<table>
<thead>
<tr>
<th>Compound</th>
<th>High molecular weight (%)</th>
<th>Low molecular weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{125}\text{I}$-EGF</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>$^{125}\text{I}$-EGF-dextran</td>
<td>23</td>
<td>77</td>
</tr>
<tr>
<td>$^{125}\text{I}$-EGF-dextran-$^{125}\text{I}$</td>
<td>48</td>
<td>52</td>
</tr>
<tr>
<td>EGF-dextran-$^{125}\text{I}$</td>
<td>64</td>
<td>36</td>
</tr>
</tbody>
</table>

* 1 h preincubation with compound, 24 h incubation during retention study

The dextran dependent increased retention may result in a substantial difference in therapeutic dose to a targeted cell, if $^{131}\text{I}$ is applied instead of $^{125}\text{I}$. Theoretical calculations showed that the dose to a tumour cell nucleus could increase approximately 100 times if $^{131}\text{I}$-EGF-dextran-$^{131}\text{I}$ was used instead of $^{131}\text{I}$-EGF. Thus, dextran seems suitable as a carrier of radionuclides aimed for therapy, and gives potential for a highly increased radiation dose.
Signalling studies

EGF normally induces signalling cascades by binding EGFR. For example, EGF can activate the MAPK signalling pathway, the PLCγ signalling pathway and the PI3K signalling pathway. In order to characterise the biologic activity of EGF-dextran, the signalling properties of the conjugate was studied in the glioma cell line U343. After stimulation of cells with EGF-dextran or EGF, the phosphorylation of signalling molecules, downstream of EGFR, was studied. The studied molecules were Erk (MAPK pathway), PLCγ (PLCγ pathway) and Akt (PI3K pathway). The phosphorylation of specific tyrosines on EGFR (Tyr 992, 1068, 1086, 1148 and 1173) was also studied.

Cultured U343 cells were stimulated with equimolar concentrations of EGF and EGF-dextran at 37°C for different amounts of time. The cells were then lysed, and proteins were immunoprecipitated. Precipitated proteins and total cell lysates were resolved by SDS-PAGE and electrotransferred to PVDF-membranes. The membranes were then blotted with antibodies recognising activated EGFR, as well as the activated downstream targets Erk, Akt and PLC-γ. The filters were also reprobed to determine the total protein content.

EGF-dextran was found to stimulate EGFR signalling in glioma cells. The extent of Erk and Akt phosphorylation was equally high after EGF and EGF-dextran stimulation. The phosphorylation status of PLCγ, however, differed significantly between EGF and EGF-dextran stimulation. As can be seen in figure 8, PLCγ was activated less efficiently after EGF-dextran stimulation than after EGF stimulation. Equal loading of the wells was verified by probing for total PLCγ (see figure 8, lower photo).

The PLCγ signalling pathway is activated after binding of PLCγ to EGFR. The major binding sites for PLCγ on EGFR are tyrosine residue 992, where the c-terminal SH2 domain of PLCγ binds, and tyrosine residue 1173, where
the N-terminal of PLCγ binds. In accordance with the low level of PLCγ phosphorylation, the phosphorylation of these tyrosine residues was also considerably lower after EGF-dextran stimulation than after stimulation with EGF. The mechanism behind the differential phosphorylation status of the tyrosine residues is not known. A possibility is that the bulky dextran moiety on EGF causes differences in orientation of the intracellular kinase domains in the EGFR dimer. This difference might result in different phosphorylation efficiency of various tyrosine residues in the c-terminal domain of EGFR.

Comments

The idea of conjugating a proteolytically undegradable carrier molecule like dextran to EGF, in order to increase the retention of radiohalogens, is elegant and appealing, and the concept has also been shown to work very well. The EGF-dextran conjugate has retained biologic activity, it binds specifically to EGFR in cell culture, and a majority of the conjugate is internalised upon binding. Furthermore, the retention of radionuclides delivered by EGF-dextran could be increased by several orders of magnitude, especially if radionuclides were coupled only to the dextran part of the conjugate.

There are, however, problems with this conjugate that have so far hindered the process of bringing it to the clinic. The main problem has been the lengthy and complicated conjugation and purification procedures that make it very difficult to adapt the production to a larger scale with retained GMP (good manufacturing practice) quality. The coupling chemistry and purification methods therefore need to be improved.

Since the intended application is to target disseminated glioma cells by local injection of the conjugate, there are also some concerns about increasing the molecular weight of the targeting agent. Large proteins are known to have a limited diffusion in tissues [89], and the addition of dextran to EGF increases the molecular weight about three times. This increase in size could be critical, since it has been shown that NGF (nerve growth factor), with a molecular weight of approximately 13 kDa, only diffuses about 2-3 mm in any direction in rat brain interstitium [75]. In comparison, 111In-labelled DOTATOC with a molecular weight of 1.4 kDa, is rapidly and extensively distributed in human brain, following intratumoural injection [87]. Adams et al. have shown that exceedingly high affinity of antibody-based molecules may impair the tumour penetration properties, due to extensive receptor binding [1]. This was, however, probably not the case for NGF, since the implantation site of the controlled release polymers containing NGF was not near any region in the brain, known to have a high concentration of NGF-receptors [75].
Treatment of cultured tumour cells with the tyrosine kinase inhibitor (TKI) gefitinib (“Iressa”, ZD1839), in combination with targeted radiotherapy using radiolabelled EGF, was investigated in paper III and IV.

Gefitinib is an anticancer drug developed by the AstraZeneca group of companies. It is a low molecular weight compound that acts by binding specifically to the tyrosine kinase domain of EGFR. It thereby inhibits mitotic signals from EGFR and thus prevents further growth of EGFR expressing tumours. However, the binding of gefitinib to EGFR is reversible, which means that tumour cells are growth inhibited only as long as they are exposed to gefitinib [147]. If the treatment is interrupted, a majority of the tumour cells will resume growth. It is therefore of interest to combine gefitinib treatment with other therapeutic modalities. Several experimental in vitro or in vivo studies have been performed, where gefitinib is combined with inhibitory antibodies, such as trastuzumab (Herceptin) [98], chemotherapy [31] or external radiation [62, 136], and many of these combinations have shown synergistic effects.

Combined treatment with radionuclide tumour targeting and TKIs like gefitinib has not been studied before. However, in vitro studies have previously indicated that gefitinib can increase the uptake of radiiodinated EGF in tumour cells with a high expression of EGFR [81]. This encouraged us to investigate the possibility of combining gefitinib treatment with radionuclide tumour targeting using radiolabelled EGF as targeting agent.

Conjugates

Recombinant human EGF labelled with $^{125}$I was used as targeting agent, for studies of the binding and cellular processing, during simultaneous gefitinib treatment. EGF was labelled with $^{125}$I using the chloramine-T (CAT) method, as previously described.

When studying the effects of combined gefitinib and targeted radiotherapy treatment, EGF was labelled with the alpha-emitter $^{211}$At. An indirect labelling method was used for labelling of EGF with $^{211}$At. The labelling was performed in two steps. First, N-succinimidyl-4[trimethylstannyl]benzoate was labelled with astatine, using CAT. The obtained
astatinated linker molecule, N-succinimidyl-[^211]At]-4-astatobenzoate, was thereafter coupled to amino groups on EGF, yielding ^211At-benzoyl-EGF.

**Binding and cellular processing**

Two EGFR rich cell lines, U343 and A431, were used in all cellular experiments. U343 is a glioma cell line which expresses approximately 0.5 million EGFR per cell. A431 is a human squamous carcinoma cell line originating from vulva, which expresses approximately 2 million EGFR per cell. Both cell lines were grown as monolayer cultures during the experiments.

The influence of gefitinib treatment on cells was first investigated. The cell growth, with or without gefitinib (1 µM), was measured for both cell lines. As seen in figure 9, A431 cells were efficiently growth inhibited by gefitinib at this concentration. If the treatment was interrupted and the cells were washed free from drug, the cells, however, resumed growth at the same rate as control cells. The glioma cell line, U343, on the other hand, was found to be resistant to gefitinib treatment, since no growth inhibition could be seen.

![Figure 9](image)

Figure 9 Influence of gefitinib on the growth of U343 cells (A) and A431 cells (B). Cells were grown in the presence or absence of 1 µM gefitinib. Growth inhibited A431 cells could recover their growth capacity if the gefitinib incubation was interrupted (after 48 h).

The binding of radiohalogenated EGF to cultured cells, simultaneously treated with gefitinib, is shown in figure 10. Both ^125I-EGF and ^211At-EGF were used. Cells were first treated with gefitinib (0-1 µM) for 48 or 24 h, before addition of ^125I-EGF or ^211At-EGF. The binding was then studied, in presence of gefitinib, for up to 24 h. The specificity of binding was assessed by adding excess amounts of unlabelled EGF, along with the radiolabelled EGF, in the binding study (figure 11).
The influence of gefitinib on cellular retention of radioactivity was also studied (figure 12). After treatment with gefitinib in combination with $^{125}$I-EGF, as described above, the incubation was interrupted after 45 minutes and the cells were washed. Fresh culture medium containing 0 or 1 µM gefitinib was added to the cells, and the retention was then studied for up to 20 h.

**Figure 10** Binding of $^{125}$I-EGF (A and C) and $^{211}$At-EGF (B and D) to U343 cells (A and B) and A431 cells (C and D), during treatment with various concentrations of gefitinib.

**Figure 11** Specificity of $^{211}$At-EGF binding in U343 cells (A) and A431 cells (B), treated with 0 or 1 µM gefitinib. The cells were incubated with $^{211}$At-EGF for 7 h. Excess amounts of unlabelled EGF were used to block EGFR specific binding.
Results of the binding study showed that the uptake of both $^{125}$I-EGF and $^{211}$At-EGF increased with increasing gefitinib concentrations (figure 10). The binding was specific, since it could be displaced by excess amounts of EGF (see figure 11). Interestingly enough, the increase in uptake could be seen with both cell lines, despite their difference in sensitivity to gefitinib. It has been suggested that the increased binding of radiolabelled EGF is due to an EGFR dimer formation in absence of ligand, caused by gefitinib binding, and leading to increased affinity for EGF [81]. This is, however, probably not the only explanation, since the cellular retention of radioactivity also was affected by gefitinib treatment (figure 12). The results from the retention study showed that in both cell lines the retention of radioactivity, after interrupted $^{125}$I-EGF incubation, was significantly prolonged by gefitinib treatment. This suggests that the cellular processing and excretion are also affected by gefitinib treatment.

**Experimental therapy in vitro**

The effect of the increased $^{211}$At-EGF uptake on cell survival was first studied using a clonogenic survival assay. U343 cells and A431 cells were treated first with gefitinib (0-1 µM) for 24 h, and thereafter with combined gefitinib and $^{211}$At-EGF for another 24 h. In addition to this, control cells were treated with an excess of EGF, in order to block binding of astatinated conjugate. This control group was used to determine the amount of cell deaths resulting from $^{211}$At-EGF in the medium. Another group of cells was treated with gefitinib only and not with $^{211}$At-EGF.

After treatment, the cells were washed, trypsinised, counted and plated for clonogenic survival. Cells that survived the treatment formed colonies which after fixation and staining could be counted by the naked eye. A colony was
counted only if it contained 50 or more cells. A treatment that killed a lot of cells consequently yielded few colonies and a low survival, whereas a less effective treatment yielded more colonies and better survival.

The results of the clonogenic survival assay are shown in figure 13. For the U343 cells, the survival after combined treatment with gefitinib and $^{211}$At-EGF, as expected, decreased with increasing gefitinib concentrations. At the highest gefitinib concentration (1 µM) the survival had decreased about 3.5-fold. No effect on survival was seen with gefitinib treatment alone. Also, there was no effect on blocked cells, which had been pre-saturated with non-labelled EGF just before addition of $^{211}$At-EGF. Thus, there was virtually no background killing due to $^{211}$At-EGF in the surrounding medium. This is important, since it shows that $^{211}$At-EGF was mainly affecting targeted cells.

The A431 cells demonstrated a dramatic decrease in survival after exposure to $^{211}$At-EGF (Figure 13). Furthermore, there was a slight decrease in survival after treatment with gefitinib alone, which might be explained by gefitinib-induced apoptosis [46]. The combined treatment with gefitinib and $^{211}$At-EGF in A431 cells, however, surprisingly resulted in an increase in survival, even though combined treatment resulted in more cell associated astatine than $^{211}$At-EGF treatment alone did. This increase was quite pronounced: 1 µM gefitinib increased the survival of A431 cells about 22-fold compared with $^{211}$At-EGF treatment alone. These results indicate that gefitinib could have a protective effect against $^{211}$At-EGF for cells that are growth inhibited by gefitinib treatment.

![Figure 13](image)

**Figure 13** Clonogenic survival assay, with U343 cells (A) and A431 cells (B). The cells were treated with 1 µM gefitinib alone (open squares) or with a combination of gefitinib and $^{211}$At-EGF (open triangles). To determine the amount of cell death due to unspecific irradiation from $^{211}$At-EGF in the surrounding medium, control cells were blocked with excess amounts of EGF, in addition to treatment with $^{211}$At-EGF and gefitinib (filled triangles).
To confirm the results and to exclude bias due to the method used, a cell growth assay was performed. U343 cells and A431 cells were treated with gefitinib (0 or 1 µM) and \(^{211}\text{At-EGF}\) for 24 h, as described above. But instead of seeding a portion of the cells for clonogenic survival, all cells were, in this assay, counted, approximately once a week, to follow the increase or decrease in cell number. In principle, the cells in the assay can be looked upon as a tumour that is treated, and then the shrinkage or regrowth of the tumour is measured.

The results of the cell growth assay, shown in figure 14, confirmed the results from the clonogenic survival assay. In U343 cells, combined gefitinib and \(^{211}\text{At-EGF}\) treatment delayed regrowth of the tumour cells longer than \(^{211}\text{At-EGF}\) treatment alone (eight days and four days growth delay, respectively, compared to untreated control cells). For the A431 cells, the concept of a gefitinib-induced protective effect was strengthened, since a clear difference in growth could be seen between combined treatment and \(^{211}\text{At-EGF}\) treatment alone. \(^{211}\text{At-EGF}\) treatment gave a growth delay of 46.5 days relative to control cells, whereas combined treatment gave only 24 days growth delay.

The reason for the protective effect of gefitinib on A431 has not yet been elucidated. It has been hypothesised that gefitinib impairs the internalisation of EGF-EGFR complexes, which can lead to the fact that \(^{211}\text{At-EGF}\), when cells are treated with gefitinib, is situated on the cell membrane instead of in the cytoplasm. The dose from \(^{211}\text{At}\) to the cell nucleus would then be significantly lower, which would lead to increased survival [57]. This explanation is, however, probably incorrect, since recent experiments with fluorescently labelled EGF indicated that EGF was internalised in A431 cells also during gefitinib treatment (results not shown). Cells were then treated
with gefitinib and EGF-Texas Red (EGF-TR) for 30 minutes, and after fixation and mounting, the staining was evaluated in a confocal microscope. A vast majority of the EGF then seemed to be internalised in endosomes. The same pattern was seen in cells not treated with gefitinib. Control cells incubated on ice during the treatment with EGF-TR seemed stained predominantly at the cell membrane.

Another hypothesis about the reason for the protective effect is related to DNA repair. It has been shown that cultured cells treated with gefitinib are arrested in $G_1$ phase [62]. A possible explanation might therefore be that the delayed cell mitosis, caused by gefitinib, enhances the possibility of successful DNA repair of $^{211}\text{At}$-EGF induced DNA damages, thereby increasing the survival. Whether this is true or not, will hopefully be revealed by future experiments, probably using external radiation.

Comments

Combining different therapy modalities is a common way to enhance the therapeutic effect on tumour cells. Radiotherapy has, for example, been combined with radiosensitising agents like cyclooxygenase-2 (COX-2) inhibitors or topoisomerase I inhibitors, in order to enhance the antitumour effect of radiation [29, 120]. The EGFR tyrosine kinase inhibitor gefitinib has been studied in combination with inhibitory antibodies, chemotherapy and radiotherapy, but no studies on combined targeted radiotherapy and gefitinib treatment had previously been performed.

The most basic, and also most important, requirement in targeted radiotherapy is to have a high uptake of radionuclides in the tumour. The finding that gefitinib could increase the uptake of $^{125}\text{I}$-EGF and $^{211}\text{At}$-EGF in tumour cells up to five times, was therefore very encouraging. The cellular retention of radioactivity could also be significantly prolonged by gefitinib. It was especially interesting that the increased uptake could be seen also with U343 cells, since this cell line was shown to be resistant to gefitinib treatment at the studied concentrations. In this cell line, the enhanced radionuclide uptake also had effect on the cell survival.

The discovery that the gefitinib sensitive A431 cell line survived combined gefitinib and $^{211}\text{At}$-EGF treatment better than treatment with $^{211}\text{At}$-EGF only was unexpected and surprising. EGFR activation has been connected with increased radioresistance, possibly via disruption of the balance of downstream signalling transduction pathways to favour survival signals. EGFR inhibitors like gefitinib have consequently been associated with radiosensitisation rather than the opposite [123]. A study on A431 cells accordingly showed that gefitinib potentiates rather than decreases the effect of external radiation [136]. However, in that study an additive effect on
clonogenic survival was seen only when gefitinib was administered after radiation. No effect was seen in cells treated with gefitinib before radiation.

The concept of using co-treatment with small effector molecules, to enhance uptake or retention of radionuclide targeting agents, is interesting and worth further exploration. Further studies with gefitinib will include animal studies, to investigate whether increased uptake of radiolabelled EGF can be achieved in vivo as well. Since our studies with gefitinib only involved two cell lines, it is of interest to see response patterns of other cell types. It would also be interesting to study if other EGFR specific TKIs have the same influence on EGF uptake as gefitinib.

Other types of molecules, with a potential to increase uptake or retention of targeting agents, may also be of interest for combined therapy. Drugs that raise the pH of lysosomes can, for example, trap internalising agents like EGF in lysosomes and other acidic compartments, thereby increasing their cellular retention [34]. Examples of such drugs are chloroquine, used against malaria, and thioridazine, an antipsychotic drug [19, 76].
EGF-chelates (paper V and VI)

Radioactive metals are known to have a good intracellular retention, and are therefore attractive to use in targeted radionuclide imaging and therapy. Commonly used radiometals for these applications are e.g. $^{111}$In, $^{99m}$Tc, $^{90}$Y, and $^{177}$Lu. Proteins are most often labelled with radiometals, using a chelating agent. Chelating agents are organic compounds that are capable of forming coordinate bonds with metals through two or more atoms. The chelating agent is, most often, first coupled to the protein, and thereafter the radiometal is added.

When chelates are used for labelling of targeting agents, intended for targeted radionuclide imaging and therapy, the stability of the radiometal-chelate complex is essential. If the stability is insufficient, it may lead to release of radiometal and subsequent increased background irradiation of normal tissues. Certain radiometals, like $^{90}$Y and $^{177}$Lu, also accumulate in bone matrix, if released from the chelating agent [80, 135, 139].

Several different chelating agents have been used for labelling of targeting agents. Both acyclic chelates, like e.g. DTPA, and macrocyclic chelates like e.g. DOTA, may be used. Generally, macrocyclic chelators form more stable radiometal-chelate complexes than acyclic chelators. The incorporation of metal is, however, often slower and more difficult for macrocyclic agents, and often requires heating. The stability of a chelator also depends on the number of bonds it can form to the metal ion, and on the radiometal used. Different radiometals may therefore need different chelating agents for optimal stability.

Conjugates

In paper V and VI we have characterised radiolabelled EGF-chelates, aimed for targeted therapy of disseminated glioma cells. A glioma patient may be burdened with a bulky residual tumour, somewhat smaller cell clusters and single cells. Such a variety of targets suggest that an improved treatment might be achieved by using a panel of several radionuclides, a “radionuclide cocktail”. It has been demonstrated in rats that a combination of $^{90}$Y and $^{177}$Lu-labelled DOTATATE (a somatostatin analogue) was superior to treatment with only one of the radionuclides. For treatment of gliomas with EGF-chelate, $^{90}$Y may be used against a bulky tumour, and $^{177}$Lu is suitable
for treatment of smaller cell clusters. For treatment of single tumour cells, the auger emitter $^{111}$In may be used [8]. The design of the cocktail approach could be to use one EGF-chelate labelled with various types of radionuclides.

EGF has previously been conjugated to the acyclic chelating agent DTPA [106, 115]. DTPA is sufficiently stable for chelating $^{111}$In, but the stability is not adequate if $^{90}$Y [118, 126, 135], or $^{177}$Lu [80, 139] is used. We therefore investigated the possibility of using Bz-DTPA instead of DTPA. DTPA is a heptadentate chelator, i.e. seven atoms are involved in chelating the metal ion (three nitrogens, and four carboxylic oxygens), and one carboxylic arm is used for coupling to the peptide (see figure 15). The octadentate Bz-DTPA should be more stable, since all five carboxylic arms can be used for chelating the metal ion. Our aim was therefore to develop and study a Bz-DTPA-EGF chelate, labelled with different, potentially cytotoxic, radionuclides.

In paper V, we compared $^{111}$In-labelled Bz-DTPA-EGF and DTPA-EGF conjugates in the glioblastoma cell line U343. In paper VI, Bz-DTPA-EGF was labelled with $^{177}$Lu and studied both \textit{in vitro} and \textit{in vivo}.

When making the EGF-chelates, the chelating agents were first conjugated to human recombinant EGF. Human EGF was used for several reasons. First, it is possible to get a higher specific activity with human EGF than with mouse EGF, since human EGF has more reactive amino groups. Second, for a future application in humans, it is less complicated to ensure purity regarding e.g. viruses, when the product is not purified from animal organs.

![Figure 15](image)

\textbf{Figure 15} Structures of the EGF-chelates used: Bz-DTPA-EGF (A) and DTPA-EGF (B).
During the conjugation, an amino group of human EGF, either at the amino terminal or in the lysine residues, was coupled to the chelating agents by mixing isothiocyanate-benzyl-DTPA or dianhydride DTPA with EGF in borate buffer at pH 9.1. After separating conjugates from unreacted chelating agents, the EGF-chelates were labelled with radionuclides. A solution of $^{111}$In or $^{177}$Lu was added to the EGF-chelates in 0.1 M ammonium acetate buffer, pH 6.0, and the reaction mixture was mixed at room temperature for 1 h.

### Binding and cellular processing

Three different EGF-chelates, $[^{111}$In]DTPA-EGF, $[^{111}$In]Bz-DTPA-EGF and $[^{177}$Lu]Bz-DTPA-EGF, were studied on U343 glioma cells, grown as monolayer cultures. The binding of EGF-chelate to cells was studied by incubating radiolabelled EGF-chelate with cells for different amounts of time, and after washing, by measuring the cell associated radioactivity. The amount of unspecific binding was measured by blocking receptors on the cells with excess amounts of unlabelled EGF, prior to addition of radiolabelled EGF-chelate. All three EGF-chelates were shown to bind specifically to EGFR on cells (figure 16). The unspecific binding of the $^{177}$Lu-labelled EGF-chelate was, however, higher than for the two $^{111}$In-labellled conjugates. The unspecific binding also increased somewhat with time in this case. The reason for this is not known.

![Figure 16](image_url)

**Figure 16** Binding of the EGF-chelates, $[^{111}$In]DTPA-EGF, $[^{111}$In]Bz-DTPA-EGF and $[^{177}$Lu]Bz-DTPA-EGF, to U343 cells (−). The specificity of the binding was assessed by blocking EGFR binding with excess amounts of unlabelled EGF (+).
The amount of binding was high for all three conjugates (figure 16), and studies of the internalisation into cells also showed that a majority of all three EGF-chelates was internalised at all time points studied (results not shown). This was studied by using an acid-wash technique developed by Haigler et al [54]. The binding kinetics of $^{111}$In-labelled Bz-DTPA-EGF appeared to be faster than for the other chelates studied, since a higher portion of the radioactivity was cell associated at early time points. This was, however, probably an artefact, due to a larger amount of cells used in this case. Otherwise, the molar amount of EGF-chelate added, was approximately equal to the amount of receptors.

The intracellular retention of radioactivity, after interrupted incubation with EGF-chelate, was also studied. The EGF-chelates were allowed to bind to cells for 5 h, and after washing, the retention was studied for up to 24 h. For all three conjugates, the retention of radioactivity was good, with 55-65% still cell associated after 45 h (figure 17). The retention of the EGF-chelates was also shown to be comparable to that of EGF-dextran, labelled with $^{125}$I on the dextran part. This indicates that very small amounts of the radionuclides diffuse out from the cells, which is important in a therapeutic application. For comparison, the retentions of EGF and EGF-dextran, directly labelled with $^{125}$I are also shown in figure 17.

![Figure 17](image-url)

**Figure 17** The retention of cell associated radioactivity, after interrupted incubation with the EGF-chelates $^{111}$In-DTPA-EGF, $^{111}$In-Bz-DTPA-EGF and $^{177}$Lu-Bz-DTPA-EGF. The radionuclide retention, after incubation with $^{125}$I-EGF and EGF-dextran-$^{125}$I, is also shown, for comparison.
Affinity determination

Affinity is a measure of how strong the binding is between e.g. a receptor and its ligand. The stronger the binding is, the lower the dissociation constant, $K_d$. The dissociation constants of the EGF-chelates were determined from saturation studies on cultured U343 cells. Dilution series of radiolabelled EGF-chelates were incubated with cells on ice for 3-4 h. The amount of EGF-chelate bound to cells was then measured and plotted against the molar concentration of added EGF-chelate. Unspecific binding, determined by EGFR blocking with excess EGF, was subtracted from the total binding. The data were analysed with GraphPad Prism 3.0, and the dissociation constant, $K_d$, was determined.

**Table 4: Binding affinity data**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Dissociation constant, $K_d$ (nM)</th>
<th>Binding maximum, $B_{max}$ (fmol / 10^5 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[$^{111}$In]DTPA-EGF</td>
<td>2.7 ± 0.1</td>
<td>94 ± 0.9</td>
</tr>
<tr>
<td>[$^{111}$In]Bz-DTPA-EGF</td>
<td>2.0 ± 0.1</td>
<td>88 ± 1.6</td>
</tr>
<tr>
<td>[$^{177}$Lu]Bz-DTPA-EGF</td>
<td>1.9 ± 0.4</td>
<td>68 ± 4.1</td>
</tr>
</tbody>
</table>

Affinity is an important parameter for a tumour targeting conjugate, since it determines the binding stability of the conjugate to a malignant cell. In table 3 the binding affinities of the studied EGF-chelates are shown. As can be seen, the dissociation constants are very similar for the three EGF-chelates, [$^{111}$In]DTPA-EGF, [$^{111}$In]Bz-DTPA-EGF and [$^{177}$Lu]Bz-DTPA-EGF. They are also similar to the $K_d$ obtained for EGF itself (1.3 nM) [40], which means that the binding capacity of EGF, to a large extent is retained, through the chelate conjugation and purification procedures. Radiolabelled octreotide derivatives that are used clinically for imaging [134] or therapy [134, 135] of tumours, also have $K_d$ values well within the range of the affinities obtained with the EGF-chelates.

**Stability studies**

Stability of radiometal-chelate complexes are important for conjugates aimed for tumour therapy, since dissociation of radiometals may lead to unspecific irradiation of normal tissues. When $^{90}$Y or a radiolanthanide like $^{177}$Lu is used, unspecific irradiation of the radiosensitive bone marrow is of great concern, since these radiometals are known to accumulate in bone [80, 135].

The stability of the EGF-chelates, [$^{111}$In]DTPA-EGF and [$^{111}$In]Bz-DTPA-EGF, was studied in vitro. The EGF-chelates were incubated in calf serum for 24 h at 37°C, and were then separated on a FPLC column. Control
samples, incubated in PBS and stored in a refrigerator, were also analysed, for comparison. The results showed that both $^{111}\text{In}$-chelates were quite stable. Approximately 5 % of the indium was transchelated to blood serum proteins, from both $[^{111}\text{In}]\text{Bz-DTPA-EGF}$ and $[^{111}\text{In}]\text{DTPA-EGF}$.

**Animal studies**

For $[^{177}\text{Lu}]\text{Bz-DTPA-EGF}$, the stability was not studied *in vitro*, but it could be assessed from a biodistribution study, performed in female NMRI mice. Even though the intention is to inject the targeting agent intracranially, it is important to study the biodistribution systemically too. A targeting conjugate administered in the brain may leak out into the blood circulation, and it is therefore of interest to estimate the risk that critical organs are exposed to radiation. Most often, the leakage to blood is relatively small, but since glioma patients because of their disease often have a disrupted blood-brain barrier, it may be of importance [88].

Mice were injected intravenously via the tail vein, with $[^{177}\text{Lu}]\text{Bz-DTPA-EGF}$ in PBS. Animals were then sacrificed at 0.5, 1, 4 and 24 h post injection. Blood was collected, and various organs were dissected, weighed and measured for radioactive content. Organ values were calculated as percent of injected dose per gram of organ (%ID/g).

**Table 5: Biodistribution of $[^{177}\text{Lu}]\text{Bz-DTPA-EGF}$ in mice (%ID/g)**

<table>
<thead>
<tr>
<th>Organ</th>
<th>0.5 h</th>
<th>1 h</th>
<th>4 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>1.6 ± 0.13</td>
<td>0.86 ± 0.25</td>
<td>0.28 ± 0.09</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Heart</td>
<td>1.0 ± 0.17</td>
<td>0.83 ± 0.18</td>
<td>0.54 ± 0.18</td>
<td>0.40 ± 0.07</td>
</tr>
<tr>
<td>Pancreas</td>
<td>2.2 ± 0.58</td>
<td>2.1 ± 0.69</td>
<td>1.7 ± 0.76</td>
<td>1.1 ± 0.14</td>
</tr>
<tr>
<td>Spleen</td>
<td>8.6 ± 2.1</td>
<td>7.6 ± 1.7</td>
<td>3.3 ± 1.8</td>
<td>4.9 ± 0.74</td>
</tr>
<tr>
<td>Stomach</td>
<td>2.9 ± 0.2</td>
<td>2.6 ± 0.44</td>
<td>2.0 ± 0.79</td>
<td>1.4 ± 0.13</td>
</tr>
<tr>
<td>Liver</td>
<td>34 ± 6.3</td>
<td>36 ± 4.1</td>
<td>22 ± 7.9</td>
<td>20 ± 4.0</td>
</tr>
<tr>
<td>Kidneys</td>
<td>24 ± 3.0</td>
<td>20 ± 7.8</td>
<td>22 ± 5.1</td>
<td>15 ± 2.4</td>
</tr>
<tr>
<td>Lungs</td>
<td>1.5 ± 0.51</td>
<td>1.1 ± 0.27</td>
<td>0.67 ± 0.53</td>
<td>0.67 ± 0.16</td>
</tr>
<tr>
<td>Small intestine</td>
<td>2.5 ± 0.60</td>
<td>2.6 ± 0.79</td>
<td>1.4 ± 0.43</td>
<td>0.66 ± 0.21</td>
</tr>
<tr>
<td>Large intestine</td>
<td>1.8 ± 0.10</td>
<td>2.0 ± 1.1</td>
<td>1.2 ± 0.39</td>
<td>0.70 ± 0.18</td>
</tr>
<tr>
<td>Skin</td>
<td>0.92 ± 0.10</td>
<td>0.79 ± 0.22</td>
<td>0.51 ± 0.20</td>
<td>0.36 ± 0.37</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.48 ± 0.03</td>
<td>0.41 ± 0.12</td>
<td>0.26 ± 0.10</td>
<td>0.16 ± 0.08</td>
</tr>
<tr>
<td>Bone</td>
<td>1.3 ± 0.22</td>
<td>2.2 ± 0.76</td>
<td>2.9 ± 0.42</td>
<td>2.7 ± 1.4</td>
</tr>
<tr>
<td>Thyroid</td>
<td>2.0 ± 0.79</td>
<td>1.5 ± 0.87</td>
<td>1.2 ± 0.32</td>
<td>0.87 ± 0.37</td>
</tr>
<tr>
<td>Submaxillary gland</td>
<td>2.0 ± 0.81</td>
<td>2.1 ± 1.5</td>
<td>1.7 ± 0.9</td>
<td>1.1 ± 0.46</td>
</tr>
<tr>
<td>Brain</td>
<td>0.07 ± 0.03</td>
<td>0.05 ± 0.03</td>
<td>0.03 ± 0.02</td>
<td>0.03 ± 0.01</td>
</tr>
</tbody>
</table>
The results of the biodistribution in normal mice are presented in table 5. As can be seen, the blood clearance was quick, with less than 1 % ID/g of whole blood remaining 1 h post injection. A majority of the radioactivity was at all time points found in liver, kidneys and spleen. The uptake in liver was probably receptor-mediated to a certain extent since hepatocytes are known to express EGFR. Uptake in kidneys was, on the other hand, probably a consequence of excretion of conjugate via kidneys to urine. Radioactivity was also taken up by pancreas, stomach, lungs, small and large intestine, bone, thyroid and submaxillary gland, although at a lower level. It was encouraging to see that the radioactivity in almost all organs decreased with time. There is, however, some concern regarding uptake in bone. Bone was the only organ in which radioactivity accumulated with time, and this may be an indication that the stability of the chelate, Bz-DTPA, is not entirely sufficient for $^{177}$Lu.

It is known that normal cells in some tissues, e.g. hepatocytes in the liver, express EGFRs. We used these organs as a model to investigate the capability of $[^{177}\text{Lu}]$Bz-DTPA-EGF to target EGFR in vivo. In order to study the specificity of binding, some mice were injected intravenously with excess amounts (100 µg) of unlabelled EGF prior to injection of $[^{177}\text{Lu}]$Bz-DTPA-EGF, to block the receptors. The mice, with or without blocked EGFR, were then sacrificed after 0.5 h, and dissected as described above.

![Figure 18 Specificity of $[^{177}\text{Lu}]$Bz-DTPA-EGF binding in normal mice. Excess amounts of unlabelled EGF was injected prior to $[^{177}\text{Lu}]$Bz-DTPA-EGF injection, in order to block EGFR specific uptake.Liver and kidney are shown separately, with a different scale on the y-axis.]
The results of selected organs are presented in figure 18. As can be seen, \[^{177}\text{Lu}\]Bz-DTPA-EGF uptake could be blocked in liver, pancreas, stomach, small and large intestine and submaxillary gland. This is quite consistent with the literature, since most of these organs have been reported to express EGFR. We could thereby confirm that \[^{177}\text{Lu}\]Bz-DTPA-EGF could target EGFR \textit{in vivo}. Interestingly enough, the uptake in kidneys was significantly increased in blocked animals. This was probably because of the larger amount of \[^{177}\text{Lu}\]Bz-DTPA-EGF excreted through kidneys, as a consequence of blocked uptake in other organs.

Recently, the biodistribution of \[^{111}\text{In}\]DTPA-EGF was also studied in Balb/c nu/nu mice with tumour xenografts. In this unpublished pilot study, mice were injected subcutaneously with EGFR-expressing A431 cells, (approximately 9 million cells per tumour) in both dorsal flanks. The tumours were allowed to grow for 12 days, and had then reached a size of approximately 0.6-0.7 cm\(^3\). A solution of \[^{111}\text{In}\]DTPA-EGF was then intravenously injected via the tail vein. The animals were sacrificed 1 h or 4 h post injection (five and four mice respectively, all with double tumours), and were dissected according to a standard protocol.

![Figure 19](image-url) **Figure 19** Tumour-to-organ ratios for blood and brain, after iv injection of \[^{111}\text{In}\]DTPA-EGF in mice with A431 tumour xenografts.

The results of the study were encouraging since a clear tumour uptake could be seen. Most of the studied organs had significantly lower uptake of radioactivity than the tumour. This was especially apparent in blood and brain, where the tumour-to-organ ratios were approximately 25 and 50, respectively (figure 19). Kidneys and liver, however, had appreciably higher uptake than tumour. This was expected, since the biodistribution of EGF-chelates in normal animals showed high uptake in liver and kidneys [142]. As discussed above, the liver uptake is probably receptor specific to some extent, whereas kidney uptake is a result of kidney excretion of EGF-chelate. Some of the organs, shown above to have a receptor-specific uptake of
[\textsuperscript{177}Lu]Bz-DTPA-EGF (pancreas, stomach, thyroid and salivary gland), accordingly had an uptake of [\textsuperscript{111}In]DTPA-EGF approximately equal to that of the tumour.

In conclusion, it was shown that intravenously injected [\textsuperscript{111}In]DTPA-EGF could target EGFR-expressing tumours in mice. Good tumour-to-organ ratios for blood and brain bode well for future locoregional targeting of disseminated glioma cells.

Comments

The EGF-chelates studied in paper V and VI generally showed excellent targeting qualities in the in vitro tests. There was a high uptake in cells, a high degree of internalisation and good specificity of binding. One exception was the lutetium-labelled Bz-DTPA conjugate, which had a somewhat increased background binding.

The most attractive feature of the EGF-chelates is perhaps the supreme intracellular retention of the radiometals. The retention was equally good to that of EGF-dextran-\textsuperscript{[\textsuperscript{125}I]}, but EGF-chelates do not have the disadvantage of a bulky extra part, increasing the molecular weight. They should therefore have better diffusion capacity than EGF-dextran in tissues. Another advantage that the EGF-chelates studied here have over EGF-dextran, is the fast and uncomplicated conjugation, labelling and purification procedures, which are essential for a drug intended for human use.

It is still uncertain whether Bz-DTPA is a sufficiently stabile chelator for \textsuperscript{177}Lu or not. The biodistribution study indicated some accumulation of \textsuperscript{177}Lu in bone, although at a low level. This may, however, be solved by using a macrocyclic chelator like DOTA or Bz-DOTA instead. We have therefore initiated studies of EGF conjugated to DOTA. An alternative may be the acyclic chelating agent, CHX-A\textsuperscript{+}, which has been shown to be suitable for chelating \textsuperscript{177}Lu [90].

The biodistribution study in normal mice also showed that two organs, liver and kidneys, at all time points accumulated a majority of the radioactivity. The highest uptake per gram of tissue was seen in liver, which was not surprising, since EGFR expression at a rather high level has been demonstrated in hepatocytes. There might, however, be a positive aspect of the liver accumulation of circulating conjugate, since this may spare more radiosensitive organs, like bone marrow and kidneys, from high radiation doses. The use of hepatic clearance has earlier been proposed, to improve tumour-to-non-tumour localisation ratios of radiolabelled, tumour targeting antibodies, by utilizing the ability of hepatocytes to excrete degradation products in the bile [48, 83, 112].
Although the application of the EGF-chelates is to target glioma cells using a locoregional administration route, it was encouraging to see that conjugates could target EGFR \textit{in vivo}, using an intravenous administration route. This was shown using both EGFR-expressing organs and tumour xenografts as models, and proves that the good quality targeting capacities of the EGF-conjugates is retained also \textit{in vivo}. Unfortunately, it is difficult to find appropriate animal tumour models to evaluate the targeting capacities of locally injected conjugate, aimed for treatment of disseminated glioma cells. Some glioma models in mice have been developed [144], but the size of these animals, makes it difficult to study the targeting approach in a realistic way.

A clinical study on glioma patients is planned, with the aim to study the targeting capacity of [\textsuperscript{111}In]DTPA-EGF. The bulky part of the tumour will be resected surgically and tested histologically for EGFR expression. If the tumour expresses high amounts of EGFR, a Rickham reservoir will be implanted, to allow injection of [\textsuperscript{111}In]DTPA-EGF into the cavity. Only trace amounts of radioactivity will initially be used. The targeting will be monitored using scintigraphic imaging. The reason for choosing DTPA as chelator over Bz-DTPA is that DTPA is commercially available and has previously been used for clinical applications [38, 129]. Hopefully, this study will elucidate whether targeting of disseminated glioma cells can be achieved, using radiolabelled EGF-conjugates as targeting agent.
Summary and future studies

Summary
The aim of this thesis was to develop and preclinically characterise radiolabelled EGF conjugates aimed for tumour targeting. The EGF receptor is overexpressed on tumour cells in several malignancies, e.g. high grade gliomas. Radiolabelled EGF conjugates may potentially be of use in treating disseminated tumour cells, which cannot be managed with current therapy methods. Three types of conjugates were studied in the thesis: EGF-dextran labelled with $^{125}$I, EGF labelled with $^{211}$At, and two EGF-chelates, DTPA-EGF and Bz-DTPA-EGF, labelled with the radioactive metals $^{111}$In and $^{177}$Lu.

When using an internalising targeting agent like EGF, it is important that the radionuclides are retained inside the targeted cell after degradation of EGF. Radiohalogens, like $^{125}$I, $^{131}$I and $^{211}$At, are known to diffuse quickly out of cells, once the targeting agent is degraded. One approach to avoid this excretion of radiohalogens is to conjugate the targeting agent to a residualising carrier molecule. In paper I, we investigated the possibility of using dextran as carrier, to improve the cellular retention of radioiodine. We studied the retention of EGF-dextran labelled with $^{125}$I on the EGF part, the dextran part or on both parts. The results showed that radioiodine retention could be significantly improved, especially if radionuclides were coupled to the dextran part of the conjugate. Approximately 75% of the radioiodine was still cell associated after 24 h. In paper II, the biologic activity of EGF-dextran was studied. We analysed the activation of EGFR, as well as three signalling molecules downstream of EGFR, after stimulation with EGF-dextran or EGF. The results showed that EGF-dextran had biologic activity and could activate EGFR in cell culture. However, the effect on one of the signalling molecules, PLC$_{\gamma}$, differed between EGF and EGF-dextran stimulation. The phosphorylation of PLC$_{\gamma}$ was significantly lower after stimulation with EGF-dextran than after EGF stimulation.

In conclusion, the EGF-dextran conjugate appears to be a good tumour targeting candidate, for potential use against e.g. disseminated glioma cells. However, the lengthy and complicated conjugation and purification...
procedures of EGF-dextran are a problem that has so far hindered clinical trials with this substance.

A better alternative might therefore be to use an EGF-chelate as tumour targeting agent. EGF-chelates can be labelled with radiometals, e.g. $^{111}$In or $^{177}$Lu, which are known to have excellent intracellular retention. In paper V and VI, two EGF-chelates, DTPA-EGF and Bz-DTPA-EGF, were developed and labelled with $^{111}$In and $^{177}$Lu. They were studied both \textit{in vitro} and \textit{in vivo} and were shown to have promising tumour targeting qualities. All EGF-chelates bound specifically and with high affinity ($K_d$ was estimated to approximately 2 nM) to EGFR in cell culture. They were also internalised into cells after binding, and the retention of radiometals was high (approximately 60% of the radiometals were still cell associated after 45 h).

A biodistribution study with $[^{177}$Lu]$Bz$-DTPA-EGF in mice showed that liver and kidneys accumulated a majority of the radioactivity. Kidney uptake was probably a consequence of the renal clearance of radioactivity, whereas a main part of the liver uptake probably was receptor mediated, since there is normal expression of EGFR in liver, as well as some other organs.

The EGFR-expressing organs were also used as a model to study the specificity of [${}^{177}$Lu]$Bz$-DTPA-EGF binding \textit{in vivo}. By blocking EGFR-mediated uptake with excess amounts of EGF, prior to the injection of EGF-chelate, the specificity of [${}^{177}$Lu]$Bz$-DTPA-EGF binding \textit{in vivo} could be confirmed. An unpublished pilot study in mice with tumour xenografts also demonstrated tumour uptake of iv injected [${}^{111}$In]DTPA-EGF, with tumour-to blood ratios of approximately 25. Thus, the EGF-chelates can be considered as interesting tumour targeting candidates, and a clinical study in glioma patients is therefore planned.

In paper III and IV, the potential of using tumour targeting with radiolabelled EGF, in combination with the EGFR TKI gefitinib (Iressa, ZD1839), was studied. Two cell lines, one resistant and one sensitive to gefitinib, were used. The cellular uptake of $^{211}$At-EGF was shown to be considerably increased by simultaneous gefitinib treatment, in both cell lines. The retention of radioactivity was also prolonged by gefitinib treatment in both cell lines. The effect of the combined treatment, however, differed between the cell lines in a quite unexpected way. In the gefitinib resistant cell line, combined treatment decreased the cell survival approximately 3.5 times relative to $^{211}$At-EGF treatment alone. In the gefitinib sensitive cells, on the other hand, combined treatment increased the cell survival relative to $^{211}$At-EGF treatment alone. The reason for this protective effect of gefitinib in A431 cells is not known. The finding is, however, important both radiobiologically and clinically, and the mechanisms behind should therefore be further investigated.
Future studies

The targeting conjugates developed and studied in this thesis have shown promising qualities as tumour targeting agents, but there are a lot of further studies that may be done, and many questions to be answered. We have planned to study the following:

1. It would be interesting to find out the mechanisms behind the protective effect of gefitinib against $^{211}$At-EGF in A431 cells. Other gefitinib-sensitive cell lines should also be studied to see whether this is a general phenomenon or not.

2. In order to study tumour targeting combined with gefitinib treatment \textit{in vivo}, we can analyse the uptake of dual labelled EGF-chelate, $[^{111}\text{In}]{\text{Bz-DTPA-EGF}}[^{125}\text{I}]$ during simultaneous gefitinib treatment, in mice with A431 tumour xenografts. By using a dual label, we will be able to obtain information about how gefitinib influences retention of both labels.

3. Since gefitinib was shown to have such an interesting impact on EGF-based targeting agents, it would be interesting to study the impact of other tyrosine kinase inhibitors too, both reversible and irreversible. Other drugs, known to increase the intracellular retention of endocytosed agents, may also be of interest to study in combination with EGF-based tumour targeting agents. For example, lysosomotrophic drugs like chloroquine and thioridazine may be used.

4. It would be very valuable to develop an \textit{in vitro} assay, in which the cell killing potential of targeting with different radionuclides could be assessed. By using the same tumour targeting device, just changing radionuclide, the effects of different radionuclides may be compared. If a three-dimensional cell cluster is used instead of cells in monolayer, one can also study the crossfire effect.

5. In order to possibly increase the stability of the $^{177}$Lu chelate, EGF can be chelated to a macrocyclic chelator like DOTA. An EGF-DOTA chelate can also be labelled with e.g. the positron-emitter $^{68}$Ga. DOTA-EGF labelled with $^{111}$In and $^{68}$Ga may then be used for imaging in tumour bearing animals, using SPECT and PET, respectively.

6. It would be very interesting to perform therapy with a $^{177}$Lu-labelled EGF-chelate in animals with tumour xenografts. The choice of animal and tumour model, however, has to be discussed, since intravenous injections are not optimal due to normal expression of EGFR in certain tissues.

7. A clinical study with intracranial administered $[^{111}\text{In}]$DTPA-EGF is planned in glioma patients. The purpose of the study is to evaluate the targeting capacity of the $[^{111}\text{In}]$DTPA-EGF conjugate. Patients with anaplastic astrocytomas (WHO grade III) or glioblastomas (WHO grade
IV) will be included in the study, provided that they have tumours overexpressing EGFR. After surgical resection of visible tumour, a Rickham reservoir will be implanted. $[^{111}\text{In}]$DTPA-EGF can then be administered via a catheter, directly into the operation cavity. Hopefully, we will then be able to monitor the ability of $[^{111}\text{In}]$DTPA-EGF to diffuse through the brain, and target disseminated glioma cells, via scintigraphic imaging. Only trace amounts of radioactivity will initially be used. However, if excellent tumour targeting is achieved, therapeutic amounts of a suitable radionuclide will be provided.
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