EPIDERMAL GROWTH FACTOR RECEPTOR: TARGET FOR DELIVERY AND SILENCING

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Epidermal growth factor receptor: target for delivery and silencing
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EPIDERMAL GROWTH FACTOR RECEPTOR: TARGET FOR DELIVERY AND SILENCING

Het uitschakelen van en afleveren via de EGFR
(met een samenvatting in het Nederlands)

EGFR: um receptor alvo para entrega e silenciamento
(com sumário em Português)

Récepteur à l'EGF: inhibition et délivrance ciblée
(avec un résumé en Français)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op maandag 8 december 2008 des middags te 2.30 uur

door

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Things are not worth for the time they last, but for the intensity they have when they happen. That is why there are unforgettable moments, unexplainable things and incomparable people.

~

O valor das coisas não está no tempo que elas duram, mas na intensidade com que acontecem. Por isso, existem momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis.

Fernando Pessoa

To my family

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Chapter 1

General Introduction

CANCER THERAPY

The principal aim of cancer therapy is to stop the proliferation and migration of malignant cells, ideally without affecting healthy cells. Conventional chemotherapy attacks the rapidly dividing cancer cells by blocking the cell cycle. Because of the rapid proliferation of cancer cells, chemotherapy is to some extent selectively toxic to tumour tissue. However, not only cancer cells divide rapidly, e.g. haematopoietic cells and hair follicles also have a rapid turnover. This explains the common side effects of these therapies. In recent years, major efforts have been made to improve the therapeutic efficacy of these conventional drugs in cancer therapy. In the field of drug delivery, for instance, the use of carriers that promote selective delivery of the drugs to cancer cells (such as immunoliposomes) has been an important strategy. In parallel with the research on targeted drug delivery systems, significant advances have been made in the field of tumour molecular biology, which have allowed the development of new therapeutic molecules that discriminate between healthy and malignant cells.

CANCER ORIGIN AND DEVELOPMENT

Cancer cells and healthy cells are different. The origin of cancer has been widely accepted as a multistep process leading to the accumulation of genetic mutations and the activation of oncogenes [1, 2]. More recently, the term *cancer stem cells* has been introduced, referring to a potent proliferative cell population also designated *tumour-initiating cells* [3]. This finding underlines the need for a better understanding of the events responsible for malignant transformation and tumour proliferation. Nevertheless, certain common characteristics can be ascribed to cancer cells, such as unlimited cell proliferation, evasion of apoptosis, stimulation of angiogenesis, tissue invasion and metastasis (Figure 1). These events do not concern tumour cells alone. They rather involve host cells and their surroundings, which include stromal fibroblasts, epithelial cells, cells forming blood and lymphatic vessels, and cells from innate and adaptive immunity, that become part of the tumour-microenvironment [4].

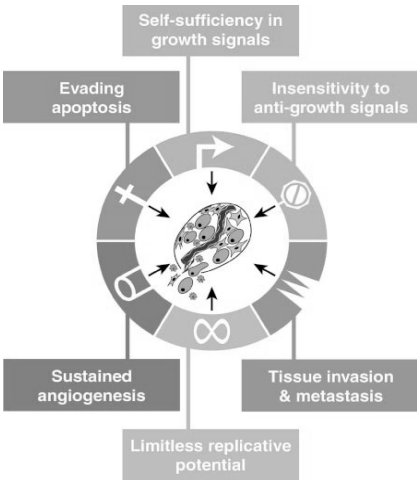

Figure 1. Capabilities acquired by cancer cells during tumour development.
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The increasing understanding of the events responsible for the initiation and progression of cancer, the key players implicated, and the communication between the different cell types involved, has enabled the identification of cancer-specific molecular targets, which allows the development of rationally designed anticancer therapeutics [5-7]. These *smart cancer drugs* are more selective in distinguishing malignant from healthy cells, and therefore their use is likely associated with less severe and debilitating side effects than the conventional cancer chemotherapy.

PROTEIN KINASES AND GROWTH FACTORS

Many of the cancer-specific molecular targets that have been recently identified are protein kinases [8]. Protein kinases are enzymes that phosphorylate certain amino acids in proteins. Kinases are generally involved in the regulation of protein activity. As protein kinases can be found in different locations within cells, they can interfere with several cellular processes, for example: at the cell surface, where they function as transmembrane receptors; inside the cell, where they act as intracellular transducers; or inside the nucleus, where they are involved in signal transduction and transcription. Overall, protein kinases play crucial roles in cell cycle progression and, therefore, these are interesting targets for rational cancer drug design [9]. As many growth factor receptors are protein kinases, tumour-associated growth factors and growth factor receptors have received widespread interest [10]. Some examples of well-explored growth factors are the epidermal growth factor (EGF), the vascular endothelial growth factor (VEGF), the transforming growth factor-alpha (TGF-α), the insulin-like growth factor (IGF), and the platelet-derived growth factor (PDGF).

EPIDERMAL GROWTH FACTOR RECEPTOR

The epidermal growth factor receptor (EGFR) is a transmembrane receptor kinase, which belongs to a family of receptors also designated as ErbB receptors. This family of receptors is involved in the regulation of many cellular processes, mediated by EGF, TGF-α, and other ligands. EGFR is expressed in all cell types, except haematopoietic cells and primary cells of epidermal origin. The majority of human epithelial cancers have dysregulated EGFR signalling, usually associated with the overexpression of EGFR. This can induce ligand-independent activation of the receptor. Also, the overexpression of some of the EGFR ligands can occur. In other cases, mutated EGFR compromises the adequate receptor activation. Understanding receptor activation and the mechanisms by which EGFR subsequently regulates cell proliferation, apoptosis evasion, angiogenesis stimulation, tissue invasion, and metastasis, has allowed the development of rationally designed EGFR-targeted therapeutics [11, 12]. These therapeutic molecules, designed to inhibit EGFR activation, can be divided into two major groups: the monoclonal antibodies (mAbs), which interfere at the extracellular level with ligand binding to the receptor, and tyrosine kinase inhibitors (TKIs), which operate at the intracellular level by inhibiting EGFR phosphorylation and activation [13, 14]. An additional strategy interferes with EGFR at the mRNA level, silencing the expression of the receptor protein.

SMALL INTERFERING RNA AND SILENCING

Small interfering RNA (siRNA) molecules are central elements of a process named RNA interference (RNAi). This discovery was well acknowledged two years ago by awarding the Nobel Prize for Physiology or Medicine to Fire and Mello [15]. RNAi was first described in worms, as double-stranded RNA (dsRNA) mediated-suppression of genes with the same sequence as the dsRNA nucleotide sequence [16]. This mechanism was actually noted earlier in plants and yeasts and referred to as reversible co-suppression of genes or quelling [17, 18]. Nowadays, RNAi is better understood and known to be a natural mechanism occurring in all eukaryotes (and possibly prokaryotes), crucial for gene regulation and cell division, as well as protection against RNA virus infections [19].
In short, RNAi involves several proteins present in the cytoplasm of cells (Figure 2). Chemically synthesised siRNA molecules can be directly introduced into the cytoplasm or, alternatively, short hairpin RNAs (shRNA) can be expressed from encoding DNA. These shRNA are then processed by an enzyme named Dicer into siRNAs, which are the functional mediators of RNAi. For RNAi to occur, these siRNA molecules need to assemble with proteins forming the RNA-induced silencing complex (RISC). While assembly takes place, one of the strands of siRNA, the *passenger strand*, is discarded. The other strand (*guide strand*) binds to Argonaute-2 (Ago-2), one of the proteins of the RISC, and the RISC is then in the active state. The activated RISC recognises the complementary sequence of mRNA. After base paring, the mRNA is subsequently cleaved and degraded by Ago-2. Thereafter, RISC unloads the *guide strand* of siRNA and RNAi can proceed by reloading with a new siRNA molecule [20]. Because this process degrades mRNA in the cytoplasm, which migrated from the nucleus after being transcribed from DNA, it is a post-transcriptional gene silencing process. RNAi has been employed by many researchers for its potential to silence the expression of virtually any protein.

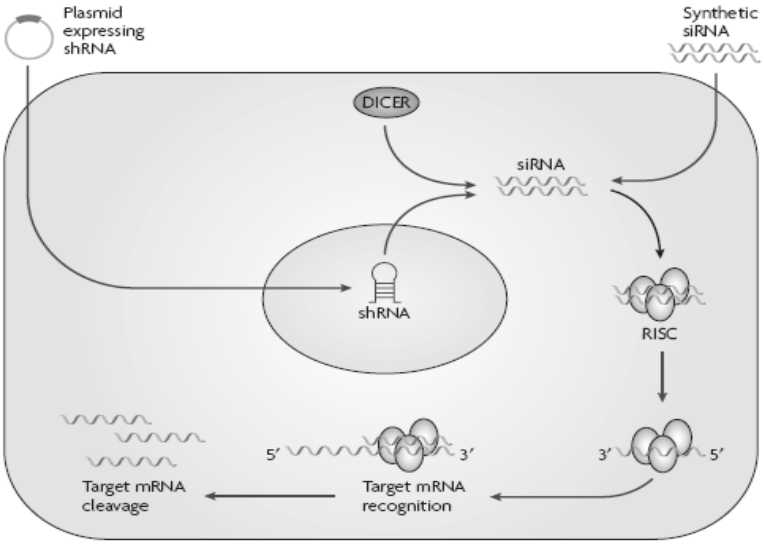

Figure 2. The mechanism of RNAi.
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DELIVERY OF SMALL INTERFERING RNA

For RNAi to occur, siRNA molecules need to be present in the cell cytoplasm. However, the physicochemical properties of siRNA (such as negative charge, hydrophilicity, as well as large size) hamper the cell entrance of siRNA molecules. Delivery systems are generally used to overcome this difficulty by interacting with the siRNA and thereby protecting it, ultimately favouring cellular uptake of siRNA. Such systems, generally based on the use of cationic lipid- or polymer-based carrier molecules [22, 23], are referred to as lipoplexes or polyplexes, respectively, and are mainly taken up by cells through endocytosis. Once inside the endosomes, siRNA needs to cross the endosomal membrane in order to reach the cytoplasm, a process known as endosomal escape. The escape from endosomes needs to occur promptly, as a delay could cause acidic degradation of siRNA molecules. Interestingly, different strategies have been reported in literature for promotion of endosomal escape for a variety of molecules. For example, peptides have been synthesised based on fusogenic domains of the influenza virus showing successful results in gene delivery. These peptides, when localised in the slightly acidic environment of late endosomes, change their conformation and consequently destabilise the endosomal membrane [24, 25]. A technique named photochemical internalisation (PCI, Figure 3), which employs photochemical reactions to disrupt endocytic vesicles, has been applied to deliver molecules such as the proteins gelonin and saporin, and the chemotherapeutic agent bleomycin into the cell cytoplasm [26].

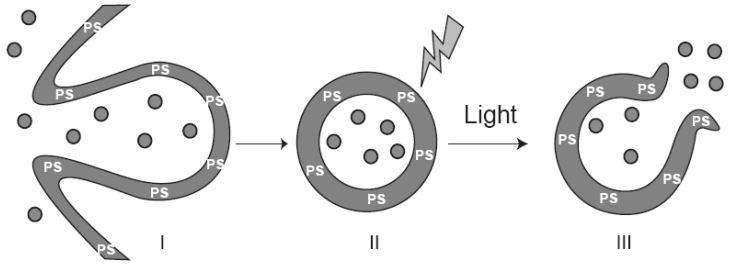

Figure 3. Photochemical internalisation (PCI).
The photosensitiser (PS) initially localises in the cell membrane. Upon endocytosis (I) PS is present in the membranes of the vesicles. After illumination (II) the PS is activated and is involved in the production of reactive oxygen species that oxidise components of the endosomal membranes, consequently releasing the content of the vesicles in the cell cytoplasm (III).

In addition to addressing the intracellular delivery problem, siRNA delivery systems should also ensure *in vivo* that siRNA molecules are stably transported towards the target tissue, that they penetrate it, and distribute to reach and be taken up by the target cell population. Some of the difficulties encountered after intravenous administration of siRNA, such as limited extravasation and accumulation at the target organ, are minimised by using local administration. However, local administration is not always possible, and for this reason delivery systems for the systemic delivery of siRNA, as well as for other therapeutic molecules, have been widely investigated [27, 28].

TARGETED LIPOSOMAL DELIVERY OF MACROMOLECULES

Considering the systemic administration of macromolecules, ideal delivery systems remain stable in the bloodstream. They ensure prolonged circulation time and protection from opsonisation by blood serum proteins that lead to rapid clearance by macrophages of the mononuclear phagocytic system (MPS). In addition, these delivery systems should enable the extravasation from blood vessels and penetration into the tissues of the target organ of interest, where the therapeutic molecules should accumulate and perform their action. By favouring the accumulation of the therapeutic molecules in the organ/tissue and cells of interest, side effects may be decreased. Several delivery systems have been developed possessing as many of these preferential characteristics as possible. The long-circulating liposomes, also referred to as *stealth* liposomes, are a good example. Long-circulating liposomes are phospholipid bilayer vesicles, with hydrophilic polymers, most often polyethylene glycol (PEG) chains, grafted onto their surface. Liposomes have been presented as attractive drug delivery systems, and a broad spectrum of therapeutic molecules have been incorporated into these vesicles [29]. The formulation Caelyx® (Doxil® in the United States) consists of doxorubicin-containing PEG-liposomes, and has demonstrated significant clinical advantage over free doxorubicin in particular regarding its capacity to decrease drug related cardiotoxicity. These long-circulating delivery systems take advantage of the well-known enhanced permeability and retention (EPR) effect. This is a passive targeting ability in which the leaky vasculature present in tumours, together with the reduced lymphatic drainage, favour the accumulation of colloidal systems and macromolecules in tumours, provided that they circulate long enough in the bloodstream [30].

Besides passive targeting, active targeting to tumour cells has been investigated through the coupling of specific targeting ligands to the liposome surface [28, 31]. Small peptides or tissue-specific ligands have been used to target delivery systems to tumour cells, via the corresponding receptor: transferrin, folate, and EGF are some examples [32]. Moreover, monoclonal antibodies (mAbs), fragments of antibodies (Fabs), or single chain variable fragments (scFv) have been coupled to liposomes (generally referred to as immunoliposomes). These have shown promising results in preclinical studies, but are still in early stage of clinical evaluation [31]. In the particular case of EGFR, several Fabs derived from mAbs clinically employed to inhibit ligand binding, have been coupled to liposomes, showing advantage over the uncoupled liposomes due to the significant increase of interaction with the target cells [33, 34]. Overall, liposomes appear to be attractive and flexible drug delivery systems, which can be relatively easily tailored to the therapeutic need and application.

NANOBODIES

Nanobodies are the smallest antigen-binding immunoglobulin fragments (4 nm height, 2.5 nm diameter, Figure 4), which were isolated from heavy chain-only camelid antibodies. These antibodies were discovered fifteen years ago [35] and have also been isolated in sharks and spotted ratfish. Nanobodies have several advantages over regular antibodies: they are ten times smaller (15 kDa); they are chemically more stable and re-fold easily upon heat-denaturation; and they are more hydrophilic than Fabs and scFv. In addition, nanobodies show a similar degree of specificity and affinity towards their antigen as mAbs, and at the same time they can be easily engineered and grown in bacteria or yeast [36].

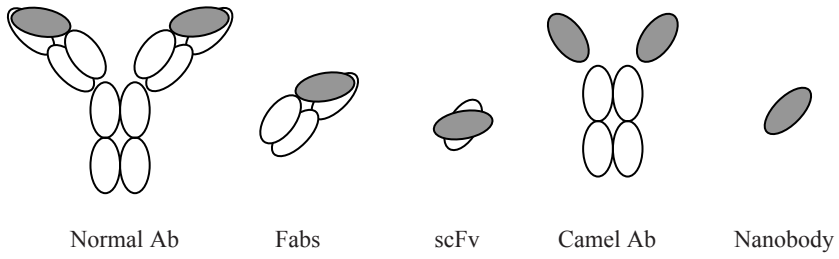

Figure 4. Schematic representation of antibodies.
From left to right: conventional antibodies (Ab); antibody fragments (Fabs); single chain variable fragment (scFv); heavy chain-only antibodies from camelids; nanobody. The heavy variable domains are represented in grey.

Because of their small size, nanobodies are excellent imaging agents, showing efficient tissue penetration and fast clearance of unbound molecules from the circulation [37]. Their small size could be a disadvantage for therapeutic applications as 15 kDa is below the threshold for glomerular filtration, leading to rapid clearance. However, strategies have been employed to increase their molecular weight and circulation time, such as coupling of two and three nanobodies, forming multivalent molecules [38]. Nanobodies have already demonstrated their potential as antitumour agents. Roovers *et al* have tested trivalent, bispecific anti-EGFR nanobodies, where two nanobodies were directed to EGFR and were linked to a third nanobody that was directed to mouse serum albumin to prolong circulation time. The bispecific trimer induced a significant delay in tumour growth [39].

Taken together, nanobodies are interesting molecules for imaging and for therapeutic applications, and they may possibly form a new class of targeting molecules for colloidal drug delivery systems.

AIM AND OVERVIEW OF THE THESIS

The epidermal growth factor receptor (EGFR) is overexpressed in many human epithelial cancers and has long been recognised as a target for cancer therapy. The principal aim of this thesis was to target the EGFR and to interfere with its function in tumour growth. **Chapter 2** reviews the relevant literature on EGFR and illustrates the attractiveness of EGFR-inhibition for use in oncology. The main approach employed in this thesis to accomplish the aim is to silence the expression of the EGFR protein, by use of siRNA. A major requirement for success with this approach is the effective delivery of siRNA to the target cell cytoplasm, combining sufficient intracellular delivery with adequate endosomal escape. Lipid-based carriers are employed to promote cell interaction and cellular uptake of anti-EGFR siRNA. The role of endosomal escape in lipoplex-mediated siRNA delivery and the resulting impact on silencing efficiency are assessed by two methods. The first, described in **Chapter 3**, involves a fusogenic peptide derived from the influenza virus. The second method is photochemical internalisation (PCI), a technique that employs photochemical reactions to disrupt endocytic vesicles. PCI is evaluated in **Chapters 4 and 5** for its capacity to enhance siRNA-mediated EGFR silencing, both *in vitro* and *in vivo*, respectively. In addition, **Chapter 5** gives an overview of the current state-of-the-art of the clinical use of siRNA, and presents successful preclinical studies involving local administration of siRNA. **Chapter 6** discusses the challenges of *in vivo* targeted delivery of siRNA, at three different targeting levels: tissue, cellular and intracellular level, giving examples of chemical modifications and carrier systems employed for meeting these challenges. In **Chapter 7**, a new targeted drug delivery system is investigated: anti-EGFR nanobodies coupled to the surface of liposomes. This new targeted delivery system is tested for its *in vitro* EGFR-targeting capacity and possible therapeutic consequences. Finally, **Chapter 8** presents the summarising discussion, where the achievements reported in this thesis are placed in perspective with the current research on EGFR for cancer therapy.

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CHAPTER 2

MOLECULAR BIOLOGY OF EPIDERMAL GROWTH FACTOR RECEPTOR INHIBITION FOR CANCER THERAPY

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ABSTRACT

Understanding the role of the epidermal growth factor receptor (EGFR) in cellular signalling processes underlying malignancy has enabled the development of rationally designed EGFR-targeted therapeutics. Strategies have been devised to interfere with the EGFR signalling at three different levels: at the extracellular level, competing with ligand binding; at the intracellular level, inhibiting the activation of the tyrosine kinase; or at the mRNA level, modulating the expression of the EGFR protein. Each of these strategies has proven to have an antitumour effect mediated by events such as inhibition of cell proliferation, induction of apoptosis, decrease of cellular invasion and migration, and/or inhibition of angiogenesis. Furthermore, the combination of these strategies with traditional chemotherapy or radiotherapy has generally resulted in enhanced antitumour effects. Likewise, the benefit of interfering simultaneously with different signalling pathways has been documented to improve tumour growth inhibition. These preclinical results have encouraged clinical studies which led to the FDA approval of three drugs. However, finding the perfect strategy for each individual patient appears to be a limiting factor, demanding further research to be able to generate relevant molecular expression profiles on a case-to-case basis. Taken together, a successful EGFR inhibition will require a better understanding of signalling pathways in combination with the development of rationally designed effective molecules.

INTRODUCTION

Recent research in oncology has been focused on understanding molecular events that turn a healthy cell into a malignant one. A better understanding of these events would open a new chapter for cancer therapy, by offering the possibility to interfere with the specific processes responsible for the malignant transformation and to exploit the molecular differences between malignant and non-malignant cells.

In cancer therapy, the principal aim is to interrupt the proliferation and migration of abnormal cells, ideally without affecting healthy cells. However, until now the traditional cytotoxic chemotherapy has not discriminated between these two different cell populations. Therefore, recent research has focused on key molecules involved in normal and abnormal cell division, cell proliferation, migration and survival. Growth factors and their receptors are among these key molecules.

The epidermal growth factor receptor (EGFR) plays a key role in many of the cellular processes involved in cancer development and has proven to be a promising target for cancer therapy [1].

This review will discuss various approaches for inhibition of the EGFR, giving special emphasis to combined strategies. By this means, the paper will provide evidences of the benefits of EGFR inhibition for cancer therapy.

EPIDERMAL GROWTH FACTOR RECEPTOR STRUCTURE AND ACTIVATION

The EGFR is a 170-kDa transmembrane glycoprotein that belongs to a family of receptors also known as the type I receptor tyrosine kinases (RTKs) or ErbB receptors family. This family is composed of four closely related receptors: the EGFR itself (ErbB1/HER1), ErbB2 (HER2/neu), ErbB3 (HER3), and ErbB4 (HER4). Each of these receptors is composed of 3 domains: the extracellular binding domain; the lipophilic transmembrane segment and an intracellular protein tyrosine kinase (TK) domain with a regulatory carboxyl terminal (CT) segment [2, 3].

The EGFR extracellular domain consists of four subdomains, two homologous large (L) domains and two cystein-rich (CR) domains, referred to as the L1, CR1, L2 and CR2 subdomains, or domains I – IV, respectively. The extracellular domain of the inactive receptor is a dynamic structure that can be organised in a closed or tethered conformation with low affinity for the ligand, or in an opened conformation with high affinity for the ligand [4] (Figure 1). These two conformations seem to be in equilibrium. In the closed conformation the CR1 region is in contact with the CR2 subdomain, whereas in the opened conformation the CR1 region has a different location, which exposes a particular segment named the dimerisation arm. In this high-affinity conformation both the L1 and L2 subdomains are involved in ligand binding to the receptor. The CR2 is presumably also involved in the targeting of the EGFR to the caveolae/lipid raft component of the plasma membrane. The transmembrane (TM) domain is followed by the intracellular domain which has a juxtamembrane (JM) region, a TK region and a CT. This CT contains tyrosine residues that modulate EGFR-mediated signal transduction after phosphorylation. The CT also has several threonine and serine residues, whose

phosphorylation seem to be involved in receptor downregulation processes and endocytosis [5].

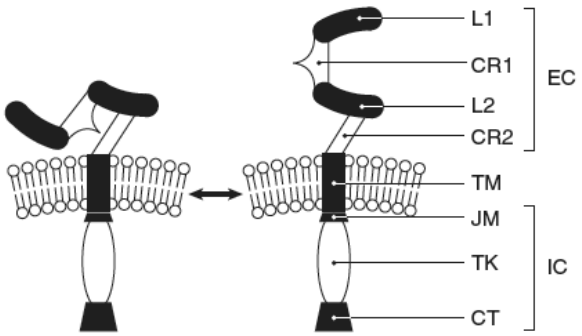

Figure 1. Structure of EGFR.
The EGFR is composed of three domains: the EC domain; the lipophilic TM segment and an IC domain. The EC domain of the inactive receptor can be present in two conformations, which are present in equilibrium: the tethered or low-affinity conformation (left), and the opened or high-affinity conformation (right). The EC domain consists of four sub-domains (I-IV, numbered from distal end to membrane), also known as L1/L2 and CR1/CR2. The TM domain is followed by the IC domain, which has a JM region, a TK region and a CT domain, responsible for recruitment and phosphorylation of proteins in pathways that are activated by EGFR. CR: Cystein-rich; CT: Carboxyl terminal; EC: Extracellular; EGFR: Epidermal growth factor receptor; IC: Intracellular; JM: Juxtamembrane; L: Large domain; TK: Tyrosine kinase; TM: Transmembrane.

EGFR activation occurs when a ligand, such as epidermal growth factor (EGF), transforming growth factor-α (TGF-α), amphiregulin, betacellulin, heparin-binding EGF, or epiregulin [3], binds to the receptor presenting the opened conformation [4]. This specific spatial arrangement of the subdomains, allows binding of the ligand between the L1 and L2 subdomains, and exposes the dimerisation arm, enabling the formation of a homo- or heterodimer by interacting with another member of the ErbB receptor family. The receptor dimerisation results in internalisation of EGFR via clathrin-coated pits, however, high concentration of ligand can stimulate an extra non-clathrin mediated pathway [6].

The ligand-induced dimerisation is also responsible for the cross- or transphosphorylation of key tyrosine residues in the C-terminal substrate domain; this culminates in the recruitment of SH2- and PTB-containing signalling proteins. This event results in the phosphorylation of several intracellular substrates, activating a number of cascades that lead the signal towards the nucleus (Figure 2). The four best known signalling routes are the mitogen-activated protein kinase (MAPK), the phosphatidylinositol-3-OH kinase (PI3K)/Akt, the signal transducer and activator of transcription (STAT)-mediated, and the phospholipase Cγ (PLCγ) pathways. Each signal will activate gene expression and ultimately induce cellular responses, such as cell proliferation, differentiation, invasion, migration, adhesion, survival and cellular repair [5].

A newly internalised activated ligand-EGFR complex can either be degraded or recycled. This fate is determined by two factors: the stability of the activated ligand-EGFR

complex in the endosome, and the presence of an ubiquitin ligase known as Cbl which will monoubiquitinate the receptor and consequently target receptors for lysosomal degradation [3, 7].

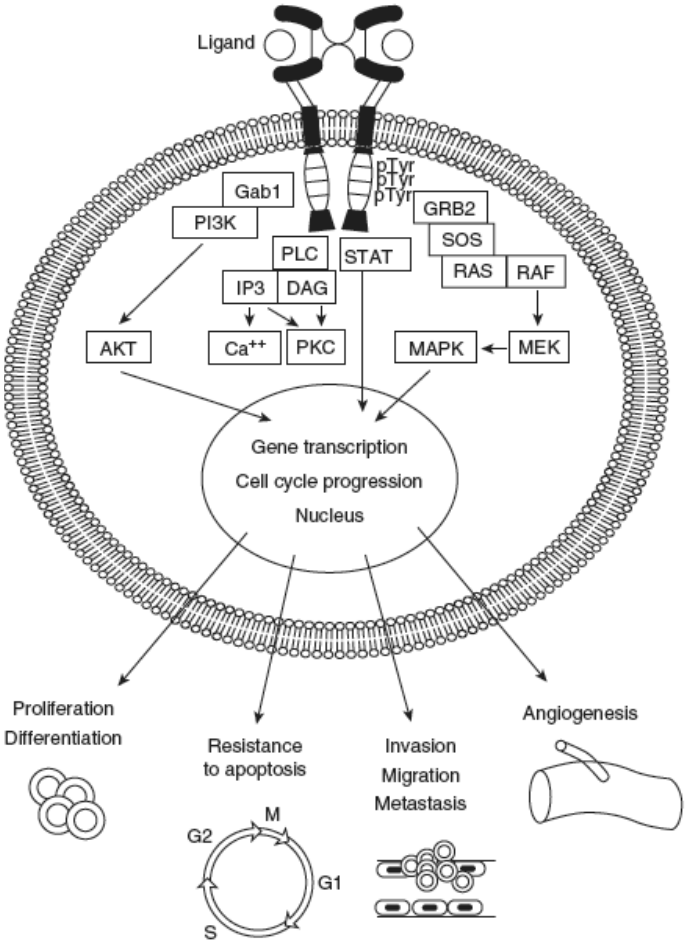

Figure 2. EGFR Activation and Signal Transduction.
Ligand binding to EGFR between regions L1 and L2 exposes the dimerisation arm, enabling the formation of a homo- or heterodimer by interacting with another member of the ErbB receptor family. The dimerisation results in phosphorylation of tyrosine residues in the CT domain, recruitment of signalling proteins and, ultimately, phosphorylation of intracellular substrates activating several signal transduction pathways. The best known signalling routes are the MAPK, the PI3K/Akt, the STAT-mediated, and the PLCγ pathways. These pathways transmit the signal to the nucleus and modulate cellular transcription and cell cycle progression. Ultimately, these effects in the nucleus translate into enhanced differentiation, proliferation, and resistance to apoptosis. Furthermore, invasion, migration and metastasis of cells are promoted and angiogenesis is stimulated. CT: carboxyl terminal; DAG: Diacylglycerol; EGFR: Epidermal growth factor receptor; IP3: Inositol triphosphate; MAPK: Mitogen-activated protein kinase; MEK: MAPK-kinase; PKC: Protein kinase C; PI3K: Phosphatidylinositol-3-OH kinase; PLCγ: Phospholipase Cγ; STAT: Signal transducer and activator of transcription.

EPIDERMAL GROWTH FACTOR RECEPTOR AND CANCER

The activation of EGFR is known to trigger many cellular events, ultimately leading to cellular proliferation, differentiation, migration, adhesion, protection from apoptosis, and induction of angiogenesis. Such processes, already vital for healthy cells, are even more relevant in uncontrolled dividing tumour cells.

Nearly 20 years ago, several researchers proposed EGFR as a target for cancer therapy. At present, several lines of evidence have further supported this claim [1, 8, 9]:

- Besides being expressed in most normal human epithelial tissues, EGFR is overexpressed in many human tumours; examples include cancers of the head and neck, breast, lung, glioblastoma, bladder, colorectal, prostate and ovarian carcinoma;
- EGFR expression has been correlated with a poor response to treatment, fast disease progression and poor survival;
- Production of ligands such as EGF and TGF-α is often found to be increased in the same tumour cells where receptors are overexpressed;
- In early studies, a series of monoclonal antibodies (mAbs) against the EGFR proved to be able to inhibit the growth of cancer cells [10-12].

Altogether, EGFR seems to be a rational target for cancer therapy. The next section will focus on strategies so far developed for interfering with the EGFR.

TARGETING EPIDERMAL GROWTH FACTOR RECEPTOR STRATEGIES

Since EGFR has proven to be involved in the initiation, growth and metastasis of many human tumours, several strategies for targeting the EGFR have been developed. Basically, three types of strategies can be distinguished according to the location where the action takes place (Figure 3):

- At the extracellular domain of the EGFR, interfering with ligand binding to the receptor;
- At the intracellular domain, inhibiting the activation of the TK protein, or
- At the mRNA level, interfering with the production of the EGFR protein.

Targeting the extracellular domain

For targeting the extracellular domain, mAbs have been used to compete with ligands to the EGFR extracellular domain. It has been suggested that even though mAbs do not bind to the same site that ligands do, they bind close enough to be able to prevent ligands from binding [13]. After binding to the receptor, mAbs are not only able to prevent activation of the MAPK, PI3K/Akt, STAT and PLCγ signal transduction pathways, but they can also cause downregulation of the receptor [14]. This seems to happen due to the high stability of the complex mAb-EGFR in the endosome, which does not dissociate when pH decreases, thereby routing the complex to lysosomal degradation [15]. In addition, mAbs have been implicated in the recruitment of certain immune cells, such as natural killer cells, through the Fc segment, contributing to the antitumour action of these molecules [16].

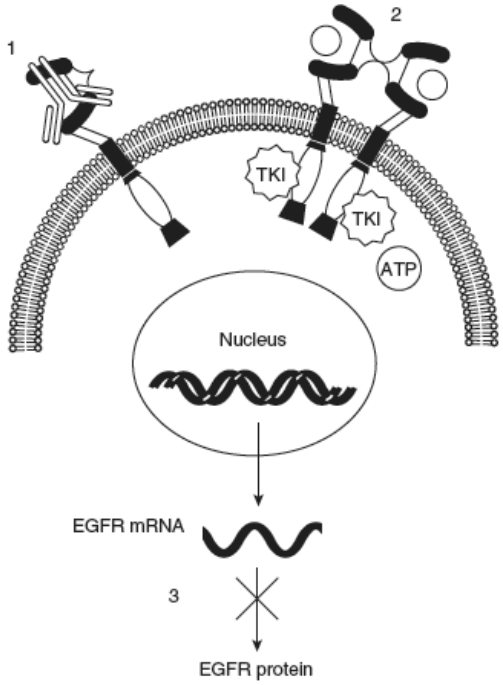

Figure 3. Strategies targeting the EGFR.
Targeting the EGFR in order to block its signal transduction can be done at three different levels: (1) at the EGFR extracellular domain, interfering with ligand binding to the receptor, for example, by using antibodies; (2) at the intracellular domain, inhibiting the activation of the tyrosine kinase protein, for example, by using TKI; or, (3) at the mRNA level, by inhibiting mRNA translation, for example, by using siRNA. EGFR: Epidermal growth factor receptor; siRNA: Small interfering RNA: TKI: Tyrosine kinase inhibitor.

Cetuximab (Table 1) is an example of anti-EGFR mAb that binds with high affinity (Kd = 0.39 nM) to the easily accessible extracellular domain of the receptor and competes with the ligand binding to the receptor. In fact, this mAb binds exclusively to a region of the L2 subdomain of the extracellular domain. As a result, it blocks the access of growth factors to their binding site on this subdomain. Even though both subdomains L1 and L2 are necessary for ligand binding, blockade of one of them seems to be sufficient to prevent the ligand from binding to the receptor [15]. Cetuximab has been going through extensive preclinical studies. Several *in vitro* and *in vivo* studies have been performed, and antitumour effects have been reported in many carcinomas, such as colorectal, prostate, renal, pancreatic, head and neck squamous cell carcinoma (HNSCC) and non-small cell lung carcinoma (NSCLC) [17]. Several examples are presented and discussed in section 5. The outcome of preclinical studies has encouraged clinical studies. Cetuximab has been going through phase I, II and III clinical trials, and was in fact approved by the US Food and Drug Administration (FDA) in February 2004 (marketed Erbitux®) for treating patients with advanced metastatic colorectal cancer, either as monotherapy or in combination with irinotecan. Recently, it was also approved, alone or in combination with cisplatin and radiation, for patients with HNSCC. Furthermore, cetuximab is being evaluated in patients with NSCLC and pancreatic cancer [18].

Two other examples of mAbs (Table 1) that are undergoing clinical development are panitumumab, which has been tested, for example, on prostate and renal carcinoma; and matuzumab, tested on cancer of the head and neck [19]. Clinical data on these antibodies have been reviewed by other authors [20].

Some antibodies can also be used against EGFR serving as carriers of radionuclides, toxins, or prodrugs. Azemar and colleagues studied the antitumour activity of recombinant single-chain antibody toxins specific for ErbB2 and EGFR against head and neck cancer cells *in vitro* and *in vivo*. The recombinant toxins consisted of variable domains of the heavy and light chains of mAbs, which permit cell recognition, genetically fused to a truncated *Pseudomonas* exotoxin A. After binding to the receptor, the toxin could be delivered intracellularly, which strongly enhanced its antitumour action. Results suggested that EGFR and ErbB2-specific antibody toxins may perhaps become valuable therapeutic reagents for the treatment of HNSCC [21]. This strategy could possibly be explored for other cancer types, increasing its promising therapeutic prospects.

Targeting the intracellular domain

For the intracellular domain, small molecular weight inhibitors of the TK protein have been developed. Such molecules compete with ATP for the intracellular catalytic site of the EGFR, thereby interfering with its activation. Contrary to mAbs, which lead EGFR to degradation, these molecules inhibit receptor phosphorylation without affecting EGFR expression. Their action, which results from preventing the activation of the MAPK, PI3K/Akt, STAT, and PLCγ signal transduction pathways, can be either reversible or irreversible, depending on reversible or covalent binding of the TK inhibitor (TKI) to the ATP binding site; and specific for one or more of the receptors from the ErbB family [14].

Many preclinical studies have been carried out with different TKIs. Gefitinib and erlotinib (Table 2) are the most intensively investigated TKIs. Gefitinib is highly selective for EGFR TK and has shown antitumour responses, in both *in vitro* and *in vivo* studies, for different carcinomas, such as NSCLC, ovarian, breast, colorectal, gastric, prostate carcinoma and HNSCC. Erlotinib also proved to have antitumour effects in, for example, NSCLC, HNSCC, pancreatic, ovarian, breast, and colorectal carcinoma [19, 22]. In section 5, some detailed examples are presented. Gefitinib and erlotinib are indeed the most advanced TKIs in clinical development, and both have been approved by the FDA. Gefitinib was approved in May 2003 for the treatment of patients with refractory advanced NSCLC. However, the results observed in large phase III clinical trials were unable to confirm the survival advantage for patients receiving gefitinib [23, 24]. These results highlighted the need for appropriate selection of patients enrolled into the trials, together with the establishment of a reliable molecular predictor for TKI sensitivity. Erlotinib was approved by the FDA in November 2004 also for advanced NSCLC [25], and similar difficulties were encountered during erlotinib clinical trials. Recently, it has been suggested that specific mutations on the TK domain of EGFR and an increased copy number of EGFR gene could predict the response to TKIs [26].

Two other examples of TKIs (Table 2) that have been involved in clinical development are canertinib, a pan-ErbB family TKI, and lapatinib [17]. Further information on clinical studies can be found in other reviews [20].

Targeting the epidermal growth factor receptor mRNA

The third group of EGFR-inhibiting strategies interferes with mRNA, decreasing the production of EGFR protein. In this approach, gene-silencing nucleic acids, such as ribozymes, DNA enzymes (DNAzymes), antisense oligonucleotides (AS-ONs), and small interfering RNAs (siRNAs), have been used as therapeutic agents. The activity of each of these nucleic acids relies mostly on their hybridisation with accessible sites within target mRNA; however, there are some differences. AS-ONs, for example, will form a double strand with the target mRNA, which will be recognised by the enzyme RNase H. This enzyme will then hydrolyse the bound mRNA, whereas ribozymes (RNA enzymes) and DNAzymes own an intrinsic catalytic core of nucleotides that will directly hydrolyse the mRNA and, therefore, inhibit its translation into the encoded gene-product. As for siRNA, once inside the cell, it will assemble into the RNA-induced silencing complex (RISC) and the siRNA antisense strand will guide the complex to the complementary sequence in target RNA, enabling the cleavage of the target mRNA.

Ciardiello and colleagues have tested several AS-ONs targeting different regions of the human EGFR mRNA. Their aim was to select the antisense sequences that could induce an optimal inhibition of EGFR production and, therefore, be interesting for a therapeutic approach. Two of the sequences (Table 3) were chosen for further studies, then modified into a mixed backbone oligonucleotides (MBO), and their antitumour properties *in vivo* were assessed. Results showed an antitumour effect of specific EGFR AS-ONs and allowed the identification of novel EGFR antisense MBOs, deserving further evaluation as potential selective antitumour agents [27].

In 1998, a study reported the use of ribozymes for inhibition of one mutated EGFR [28]. This study was based on evidence suggesting that the expression of aberrant EGFR mRNA is a determinant factor for continuous and aggressive growth of malignant gliomas. Appropriate ribozymes were designed and directed to specific sequences of the corresponding aberrant EGFR mRNA. Results showed the inhibitory effect of the designed ribozyme on the growth of transformed cells *in vivo* and, therefore, it was stated that such ribozymes could be interesting for the degradation of aberrant EGFR mRNA as a gene therapy approach for malignant glioma.

Last but not least, siRNA has been recently introduced as a strategy for EGFR inhibition. The first reported study using siRNA (Table 3) to silence EGFR expression was presented in 2003 [29], explaining why this approach has received comparably little attention as yet.

As the activity of gene silencing nucleotides relies on the hybridisation to specific and accessible sites within the target mRNA, the prediction of the targeted nucleotide sequence can be difficult. In 2003, Beale and colleagues focused on this problem and by using scanning arrays they designed ribozymes, DNA enzymes and siRNA molecules. Results showed the effectiveness of their method, by demonstrating the inhibition of EGFR expression in cultured A431 cancer cells [30].

In addition to the structure of the target mRNA, the physicochemical characteristics of gene-silencing nucleotides pose significant problems for ultimate use in humans, as they are large, negatively charged and still require intracellular delivery. Therefore the design of specific delivery systems will be essential in further development. This delivery issue seems less of a problem for the other two strategies involving small molecular weight drugs and molecules which act extracellularly.

Besides these three types of targeting strategies, recent reports have referred to other specific molecules that can be used to interfere with EGFR signalling; for example, Argos, a protein secreted by *Drosophila melanogaster*, which seems to be able to neutralise EGFR ligands, and Decorin, a soluble proteoglycan, which was reported as inducing downregulation of EGFR by protracted internalisation and degradation. Such reports highlight the possibility of developing additional promising strategies for interfering with EGFR in cancer therapy [31, 32].

Table 1. Targeting the extracellular domain of EGFR: monoclonal antibodies.

Agent	Other designation	Sponsor
Cetuximab	IMC-C225, Erbitux®	ImClone Systems/Bristol-Myers Squibb
Panitumumab	ABX-EGF	Abgenix
Matuzumab	EMD-72000	Merck

EGFR: Epidermal growth factor receptor

Table 2. Targeting the intracellular domain of EGFR: tyrosine kinase inhibitors.

Agent	Other designation	Sponsor
Gefitinib	ZD1839, Iressa	AstraZeneca
Erlotinib	CP-358-774, OSI-774, Tarceva	Genentech/OSI/ Roche
Canertinib	CI-1033	Pfizer
Lapatinib	(pan-ErbB family TKI)	GlaxoSmithKline

EGFR: Epidermal growth factor receptor

Table 3. Targeting the EGFR mRNA: gene silencing nucleic acids.

Description	Sequence (5’ to 3’)	Ref.
AS-ON location EGFR mRNA 2457-2476 mRNA 4614-4633	TATGATCTGTCACAGCTTGA TGCTAGGTTTCCTCCCTTTC	[27]
siRNA ErbB1-1 siRNA ErbB1-3	5’-CUCUGGAGGAAAAGAAAGUTT-3’ 3’-TTGAGACCUCCUUUUCUUUCA-5’ 5’-CACAGUGGAGCGAAUUCCUTT-3’ 3’-TTGUGUCACCUCGCUUAAGGA-5’	[29]
pEGFR-antisense	CCGGCCGTCCCGGAGGGTCGCATCGCTGCTCCCCGAAG	[33]
AS-ON	CGGAGGGTCGATCGCTG	[34]
AS-ODN location EGFR mRNA 760-779	CCCCAGCAGCTCCCATTGGG	[35]

Table 3 does not show all examples mentioned in the text as some articles do not disclose sequence information.
EGFR: Epidermal growth factor receptor; AS: Antisense; ODN: Oligodeoxynucleotide; ON: Oligonucleotide; pEGFR: Phosphorylated EGFR; siRNA: Small interfering RNA.

FUNCTIONALITY OF EPIDERMAL GROWTH FACTOR RECEPTOR INHIBITION

This section deals with the responses at the cellular and tissue level resulting from the interference with EGFR function.

The activation of EGFR by one of the possible ligands can initiate a number of signalling cascades: the MAPK, PI3K/Akt, STAT and PLCγ pathways; culminating in cellular responses such as cell proliferation, differentiation, migration and survival. Approximately 20 years ago, mAbs were first produced against EGFR and used to better understand two functions of the EGFR:

- EGF binding to the receptor and its subsequent stimulatory or inhibitory effect on cell proliferation; and
- the TK activity, whether it retained activity when both EGF and mAb were present [10-12].

These studies have increased knowledge on the cellular processes occurring upon activation of EGFR by EGF. From that time on, a broad research area has been covered in order to elucidate the role of all participants in cellular processes involving EGFR.

Cell cycle arrest, cell proliferation inhibition and apoptosis induction

Cell cycle designates a very complex process responsible for the duplication of the genetic material of one cell and division into two identical daughter cells. In fact, it is a systematic sequence of events, grouped in four phases, G1, S, G2 and M. In the S phase (synthesis) the genetic material is duplicated, in the M phase (mitosis) the duplicated genetic material, that is, the chromosomes, are distributed equally to the two daughter cells. In phase G1 and G2 the cell is preparing itself for the following phases, S and M, respectively. A cell that is not active, but is in a quiescent or resting state is said to be in G0 phase. The transition from one phase to the next one of the cell cycle is efficiently regulated by different classes of cellular proteins, and before moving to the next phase there are checkpoints, which are pathways controlling the order and timing of phase progression. In fact, such checkpoints will delay the cell cycle whenever the running phase is not entirely and/or correctly finished or can even activate the apoptotic program if no repair is possible [36].

Cancer cells are known to be uncontrolled-dividing cells. In normal cells, a continuous stimulation of proliferation usually activates apoptosis. A tremendous effort has been made in order to understand why controlling pathways and key regulators of the cell cycle sometimes fail to regulate cell division in cancer.

A few years after the production of the first EGFR mAbs [10-12], Mendelsohn reported inhibition of cell proliferation of some human tumour cells by two murine mAbs, 528 IgG2a and 255 IgG1 [37]. In 1995 the mAb 225 proved to be able to inhibit binding of EGF to its receptor and, consequently, to block the activation of the TK in human colorectal carcinoma cell line (DiFi) in *in vitro* studies. As a result, a G1 cell cycle arrest and induction of apoptosis were observed [38]. Later, such G1 arrest in the cell cycle was better understood. It was in fact associated with a decrease in the activity, but not the protein content, of specific kinases that are required for G1 phase progression and entry into the S phase, as well as an increase in kinase inhibitors, such as $p27^{KIP1}$, due to increased transcription and translation [39, 40]. For this murine mAb 225 a human /

murine chimeric version was produced in order to decrease the generation of human anti-mouse antibodies in receivers [41]. This antibody is known as cetuximab (Table 1).
In 1997 an irreversible TKI – CP-358,774 – proved to block cell cycle at phase G1 and induce apoptosis of DiFi cells, *in vitro* [42]. This TKI is nowadays known as erlotinib (Table 2), which has recently shown antitumour effect with cell cycle arrest on hepatocellular cancer cells [43]. Other examples of TKIs capable of a G1 arrest in cell cycle were reported by Busse *et al* [44]. Gefitinib, one of the most investigated TKIs, has also proven to provoke cell cycle arrest, with up-regulation of the $P27^{KIP1}$ kinase inhibitor; inhibition of cell proliferation, and apoptosis induction during *in vitro* and *in vivo* studies on different cancer types, such as esophageal and oral squamous cell carcinoma [45, 46], lung adenocarcinoma [47], breast [48] and prostate carcinomas [49].
Regarding the third strategy, which employs therapeutic nucleic acids to interfere with the EGFR mRNA, the first predictable cellular effect obtained would be a decrease in the EGFR protein expression. Similar types of cellular effects reported for mAbs and TKIs would then be expected. Indeed, in 1998 a group of researchers reported *in vivo* inhibition of tumour growth, suppression of EGFR protein expression and increased apoptosis after delivery of AS-ONs plasmids (Table 3) for EGFR on HNSCC [33]. Ciardiello and colleagues also demonstrated cell proliferation inhibition and apoptosis induction using AS-ONs (Table 3) to target the EGFR on different cell lines originating from human colon, breast and ovarian cancers [27]. In 2003, results were published showing that siRNA (Table 3) against EGFR is able to decrease the expression of EGFR protein by 90%, in A431 human epidermoid carcinoma cells, as well as to reduce cell proliferation and induce apoptosis [29].

Cell adhesion modulation, cellular invasion and metastasis inhibition

Formation of tumour metastases involves several processes, such as cellular migration and invasion of surrounding tissues, lymph or blood stream. These processes depend on cell adhesion properties, which are regulated by adhesion molecules. E-cadherin, for example, is a calcium-dependent cell adhesion molecule, and it has been suggested as being involved in non-invasiveness of tumours. In 1998, results were published showing EGFR regulating the cell-cell adhesion by modulating E-cadherin and actin cytoskeleton interaction. These results suggested that EGFR activation results in loss of cell adhesion, due to the decoupling of actin from the E-cadherin adhesion complex. This would in turn enable invasion of surrounding tissues [50]. Integrins, another group of adhesion molecules, are transmembrane cell surface receptors that mediate adhesion of cells to the extracellular matrix (ECM). Several studies have reported the importance of integrins, growth factors and their receptors, such as EGFR, in cellular adhesion and migration. In fact, activation of EGFR was reported as leading to an enhanced adhesion of cells to proteins of the ECM, consequently allowing cellular invasion in breast cancer [51, 52]. Besides adhesion molecules, matrix metalloproteinases (MMPs) have a key role in cellular invasion. These enzymes are secreted by tumour and/or host cells and have a proteolytic activity on ECM components. MMP-9, for example, was reported as being up-regulated in HNSCC, due to activation of the EGFR, together leading to an increased tumour cell invasion [53, 54]. Thus, EGFR activation, and its influence in cellular

migration and invasion, gives another perspective of the effects that EGFR inhibitors can induce in tumour progression.
A mAb, ICR62, was used to block the EGFR on HNSCC, and results showed inhibition of MMP up-regulation, as well as inhibition of migration and invasion, usually induced by EGFR activation [53, 55]. In small-cell lung cancer, similar effects were obtained using mAb 528 as an EGFR inhibitor [56]. The mAb 225 was able to block the EGFR on breast cancer cell lines, inducing an increase in E-cadherin-dependent cell adhesion, suggesting a decrease in cellular invasion [50]. The human/mouse chimeric antibody of this mAb 225, the C225 also referred to as cetuximab (Table 1), was employed for studies on HNSCC, which revealed *in vitro* inhibition of cell migration and *in vivo* decrease of the metastatic potential, concomitant with down-regulation of the expression of MMP-9 [57].
Several *in vitro* and/or *in vivo* studies with different TKIs, have shown cell adhesion re-enforcement, in some cases concomitant with a decrease in expression of some integrins and/or a down-regulation of MMP, decreased cellular invasion and metastasis inhibition. For example, the TKI PD130305 induced such effects on breast cancer cell lines [50] and gefitinib on oral squamous cell carcinoma [58].
In 2004, a study was reported in which three TKIs were used - gefitinib, erlotinib and canertinib (Table 2) - on malignant mesothelioma cell lines. The third TKI, contrary to the first two, which are EGFR specific, is a pan-EGFR family TKI. Results showed effective inhibition of cellular migration and MMP-9 production, for all three TKIs, without significant differences between their responses [59].
The third strategy, interfering with EGFR mRNA, also proved to modulate cell adhesion. The decrease in EGFR protein expression, due to the action mechanism of gene-silencing nucleic acids, proved to have the ability to interfere with the production of adhesion molecules. For example, in ovarian carcinoma cells, the expression of EGFR antisense, after inserting an expression vector containing the human EGFR complementary DNA in an antisense orientation into cells, resulted in reduction of EGFR expression. Consequently, a decrease of E-cadherin expression and invasiveness were detected [60]. A decrease in MMP-9 activity was also reported when using this strategy [61].

Angiogenesis inhibition

Angiogenesis, which is the formation of new blood vessels from pre-existing ones, is essential to supply tumour cells with oxygen and nutrients, and therefore is required for tumour growth, invasiveness and development of distant metastases. Angiogenesis is regulated by pro- and antiangiogenic factors; therefore, one way of monitoring this process is by determining the expression of these factors, before and after treatment. Key regulators of angiogenesis are vascular endothelial growth factor (VEGF), interleukin-8 (IL-8) and basic fibroblast growth factor (bFGF), which stimulate cell proliferation, migration and functional differentiation of activated endothelial cells. The contribution of EGFR activation to angiogenesis was supported by studies showing that EGF binding to EGFR upregulated VEGF mRNA expression in human cancer cells [62], as well as studies showing downregulation of VEGF production following EGFR inhibition by mAb 225 [63].

While the antitumour effects of mAb C225 were further investigated, a decrease in expression of three angiogenic factors (VEGF, IL-8 and bFGF) in human transitional cell carcinoma of the bladder was observed. This was translated into inhibition of angiogenesis, together with tumour growth reduction and inhibition of metastasis [64]. Huang *et al* also examined the effect on angiogenesis of mAb C225 using an *in vivo* tumour xenograft neovascularisation model of angiogenesis. After systemic treatment, C225 was able to reduce tumour growth and number of tumour capillaries, and also delayed the growth of established vessels towards the tumour [57].
TKIs also revealed the ability to interfere with the expression of key angiogenic factors. For example, gefitinib was used by Ciardiello and co-workers in order to evaluate its antiangiogenic and antitumour activity in human colon, breast, gastric and ovarian cancer cells. Indeed, they determined a decrease in VEGF as well as bFGF expression, with a concomitant decrease in blood vessel counts [65].
Not only mAbs and TKIs have shown ability to inhibit angiogenesis after EGFR inhibition, but also AS-ONs targeting EGFR mRNA proved such effect. EGFR AS-ONs (Table 3) were able to decrease VEGF expression on HNSCC [34].

Summary
Taken together, these results suggest that interfering with EGFR signalling, either by using mAbs, which inhibit ligand binding; using TKIs, which block TK activation; or interfering with EGFR mRNA, which prevents EGFR expression, produces antitumour activity mediated by several cellular effects:
- Cell cycle arrest, induction of apoptosis and decrease of cell proliferation;
- Modulation of the expression and activity of adhesion proteins, and MMP, leading to a reduction of invasiveness and therefore reduction of metastatic capacity; and,
- Inhibition of angiogenesis, through downregulation of proangiogenic factors.

COMBINED STRATEGIES

The following sections will present examples of enhanced antitumour activity resulting from the combination of EGFR inhibition with other strategies.

Combination of epidermal growth factor receptor inhibition with chemotherapy or radiation therapy

Conventional cancer therapies do not discriminate between healthy and malignant cells. Nonetheless, as an attempt to improve response to traditional chemotherapy and radiation therapy, numerous studies have tested their combination with EGFR inhibitors.
Several studies involving mAb 225 and, later, its human / murine chimeric version C225 (Table 1) were able to show an increased antitumour effect on different cancer types, both *in vitro* and *in vivo*, when combined with different classes of chemotherapeutic agents, including cisplatin, doxorubicin, paclitaxel, topotecan and gemcitabine [66]. An enhanced antitumour effect was also detected with cetuximab when applied concomitantly with ionising radiation [22].

TKIs were also reported as being capable of enhancing the antitumour activity of conventional chemotherapeutic agents, in both *in vitro* and *in vivo* studies [1]. As an example, gefitinib potentiated paclitaxel antitumour effects on colorectal carcinoma [65]. Gefitinib was part of several *in vitro* and *in vivo* studies combined with radiotherapy, which resulted in increased cytotoxic effects on head and neck cancer, breast, ovarian, colorectal and non-small cell lung carcinomas [66].
Ciardiello and colleagues, while working with AS-ONs (Table 3) for interfering with EGFR, also tested several cytotoxic drugs in combination with this strategy. They reported an improved tumour growth inhibition for combinations with cisplatin, doxorubicin, paclitaxel or topotecan [27]. In addition, a beneficial combination of docetaxel and AS-ONs (Table 3) targeting the EGFR has given rise to a possible effective and less toxic treatment for HNSCC [35].
In general, combining EGFR inhibition and traditional chemotherapy or radiotherapy, results in stronger antitumour effects. Such a combination could decrease the toxicity usually associated with conventional therapy by decreasing the necessary dose of cytotoxic drug. In fact, this approach has already been tested in many clinical trials [20].

Combination of epidermal growth factor receptor inhibitors

Recently, the combination of two different EGFR inhibitors was suggested. The purpose was to investigate whether interfering with EGFR by two different strategies would give better results than one EGFR inhibitor alone.
Huang and colleagues reported beneficial results concerning the antitumour activity of two combinations: the mAb cetuximab with one of the two TKIs, either gefitinib or erlotinib, on different cancer types (HNSCC, vulvar squamous cell carcinoma, prostate and NSCL carcinomas). *In vitro* and *in vivo* results of both associations, compared to single-drug treatment, showed a general enhancement of cell proliferation inhibition, enhanced apoptosis induction, and a generally more profound tumour regression. They were also able to show that cetuximab-resistant cells still respond to TKI treatment, suggesting that EGFR inhibitors associations could overcome certain limitations of one EGFR inhibitor alone, such as acquired resistance [67].
Another experiment combining cetuximab and gefitinib on different cell lines (of human head and neck cancer, and vulvar carcinoma) was less successful. Some controversial results were obtained, suggesting that the effect of such associations is not yet completely clear and, therefore, new attempts of mAb and TKI combinations should be considered with caution [68]. Similar conclusions were drawn after testing other associations, such as gefitinib and trastuzumab (a mAb specific for ErbB-2) on prostate and breast cancer [69].
Results are not always consistent, which shows how difficult the interpretation of the results from these associations can be. Nevertheless, some studies have demonstrated an enhanced antitumour effect in particular cases [70]. These suggest that the outcome of a combination could depend on the cancer type. Further investigation is therefore required in order to determine which associations of EGFR inhibitors can be beneficial and for which cancer types.

Combination of epidermal growth factor receptor and vascular endothelial growth factor receptor inhibitions

Understanding tumour angiogenesis and its regulation by angiogenic factors (e.g., EGF and VEGF) stimulated attempts to interfere with more than one factor simultaneously. Consequently, the combination of both EGFR and VEGFR inhibition seemed to be a promising approach.

The human / mouse chimeric mAb C225, whose antiangiogenic effect had been demonstrated concomitantly with a decrease of VEGF expression, as well as other angiogenic factors [64], was used in combination with another mAb, DC101, directed to VEGF-receptor-2 (VEGFR2), on gastric cancer. The combined treatment resulted in a significant enhanced suppression of tumour growth, as well as a further decrease in cell proliferation and vascularity [71].

Another approach was tested by Ciardiello and colleagues on colorectal carcinoma. The same mAb C225 was used to inhibit EGFR; however, for interfering with VEGFR signalling they used AS-ONs for VEGF mRNA, decreasing the VEGF protein content. Results of combined treatment showed prolonged inhibition of tumour growth, reduction of microvessel number and improved mice survival [72].

Studies involving EGFR TKIs in association with VEGFR inhibitors have also been reported. For instance, gefitinib was tested alone and in combination with PTK787/ZK222584, an inhibitor of VEGFR TK. The simultaneous blockade of both signalling pathways resulted in a cooperative antitumour effect [73].

Some specific TKIs have been reported as being effective not only for inhibition of the TK of EGFR but also for inhibition of the TK of the VEGFR. ZD6474 is a potent reversible inhibitor of ATP binding to VEGFR-2 TK, which also inhibits EGFR TK activity. Therefore, it is able to interfere with two key pathways in tumour growth and, consequently, to produce a potent antitumour effect. This effect has been reported, for example, in pancreatic, prostate, ovarian, breast and lung carcinomas [74]. AEE788 is another inhibitor of TKs of both EGFR and VEGFR, and whose antitumour effect has been reported on colon carcinoma [75], prostate [76] and thyroid carcinoma [77]. Furthermore, an enhanced antitumour effect was obtained once each molecule was combined with chemotherapy or radiation therapy.

Raben *et al* presented another example of an approach for interfering with both the angiogenesis process and the EGFR signalling pathway. In this case, together with gefitinib – a TKI for EGFR – a novel vasculature-targeting agent was used. ZD6126 is a vascular-targeting agent which selectively disrupts intratumoral immature vasculature, and the combination of both drugs resulted in a stronger antitumour activity [78].

All of these examples demonstrate the benefit of combining the inhibition of two key signalling pathways involved in tumour progression. Both angiogenesis and activation of the EGFR proved to be essential for cellular progression, invasion and development of metastases. However, other pathways may have a contribution to tumour progression, increasing the number of possible combinations.

Besides the combined inhibition of EGFR and VEGFR, other combined strategies have been tested and encouraging results have been published. For example, the combined administration of antibodies to block both EGFR (ABX-EGF) and IL-8, which has been

correlated with the progression of some human tumours, has been suggested as an effective approach for treatment of metastatic human breast carcinoma [79]. The simultaneous blockade of EGFR and insulin-like growth factor receptor (IGFR) signalling pathways has also been suggested as being a more efficacious strategy for tumour therapy [80].
Further research on this issue will continue in order to find additional combined strategies that efficiently interfere with tumour progression.

EXPERT OPINION AND CONCLUSION

Over the past two decades, efforts have been made to better understand the cellular processes behind malignancy. Since the idea of targeting EGFR as an option for cancer treatment was first proposed, a large number of EGFR inhibitors have been developed and their antitumour potentiality has been investigated. The inhibition of EGFR has proven to result in a multitude of cellular effects, leading to a general antitumour effect, consequently giving evidences of the benefits in exploiting EGFR inhibitors for cancer therapy.
Preclinical studies have encouraged clinical trials, which have been investigating the potential of mAbs and TKIs as single anticancer agents, or in combination with chemotherapeutic drugs or radiation therapy, in different cancer types. Nevertheless, a reliable molecular marker for an adequate selection of responsive patients to a specific treatment remains to be established. Furthermore, a better understanding of resistance mechanisms observed in clinical trials point to areas of further investigation.
The fact that not only EGFR signalling pathways can drive cell proliferation, and that cancer cells may use multiple and redundant intracellular pathways to overcome blockade of a particular receptor or protein signal, cautions against inhibition of single pathways. Therefore, inhibition of alternative pathways and combinations between EGFR inhibitors and other targeted drugs are expected to show a clear overall benefit. However, one should bear in mind that combining different drugs could give rise to pharmacokinetic / pharmacodynamic issues associated with formulations. In this context, gene-silencing nucleic acids seem promising, as only the sequence of nucleotides change while the same carrier can be used for delivery.
Ultimately, this rationale approach would lead to specific combinations of targeted therapeutics, based on molecular profiling of gene expression of an individual patient, to tailor inhibition of precise pathways that drive tumour proliferation.
Developing such tailored cancer therapy clearly requires further research. In coming years, we may face a transition from cytotoxic therapies to individual combinations of multiple inhibitors of key regulatory pathways, where EGFR inhibition will play a major role.

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CHAPTER 3

FUSOGENIC PEPTIDES ENHANCE ENDOSOMAL ESCAPE IMPROVING siRNA-INDUCED SILENCING OF ONCOGENES

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ABSTRACT

Small interfering RNA (siRNA) molecules are the functional mediators of a post-transcriptional gene silencing process known as RNA interference (RNAi). The siRNA-mediated silencing of genes involved in diseases is considered a very promising therapeutic strategy. However, as for all the nucleic acid based therapeutics, these negatively charged and hydrophilic molecules do not readily cross biological membranes. The use of cationic carriers generally results in positively charged complexes which are taken up by cells through endocytosis. Still, for gene silencing, these complexes need to escape through the endosomal membrane, thereby reaching the cell cytoplasm where all the RNAi machinery is present. One of the strategies developed to facilitate endosomal escape mimics the fusion of viral envelopes with host cell endosomal membranes, which occurs during viral infections. Several synthetic fusogenic peptides have been synthesised based on the fusion domain of the influenza virus. In this study, we evaluated the effects of the influenza-derived fusogenic peptide diINF-7 on gene silencing efficiency of siRNA lipoplexes targeting the K-ras and the epidermal growth factor receptor (EGFR) oncogenes. In general, significant enhancement of gene silencing activity was noted after addition of diINF-7 fusogenic peptide, identifying endosomal escape as a limiting factor for siRNA silencing efficiency.

INTRODUCTION

RNA interference (RNAi) is regarded as an attractive and potent mechanism for silencing gene expression in a sequence-specific manner. Either used for understanding the function of genes or interfering therapeutically with aberrant gene expressions, this technique has captured the interest of many researchers.

Short double-stranded RNA molecules, known as small interfering RNA (siRNA) are the functional mediators of RNAi. For RNAi to occur, siRNA molecules need to be present in the cell cytoplasm, where the post-transcriptional RNAi-silencing machinery is available. When siRNA assembles into the RNA-induced silencing complex (RISC), it can interact with and degrade complementary mRNA sequences, thereby interrupting the translation of specific proteins. However, the physicochemical properties of siRNA molecules, such as the relative large size (approximately 14 kDa), the negative charge and hydrophilicity, make it difficult for siRNA molecules to cross cellular membranes and to reach the cytoplasm. As a consequence, several viral and non-viral carrier systems have been developed in order to deliver chemically synthesised siRNA molecules into the cells. Non-viral systems are usually based on electrostatic complexation of negatively charged siRNA with positively charged polymers or lipids. The resulting complexes generally have a net positive charge, which facilitates the interaction with the negatively charged cellular membrane, and are likely taken up by cells through endocytosis. Once inside the endosomes, these complexes or their siRNA should be able to escape through the endosomal membrane, in order to avoid degradation and to allow RNAi to occur [1].

A number of strategies have been proposed to facilitate endosomal escape: pore forming peptides, flip-flop of phospholipids, pH-buffering capacity by protonable groups (*proton sponge*), and photochemical internalisation are some of the examples [2-4]. This present research employs another strategy to facilitate endosomal escape: fusogenic peptides.

Since the early eighties, many studies have been performed on membrane fusion activity of animal viruses and, as a result, many viral fusogenic peptide sequences have been identified [5, 6]. The functional role of fusogenic peptides lies in the fusion process occurring between the viral envelope and host cell endosomal membrane, to transport the viral genome into the cytoplasm, after receptor-mediated endocytosis. The influenza virus hemagglutinin protein has an N-terminal fusion domain on the HA2 subunit which becomes protonated upon acidification of the endosomes. As a result, this hydrophobic fusion peptide domain changes its conformation and moves to the outside of the protein where it will interact with the endosomal membrane, causing its destabilisation [7]. Several synthetic fusogenic peptides have been synthesised based on the fusion domain of the influenza virus. Among those, the INF-7 peptide has demonstrated its fusogenic capacity by improving the transfection efficiency of non-viral gene delivery systems [8, 9] and the corresponding dimeric peptide, diINF-7, proved to be able to enhance cytosolic delivery of macromolecules entrapped in immunoliposomes [10, 11].

In this study, we evaluated the effects of the influenza-derived fusogenic peptide diINF-7 on the silencing efficiency of siRNA lipoplexes targeting two genes frequently involved in malignancies: K-ras and epidermal growth factor receptor (EGFR). Mutations in K-ras gene are associated with one third of all human cancers and 35% of colorectal cancers

[12], and many human epithelial cancers are marked by the overexpression of EGFR protein (e.g. lung, head and neck, breast, colon, prostate, and ovarian carcinomas); both are therefore relevant targets for RNAi application.

SILENCING THE K-RAS ONCOGENE

Murine colon carcinoma cells C26 were cultured in DMEM medium containing 3.7 g/l sodium bicarbonate and 4.5 g/l glucose, supplemented with antimicrobial agents, 2 mM L-glutamine and 10% (v/v) foetal bovine serum, at 37 °C in a humidified atmosphere containing 5% CO_2.

One day after seeding $3x10^5$ cells per well, complexes of anti-K-ras siRNA (Eurogentec) and Lipofectamine 2000 (LF) were prepared as recommended by the manufacturer (Invitrogen), using two different siRNA concentrations (450 and 600 pmol/well). The size of these anti-K-ras siRNA/LF complexes was in the range of 110-120 nm. Immediately after preparing the lipoplexes, the diINF-7 peptide, which had been synthesised as previously described [10], was added to the particles at a concentration of 12 µg/µl LF, forming the anti-K-ras siRNA/LF/diINF-7 complexes by electrostatic interactions. The addition of diINF-7 fusogenic peptide had no effect on particle size, and only a slight effect on surface charge (a consistent decrease of 5 mV), compared to the anti-K-ras siRNA/LF complexes. Both the complexes were added to the cells and incubated for 5 h after which the medium was refreshed. Forty-eight hours after, cells were detached, the K-ras protein expression was assessed by western blotting as previously described [13]. Enhanced chemiluminescence was used for detection and the intensity of the bands was analysed and quantified with Gel-Pro Analyser software (INTAS).

The analysis of the films (Figure 1) revealed a decrease of K-ras protein expression of approximately 25% by the anti-K-ras siRNA/LF complexes and a decrease of approximately 80% by the anti-K-ras siRNA/LF/diINF-7 complexes, for the highest siRNA concentration (600 pmol siRNA/well) (Figure 2). Thus, a 3.5-fold enhancement of siRNA silencing efficiency was obtained when diINF-7 peptide was associated with the complexes. Interestingly, the anti-K-ras siRNA/LF/diINF-7 complexes, at 450 pmol/well, induced a better silencing of K-ras (approximately 55%) than the complexes with the higher dose (600 pmol/well) without fusogenic peptide (approximately 25%). Complexes of control siRNA and LF, with or without the fusogenic peptide, showed no effect on K-ras knockdown (Figure 1).

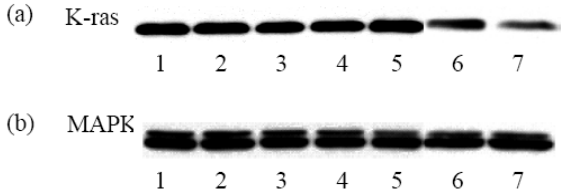

Figure 1. Silencing K-ras protein expression.
(a) Western blot: one day after seeding $3x10^5$ C26 cells per well, these were incubated with (1) anti-K-ras siRNA/LF (450 pmol/well); (2) anti-K-ras siRNA/LF (600 pmol/well); (3) anti-NS siRNA/LF (450 pmol/well); (4) anti-NS siRNA/LF (600 pmol/well); (5) buffer; (6) anti-K-ras siRNA/LF/diINF-7 (450 pmol/well); and (7) anti-K-ras siRNA/LF/diINF-7 (600 pmol/well) complexes, in six-well plates. Samples were prepared for WB two days later. (LF – Lipofectamine; NS – non specific); (b) MAP kinase was used as a loading control.

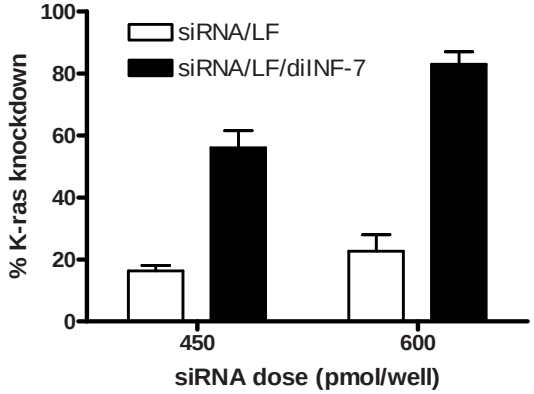

Figure 2 Silencing K-ras protein expression.
Knockdown of K-ras protein (in %) after analyses of three different western blot films, using the Gel-Pro Analyser software. Two different complexes were incubated with C26 cells: the anti-K-ras siRNA/LF and the anti-K-ras siRNA/LF/diINF-7. Both complexes were tested in two doses, 450 and 600 pmol siRNA per well.

SILENCING THE EGFR ONCOGENE

Human epidermoid carcinoma cells A431 were subcultured in Dulbecco's Modified Eagle's medium (DMEM) containing 3.7 g/l sodium bicarbonate and 4.5 g/l glucose, supplemented with antimicrobial agents, 2 mM L-glutamine and 7.5% (v/v) foetal bovine serum, at 37 °C in a humidified atmosphere containing 5% CO_2. Human head and neck squamous carcinoma cells 14C were subcultured in similar conditions, except for the DMEM, which was supplemented with antimicrobial agents and 5% (v/v) foetal bovine serum.

One day after seeding $4x10^4$ cells per well, complexes of anti-EGFR siRNA (Eurogentec) and Lipofectamine 2000 (LF) were prepared as recommended by the manufacturer (Invitrogen). These anti-EGFR siRNA/LF complexes had an average size of 120 nm. The anti-EGFR siRNA/LF/diINF-7 complexes were prepared as described for anti-K-ras

siRNA. In a similar way, only their charge was slightly affected being less positive than for the anti-EGFR siRNA/LF complexes. Both the complexes were added to the cells and incubated for 5h, after which the medium was refreshed. After forty-eight hours, cells were detached, incubated with an anti-EGFR monoclonal antibody labelled with FITC (Santa Cruz Biotechnology Inc.), and the expression of EGFR was accessed by flow cytometry, using a FACScalibur (Becton & Dickinson).

Figure 3 presents the expression of EGFR determined by flow cytometry for A431 cells treated with both complexes (anti-EGFR siRNA/LF and anti-EGFR siRNA/LF/diINF-7) and with each component separately, as controls. Figure 4 shows the percentages of knockdown of EGFR expression for cells treated with both complexes relatively to the controls. There is a clear increase in knockdown of EGFR expression, of approximately 2-fold, for the complexes which contained diINF-7 fusogenic peptide compared to the ones lacking the fusogenic peptide. Each of the elements alone showed no effect on EGFR expression.

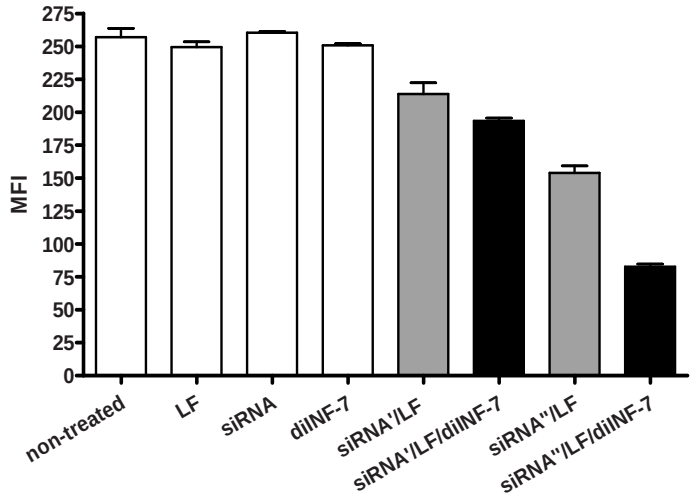

Figure 3. Expression of EGFR.
One day after seeding $4x10^4$ A431 cells, these were incubated with different complexes - the anti-EGFR siRNA/LF and the anti-EGFR siRNA/LF/diINF-7, both in 20 and 40 pmol/well doses (siRNA' and siRNA'', respectively) – and with each element separately (Lipofectamine, LF; anti-EGFR siRNA; diINF-7 fusogenic peptide), in 24-well plates. Two days later, the values of mean fluorescence intensity (MFI) were determined by flow cytometry and correspond to the mean of three different measurements. NT – non-treated.

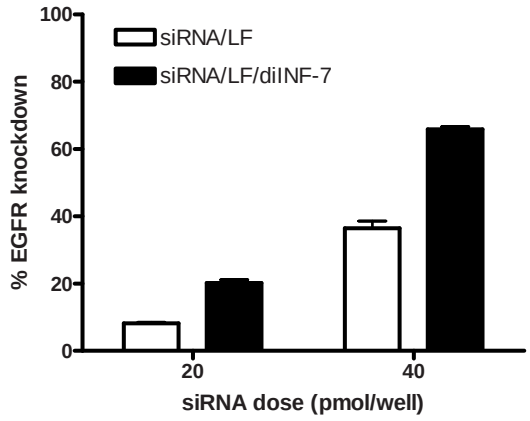

Figure 4. Silencing EGFR protein expression on A431 cells.
The percentage of EGFR expression is calculated using the mean fluorescence intensity (MFI). Controls were set to 100% and the silencing efficiency of the samples was calculated by determining the ratio of fluorescence.

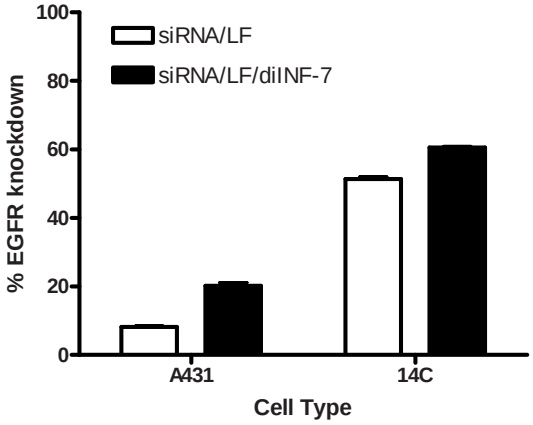

Figure 5. Silencing EGFR protein expression on A431 and 14C cells.
A431 and 14C cells were treated as described earlier with lipoplexes of anti-EGFR siRNA/LF or anti-EGFR siRNA/LF/diINF-7, at 20 pmol siRNA/well. The MFI was determined and the percentage of EGFR knockdown was calculated relatively to the controls.

Figure 5 shows differences in EGFR-silencing efficiencies of the lipoplexes for two different cell lines. The anti-EGFR siRNA/LF complexes induced stronger knockdown of EGFR on 14C cells, likely due to their lower expression of EGFR as compared to A431 cells. The enhancement of silencing efficiency by diINF-7 peptide is smaller (1.2-fold) in 14C cells compared to A431 cells (2-fold).

DISCUSSION

In this study we demonstrate that the synthetic influenza-based diINF-7 peptide can enhance the endosomal escape of complexes composed of siRNA and Lipofectamine. The improvement of the silencing efficiency that we observed is certainly related to the cationic carrier used. Here, Lipofectamine, a commercially available transfectant for nucleic acids, is capable of promoting the escape from the endosomes to a certain extent without additional helper molecules, such as fusogenic peptides. However, our data show that improvement is clearly possible. This is in agreement with the study by Veldhoen *et al* where it is reported that only a fraction of the total number of siRNA molecules that enter the cells with Lipofectamine is responsible for the silencing of the target gene, while the rest remains entrapped in vesicles spread throughout the cytoplasm of the cells [14].

Other carriers, unable to escape from the endosomes on their own, could particularly benefit from this fusogenic peptide, and as a result become attractive carriers for siRNA. Furthermore, such enhancement of siRNA silencing efficiency, would allow the use of lower concentrations of siRNA which has been shown to reduce the non-specific effects of siRNA treatment [15, 16]. It would also prevent the saturation of the RNAi machinery, which has been co-related with a reduction of RNAi efficiency and the disturbance of the endogenous miRNA pathways [17].

The silencing-enhancement by diINF-7 on EGFR was not constant for different tumour cell lines. Silencing of EGFR in A431 cell benefited more from diINF-7 compared to 14C cells. This could be related to target protein expression: A431 cells strongly overexpress EGFR [18], and therefore anti-EGFR siRNA/LF could be relatively inefficient, thus leaving more room for improvement by addition of the peptide. Alternatively, differences between cell lines may account for the observed variations in silencing-enhancement.

Our studies focus on silencing the expression of proteins which are known to be involved in tumour progression and, therefore, are relevant targets for RNAi application. Enhancing the silencing efficiency of siRNA targeting these two genes certainly improves therapeutic applications targeting the EGFR and K-ras oncogenes.

In conclusion, this study emphasises the endosomal escape as a limiting factor for siRNA-mediated silencing and shows that fusogenic peptides can be used to efficiently enhance the endosomal escape of siRNA-Lipofectamine lipoplexes.

ACKNOWLEDGMENTS

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CHAPTER 4

PHOTOCHEMICAL INTERNALISATION ENHANCES SILENCING OF EPIDERMAL GROWTH FACTOR RECEPTOR THROUGH IMPROVED ENDOSOMAL ESCAPE OF SIRNA

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ABSTRACT

Photochemical internalisation (PCI) has been employed as a tool for site-specific intracellular delivery of a variety of molecules. In this study, for the first time, PCI has been employed to facilitate the endosomal escape of small interfering RNA (siRNA) molecules, which are the functional mediators of RNA interference (RNAi). In order to interact with the machinery that will induce post-transcriptional gene silencing, siRNA molecules need to enter the cytoplasm of the cells. This study shows that one of the important rate-limiting steps of siRNA silencing efficiency is the ability of siRNA molecules and/or complexes to escape from the endosomes into the cell cytoplasm. The target of this study, the epidermal growth factor receptor (EGFR), is known as an attractive target for cancer therapy. In this study, a 10-fold increased efficiency in knockdown of the EGFR protein was obtained when anti-EGFR siRNA treatment was combined with PCI as compared to siRNA treatment alone. The fact that this combined treatment resulted in a stronger silencing efficiency indicates that lower doses of siRNA can be used when PCI is employed to augment siRNA delivery. Lowering doses of siRNA would prevent saturation of the RNAi machinery and reduce off-target effects. In addition, local illumination of target tissue would only induce PCI in the desired cells, which can further increase the specificity of the treatment, supporting PCI as an attractive strategy to improve siRNA silencing efficiency.

INTRODUCTION

The epidermal growth factor receptor (EGFR) is a recognised target for cancer therapy. This transmembrane glycoprotein is known to be overexpressed in many tumours (e.g. colorectal, head and neck, lung, and ovarian carcinomas), and its signalling pathway is involved in cell differentiation, proliferation, migration, development of angiogenesis, and apoptosis inhibition [1]. Furthermore, expression of EGFR is associated with poor treatment responses and fast disease progression. Strategies have been developed to interfere with EGFR-signalling; in particular, monoclonal antibodies (mAbs) and tyrosine kinase inhibitors (TKIs) have been broadly explored [2]. Alternative strategies, such as RNA interference (RNAi), aim at reducing the expression of EGFR protein [1].

Small interfering RNA (siRNA) molecules are the functional mediators of RNAi, a mechanism able to interrupt the translation of a specific protein by inducing post-transcriptional gene silencing. The cleavage of mRNA, induced by siRNA molecules with a complementary sequence, gives the opportunity to design siRNAs that inhibit the expression of specific proteins responsible for pathologies [3], such as EGFR. Nevertheless, in order to functionally silence EGFR expression, siRNA molecules need to be present in the cytoplasm of the EGFR-expressing tumour cells to interact with the RNAi machinery. The physicochemical properties of siRNA, featured by relatively high molecular weight and strong negative charge, impede cell membrane translocation. As a consequence, a variety of (viral and non-viral) delivery systems have been proposed [3]. Non-viral based carriers, like cationic lipoplexes and polyplexes, are usually taken up by cells through charge-based binding and endocytosis. Once inside the endosomes, these complexes need to efficiently escape into the cell cytoplasm, prior to acidification, in order to avoid lysosomal degradation. Some of the carrier materials are known to be able to destabilise the endosomal membrane by themselves, by acting as a proton sponge or a fusogen [4]. Nevertheless, a number of additional mechanisms have been proposed to facilitate the escape of particles from the endosomes, such as photochemical internalisation (PCI).

PCI was first presented in 1999 [5] as a novel technology for delivery of a variety of therapeutic molecules into the cell cytoplasm. Since then, several studies have been published, demonstrating that this technique can be used, both *in vitro* and *in vivo* [6-8], for site-specific delivery of macromolecules, namely proteins [5], peptides [5], peptide nucleic acids [9, 10], and plasmid DNA carried by non-viral [5, 11, 12] and viral-based carrier systems [13]. PCI technology employs specific, preferably amphiphilic [14], photosensitising compounds which accumulate in the membranes of the endocytic vesicles. Upon illumination, such photosensitisers (PS) become excited, and subsequently induce the formation of reactive oxygen species, primarily singlet oxygen. This highly reactive intermediate can damage cellular components, but the short range of action and short life-time, confine the damaging effect to the production-site. This localised effect induces the disruption of the endocytic vesicles, thereby releasing the entrapped therapeutic molecules into the cell cytoplasm [5].

The aim of this study was to determine whether siRNA-mediated silencing of EGFR could be enhanced using PCI as a tool to improve the endosomal escape of anti-EGFR siRNA and, if so, to determine the extent of the silencing improvement.

MATERIALS AND METHODS

Cell line and culture conditions

The human epidermoid carcinoma cell line A431 was kindly given by Dr. Paul M. P. van Bergen en Henegouwen (Institute of Biomembranes, Utrecht University, The Netherlands). A431 cells were subcultured in Dulbecco's Modified Eagle's medium containing 3.7 g/liter sodium bicarbonate, 4.5 g/l L-glucose (Gibco BRL, Breda, The Netherlands) and supplemented with L-glutamine (2 mM), 7.5% (v/v) foetal bovine serum, penicillin (100 IU/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml), at 37 °C in a humidified atmosphere containing 5% CO_2.

Photosensitiser and light source

The photosensitiser (PS) $TPPS_{2a}$, meso-tetraphenylporphine with two sulfonate groups on adjacent phenyl rings, was kindly provided by Dr. Anders Høgset (PCI Biotech AS, Oslo, Norway). The PS was light protected and stored at -20 °C until use. Cells were exposed to blue light from the LumiSource®, a bank of four light tubes emitting light in the region of 375-450 nm, with 13 mW/cm^2 irradiance (PCI Biotech AS, Oslo, Norway).

Preparation of siRNA/LF complexes and treatment of cells

Complexes of anti-EGFR siRNA and Lipofectamine (anti-EGFR siRNA/LF) were prepared by gently mixing 20, 40 and 80 pmol of anti-EGFR siRNA (Eurogentec, Maastricht, The Netherlands) in 50 µl of 5 mM HEPES (pH 7.4), with 1, 2 and 4 µl Lipofectamine (Invitrogen, Breda, The Netherlands) in 50 µl of 5 mM HEPES (pH 7.4), respectively. The solutions were incubated for 15 min at room temperature (RT) and then added to the cells, resulting in a final concentration of 40, 80 and 160 pmol/ml of anti-EGFR siRNA in the wells. One day after seeding, the cells were incubated with 0.1-1.0 µg/ml PS, in complete culture medium, for 18 h at 37 °C. After this, the medium was removed, cells were washed and fresh medium was added to the wells. Thereafter, the anti-EGFR siRNA/LF complexes were added to the cells and incubated for 5 h. Then, the medium was removed, cells were washed and fresh medium was added to the wells. Cells were then exposed to the light source for 60, 75, or 90 s, and then incubated at 37 °C for 2 days, after which the EGFR protein expression was analysed, either by flow cytometry or western blot.

Complexes of non-specific (NS) siRNA were prepared with anti-Luc siRNA (Qiagen, Venlo, The Netherlands) and Lipofectamine as described above.

Cell proliferation assay

Four thousand cells were seeded per well of 96-well plates and treated as described above, except for the addition of anti-EGFR siRNA/LF complexes. Immediately after illumination of the cells, BrdU reagent was added to the cells and 24 h later the ELISA BrdU-colorimetic immunoassay (Roche Applied Science, Penzberg, Germany) was performed as recommended by the manufacturer. After adding 25 µl of 1 M H_2SO_4 to interrupt the colorimetric reaction, the absorbance at 450 nm was measured, with the reference wavelength set to 655 nm, using a Bio-Rad Novapath Microplate Reader (Bio-Rad Laboratories, Veenendaal, The Netherlands).

Confocal microscopy

For confocal laser-scanning microscopy analysis, $6x10^4$ cells were seeded per well of 12-well plates, on gelatine (Sigma-Aldrich, Zwijndrecht, The Netherlands) coated coverslips. The cells were incubated with 0.5 µg/ml of PS and washed as described above. For this experiment the complexes were prepared with Alexa633-labelled siRNA (Eurogentec, Maastricht, The Netherlands) and Lipofectamine, in the same way as described earlier, with a final concentration of 100 pmol/ml of Alexa633-labelled siRNA per well. EGF-Alexa488 (Molecular Probes, Breda, The Netherlands) was added to the wells (0.25 µg/ml) together with the complexes. The cells were fixed with a 4% formaldehyde (Fluka, Zwijndrecht, The Netherlands) solution in PBS, for 30 min at RT, after 4 h incubation or few minutes after the 75 s illumination step. After fixation, cells were washed twice with PBS and mounted on glass cover slides, using FluorSave (Calbiochem, San Diego, CA, USA). The cells were then analysed on a Leica TCS-SP confocal laser-scanning microscope (Leica, Heidelberg, Germany) equipped with three lasers: 488-nm Argon, 568-nm Krypton, and 647-nm HeNe laser.

Flow cytometry analysis

Forty thousand cells were seeded per well of 24-well plates and treated as described earlier. Two days later, the cells were trypsinised, washed with PBA buffer (phosphate buffered saline supplemented with 0.3% BSA and 0.03% sodium azide) and resuspended in 100 µl PBA buffer. The cells were then incubated with either a specific anti-EGFR monoclonal antibody, or a non-specific antibody, both labelled with FITC (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), for 30 min. Thereafter, the cells were washed twice with PBA, resuspended in 300 µl PBA and analysed using a FACScalibur (Becton & Dickinson, Mountain View, CA, USA). Dead cells were discriminated from viable cells by adding propidium iodide (Molecular Probes, Breda, The Netherlands) to a final concentration of 1 µg/ml. The data were analysed with Summit® software (Dako-Cytomation, Fort Collins, CO, USA) and for relative quantification, the mean fluorescence intensity times the percentage of positive events was used. Generally 10,000 cells were analysed during each measurement, and samples were prepared in triplicates.

Western blot

Eighty thousand cells were seeded per well of 6-well plates and 48 h after the previously described treatment with anti-EGFR siRNA/LF complexes, cells were lysed with RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS, containing 1 mM PMSF and 150 µl/ml of complete EDTA-free mini tablet protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany), for at least 20 min on ice. The lysates were collected and concentrated using centrifugal filters with a molecular weight cut-off of 10 kDa (Microcon, Millipore, Amsterdam, The Netherlands). The total protein content was measured using the Micro BCA assay (Pierce, Rockford, USA), and samples were prepared with 30 µg of total protein, thereafter loaded in each lane of a 7.5% polyacrylamide gel. Electrophoresis was performed at 10-15 mA and subsequently proteins were electrotransfered on a

nitrocellulose membrane (Amersham Bioscience, Buckinghamshire, UK), 1h at 100mA, with a Scie-Plas semidry blotter (Scie-Plas, Warwickshire, UK). The non-specific binding to the membrane was blocked using 5% BSA in PBS-T buffer (PBS with 0.1% Tween-20) for 1 h at RT, with constant shaking. Thereafter, the membrane was incubated with the primary antibody, rabbit polyclonal anti-EGFR (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a dilution of 1:250 in PBS-T buffer, followed by incubation with the secondary antibody, goat anti-rabbit antibody labelled with Cy5 dye (Amersham Pharmacia Biotech, Little Chalfont, UK) at a dilution 1:1250 in PBS-T buffer. For fluorescence visualisation, a Typhoon 9400 scanner (Amersham Biosciences, Buckinghamshire, UK) was used. The intensity of the bands was quantified by the ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA, USA).

RESULTS AND DISCUSSION

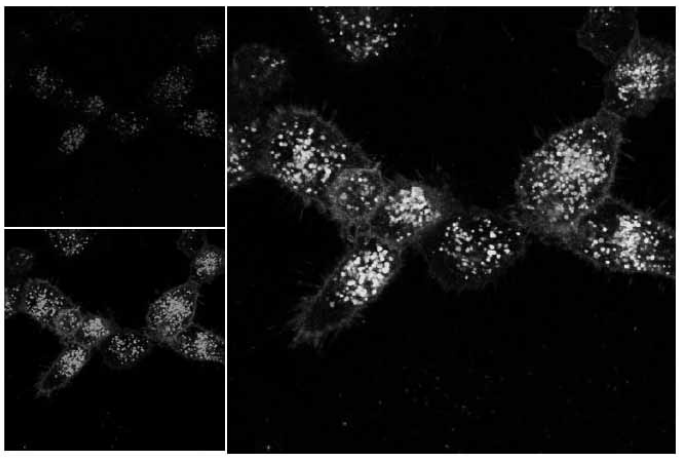
In principle, PCI is able to trigger endosomal escape of intact molecules, captured in endocytic vesicles, by disrupting the membrane of these vesicles through photochemical treatment. This technology is preferably performed with amphiphilic PS which accumulate in the membranes of the endocytic vesicles [14]. Preliminary studies confirmed the localisation of the PS used in this study, $TPPS_{2a}$ (meso-tetraphenylporphine with two sulfonate groups), in endocytic vesicles prior to illumination (data not shown). Our preliminary observations are consistent with those reported previously [14-16].

To be functional, the PCI technique requires the accumulation of macromolecules inside endocytic vesicles. Confocal microscopy images presented a punctuated fluorescent pattern 4 h after incubation of A431 cells with complexes made of Lipofectamine and Alexa633-labelled siRNA (siRNA-Alexa633/LF) (red vesicles), and with Alexa488-labelled Epidermal Growth Factor (EGF-Alexa488) (green vesicles) (Figure 1A). This fluorescent complex of EGF was used to visualise the cellular membrane and endosomes, resulting from the EGF-EGFR complex formation and trafficking. The punctuated pattern, together with several areas where red and green vesicles co-localise (yellow vesicles), indicates the accumulation of the complexes in endocytic vesicles. When illumination is applied after 4 h incubation of A431 cells with siRNA-Alexa633/LF complexes and EGF-Alexa488, a reduction of the punctuated fluorescent pattern is observed (Figure 1B). The observed change from punctuated to diffuse fluorescence pattern of Alexa633 (red) throughout the cytoplasm of the cells, suggests the release of siRNA molecules and/or siRNA/LF complexes from the endosomes, as a result of a photo-induced disruption of the endocytic vesicles. This indicates that PCI is a useful tool to facilitate the endosomal escape of siRNA.

The next step was to determine whether PCI, apart from improving the endosomal escape of complexes made of anti-EGFR siRNA and Lipofectamine (anti-EGFR siRNA/LF), would enhance the silencing efficiency of siRNA. The release of the entrapped complexes from the endocytic vesicles is known to be mainly dependent on the intensity of the photochemical reactions (photochemical dose) [17]. Therefore, the amount of photosensitising compound and the duration of light exposure are decisive variables.

Figure 3 shows the knockdown of EGFR expression, resulting from siRNA-mediated silencing treatment, when combined with different photochemical doses, determined by flow cytometry analysis (Figure 2).
Increasing concentrations of the photosensitiser employed in PCI, resulted in increased knockdown of the EGFR, up to 70% (Figure 3A). The same trend was detected for increasing duration of light exposure (Figure 3B). Indeed PCI is able to enhance gene silencing and, in fact, these results confirm that higher photochemical doses induce more efficient release of the complexes from the vesicles.

A



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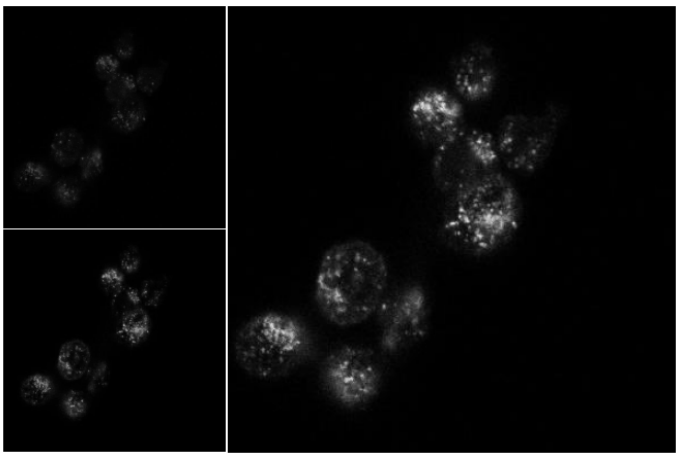

Figure 1. Release of siRNA in the cytoplasm after PCI.
Confocal Microscopy Images show cells incubated for 4 h with siRNA-Alexa633/LF complexes (red) and EGF-Alexa488 (green) (A), and cells which were illuminated after the 4 h-incubation (B). The punctuated pattern (A), most likely indicating localisation in endocytic vesicles, as indicated by co-localisation (yellow, in the overlay image) of complexes and EGF-Alexa488, gives place to a less punctuated and more diffuse pattern (B) where the fluorescence is spread throughout the cytoplasm of the cells. Cells were previously incubated with PS, as described in Materials and Methods.

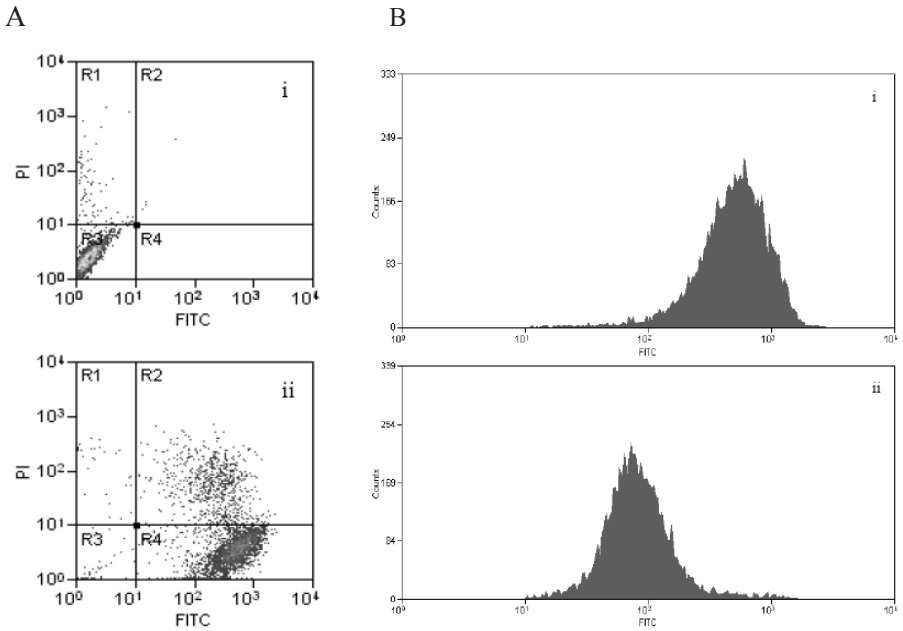

Figure 2. Flow cytometry analysis.
Dot plots from flow cytometry measurements show FITC fluorescence (FITC-labelled anti-EGFR antibody) versus PI (labelling dead cells) (A). Two controls are shown: (i) a sample of cells incubated with non-specific FITC-labelled antibody, and (ii) a sample of cells previously subjected to photochemical reactions (0.4 µg/ml PS; 60 s illumination) followed by incubation with FITC-labelled anti-EGFR antibody. The cells from quadrant R4, which are negative for PI fluorescence and positive for FITC fluorescence, were considered for analysis. These correspond to living cells which are labelled with the anti-EGFR antibody. Histograms of FITC fluorescence (B), from cells displayed in R4 quadrant only, are used for relative quantification of the expression of EGFR. Two examples are given: (i) a sample of cells previously subjected to the same photochemical dose as mentioned above, and (ii) a sample of cells treated with anti-EGFR siRNA/LF complexes (160 pmol/ml of anti-EGFR siRNA) combined with PCI (same photochemical dose). The shift towards lower values of fluorescence intensity indicates a decrease in EGFR expression.

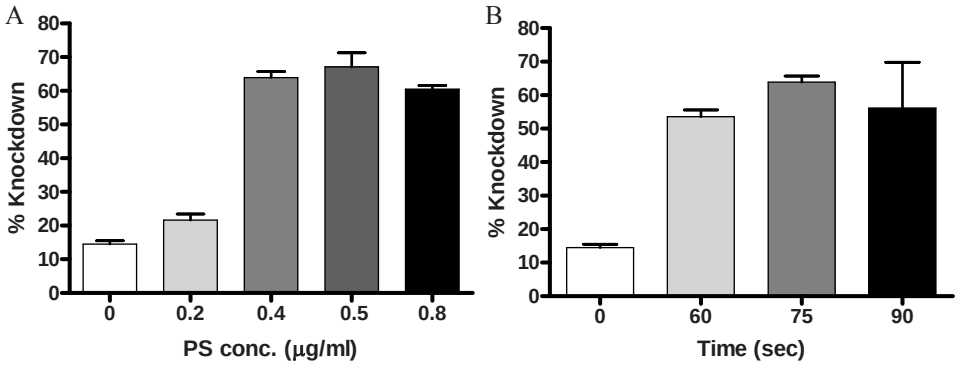

Figure 3. Knockdown of EGFR expression following PCI-facilitated siRNA-mediated silencing.
The percentage of knockdown of EGFR protein expression, calculated based on the mean fluorescence intensity times the percentage of positive events (see Figure 2), is plotted as function of (A) the concentration of PS in µg/ml (for anti-EGFR siRNA/LF complexes with a final concentration of 40 pmol/ml of anti-EGFR siRNA, and 75 s of illumination), and (B) the interval of illumination in seconds (for anti-EGFR siRNA/LF complexes with a final concentration of 40 pmol/ml of anti-EGFR siRNA, and 0.5 µg/ml of PS concentration).

Increasing photochemical doses can, however, be associated with a higher degree of cytotoxicity. The combination of photosensitising compounds and light has already been explored in photodynamic therapy (PDT) [18]. In this strategy, a general cytotoxic effect is induced by photochemical reactions in order to eradicate cells. On the contrary, PCI aims at inducing photochemical reactions only in the membranes of endocytic vesicles, for site-specific intracellular delivery. Though, PS usually employed for PCI tend to accumulate in endocytic membranes [14], a certain cytotoxicity is inevitably associated with PCI. To study the cytotoxicity induced by the photochemical treatment, the proliferation capacity of A431 cells was assessed, at increasing concentrations of PS and increasing intervals of illumination. As expected, higher amounts of photosensitising compound induced a reduction in cell proliferation and the reduction in cell proliferation was more pronounced for longer illumination intervals (Figure 4). No effect was detected when light alone was applied (0.0 μg/ml of PS) and, notably, a low dark toxicity (at 0 s illumination) was associated to the highest PS concentrations (> 0.8 μg/ml of PS). Since photochemical reactions are, in both PDT and PCI, essential, the determination of the right balance between cytotoxicity and delivery is critical. Obviously, in case of tumour cell treatment, the cytotoxicity induced by PCI is less of a concern, and could even be considered an adjuvant. However, giving that the first goal of this study was to evaluate PCI as a tool for improving siRNA delivery into the cell cytoplasm, the subsequent experiments were performed employing PCI under conditions that induce a low cytotoxicity, i.e. 0.4 μg/ml PS and 60 sec illumination. These conditions (photochemical dose) resulted in a modest reduction of cell proliferation (less than 10%) compared to non-illuminated controls (Figure 4).

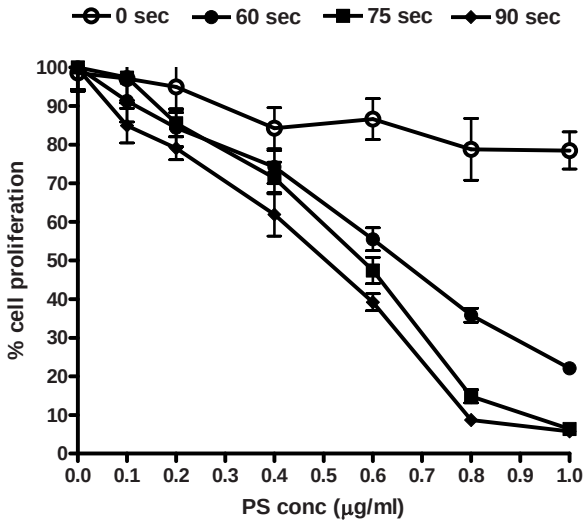

Figure 4. Reduction of cell proliferation by photochemical treatment.
The percentage of cell proliferation, determined 24 h after illumination using BrdU incroporation, is plotted as function of PS concentration (μg/ml) with which cells were incubated for 18 h. Three different illumination intervals were tested: 60 (●), 75 (■) and 90 s (♦). No significant effect was determined by illumination alone (0.0 μg/ml PS), and only a weak dark toxicity (0 s, ○) was associated to the highest PS concentrations.

Figure 5B shows the analysis of Western blot (Figure 5A) prepared with cell lysates obtained from samples treated with anti-EGFR siRNA/LF complexes, with and without combination with the PCI technique performed under the conditions mentioned above. Here a 70% knockdown of EGFR expression was obtained when PCI was part of the treatment, compared to 20% knockdown when the treatment consisted of anti-EGFR siRNA/LF complexes alone. Figure 5B also shows no significant effect on the expression of EGFR protein for cells subjected to photochemical treatment alone (bar 3), cells treated with free anti-EGFR siRNA (bar 5), and complexes made of non-specific siRNA (bar 6). The increase in silencing efficiency, detected with Western blot, is lower than the enhancement determined by flow cytometry in Figure 3A (approximately 10% EGFR knockdown for anti-EGFR siRNA/LF complexes alone, and approximately 70% EGFR knockdown when combined with PCI), albeit that flow cytometry analysis was performed after a higher photochemical dose (0.5 μg/ml PS and 75 s illumination). These results, although detected with different techniques, support the notion that PCI has an increased effect when inducing higher cytotoxicity. However, for delivery purposes it is important to avoid higher cytotoxicity and to keep cytotoxicity as lower as possible.

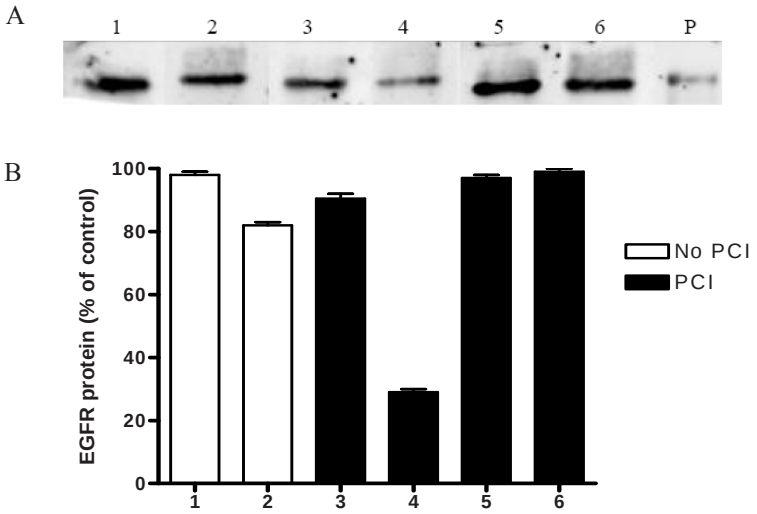

Figure 5. Knockdown of EGFR expression following PCI-facilitated siRNA-mediated silencing.
Western Blot (A): cells were treated, as described in the Materials and Methods, with 0.4 μg/ml $TPPS_{2a}$ and complexes with a final concentration of 40 pmol/ml of siRNA made of anti-EGFR siRNA or non-specific siRNA. After 5 h incubation with the complexes or free anti-EGFR siRNA, cells were illuminated for 60 s. Band Analysis (B): the fluorescence intensity of the bands, from two nitrocellulose membranes, is plotted in percentages (%) compared with non-treated cells as controls. Lane/Bar 1 and 3 – non-treated cells; lane/bar 2 and 4 – anti-EGFR siRNA/LF complexes; lane/bar 5 – free anti-EGFR siRNA; lane/bar 6 – non-specific siRNA/LF complexes; P – EGFR protein (250 ng, Sigma-Aldrich, Zwijndrecht, The Netherlands); open bars – without PCI; filled bars – with PCI.

To determine the added value of PCI for siRNA silencing efficiency, three different doses of anti-EGFR siRNA/LF complexes were tested. As shown in Figure 6B, which was obtained from analysis of Figure 6A, the highest concentration of anti-EGFR siRNA,

combined with PCI, induced up to 80% knockdown of the EGFR. An equal concentration of anti-EGFR siRNA showed a silencing efficiency of approximately 40% without applying PCI. Additionally, Figure 6A confirms that the photochemical reactions induced by PCI have no significant effect on the expression of EGFR of non-treated cells, as well as cells incubated with complexes made of non-specific siRNA.

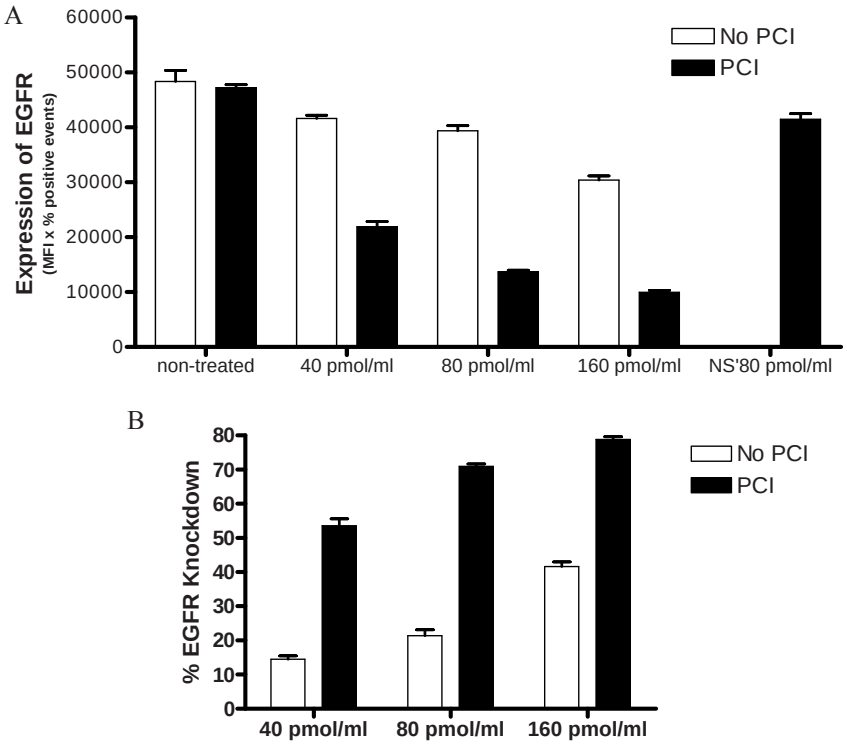

Figure 6. Effect of PCI on the relationship between concentration of siRNA/LF complexes and silencing efficiency.
Expression of EGFR (A), plotted as mean fluorescence intensity times the percentage of positive events (as described in Figure 2), was determined for cells treated with different concentrations of anti-EGFR siRNA/LF complexes, with or without combination with PCI (for 0.4 μg/ml of PS concentration and 60 s of illumination time). Non-treated cells and cells treated with complexes made of non-specific siRNA were used as controls. Percentage of knockdown of EGFR protein expression (B) is plotted as function of the final concentration of anti-EGFR siRNA present in the wells.

Considering that dose-response curves for anti-EGFR siRNA/LF complexes are likely sigmoidal, it can be calculated that a 50% silencing efficiency is reached when 35 pmol/ml (95% confidence interval 32 to 38 pmol/ml) anti-EGFR siRNA/LF complexes is combined with PCI, whereas 341 pmol/ml (95% confidence interval 317 to 368 pmol/ml) anti-EGFR siRNA/LF complexes are needed to reach 50% silencing without PCI ($p<0.0001$ (F-test)). This corresponds to a PCI-mediated enhancement of silencing efficiency of one order of magnitude. These results contrast favourably with some of the results obtained for PCI experiments with cationic lipids as carriers for

plasmid DNA. As previously reported by Høgset *et al*, the effect of PCI on transfection efficiency of lipid-based carriers is not as predictable as for polymer-based carriers, where PCI usually has a positive effect (e.g. polylysine, polyethylenimine) [17]. In the case of transfection with cationic lipids, some of the studies have reported a reduction of transfection efficiency, which seems to vary according to the cell type and lipid composition. In contrast, our results clearly indicate that lipid-complexed nucleic acids are delivered to a higher degree in the cell cytoplasm when PCI is applied. This could be explained by the fact that RNAi is processed in the cytoplasm, which, in this case, would be a major advantage, as compared to plasmid DNA for which intracellular trafficking to the nucleus consists of an additional limiting step for transfection to occur.

Taken together, the present study identifies endosomal escape as an important limiting step for siRNA silencing efficiency. Additionally, this study demonstrates that lipid-based carriers of nucleic acids can benefit from PCI, provided that the site of activity of the nucleic acid concerned is localised in the cytoplasm. Importantly, the 10-fold enhancement of silencing efficiency, obtained by virtue of the application of PCI, would enable the minimisation of siRNA concentrations to achieve satisfactory silencing activities. The use of high doses of siRNA has been related to off-target effects [19] and to the saturation of the RNAi machinery, which has already been referred and correlated with a reduction of the degree and the duration of siRNA-mediated silencing [20]. Therefore any attempt to decrease siRNA concentrations needed for silencing should be taken into account.

One of the major advantages of PCI as a delivery tool is its intracellular site-specific action. The fact that the photochemical-induced delivery of the specific macromolecule is confined only to the illuminated area is an advantage for *in vivo* studies. siRNA delivery would be limited to the desired cells, thereby further reducing non-specific effects. However, the major challenge for this technique is the difficulty of light to deeply penetrate skin. Nevertheless, this has been attenuated by the development of lasers that can be coupled into fibre optics, which in their turn can be inserted into endoscopes. Nowadays it is possible to illuminate regions in the human body, previously inaccessible, e.g. lungs, gastrointestinal tract, bladder [21].

This report demonstrates for the first time that PCI can be used to enhance the endosomal escape of anti-EGFR siRNA/LF complexes. The following step will be to determine the applicability of PCI *in vivo* for enhancing antitumour effects of anti-EGFR siRNA, with limited non-specific effects.

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CHAPTER 5

DELIVERY OF SIRNA TO THE TARGET CELL CYTOPLASM: PHOTOCHEMICAL INTERNALISATION FACILITATES ENDOSOMAL ESCAPE AND IMPROVES SILENCING EFFICIENCY, *IN VITRO* AND *IN VIVO*

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ABSTRACT

The prospect of introducing siRNA in a cell, to induce silencing of the corresponding gene, has encouraged research into RNAi-based therapeutics as treatment for human diseases. At present, the siRNA molecules that are in a more advanced stage of clinical evaluation have a common factor: all are delivered locally at the site of the disease. Thus, the state of the art in delivery of siRNA appears to be the local administration. This can certainly be attributed to the characteristics of siRNA molecules, such as relatively high molecular weight, negative charge, and susceptibility to nuclease degradation, which make systemic application as a drug molecule difficult. When focusing on local administration, the main concerns for siRNA delivery can be restricted to the trafficking of siRNA molecules from the vicinity of the target cells to the intracellular compartment where RNAi takes place, i.e. the cytoplasm.

This contribution is focused on the barriers and challenges in trafficking of siRNA upon local delivery. First, an overview is given on the current state of the art for siRNA delivery in clinical trials. Second, recent successful preclinical studies, involving direct and local administration of siRNA, are reviewed. Third, emphasis is given to the endosomal escape. Some of our recent work is presented: the application of photochemical internalisation (PCI) to improve the endosomal escape of siRNA lipoplexes *in vivo*. Finally, concluding remarks focus on the advantages of employing a technique such as PCI to enhance the endosomal escape of siRNA molecules.

INTRODUCTION

Early after RNA interference (RNAi) was first described in worms in 1998 [1], small interfering RNA (siRNA) was identified as the key mediator of RNAi in mammalian cells [2]. Its potential as a tool for understanding gene function and promise for therapeutic applications were immediately recognised. The prospect of introducing siRNA in a cell, leading to the degradation of complementary messenger RNA (mRNA) and to the silencing of the corresponding gene, rapidly captured the interest of many researchers. Several research institutes and companies have been seeking for ways to develop RNAi-based therapeutics as treatment for virtually every human disease that is caused or promoted by a single or a few genes.

Six years after the publication of Fire and Mello [1], RNAi moved into clinical trials. This extremely rapid progress from discovery to clinical studies is striking. A closer look at the three siRNA molecules that are in a more advanced stage of clinical evaluation, show a common factor: all three siRNA molecules are delivered locally at the site of the disease. Bevasiranib or Cand5 (2004, OPKO Health) and AGN-745 or Sirna-027 (2004, Sirna Therapeutics/Allergan) are both developed for age-related macular degeneration (AMD), involving direct application of siRNA in the vitreous compartment. ALN-RSV01 (2005, Alnylam Pharmaceuticals), developed for treating respiratory syncytial virus (RSV), involves direct delivery of siRNA into the lungs [3]. This reflects that the state of the art in siRNA delivery is the local administration at the site of the disease. However, this situation is rapidly changing. Recently started Phase I clinical trials involve systemic delivery of siRNA (November 2007, Quark Pharmaceuticals / Silence Therapeutics), and of siRNA formulated as a targeted nanoparticle (June 2008, Calando Pharmaceuticals) [4].

At present, however, only local delivery of siRNA is in advanced stages of clinical evaluation. This is undoubtedly caused by the characteristics of siRNA molecules. Their relatively high molecular weight, charged character and vulnerability towards degradation, which generally are mandatory for a carrier system, together with their intracellular site of action, makes the application of siRNA as a drug molecule difficult (Figure 1). Therefore delivery problems encountered during the transport of siRNA after administration, in order to reach the target cell within the target tissue and target organ of the patient, are preferentially minimised [3, 5-7]. Employing local administration in the diseased site, the main delivery issue can be restricted to the trafficking of siRNA molecules from the extracellular space surrounding the target cells, to the intracellular compartment where RNAi takes place, i.e. the cytoplasm.

In this contribution, we will give an overview of the current state of the art of siRNA delivery in clinical trials, focusing on local injection. This will be related to observations in a number of preclinical studies. Barriers and challenges in trafficking of siRNA upon local delivery will be addressed, with particular emphasis on the endosomal escape-issue. Our recent work focuses on the application of photochemical internalisation (PCI) to improve the endosomal escape of siRNA lipoplexes *in vivo*. Finally, concluding remarks will focus on the advantages of employing a technique such as PCI to enhance silencing efficiency.

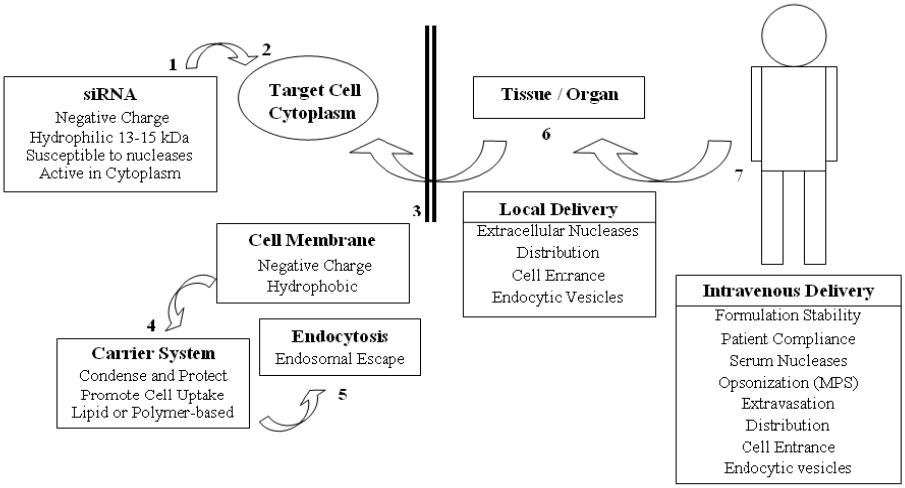


Figure 1. Schematic representation of challenges in siRNA delivery, local and intravenously.
siRNA molecules (1) mediate RNAi in the cytoplasm of the target cells (2). However, their phys-chemical properties are generally inappropriate for simple diffusion through the cell membrane (3). For this reason carrier systems are employed, mainly lipid- or polymer-based (4). These are generally positively charged and form complexes (lipoplexes or polyplexes, respectively) by charge interactions. The resulting positive charge on complexes surface promotes cell interaction and complexes are generally taken up by endocytosis (5). This however leads to the entrapment in endocytic vesicles, and to degradation upon acidification, unless endosomal escape mechanisms occur. Local delivery of siRNA (6) minimises some of the problems encountered during the transport of siRNA after intravenous administration (7), in order for siRNA to reach the target cell within the target tissue and target organ of the patient.

DELIVERY OF SIRNA – CLINICAL STATE OF THE ART

Since the first siRNA molecule (Sirna-027) has entered Phase I clinical trials in 2004 [8], a few more molecules have followed and almost ten are currently in clinical studies [4]. The siRNA molecules that have further progressed in clinical trials are delivered locally at the diseased site.

Bevasiranib (the former Cand5 from Acuity Pharmaceuticals) is at present the most clinically advanced siRNA. It is being tested in Phase III trials sponsored by OPKO Health, a company focused on ophthalmic disorders. This siRNA is administered by intravitreal injection, in a free and unmodified form, and targets the vascular endothelial growth factor (VEGF) for treatment of wet age-related macular degeneration (AMD). In this disorder, the surplus angiogenesis of the retinal capillaries is responsible for loss of vision.

AGN-745 (the former Sirna-027 from Sirna Therapeutics) was also developed for treatment of wet AMD, however, with the cognate receptor for VEGF as a target, i.e. VEGF receptor-1 (VEGF-R1). At present, it is in Phase II clinical trials supported by Allergan. This siRNA is chemically modified and, like bevasiranib, administered as aqueous solution directly in the vitreous compartment.

A third siRNA molecule, RTP801i-14, developed for treatment of wet AMD, is currently in Phase I/IIA clinical trials (Quark Pharmaceuticals/Pfizer). This drug is designed to

inhibit the expression of the hypoxia-inducible gene RTP801. This siRNA is also chemically modified and delivered as aqueous solution by intravitreal injection.
ALN-RSV01 was developed by Alnylam Pharmaceuticals for the treatment of a serious respiratory infection, caused by the respiratory syncytial virus (RSV). This siRNA silences the RSV nucleocapside-N gene and is currently in phase II clinical trials, being administered by nebulizer directly into the lungs [3].
The siRNAs mentioned above are the most advanced molecules under clinical evaluation, and are delivered as aqueous solutions directly into the vicinity of the cells where RNAi should occur. This local delivery of siRNA appears attractive as: (1) both the vitreous compartment and lung environment contain relatively low levels of nucleases, reducing the chance for rapid degradation. Along with chemical modifications (e.g. included in AGN-745), stability and protection are further ensured; (2) both cells from the retina and lung epithelium appear to be capable of uptake of siRNA in the free form (called *naked* siRNA, i.e. devoid of carrier system), despite the unfavourable phys-chemical characteristics [9-12]. The mechanism by which these cells take up free siRNA is not yet known, but may be related to the expression of the SID-1 homologue, the receptor FLJ20174 [13]. SID-1 is a transmembrane protein that enables cellular passive uptake of dsRNA in *Caenorhabditis elegans* [14], and the mammalian homologue, FLJ20174, was found to enhance internalisation of siRNA in mammalian cells. This receptor is likely not present (or not expressed to a sufficient extent) in all cell types, given the numerous studies reporting lack of silencing effect after administration of *naked* siRNA. Controversially, recent findings by Kleinman *et al* suggest a different explanation for the antiangiogenic activity of siRNAs delivered directly into the vitreous compartment [15].
Taken together, local delivery of siRNA appears to be the more rapid and less problematic manner to develop RNAi therapeutics. The next section will present some preclinical studies, examples of successful local delivery of siRNA in which other target tissues are investigated.

LOCAL DELIVERY OF SIRNA – EXAMPLES OF PRECLINICAL SUCCESS

When local delivery of siRNA is considered, extra- and intracellular trafficking of these molecules become major concerns (Figure 1). siRNAs are double stranded molecules of 19 to 23 nucleotides in length. They are relatively large molecules (~ 14 kDa), with a hydrophilic backbone and each base bears a negative charge. Once siRNA is present in the vicinity of the target tissue, it has to travel in extracellular fluids towards the target cells, and to reach the cytoplasm of the cells where RNAi should occur, without being degraded by nucleases. As crossing of the hydrophobic and negatively charged cell membrane is, in the majority of cases, not likely to occur efficiently, many carrier systems have been developed. These systems are mainly divided into polymeric-based and lipid-based carriers, and have been extensively reviewed [3, 16, 17]. In general, these carriers ensure siRNA protection from nucleases, promote cell association, and trigger cellular uptake.
Local delivery of siRNA was the approach chosen by Palliser and co-workers for treating herpes simplex virus 2 (HSV-2) infection [18]. Intravaginal application was employed to

deliver two distinct siRNA molecules, one targeting the HSV-2 UL27 gene, which encodes for an envelope glycoprotein, and the other targeting the HSV-2 UL29 gene encoding for a DNA binding protein. Oligofectamine, a cationic lipid-based material from Invitrogen, was employed as carrier for both siRNA molecules. Efficacious results were obtained, as the mice were protected from lethal infection upon vaginal challenge with a fatal dose of HSV-2. The treatment was well tolerated, it did not cause inflammation, and no induction of interferon-responsive genes was detected. This report, the first on intravaginal siRNA delivery, gives hope for the mucosal treatment of sexually transmitted viral and parasitic infections. Further reports employing mucosal delivery of siRNA, confirm that mucosal uptake of siRNA can be particularly efficient, involving cellular uptake mechanisms which may differ from those present in internal organs. Zhang and co-workers showed that RNAi can be used to manipulate gene expression in the rectal mucosa [19]. In their study, Lipofectamine from Invitrogen was used as carrier for siRNA targeting tumour necrosis factor alpha (TNF-α), and siRNA/carrier were delivered by intracolonic administration. The murine model for inflammatory bowel disease, a disease in which the digestive tract becomes inflamed causing severe diarrhea and abdominal pain, showed resistance to the induction of experimental colitis after the treatment. Similarly to Palliser observations, neither toxicity nor interferon response were detected after the siRNA/lipid treatment.

Another example of successful local delivery of siRNA was published by Nogawa *et al* In their study they focussed on bladder cancer and delivered siRNA via the urethra, using cationic liposomes as carrier [20]. Their target, the polo-like kinase-1 (PLK-1), is an important regulator of cell division in mammalian cells. Results showed inhibition of cancer growth in the murine orthotopic bladder cancer model, after transurethral administration of siRNA/carrier. These results showed promise for the development of bladder cancer RNAi therapeutics.

Maeda *et al* showed that effective local administration of siRNA is also possible directly into the ear [21]. The target protein of the study, gap junction beta 2 (GJB2), is important in audition and mutations in this gene are a common cause of deafness. After introducing the mutant gene in the mouse ear, decreased expression of the target gene and prevention of hearing loss was obtained, using DOTAP-Cholesterol liposomes to carry siRNA.

Local administration of siRNA does not always involve lipid-based carriers. Kinouchi *et al* have used atelocollagen (ATCOL) [22]. ATCOL is also known as atelopeptide type 1 collagen, as it lacks the telopeptides in N and C terminus that confer antigenicity. This biomaterial, besides its strong positive charge, has an interesting property, i.e. it is liquid at 4 °C and a gel at 37 °C, possibly allowing a prolonged release of siRNA after local administration, as documented for plasmid DNA [23]. siRNA/ATCOL was injected intramuscularly (i.m.) to treat muscular dystrophy. The target protein was myostatin, a negative regulator of skeletal muscle growth. The authors reported a marked increased in muscle mass within a few weeks after i.m. administration, showing the potential of this approach for treatment of muscular atrophy.

Studies discussed so far involve direct delivery of siRNA into the organ and tissue of interest, at the orthotopic location. However, this is not always feasible to perform in preclinical settings, e.g. in cancer research. The following examples focus on ectopic

xenograft tumour models. In these models, intratumoral injection is widely applied. Intratumoral injection has also been used in clinical trials, for instance in gene therapy [24, 25].

ATCOL was used for intratumoral delivery of siRNA targeting VEGF in a subcutaneous human prostate xenograft cancer model [26]. The authors demonstrated that ATCOL was able to extend the half-life of siRNA, by protecting it from nuclease degradation when embedded in the tumours, for at least one week. Furthermore, a clear inhibition of tumour angiogenesis and tumour growth was observed, and no toxicity or antigenicity was detected after the treatment. More recently, ATCOL was used in a xenograft model of human pancreatic cancer [27]. Here intratumoral injection of siRNA/ATCOL was directed to silence proteinase-activated receptor-2 (PAR-2). PAR-2 is a G protein-coupled receptor, which is activated in inflammatory processes and cell proliferation, and is expressed in colon, gastric, pancreatic and gallbladder cancers. The authors reported a dramatic suppression of tumour growth in this xenograft model, thereby showing that PAR-2 blockade *in vivo* may be an effective way to interfere with pancreatic tumour growth.

A new type of core-shell nanoparticle has been used for intratumoral delivery of siRNA by de Martimprey *et al* [28]. Here, preformed nanoparticles composed of a biodegradable polymeric core and a shell of the positively charged polysaccharide chitosan were mixed with siRNA forming apparently stable nanoplexes. The papillary thyroid carcinoma, the most frequent in radiation-associated thyroid carcinomas, was induced in mice as a xenograft tumour model, and Ret/PTC1 was the targeted oncogene of the study. Ret is a membrane tyrosine kinase receptor, which is involved in regulation of cell proliferation, survival and migration, though normally not expressed in thyroid follicular cells, Ret/PTC1 is expressed in the case of radiation-associated thyroid carcinomas. The authors showed a significant inhibition of tumour growth after intratumoral administration of their formulation.

Overall, the studies referred to in this section show effective delivery of siRNA locally to the site of disease, with successful silencing. In general, the carriers used are positively charged, enabling charge interaction and complexation with the negative siRNA. This usually results in a net positive charge at the surface of the complex, allowing cell association by electrostatic interactions, which likely triggers endocytic uptake mechanisms. It is generally accepted that lipoplexes and polyplexes are taken up by cells via endocytosis, though various endocytic pathways exist in eukaryotic cells (clathrin-and caveolae-mediated endocytosis, macropinocytosis) [29]. For RNAi to occur, siRNA needs to access the cytoplasm, making endosomal escape an essential element in the intracellular trafficking process of siRNA.

TRAFFICKING OF SIRNA - ENDOSOMAL ESCAPE

Poor or lack of endosomal escape is not a new concern. It is important for therapeutic molecules which have characteristics that oppose spontaneous trafficking to the intracellular site of activity [30]. Many of these therapeutic agents are large molecules (e.g. plasmid DNA, proteins), while some other are smaller (e.g. bleomycin) but still need

a mechanism to promote delivery in the right intracellular compartment. In the case of gene delivery, where lipid- or polymer-based carrier systems are employed (forming lipoplexes or polyplexes, respectively), several reviews address the many strategies employed to prevent these agents to remain trapped in lysosomes [30-32].

Our institute is focused on the design of delivery systems for a variety of macromolecules and, as a result, has been concerned with the issue of promoting endosomal escape. Mastrobattista *et al* investigated the incorporation of a fusogenic peptide, diINF-7, derived from the influenza virus hemagglutinin HA-2 domain, in an immunoliposome formulation for delivery of diphtheria toxin A chain (DTA). DTA is cytotoxic when present in the cytoplasm. The DTA-immunoliposomes were taken up by the cells, but cytotoxicity was only detected when both fusogenic peptide and DTA were encapsulated in the immunoliposomes, reflecting the endosomal escape promoted by the incorporated fusogenic peptide [33]. The same fusogenic peptide was coupled to polymers for gene delivery, showing a clear advantage of the grafted polymers in transfection efficiency, compared to the polymers without the peptide grafted [34].

Recently, this diINF-7 fusogenic peptide was employed to determine whether endosomal escape could be a limiting factor for the efficient *in vitro* delivery of siRNA lipoplexes (siRNA complexed with Lipofectamine, LF from Invitrogen) [35]. In these studies the fusogenic peptide was mixed with the preformed lipoplexes, and two different oncogenes were targeted: K-ras and EGFR. K-ras, which is part of the Ras family of proteins, is a signal transduction protein known to modulate cell proliferation and differentiation, and mutations in this gene result in unregulated proliferation and impaired differentiation. Epidermal growth factor receptor (EGFR) is a recognised target for cancer therapy, as the activation of this receptor is involved in many pathways that promote tumour development such as cell proliferation, migration and inhibition of apoptosis [36]. Two main approaches have been employed to interfere with EGFR signalling pathway, both in advanced clinical development: at the extracellular level using monoclonal antibodies that prevent binding of their ligands, and at the intracellular level using tyrosine kinase inhibitors which compete with ATP molecules and prevent the phosphorylation of the receptor, thereby blocking its activation [37]. Our approach, interfering at the mRNA level with siRNA, was until now less explored. In our studies, both K-Ras and EGFR oncogenes were knocked down to a greater extent (up to 3.5 fold enhancement) when the fusogenic peptide was associated with the lipoplexes, as compared to the lipoplexes alone. These results confirmed that endosomal escape of siRNA is important and can be facilitated by this fusogenic peptide.

Lately, other reports have been published highlighting the importance of endosomal escape of siRNA and studying different strategies for enhancing it. For instance, Lundberg *et al* used endosomolytic cell-penetrating peptides [38], Wang *et al* developed new polymerisable surfactants with pH-sensitive amphiphilicity [39], Kwon *et al* employed a membrane-lytic peptide, from the endodomain of HIV gp41, coupled to polyethylenimine (PEI) [40], and Ghosn *et al* developed an imidazole acetic acid-modified chitosan to introduce secondary and tertiary amines in the polymer for endosomal escape enhancement [41].

Our group has been employing photochemical internalisation (PCI) to facilitate endosomal escape [42]. PCI was first described in 1999 [43] and has been used as an endosomal escape-enhancer strategy for a variety of therapeutic molecules, such as the proteins gelonin [43, 44], and saporin [43, 45], the chemotherapeutics agents bleomycin [46], and doxorubicin [47], as well as for peptide nucleic acids [48], and plasmid DNA [49, 50], using either viral or non-viral carriers [51].
PCI induces the disruption of endocytic vesicles through photochemical reactions. For that purpose, PCI combines a photosensitising compound (photosensitiser, PS) with visible light (L). The PS usually has amphiphilic characteristics, and preferentially localises in the membranes of endocytic vesicles. Upon illumination (or light exposure) reactive oxygen species are formed, most importantly singlet oxygen. This molecular species has a short lifetime (0.01-0.04 μs) and a short diffusion range (10-20 nm), proceeding with oxidising reactions in the vicinity of the production area (i.e. endocytic membranes). In this manner, PCI can induce the disruption of intracellular membranes enabling the release of endosomal contents into the cell cytoplasm.
Fretz *et al* used PCI to deliver saporin, a cytotoxic protein, directly into the cytoplasm of the cells. This protein was encapsulated in different liposome formulations and the authors showed that targeting of the liposomes, either by charge or coupled ligand, was necessary for cell uptake of the saporin-liposomes. Only in case of cellular internalisation, PCI was able to deliver saporin to the cytoplasm and induce cell death [42].
Recently, we also have demonstrated that PCI can be employed *in vitro* to facilitate the endosomal escape of siRNA lipoplexes targeting the EGFR [52]. The improved cytosolic delivery of siRNA lipoplexes resulted in an enhanced silencing efficiency of the EGFR protein, up to 10-fold for the treatment combined with PCI as compared to the treatment of siRNA lipoplexes alone.
These *in vitro* studies provide evidence that promoting endosomal escape of siRNA is an attractive strategy. Interestingly, PCI has already shown potential applicability *in vivo*, facilitating the delivery of gelonin [53], bleomycin [46], and glucosylated PEI with a plasmid encoding the p53 gene [54]. Motivated by our results derived from the *in vitro* application of PCI, we decided to perform *in vivo* studies in which PCI would be applied to facilitate the delivery of siRNA lipoplexes.

PHOTOCHEMICAL INTERNALISATION *IN VIVO*

The application of PCI, both *in vitro* and *in vivo*, involves the use of a photosensitiser (PS) and light (L). For *in vivo* application the light needs to penetrate the skin in order to reach the tumour tissue. This is possible for wavelengths in the range of 600-800 nm. Consequently, the excitation wavelength of the PS employed needs to be compatible with this wavelength range. Here, the disulfonated aluminum phthalocyanine $AlPcS_{2a}$ (670 nm) was selected. Previous protocols of *in vivo* application of PCI [46, 53, 54] have been adapted to perform our studies, as depicted in Figure 2.

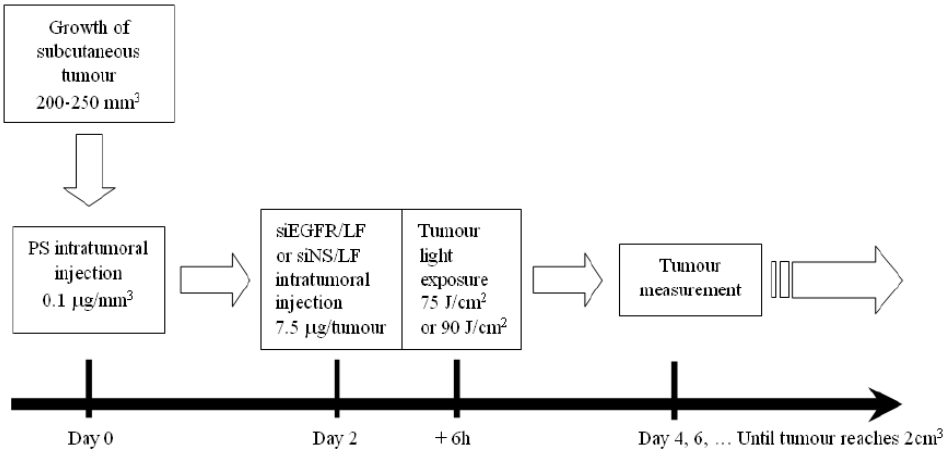
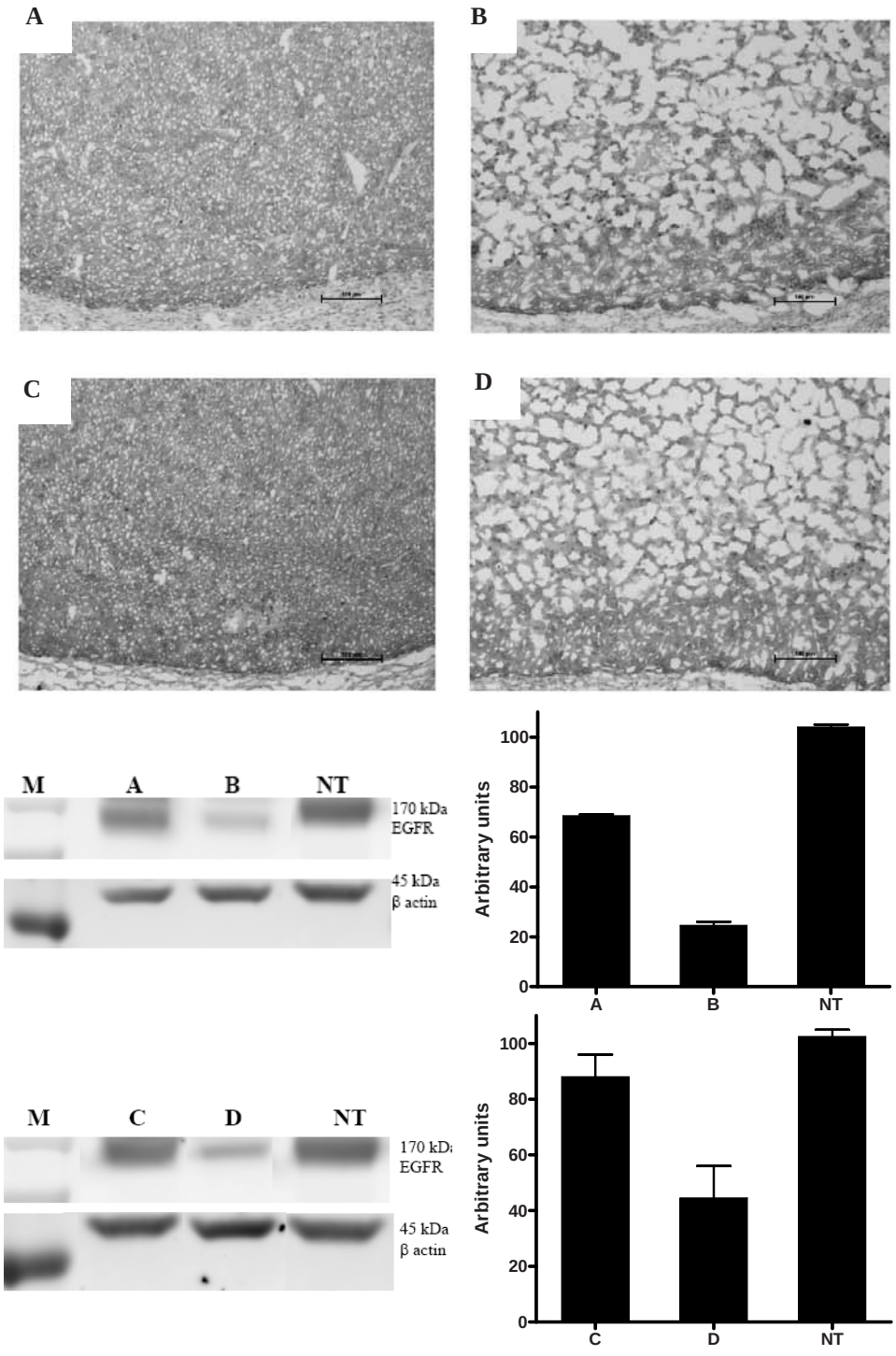


Figure 2. Illustrative time schedule for PCI application *in vivo*.
Subcutaneous tumours were induced by s.c. inoculation of A431 tumour cell suspension. Tumours were measured with a digital caliper, and tumour volume (V, in mm^3) was calculated with the formula $V = \pi/6 \times L \times S^2$, where L is the largest, and S the smallest superficial diameter. When tumours reached 200-250 mm^3, mice were included in the study, 5-8 mice per group. Treatment consisted of intratumoral (i.t.) injection of photosensitiser (PS) at day 0, followed 2 days later by i.t. injection of siRNA lipoplexes (anti-EGFR – siEGFR/LF, or non-specific – siNS/LF), and 6h later by light exposure of the tumours with a dose of 75 or 90 J/cm^2. Tumour volume was assessed every second day until volume approached 2 cm^3.

In short, the treatment consisted of intratumoral (i.t.) injection of the PS at day 0, followed two days later, by i.t. injection of the siRNA lipoplexes (either anti-EGFR siRNA or non-specific siRNA complexed with Lipofectamine, siEGFR/LF or siNS/LF, respectively). Six hours after the injection of lipoplexes, the tumour area was exposed to L. In this study, two different L doses were employed, 75 J/cm^2 or 90 J/cm^2; all other parameters were kept constant. Tumour volume was assessed every second day and when volume approached 2 cm^3, mice were removed from the study.
To determine the silencing efficiency, tumours were harvested two and three days after the treatment (i.e. at day 4 and day 5 according to Figure 2) and immunohistochemistry techniques (IHC) were employed to detect the EGFR expression in tumour tissue sections. In parallel, tissue lysates were prepared and western blot (WB) techniques employed for relative quantification of the EGFR protein expressed in the tumours. Figure 3 shows four different sections (A, B, C, D) corresponding to mice subjected to different treatments, as depicted in the corresponding panel.

Panel 1 - Figure 3. Characteristics of performed treatments.

IHC & WB	PS dose	siRNA 7.5µg	Light dose
A	0.1 µg/mm^3	EGFR	-
B	0.1 µg/mm^3	EGFR	75 J/cm^2
C	0.1 µg/mm^3	NS	90 J/cm^2
D	0.1 µg/mm^3	EGFR	90 J/cm^2


A
B
C
D
M
A
B
NT
170 kDa
EGFR
45 kDa
β actin
Arbitrary units
100
80
60
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EGFR
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Figure 3. Detection and relative quantification of EGFR expression on tumour tissue.
Tumours harvested two (A, B) and three days (C, D) after the treatment (i.e. days 4 and 5) according to Figure 2. Panel summarising the characteristics of the treatments performed. Sections of snap frozen tumours were subjected to IHC staining where brown colour indicates EGFR protein. Tissue lysates were prepared and subjected to a WB protocol. Bands at 170kDa correspond to EGFR protein and bands at 45kDa to β actin, used as a loading control. Graph bars, in arbitrary units, obtained from analysis of fluorescence intensities of the WB bands. (PS – photosensitiser, M – Marker, NT – non-treated).

These figures, together with quantitative WB analysis, indicate that treatment with siEGFR/LF alone (A) induced 30% knockdown in murine tumours. When combined with PCI at 75 J/cm^2 light dose (B), approx. 80% knockdown was detected. These results demonstrate that PCI increases silencing effects *in vivo*. Tumours from mice treated with 90 J/cm^2 light dose, achieved comparable silencing efficiencies. The silencing efficiency decreased over the following 24 h (D), underlining the temporal nature of RNAi. Importantly, siNS/LF combined with PCI at 90 J/cm^2 light dose (C) did not induce a significant effect on EGFR expression, nor a necrotic pattern visible in samples B and D. These observations suggest that more than 30% knockdown of EGFR is needed to obtain this necrotic pattern. The enhancement in silencing efficiency induced by PCI is likely caused by enhanced endosomal escape of siRNA, in line with the earlier mentioned *in vitro* outcome [52].
The 80% knockdown of EGFR and resulting necrotic pattern suggest the possible occurrence of a strong antitumour response. However, contrary to expectations, PCI at 75 J/cm^2 light dose combined with siEGFR/LF treatment did not translate into an effect on tumour volume. Only a small effect on tumour growth rate was observed, as depicted in Figure 4. The minor delay in tumour growth of approximately 3-4 days for the group treated with siEGFR/LF and PCI at a light dose of 75 J/cm^2, is most likely attributable to the downregulation of EGFR, as there are no apparent differences between tumour growth of the control group and that of the group to which non-specific siRNA lipoplexes (siNS/LF) were administered combined with PCI. Taken together, PCI with a light dose of 75 J/cm^2 has a small effect on tumour growth, while nevertheless a strong downregulation of EGFR was induced, as revealed by WB analysis (Figure 3B). This small overall effect on tumour growth may indicate a rapid recovery of tumour cells upon EGFR silencing. The recovery of EGFR expression is expected, as the effect of siRNA is known to be temporary [55]. The observed retardation in tumour growth results in an increase in tumour doubling time of approximately 4 days, which is considered to be in the range of the proposed length of activity of a single dose of siRNA (5 days).

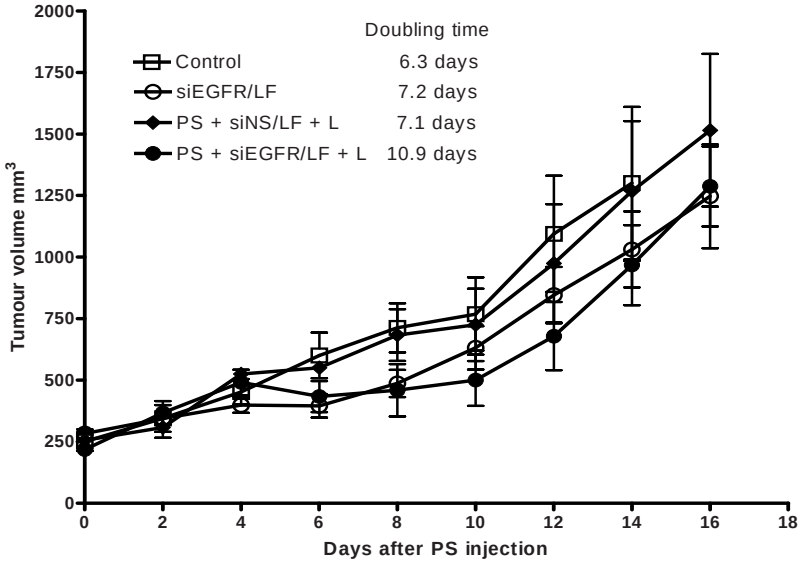

Figure 4. Tumour growth.
Mice subjected to treatment according to schedule in Figure 2 with a light dose of 75 J/cm^2. Every second day, their tumours were measured with a digital caliper and tumour volume was calculated with the formula $V = \pi/6 \times L \times S^2$, where L is the largest, and S the smallest superficial diameter.
Non-treated group (□); group treated with siEGFR/LF (○); group treated with PS, siNS/LF and L (◆); and group treated with PS, siEGFR/LF and L (●). Values plotted correspond to the mean and SEM. ($p<0.05$ for PS+siEGFR/LF+L versus Control, 1-way ANOVA).

The complete recovery of EGFR expression is confirmed by IHC techniques and WB analysis of tumours harvested at the last days of the study, i.e. when tumours approached 2cm^3. Figure 5 shows representative IHC micrographs and graph bars of WB analysis, indicating comparable EGFR expression levels for non-treated mice (NT), mice treated with siNS/LF and PCI (A), and mice treated with siEGFR/LF and PCI (B). Two other IHC micrographs are presented (C and D) where tumour tissue sections were treated for detection of CD31, a marker of endothelial cells. Here a different pattern of blood vessel arrangement and blood vessel dimensions are noticed. The tumours treated with anti-EGFR siRNA and PCI seem to have larger blood vessels that are localised at the tumour periphery (D and picture 2), as compared to the tumours treated with non-specific siRNA and PCI (C and picture 1). A similar pattern has been previously reported by Viloria-Petit *et al* in A431 tumour xenografts, which appeared to be related with an altered VEGF expression [56]. Such large blood vessels are referred in literature as a hallmark of pathological VEGF-dependent angiogenesis. VEGF signalling is closely related with EGFR. In fact, normal VEGF signalling is up-regulated by EGFR expression, and studies suggest that increased expression of VEGF is involved in resistance to anti-EGFR therapy [57].

Panel 2 - Figure 5. Characteristics of performed treatments.

IHCi & WB / IHCii / photo	PS dose	siRNA 7.5µg	Light dose
A / C / 1	0.1 µg/mm^3	NS	90 J/cm^2
B / D / 2	0.1 µg/mm^3	EGFR	90 J/cm^2

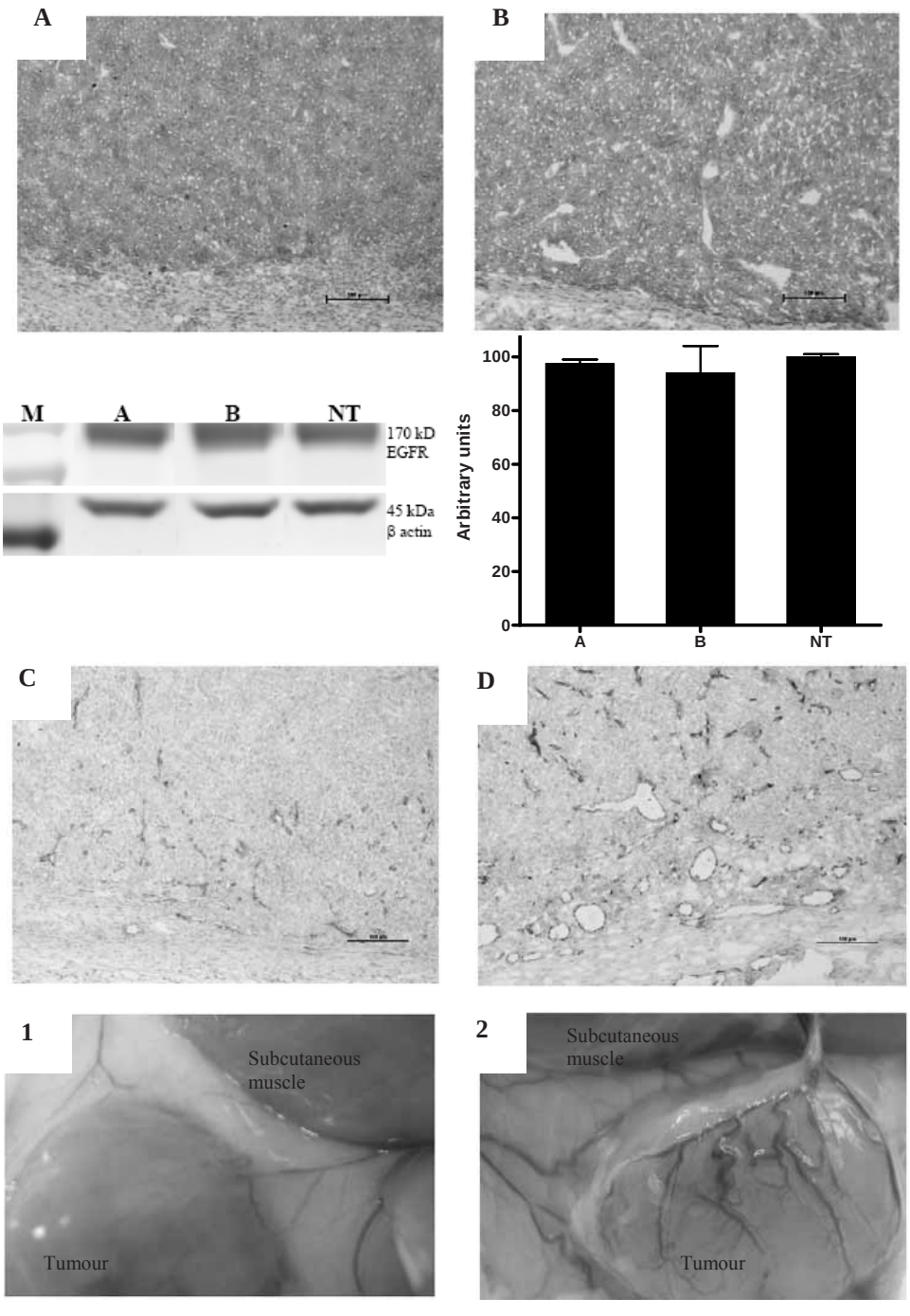

Figure 5. Detection of EGFR expression and blood vessels on tumour sections.
Tumours harvested at the last days of the experiment, when tumour size approached 2 cm^3, according to Figure 2. Panel summarising treatment settings. Sections of snap frozen tumours were subjected to IHC staining where brown colour indicates EGFR protein (A, B). Tumour tissue lysates were prepared and subjected to a WB protocol. Bands at 170kDa correspond to EGFR protein and bands at 45kDa to β actin, used as a loading control. Graph bars, in arbitrary units, obtained from analysis of fluorescence intensities of the WB bands. IHC staining for CD31, marker of endothelial cells, indicating blood vessels in brown (C, D). Pictures (1, 2) taken when tumours were harvested. (PS – photosensitiser, M – Marker, NT – non-treated).

The fast recovery of EGFR expression would probably be delayed if multiple injections of siEGFR/LF combined with PCI would have been applied, circumventing the transitory effect of siRNA. Also, the silencing of a combination of different targets, such as VEGF, or other proteins downstream to the EGFR activation signalling pathway, would likely induce a stronger antitumour effect. In a different approach, the combination of two inhibitory strategies would certainly be an advantage towards improved antitumour effects. Huang *et al* combined a tyrosine kinase inhibitor (TKI, gefitinib or erlotinib) with a monoclonal antibody (MAb, cetuximab) [58] for inhibition of EGFR, at both the intracellular and extracellular level, respectively. The combined treatments resulted in a more pronounced antitumour effect than the two strategies administered separately. The application of one of these (MAb or TKI) following the anti-EGFR siRNA treatment would probably induce a greater antitumour effect than the two independently, by blocking the activation of the remaining EGFR protein. In this context O'Grady *et al* have reported promising results on the combination of anti-EGFR RNAi and the tyrosine kinase inhibitor AG1478 [59].
Alternatively, one could apply PCI with such intensity that the photochemical treatment induces an effect comparable to photodynamic therapy (PDT) as suggested by Høgset *et al* [60]. Figure 6 shows tumour growth of non-treated mice, mice treated with siNS/LF and siEGFR/LF both with PCI at 90 J/cm^2 light dose. Here a clear antitumour effect and slower (re)growth of tumours is visible for mice treated with anti-EGFR siRNA in combination with PCI. However, a delay of tumour growth is also visible for mice treated with non-specific siRNA, likely resulting from the combination of PS with the slightly stronger dose of L (90 J/cm^2), which may have induced a PDT-effect. It is known that PDT can have an effect on vasculature [61], besides the direct cytotoxic effects on tumour cells. Nevertheless, the effect induced by siEGFR/LF and PCI is significantly stronger ($p<0.05$), most likely due to the added effect of EGFR silencing.

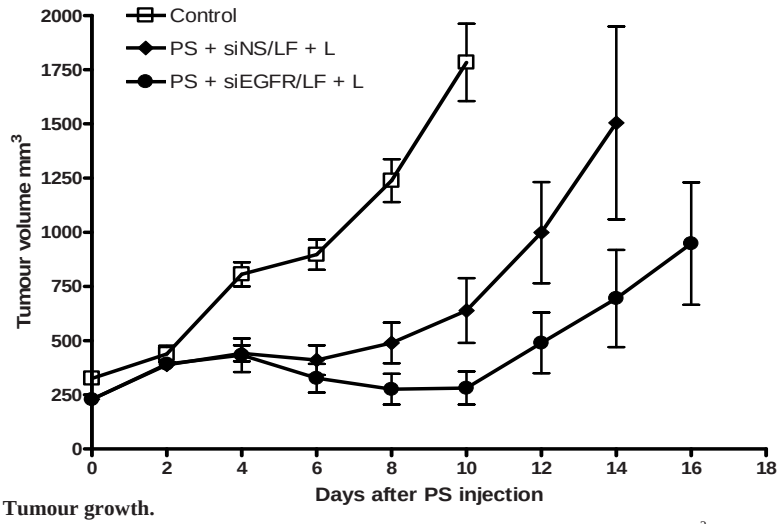

Figure 6. Tumour growth.
Mice subjected to treatment according to schedule in Figure 2. with a light dose of 90 J/cm^2. Every second day, tumours were measured with a digital caliper and tumour volume was calculated with the formula $V = \pi/6 \times L \times S^2$, where L is the largest, and S the smallest superficial diameter.
Non-treated group (□); group treated with PS, siNS/LF and L (◆); and group treated with PS, siEGFR/LF and L (●). Values plotted correspond to the mean and SEM. ($p<0.05$ for PS+siEGFR/LF+L versus PS+siNS/LF+L, 1-way ANOVA).

In conclusion, this study demonstrates the *in vivo* feasibility of PCI for enhancing the delivery of siRNA into the target cell cytoplasm, and consequently improving the knockdown of the target protein, EGFR, which is a relevant target for tumour therapy. Furthermore, this study suggests that more attention should be dedicated to increase the endosomal escape of siRNA *in vivo*, to enhance the silencing efficiency of locally administered siRNA lipoplexes.

CONCLUSION AND PERSPECTIVES

In this contribution, we demonstrate the applicability of PCI *in vivo* for local siRNA delivery. PCI was successfully employed to enhance the silencing efficiency of i.t. injected siRNA lipoplexes, and consequently, inducing a greater knockdown of the target protein, the EGFR. These studies were conducted following a protocol adapted from previously published studies in which PCI was applied *in vivo* [46, 53, 54]. However, parameters such as dose of photosensitiser and dose of light, time interval between PS and lipoplex administration, dose of lipoplexes, target protein, type of carrier used, and number of treatments, are some of the variables which require optimisation for different applications.

Furthermore, besides local delivery of lipoplexes, systemic delivery may be combined with PCI, with the advantage of local illumination ensuring delivery of the molecules to the target cells within the target tissue/organ. Alternative means of delivering light deeper in the tissue/body cavities by the development of lasers coupled to fibre optics makes it

possible to illuminate all tissues that are reachable by endoscopes [62]. These developments allow wider use of this technique in the future, increasing target specificity and preventing side-effects related to non-targeted organs. Moreover, in particular cases such as cancer therapy, the photochemical treatment can be adjusted for inducing a rather mild or even stronger PDT effect, provided that this combination is beneficial.
Overall, the application of PCI shows promise and, in fact, doses currently reaching the target tissue may be more effective if combined with an endosomal escape-enhancer strategy such as PCI.

Many research institutes and companies are developing RNAi therapeutics and these have shown promise in patients. The most successful siRNA molecules that are closest to the final stages of clinical evaluation are delivered locally at the site of the disease. This facilitates delivery and reduces doses which may prevent undesired effects in non-target tissues. For a more general application of RNAi therapeutics, by intravenous delivery for instance, additional problems are encountered during the transport phase of siRNA to the target cell, within the target tissue/organ of the patient (Figure 1) [3, 5-7]. For such applications, aspects like stability of the formulation in the bloodstream, circulation time, opsonisation by blood serum proteins, rapid clearance by macrophages of the mononuclear phagocytic system (MPS), extravasation from blood vessels and penetration into the tissues of the target organ, are critical for systemic delivery. Besides these aspects, reasonable production costs, formulation stability, and patient tolerability are important.
In conclusion, local delivery of siRNA has shown significant success and may soon be common clinical practice. As the therapeutic efficacy of siRNA is limited by intracellular trafficking, strategies to increase the delivery efficiency of locally administered siRNA molecules are of great importance. These strategies may further help the development of systemically injected siRNA formulations, where ultimately endosomal escape may be a critical determinant of therapeutic success.

ACKNOWLEDGMENTS
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CHAPTER 6

TARGETED DELIVERY OF SIRNA

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ABSTRACT

Therapeutic application of siRNA requires delivery to the correct intracellular location, to interact with the RNAi machinery within the target cell, within the target tissue responsible for the pathology. Each of these levels of targeting poses a significant barrier. To overcome these barriers several strategies have been developed, such as chemical modifications of siRNA, viral and non-viral nucleic acid delivery systems. Here, we discuss progress that has been made to improve targeted delivery of siRNA *in vivo* for each of these strategies.

INTRODUCTION

The functional mediators of RNA interference (RNAi) are small interfering RNAs (siRNA) [1, 2]. These double-stranded RNA molecules are typically 19 to 23 nucleotides in length, and consequently have a molecular weight of approximately 13 to 15 kDa and 38 to 46 negative charges. As a consequence, passive transport over the lipophilic cell membrane is poor [3–5]. At the same time, intracellular entry and translocation into the cytoplasm (and/or nucleus), where the RNAi machinery is located, is a prerequisite, for gene silencing activity [6–9]. More importantly, for *in vivo* applications, intracellular entry into the target cell within the diseased tissue is required and should lead to appearance in the cytoplasm to silence the mRNA of interest (Figure 1). Ideally, siRNA should therefore be targeted to three levels: to the target tissue, the target cell type, and the subcellular compartment.

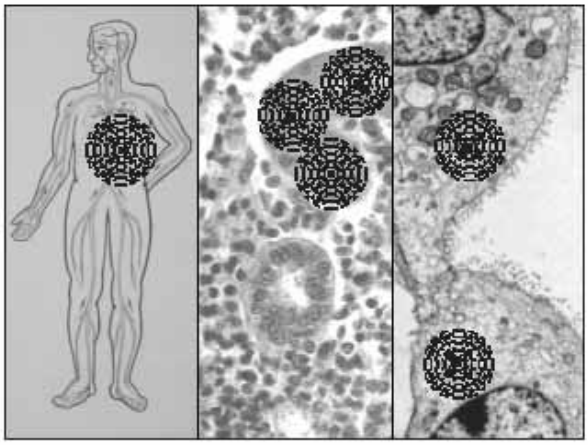

Figure 1. Three levels of targeting.
Preferably, siRNA should be targeted to the diseased tissue (I).Within this tissue it should be delivered to the correct cell type for silencing the mRNA of interest (II). Following entry of the target cell, siRNA should be delivered to the cytoplasm (and/or nucleus) to interact with the components of the RNAi machinery (III).

Primary obstacles for achieving this *in vivo* include competitive uptake by non-target cells, excretion in urine, degradation by nucleases, and endosomal trapping.

Some literature reports claim entry of siRNA in the target cells of the target tissue after intravenous injection [10]. The observations have been attributed to translocation of siRNA over the cell membrane by a dsRNA-receptor, referred to in *Caenorhabditis elegans* as SID-1, which is responsible in this organism for systemic spreading of the silencing effects [11]. Indeed, overexpression of the mammalian homologue increases the intracellular uptake of siRNA [12]. In contrast, *naked* siRNA is used by many researchers as a negative control which fails to produce silencing effects after injection *in vivo* and even after prolonged incubation of cells with high siRNA concentrations *in vitro*. This lack of activity of *naked* siRNA indicates that not all cell types express (enough of) the SID-1 homologue, to observe silencing effects. In addition, the rapid removal of *naked* siRNA after intravenous administration from the circulation, with more than 99% of the

injected dose renally excreted and taken up by liver Kupffer cells within minutes, makes a very small percentage of the administered dose available for the target tissue. This small percentage is additionally subject to nuclease degradation. Therefore, intravenous injection of naked siRNA relying on passive targeting of the diseased tissue, and SID-1 homologue-mediated target cell uptake seem to be inefficient and as yet unpredictable.
Local injection at the site of pathology avoids many of the difficulties encountered after intravenous administration, most notably the rapid elimination, and is therefore a popular approach to increase target tissue concentrations of siRNA. With this approach chances of obtaining sufficient intracellular levels of siRNA for therapeutic effects are increased [13, 14]. Furthermore, helper molecules (like cationic lipids or polymers) or physical methods (like electroporation, sonoporation, or hydrodynamic pressure) can be employed to facilitate intracellular entrance of siRNA [13, 15–19]. In addition, local production of siRNA by genes encoding for short hairpin RNA (shRNA) can ensure prolonged levels of the dsRNA intracellularly [20, 21]. The encoding genes can be delivered by viral vectors or one of the aforementioned non-viral methods.
Unfortunately, local administration is not always feasible because the target tissue cannot be reached, or covers an area that is too large to be feasible for a local injection protocol. In addition, using local injection (possibly supplemented with helper molecules or physical stimuli) selectivity in delivery to non-target and target cell types has usually not been taken into account. This is an important aspect when considering the nonspecific effects that can be induced by dsRNA. Over the past few years, it has become clear that cells can respond strongly to siRNA by different proinflammatory reactions depending on cell type, siRNA sequence, and intracellular location [22–24]. These effects can be intensified by employing cationic helper molecules [25], an effect probably mediated by a change in the intracellular trafficking of the dsRNA [23]. As a consequence, non-target cells may also take up siRNA and respond with induction of proinflammatory pathways in addition to the therapeutic RNAi effects within the target cells at the diseased site.

In this review we will focus on strategies for targeted delivery of siRNA, which are designed to improve accumulation of siRNA at three *in vivo* levels of delivery: at the target tissue, the target cell, and at the intracellular target site of action. We will concentrate on delivery approaches for systemic administration as such systems have broadest applicability. Three approaches will be discussed: chemical modifications of siRNA, viral nucleic acid delivery systems, and nonviral nucleic acid delivery systems. Most attention will be given to *in vivo* delivery strategies, as *in vitro* targeting studies often do not represent a fair evaluation of the many barriers that exist *in vivo*, possibly impeding efficient and site-specific delivery (eg, phagocyte uptake, uptake by competing cell types, excretion, intracellular processing, and siRNA (delivery system) stability).

CHEMICAL MODIFICATIONS

The nuclease sensitivity and poor tissue distribution/membrane permeation qualities of siRNA provide a reason to investigate possible chemical modifications that would improve these characteristics which would not interfere with the silencing efficiency of the siRNA molecules. Several strategies have been proposed to improve nuclease resistance and target cell uptake.

Increasing nuclease resistance

Chemical modifications in the nucleobases, sugars, and the phosphate ester backbone of siRNA can reduce siRNA sensitivity to nucleases [26–28]. Initial studies centred on the tolerance of the RNAi system for modifications in the two RNA strands [29]. A number of chemical modifications have been proposed to increase nuclease resistance, like boranophosphates [30], 4'-thioribonucleosides, phosphorothioates, 2'deoxy-2'-fluorouridine, 2'-O-methyl, 2'-O-(2-methoxyethyl), and locked nucleotides [31–36]. All of these chemically modified siRNAs were still able to induce siRNA-mediating gene silencing provided that the modifications were absent in specific regions of the siRNA and included to a limited extent. These specific restrictions regarding position and degree of modifications were dependent on the characteristics of the incorporated modification. Although increased nuclease resistance of siRNA would be expected to increase *in vivo* silencing effects, Layzer *et al* showed that this is not necessarily the case. They studied silencing effects in the liver after hydrodynamic injection, and showed that unmodified siRNA had a similar potency as the stabilised version [36].

In contrast, chemical modifications were shown to enhance therapeutic effects in a mouse model of hepatitis B virus infection. Chemically modified siRNA designed against a conserved region of the hepatitis B virus was shown to decrease viral DNA, hepatitis B surface antigen levels in serum, as well as viral RNA levels in liver over 1000-fold as compared to chemically modified control siRNA and buffer-treated animals in a hydrodynamic injection protocol. The benefit of chemically modified siRNA was supported by the fact that indicators of viral infection were 30-fold higher in animals treated with unmodified siRNA [37].

Increasing intracellular uptake

Several approaches have been developed where chemical modifications have been introduced to enhance intracellular uptake of siRNA. Liao and Wang developed poly-2'-O-(2,4-dinitrophenyl) modified siRNA. The enhanced lipophilicity of this siRNA allows passive diffusion over the cell membrane, while at the same time enhancing nuclease resistance. This approach has only been investigated *in vitro* and shows that chemical modification can enhance siRNA potency at both fronts. As a result the silencing effects of the chemically modified siRNA specific for insulin-like growth factor receptor were strongly enhanced as compared to unmodified siRNA [38].

In a similar setup, membrane permeant peptides (penetratin and transportin) were coupled to siRNA to facilitate their intracellular uptake. By coupling the peptide via a reducible disulfide linker, the bulky peptides are expected to be cleaved-off liberating the siRNA in

the cytoplasm. The approach was validated *in vitro*, thus supporting wide application of the basic technology. Nevertheless, cell specificity is lacking [39].

Furthest developed work on chemically modified siRNAs has been reported by Soutcheck *et al* [40]. They have developed siRNAs with partial phosphorothioate backbone modifications and 2'-O-methyl sugar variations on the sense and antisense strands to promote nuclease resistance, while at the same time cholesterol was conjugated to the 3' end of the sense strand using a pyrrolidine linker to change tissue distribution. The cholesterol-modified siRNA silenced reporter gene expression *in vitro* in the absence of transfection agents, something not observed for unmodified siRNAs. Probably the interaction of the cholesterol with serum components in the culture medium improved siRNA translocation over the cellular membrane. The cholesterol modification particularly enhanced binding of siRNA to serum albumin, probably explaining the prolonged circulation half-life measured after intravenous injection as compared to unmodified siRNA. In addition to a prolonged presence in the circulation, cholesterol-modified siRNAs were detected in liver and jejunum at concentrations of 100–200 ng per gram tissue 24 hours after the last injection of 50 mg/kg doses. These levels were sufficient to reduce the levels of the apolipoprotein apoB-100 in plasma by 31–68%. This reduction was paralleled by a 37% reduction in overall cholesterol levels, and reduction in high-density lipoprotein, low-density lipoprotein, and chylomicron levels. Despite these impressive results using relatively simple modifications, the doses of chemically modified siRNAs needed are relatively high and seem to underline that changing tissue distribution of siRNA in favour of target cell uptake by conjugation with comparatively small chemical groups is difficult to achieve. At the same time, small molecular weight modifications seem to be needed to preserve correct interaction with the RNAi enzymes. Cleavable linkers for coupling of bulkier modifications may be an approach to avoid these problems.

In conclusion, chemical modifications do promise important advances regarding nuclease resistance and reduced induction of the stress response. Invitrogen has developed a second-generation siRNA, known as Stealth RNAi, in which chemical modifications are designed to increase the specificity of RNAi effects by allowing only the antisense strand to efficiently enter the RNAi pathway and eliminating induction of interferon-related pathways. Others have also demonstrated that sequence and modifications can strongly influence intensity of silencing efficiency and inflammatory reactions, providing tools to optimise these [41, 42].

Taken together, chemical modifications can markedly increase nuclease resistance of siRNA improving cellular persistence and conjugation with translocating / hydrophobic functional groups can increase membrane permeation. Strategies to affect tissue distribution profiles of siRNA with chemical modifications seem more difficult.

VIRAL NUCLEIC ACID DELIVERY

Viruses are at present the most efficient gene delivery vectors. After cell binding they are capable of delivering their nucleic acid payload intracellularly in a proficient way along with nuclear localisation. Although virus-mediated delivery methods are usually based on delivery of genes encoding shRNA, few approaches used viruses to deliver chemically synthesised siRNA *in vivo* [43, 44].

Delivery of chemically synthesised siRNA

In this approach, reconstituted viral envelopes derived from influenza virus are used to encapsulate and deliver siRNAs. The reconstituted membrane vesicles contain the influenza virus spike protein hemagglutinin and additionally added cationic lipids. This protein is responsible for binding to and fusion with cellular membranes. The siRNA-loaded vesicles are taken up by receptor-mediated endocytosis, and are able to escape endosomal degradation by fusion with the endosomal membrane. Functional siRNA delivery was demonstrated *in vitro*, while *in vivo* uptake by macrophages in the peritoneal cavity was demonstrated after intraperitoneal injection. A similar approach, described siRNA delivery by simian virus SV40-based particles *in vitro* in lymphoblastoid cells [44]. As with many viral approaches, drawbacks of the systems are the difficulties of repeated administration and limited control over transduced cell type.

Delivery of DNA encoding siRNA/shRNA

A number of studies investigated the use of DNA encoding for shRNA delivered by viruses for gene silencing *in vivo*. Intravenous injection of 5×10^9 plaque forming units (pfus) recombinant adenovirus expressing shRNA targeting hepatitis B virus transcripts in mice with active replication of the hepatitis B virus, showed almost complete inhibition of viral protein production [45]. This in turn led to arrest of viral replication at day 17 after viral infection. The inhibitory effect persisted for at least 10 days. Interestingly, there appeared to be a fraction of viral protein that was not susceptible to RNAi-mediated silencing, which is suggested to be attributable to protection through binding of their mRNA to specific proteins. The exact nature of this protection and its possible involvement in RNAi resistance remains to be determined.

Uchida *et al* used expression of two separate siRNA strands against survivin by adenoviral transduction to inhibit tumour growth. Survivin is a protein that inhibits cancer cell apoptosis. Mice bearing subcutaneous U251 glioma tumours were treated with intratumoral injections of 10^{10} viral particles on three consecutive days every twenty days, ultimately leading to 4-fold smaller tumours at day 48 after start of treatment as compared to empty adenoviral vector and adenoviral vector expressing irrelevant siRNA [20].

These studies demonstrate the possibilities for single intravenous or multiple local injections of virally delivered DNA encoding si/shRNA for gene silencing. This strategy has been further confirmed in a number of different *in vivo* models and with a number of different vectors, like intracranial delivery of lentivirus-produced shRNA for inhibition of reporter gene expression in cortical neurons [46], intraperitoneal delivery of lentivirus-produced shRNA for inhibition of viral cyclin to prevent primary effusion lymphoma in

mice [47], intramuscular or intraspinal delivery of lentivirusproduced shRNA for inhibition of mutant SOD1 in amyotrophic lateral sclerosis [48, 49], and ex vivo delivery of lentivirus-produced shRNA for inhibition of CC-chemokine receptor 2 in hematopoietic cells in mice [50].

Taken together, the viral DNA-based sh/siRNA delivery process is very efficient: binding to the target cell surface and subsequent transduction, carrier stability, and protection against nucleases appear satisfactory [51–54]. However, as the discussed approaches illustrate, viruses usually lack selectivity for the target cell type. To improve specificity, the natural tropism of viruses for certain cell types may be used. Currently, much attention is focused on redirecting the natural preferred cell type of viruses towards therapeutically interesting receptors on the surface of target cells. Examples include the retargeting of murine coronavirus to the human epidermal growth factor receptor [55], directing adenovirus via fibroblast growth factor ligand towards its associated receptor (FGFR1) for delivery to glioma, or adenoviral delivery to angiogenic endothelium via RGD-peptides binding alpha v-integrins [56]. However, such approaches have not been tried as yet in combination with RNAi-mediated gene silencing *in vivo*.

The strength of the viral delivery approach is the efficient transduction of cells. Challenges that remain are the control over transduced cell type, especially after systemic administration. In addition, inflammatory reactions, immunogenicity, and oncogenic transformations continue to be important safety considerations for viral vectors that need to be addressed [57, 58].

NON-VIRAL NUCLEIC ACID DELIVERY

Whereas viral vectors possess many of the desired characteristics for efficient nucleic acid delivery, non-viral vectors possess several advantages. Important benefits of synthetic vector systems are the safety (related to their lack of immunogenicity and low frequency of integration) and ease of large scale production. In addition, they can accommodate a wide variety of nucleic acid sizes and they allow easy modification. On the downside, transfection efficiency can be a limiting factor.

To face this weakness, many functional groups need to be incorporated into non-viral nucleic acid delivery systems. A cationic functional group is usually required to bind and condense the nucleic acid, thereby protecting it against nucleases and (important for siRNA) increasing the apparent molecular weight above the renal clearance cut-off. In addition, some cationic compounds are being used as endosomal escape enhancers. Due to the resulting positive charge, complexes tend to form aggregates by binding in the blood stream to negatively charged biomolecules. As a result, their clearance is usually rapid. Moreover, such cationic complexes possess a propensity to interact with virtually any cell type they encounter, creating a need to insulate the interactive surface of the particle to promote specificity. For that purpose, shielding groups can be added to enhance colloidal stability and reduce surface charge thereby avoiding nonspecific cell uptake. To restore cell interaction in a target-specific manner targeting ligands can be coupled to induce site-specific binding and uptake. In the case of delivery of DNA encoding for shRNA by non-viral delivery systems, nuclear translocation of the DNA is

often inadequate. As such, the cytoplasmic site of activity of chemically synthesised siRNA provides an important advantage.

Delivery system based on RNA

A system consisting completely of RNA was proposed by Guo *et al* [59]. Their system is based on the packaging RNA of the DNA-packaging motor of bacteriophage phi29, which can spontaneously form dimers via interlocking right- and left-hand loops. By attaching the siRNA to one loop and an RNA aptamer to CD4 to the other, a cancer cell targeted system was created that could silence survivin gene expression *in vitro*. Alternatively, the system could also be targeted by folate.

Cationic delivery systems

Unshielded, untargeted complexes of siRNA with cationic polymers or lipids, can provide local or systemic transfection of a sufficient number of target cells for therapeutic effects. Several studies employed cationic lipids to complex siRNA to silence, amongst others, TNF-alpha in intraperitoneal macrophages after intraperitoneal administration [60], delta opioid receptor in spinal cord and dorsal root ganglia after intrathecal administration [61], polo-like kinase-1 in bladder cancer after intravesical administration, and c-raf-1 in prostate cancer cells after intravenous administration [62]. Although a sufficient number of cells must have been reached, as silencing is observed, it is fair to assume that a large part of the dose will arrive in non-target cells. In view of the nonspecific effects that can be induced by cationic lipids themselves and in particular in combination with dsRNA, this may severely hamper therapeutic application [25, 63].

A variety of other cationic compounds have also been investigated for siRNA-delivery purposes. A linear low molecular weight form of the cationic polymer polyethylenimine (PEI) has been used for treatment of (subcutaneously implanted) ovarian carcinoma in mice [64]. After intraperitoneal administration, complexed siRNA was primarily recovered from muscle, liver, kidney, and tumour. Interestingly, the major organ where PEI nucleic acid-complexes are usually recovered, the lung, was largely avoided. Importantly, silencing of Her-2 with these polyplexes inhibited ovarian carcinoma growth *in vivo*.

Atelocollagen (a highly purified type-I collagen of calf dermis digested by pepsin), was shown to be a suitable vehicle for local delivery of siRNA [17, 65]. In addition, when administered intravenously, atelocollagen-siRNA was able to localise at sites of tumour metastases and inhibit metastasis outgrowth [66]. More specifically, tumour levels increased 6-fold as compared to levels after *naked* siRNA administration (from 0.7 to 4.3 ng/mg after injection of 25 μg siRNA). This effect was, albeit less pronounced, also seen in the other organs investigated (ie, liver, lungs, kidneys, and spleen) demonstrating that the enhanced tissue uptake is not exclusively tumour-specific. Nevertheless, delivery of these levels of siRNA silencing EZH2 (enhancer of zest homologue-2, a gene overexpressed in hormone-refractory metastatic prostate cancer) or p110-α (a phosphatidylinositol 3-kinase regulating cell survival, proliferation, and migration) resulted in strong inhibition of growth of bone metastases of prostate cancer

cells. Importantly, siRNA-atelocollagen complexes failed to induce nonspecific proinflammatory responses like secretion of IFN-α and IL-12.

Targeted cationic delivery systems

A targeted amino-acid-based system was based on the cationic peptide protamine [67]. To the system's protamineblock the C-terminus of the heavy chain Fab fragment of an HIV-1 envelope antibody was coupled to form a protein construct known as F105-P. This system was highly efficient in binding to and transfection of cells expressing HIV-envelope protein, although it is unclear why the HIV-envelope protein would be internalised. Importantly, expression of interferon-β, 2', 5'-oligoadenylate synthetase, and Stat-1, as indicators of nonspecific effects, were not increased upon siRNA transfection of HIV-envelope-expressing melanoma cells. In addition, when these cells formed subcutaneous tumours *in vivo*, 30% of cells took up fluorescent siRNA when delivered by F105-P after intravenous administration. Naked siRNA was not taken up, nor was F105-P-siRNA delivered to cells that were envelope-protein negative. Delivery of a combination of siRNAs against c-myc, MDM2, VEGF strongly inhibited tumour growth *in vivo* when delivered using the F105-P system. This combination of siRNAs attacking the tumour at multiple fronts is an important advantage of the siRNA technology as it allows simultaneous interference with a number of different pathways, while the delivery problem for each individual drug molecule (siRNA) remains the same. The versatility of this targeted system was demonstrated by exchanging the HIV-envelope antibody for an ErbB2-antibody changing the specificity of the system to ErbB2-positive breast carcinoma cells.

In a cationic lipid-based approach, Pirollo *et al* coupled a transferrin receptor single chain Fv region antibody fragment to the surface of cationic DOTAP: DOPE complexes containing siRNA [68]. They evaluated the targeting potential of these systems in different murine tumour models: an orthothopically implanted pancreatic carcinoma (that produced spontaneous metastases), an orthotopically implanted prostate carcinoma, and intravenously administered breast carcinoma cells giving rise to metastases in the lung. In all these models, specific accumulation of fluorescently labelled siRNA complexed to the targeted cationic lipid particles at the site of the malignancy could be demonstrated as compared to surrounding normal tissue and liver. The question whether targeted delivery resulted in gene silencing was not addressed.

Shielded targeted cationic delivery systems

Targeted cationic systems have the important advantage that they possess a recognition signal for specific interaction with the target cell type. However, the cationic surface may also be able to interact with biomolecules or non-target cells. As such, shielding of the cationic surface may further enhance target cell specificity by reducing non-target tissue uptake and may additionally increase colloidal stability of the siRNA complexes.

In our studies we focused on the cationic polymer PEI coupled to PEG as shielding polymer. To the distal end of the PEG-chain a cyclic RGD-peptide was coupled. This peptide is a high-affinity ligand for alpha v-integrins that are overexpressed on angiogenic endothelial surfaces [69]. Tissue distribution studies *in vivo* of fluorescently

labelled siRNA in subcutaneous neuroblastoma-bearing mice showed that injection of *naked* siRNA did not produce appreciable tumour levels, but rather rapid clearance into the urine. PEI-siRNA complexes also lacked the production of high fluorescence in the tumour, but did increase liver and especially lung levels. The fluorescence appeared punctuate in both latter tissues, probably reflecting formation of aggregates in the circulation. When the PEG-shielded, targeted nanoparticles were used, a higher level of specificity for the tumour and lower levels of fluorescence in the lung and liver were observed. In a therapeutic setting, siRNA against murine VEGF receptor-2 was used, since the receptor is one of the driving factors of tumour angiogenesis. Delivery to host tumour endothelium is required to inhibit tumour proliferation. Efficacy studies with VEGFR2-specific siRNA complexed in RGD-PEG-PEI nanoparticles resulted in strong inhibition of sc neuroblastoma growth rate, which was sequence-specific. These experiments suggest that the targeted shielded nanoparticles indeed deliver the siRNA to the angiogenic endothelial cells. In line with these findings, the reduced tumour growth rate was paralleled by a reduction in blood vessels in the periphery of the tumour and changes in vascular morphology of remaining vessels, supporting an antiangiogenic mechanism of action. These results were supported by studies in a model of pathological angiogenesis in the eye [70], again demonstrating vasculature-specific delivery and inhibition of angiogenesis leading to therapeutic effects. Importantly, the studies in the eye also showed that combining siRNAs against different driving factors in the VEGF-pathway in the same delivery system improved therapeutic effects. Attacking the various receptors and growth factors simultaneously seems to offer advantages. Especially in multifactorial diseases, where functional redundancy is likely, this cocktail approach seems to offer important benefits.

Synthetic nonviral delivery systems are a diverse class of molecules used in different nucleic acid delivery strategies that range from relatively simple cationic complexation for local administration to targeted shielded systems for intravenous injection. Their adaptability to specific targeting requirements is an important advantage, although optimisation of delivery efficiency continues to remain important.

FINAL REMARKS

Over the last decades, research on the promises of nucleic acids for therapeutic intervention and the difficulties encountered in turning these promises into clinical reality have provided a clearer picture of the development steps that are needed to transform nucleic acids into actual drug molecules. As a result siRNA has been able to make a remarkable rapid progress from initial discovery as functional mediator of RNA interference in mammalian cells in 2001 to three clinical trials at the end of 2005: two in age-related macular degeneration, the other in respiratory syncytial virus infection [71]. Nevertheless, the choice of the diseases also reflects the delivery difficulties encountered for this class of nucleic acids. These diseases were selected partly because the target cell delivery problems are relatively low as these pathologies are confined to specific and accessible sites. To further improve target specificity, also in view of possible adverse effects occurring when siRNA is processed by non-target cells, and to allow application

of siRNA for systemic treatment several strategies can be proposed (Figure 2). Taken together they serve to increase nuclease resistance, to reduce renal excretion/specific cell uptake, to promote uptake by the target cells, and to ensure correct intracellular trafficking to the site of action. As the first preclinical proofs of principle have been delivered showing therapeutic effects of locally and systemically delivered siRNAs, it is expected that these strategies will soon translate into viable clinical development programs.

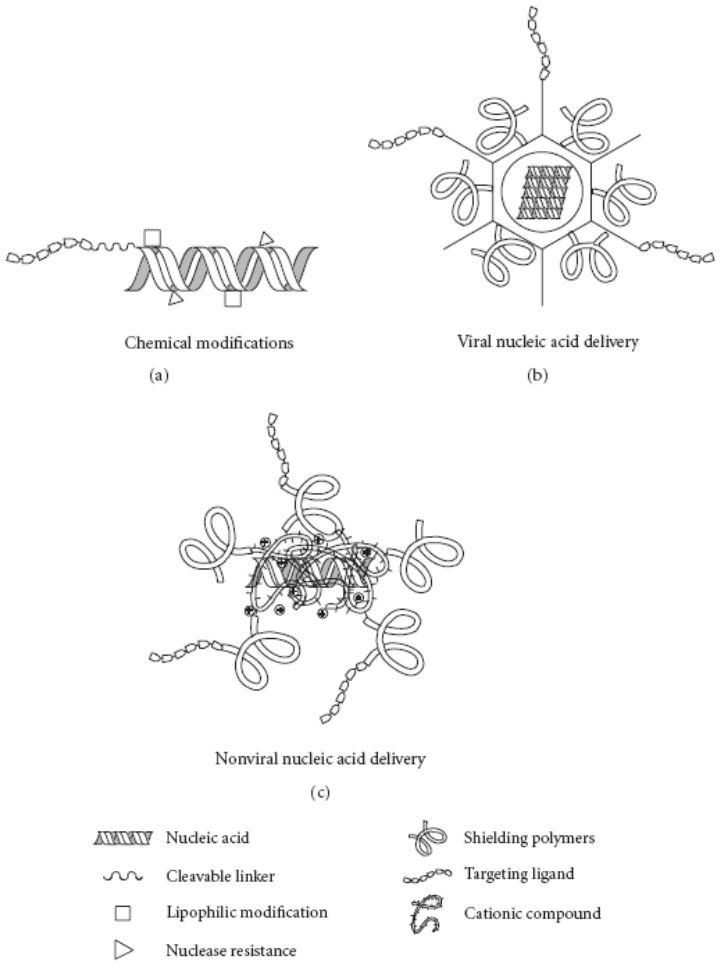

Figure 2. Strategies for siRNA delivery.
Strategies are based on (a) chemical modifications of siRNA, (b) targeting of siRNA using viral vectors, or (c) nonviral delivery systems.

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CHAPTER 7

ANTI-EGFR NANOBODY COUPLED TO LIPOSOMES: A NOVEL TARGETED DRUG DELIVERY SYSTEM WITH INTRINSIC THERAPEUTIC POTENTIAL

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ABSTRACT

The discovery of antibodies devoid of light chains in camelids led to the identification of the smallest functional antigen-binding immunoglobulin fragments, which correspond to the variable domains (VH) of these heavy (H) chain-only antibodies (VHH). Due to their dimensions, these VHH are generally named nanobodies. Despite their small size, nanobodies bind to their antigens with similar specificity and affinity as monoclonal antibodies (mAbs), and they have already been employed for both diagnostics and therapeutic applications. Here, nanobodies are coupled to liposomes for the first time and this system is presented as a novel and active targeted drug delivery system. The nanobody employed, EGa1, is an antagonist to the epidermal growth factor receptor (EGFR), a receptor overexpressed in many of the human epithelial tumours. The anti-EGFR nanobody-liposomes (EGa1-liposomes) indeed present enhanced cell association and cellular uptake compared to non-coupled liposomes. Surprisingly, a remarkable removal of the cell surface EGFR is observed right after a *pulse* incubation of 4 h: more than 90% of EGFR is undetectable; and after a period of *chase* of three days, cell surface expression of EGFR is still reduced by 50%. The sequestration of EGFR from the cell surface is found to be independent of kinase activity and to induce receptor downregulation: a 55% reduction of the total cellular EGFR is observed after two days of *chase*. These exceptional results have encouraged the investigation of the therapeutic potential of this targeted delivery system, which is indeed confirmed *in vitro*. The observed inhibition of tumour cell proliferation, induced by empty anti-EGFR nanobody-liposomes, underlines the value of this new and active drug delivery system for future therapeutic applications.

INTRODUCTION

The interest in immunotechnology and antibody development for antibody-based tumour therapy, dates back to 1980-90, when the administration of mouse monoclonal antibodies in humans was recognised to be immunogenic and to be associated with relatively poor tumour penetration [1-3]. Progress in genetic engineering techniques allowed the production of chimeric and humanised antibodies, and later of smaller antibody species, such as antibody fragments (Fabs, 55 kDa) and single chain variable fragments (scFv, 30 kDa) [4].

The accidental discovery, in 1993, of heavy chain-only antibodies in the blood of camelids [5] was a significant contribution to the field of immunology. These antibodies devoid of light chains consist of two disulfide-linked heavy chains, each composed of one variable domain (VHH – variable domain (VH) of heavy (H) chain-only antibodies) and two constant domains (CH2 and CH3). The absence of light chains makes these antibodies smaller than the conventional ones (approximately 90 kDa, compared to 150 kDa), and the cloning and isolation of their VHH allowed the identification of the smallest functional antigen-binding immunoglobulin fragment [6]. Due to their exceptional small size (4 nm height, 2.5 nm diameter), these VHH are generally referred to as nanobodies.

Nanobodies possess several attractive characteristics when compared with other antibodies [7]. Nanobodies show a similar degree of specificity and affinity towards their antigen as monoclonal antibodies (mAbs), having dissociation constants (K_d) in the low nanomolar to picomolar range. However, because nanobodies are 10-times smaller than mAbs and have a more concave-shape, they can recognise epitopes that are inaccessible for whole mAbs, e.g. the active site of enzymes [7]. Also, nanobodies re-fold very efficiently after heat-denaturation [8] and possess better solubility (hydrophilicity) than Fabs and scFv. Nanobodies can be isolated from immunised animals, or alternatively, they can be selected from a naive VHH phage-display library. Together with the facility to be engineered and the rather cheap and fast production in bacteria or yeast, nanobodies show great potential for a wide range of applications [9]. Nanobodies have already been successfully employed as tumour imaging agents. Their small size is ideal for tissue penetration and fast clearance of unbound molecules [10-13]. For therapeutic applications, however, their small size could be a disadvantage. Their molecular weight is below the threshold for kidney glomerular filtration, leading to rapid clearance of the nanobody from the bloodstream after intravenous administration. The strategy generally employed to overcome this is to fuse two or three nanobodies, forming bi- and trivalent molecules [13-15]. One study has demonstrated efficient inhibition of tumour growth employing trivalent, bispecific nanobodies targeting the epidermal growth factor receptor (EGFR) and albumin for longer serum half-life [15]. EGFR has long been recognised as a target for tumour therapy and, in fact, several mAbs have been developed to prevent ligand binding and, consequently, receptor activation. Together with tyrosine kinase inhibitors (TKIs), mAbs have been one of the main strategies employed for EGFR inhibition, and some of these are nowadays in clinical use [16].

In this study we combined nanobodies with liposomes. Liposomes are attractive drug delivery systems that can accommodate a broad spectrum of therapeutic molecules

(for review see [17]). The liposome surface can be modified to increase circulation time and tumour accumulation, for example with polyethylene glycol (PEG), and/or to promote cell-specific internalisation of liposome-encapsulated drugs by targeting ligands. Antibodies have been employed as targeting ligands on the surface of liposomes for many years (for review see [18]). The idea was first suggested in the 1970s [19] and many studies have followed. Recently, for example, Mamot *et al* have employed fragments of anti-EGFR antibodies coupled to liposomes (immunoliposomes) showing specific and efficient drug delivery to tumour cells over-expressing EGFR, both *in vitro* and *in vivo* [20-22].

The present study describes for the first time the use of anti-EGFR nanobodies coupled to the surface of liposomes as a new targeted drug delivery system. The nanobody employed here, EGa1, has previously been described as an antagonist to EGFR [23]. Based on the attractive characteristics of nanobodies and on successful studies that employed antibody fragments coupled to liposomes, nanobody-liposomes are proposed to represent a new and efficient drug delivery system. Remarkably, this study shows that this new carrier system has exceptional properties: the nanobody-liposomes targeting the EGFR can alone induce EGFR downregulation and this effect translates into inhibition of tumour cell proliferation. This study highlights the potential of nanobody-liposomes as a targeted drug delivery system for tumour therapy.

MATERIALS AND METHODS

Preparation of liposomes

Dioleoyltrimethylammonium propane, DOTAP (Avanti Polar Lipids, Birmingham, AL, USA), dioleoylphosphatidylethanolamine, DOPE (Lipoid, Ludwigshafen, Germany), and maleimide-polyethyleneglycol 2000 distearoylphosphatidylethanolamine, mal-PEG-DSPE (Avanti Polar Lipids, Birmingham, AL, USA) were dissolved in chloroform: methanol (1:1, v/v) in a round-bottom flask in a molar ratio of 1.43:1.29:0.28, respectively. Lissamine-rhodamine-phosphatidylethanolamine, Rho-PE (Avanti Polar Lipids, Birmingham, AL, USA), was added at 0.2 mol % for fluorescent labelling of the liposomes when needed. A lipid film was prepared under reduced pressure and rotary evaporator, followed by exposure to a stream of nitrogen until complete dryness. Liposomes were formed by rehydration of the lipid film with 10 mM Hepes / 135 mM NaCl pH 7.4 buffer. Liposomes size was reduced by multiple extrusion steps through polycarbonate membranes (Nuclepore, Pleasanton, CA, USA) with a final pore size of 100 nm.

Coupling of nanobody

The nanobody EGa1 is an antagonist of EGFR and has recently been described by Hofman *et al* [23]. EGa1 was first modified (mEGa1) in order to introduce protected sulfhydryl groups, by reaction with *N*-Succinimidyl-S-acetylthioacetate (SATA) (Pierce Biotechnology, Rockford, IL, USA). These groups were de-protected in an aqueous solution of 0.5 M Hepes / 0.5 M hydroxylamine-HCl / 0.25 mM EDTA of pH 7.0 for 30 min at room temperature (RT), generating a thioacetylated protein which is

reactive towards the maleimide crosslinker present in the mal-PEG-DSPE of the preformed liposomes. The activated mEGa1 was then incubated overnight at 4 °C with the liposomes (0.4 nmol nanobody per μmol total lipid, or 0.8 nmol/μmol when indicated) forming a thioether bond with the maleimide-PEG-DSPE incorporated in the liposomes. The molecular ratio of nanobody to total lipid was established according to previous studies [24] and was estimated to be approximately 30 nanobody molecules per liposome. Next day, Liposomes were purified and non-coupled nanobody was removed by two step washing with 10 mM Hepes / 135 mM NaCl pH 7.4 buffer, followed by separation and concentration with Vivaspin tubes (Sartorius, Epsom, UK) with a molecular weight cut-off membrane of 100 kDa.

Characterisation of liposomes

The phosphate content of the liposomes was determined with a phosphate assay according to Rouser *et al*, and from that the value total lipid (TL) content was determined [25]. In general, the values obtained were 30 mM TL for the Liposomes (2-5 % variation due to loss of lipids during extrusion), and 20 mM TL for the nanobody-liposomes (10-30% variation depending on the efficiency of the concentration step with the Vivaspin tubes). The mean particle size distribution of the liposomes and the polydispersity index (PDI) were determined by dynamic light scattering in a Malvern ALV CGS-3 with a He-Ne laser source (Malvern Instruments, Malvern, UK). The surface charge of the liposomes, ζ potential, was determined using a Malvern zetasizer Nano-Z (Malvern Instruments, Malvern, UK). Samples were prepared by dilution with 10 mM Hepes (1:4, v/v) and placed in a flow-through-cell for the measurements.

Detection of nanobody coupled to liposomes

Samples of nanobody, liposomes and nanobody-liposomes were prepared under reducing conditions for gel electrophoresis. Uncoupled nanobody was used as a control (0.25 μg) and samples of liposomes were prepared with nanobody content equivalent to 1.0 μg (165 nmol TL, considering 0.4 nmol nanobody per μmol TL). These samples were loaded on a NuPAGE Novex Bis-Tris mini gel (4-12% gradient, 1.0 mm) (Invitrogen, Breda, The Netherlands) and proteins were separated according to size, using the XCell SureLock mini-cell (Invitrogen). Subsequently proteins were electro-transferred to a nitrocellulose membrane using the iBlot Dry Blotting system (Invitrogen). The non-specific binding to the membrane was blocked overnight with 1% BSA in PBS-T buffer (PBS with 0.05% Tween-20) at 4°C on a roller bench. Next, the membrane was incubated with a rabbit anti-VHH (Unilever Research, Vlaardingen, The Netherlands) in 0.1% BSA in PBS-T buffer, for 1 h at RT on a roller bench. As a secondary antibody a goat anti-rabbit antibody labelled with Cy5 dye (Amersham Pharmacia Biotech, Little Chalfont, UK) was used, in 0.1% BSA in PBS-T buffer. Fluorescence was visualised on a Typhoon 9400 scanner (Amersham Biosciences, Buckinghamshire, UK).

Binding capacity – phage competition ELISA

Competition between the EGa1 nanobody expressed on the tip of filamentous phage and soluble EGa1 nanobody or nanobody-liposomes for binding to EGFR was tested in an ELISA setup. Rabbit-anti-Human IgG (Dako, Glostrup, Denmark, 1:2000 in PBS) was coated overnight at 4 °C in a Maxisorp (Nunc, Roskilde, Denmark) 96-well ELISA plate. The plate was blocked with 2% Marvel (skimmed milk powder) in PBS (MPBS) for 30 min at RT while shaking, and subsequently incubated with 100µl EGFR-ECD-Fc extracellular domain fusion protein (1.7 µg/ml) in MPBS for 1 h at RT while shaking. The plate was washed at least 4 times with PBS between every incubation step. Roughly 10^{10} CFU (colony forming units) of EGa1 phage, prepared as described by Marks *et al* [26], were premixed with a 1000 fold molar excess of soluble nanobody, or equivalent amount of nanobody-liposomes (considering 0.4 nmol nanobody per µmol TL), added to the ELISA plate and the mixture was incubated for 1 h at RT while shaking. Thereafter, the excess of unbound phage / nanobody / nanobody-liposomes was removed and bound phage was detected with a HRP-coupled mouse-anti-M13 (GE Healthcare, Uppsala Sweden 1:10000) in MPBS for 1 h at RT. Plates were developed with 100µl OPD/H_2O_2 and stopped with 50µl 1M H_2SO_4. The OD was read at 490 nm. As indicated in the results section, the same setup was employed for a different nanobody (7D12) and for a single chain fragment antibody (scFv 425).

Cell line and culture conditions

The human head and neck squamous cell carcinoma (HNSCC) cell line UM-SCC-14C (developed by Dr. T.E. Carey, Ann Arbor, MI, USA), abbreviated as 14C, was kindly provided by Prof. Dr. G.A.M.S. van Dongen (Department of Otolaryngology, Head and Neck Surgery, VU Medical Center, Amsterdam, The Netherlands). 14C cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2, in Dulbecco's Modified Eagle's medium containing 3.7 g/liter sodium bicarbonate, 4.5 g/liter L-glucose, L-glutamine (PAA, Pasching, Austria) and supplemented with 5% (v/v) foetal bovine serum, penicillin (100 IU/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml). Tests for mycoplasma infection were regularly performed and cells were consistently found to be mycoplasma-free.

Cell association studies

Nearly confluent monolayers of 14C cells were detached and collected. A suspension of $4x10^5$ cells/ml was prepared with complete medium and 250 µl of this suspension placed into FACS tubes. Rhodamine-liposomes (RhoL) and EGa1 nanobody-rhodamine-liposomes (EGa1-RhoL) were added to the cells at a final concentration of 0.025–0.5 mM TL, subsequently incubated in the dark for 1 h at 4 °C. At the end, cells were pelleted and washed 3 times with 1% BSA in PBS (PBA) and finally re-suspended in 300µl PBA. The mean fluorescence intensity was determined on a FACSCalibur (Becton & Dickinson, Mountain View, CA, USA). Generally, 10.000 events were acquired per sample, and samples were prepared in triplicates. Data were analysed with WinMDI 2.8 software.

Confocal microscopy

For confocal laser-scanning microscopy analysis, 8000 cells/well were seeded in a 16-well chamber slide, coated with 2% gelatine solution (Sigma-Aldrich, Zwijndrecht, The Netherlands). One day after seeding the cells were incubated with the liposomes at 0.5 mM TL concentration for either 1 h at 4 °C or 4 h at 37 °C. For these experiments, fluorescently labelled liposomes were employed (RhoL and EGa1-RhoL as mentioned above). After the incubation period, cells were washed with PBS and then fixed with 4% formaldehyde (Fluka, Zwijndrecht, The Netherlands) solution in PBS, for 30 min at RT. Next, cells were washed with PBS and incubated with Draq5 (Biostatus, Leicestershire, UK) for nuclear staining. After washing with PBS, confocal slides were mounted on glass cover slides using FluorSave (Calbiochem, San Diego, CA, USA). The following day, cells were analysed on a Leica TCS-SP confocal laser-scanning microscope (Leica, Heidelberg, Germany) equipped with three lasers: 488-nm Argon, 568-nm Krypton, and 647-nm HeNe laser.

Detection of EGFR levels at the cell surface

One day after seeding $4x10^4$ cells/well in 24-well plates (Greiner Bio-One, Frickenhausen, Germany), cells were incubated with different liposome formulations, in a concentration range 0.025 – 0.5 mM of TL. The incubation was performed at 37 °C for a period of 4 h. After the 4 h incubation, medium was refreshed and cells were further incubated for various time periods. At the end of the experiment, cells were detached by trypsinisation, washed with PBS buffer containing 0.3% BSA and 0.03% sodium azide, and resuspended in 100 µl of this buffer. For detection of EGFR at the cell surface, cells were incubated in the dark for 1 h at 4°C with either a specific anti-EGFR monoclonal antibody or a non-specific antibody, both labelled with Alexa488 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Thereafter, the cells were washed twice with buffer and finally resuspended in 300 µl buffer and analysed using a FACScalibur (Becton & Dickinson, Mountain View, CA, USA). Dead cells were discriminated from viable cells by adding propidium iodide (Molecular Probes, Breda, The Netherlands) to a final concentration of 1 µg/ml. For each sample, 10.000 gated events were analysed and samples were prepared in triplicates. Data were processed with WinMDI 2.8 software. For relative quantification, the mean fluorescence intensity (MFI) of the non-treated samples was set to 100 % of EGFR cell surface level. Experiments were repeated two or three times with liposomes prepared at different time points.

Detection of total cellular EGFR levels

In 6-well tissue culture plates (Greiner Bio-One, Frickenhausen, Germany) $2x10^5$ cells/well were seeded. The next day, liposomes were added and incubated at 37 °C for 4 h. After this incubation, medium was refreshed and cells were further incubated for 1, 2 and 3 days, unless otherwise mentioned. At the end of the experiment, cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS, containing 1 mM PMSF and 150 µl/ml of complete EDTA-free mini tablet protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany), for at least 20 min on ice. The lysates were collected and

the total protein content was determined using the Micro BCA assay (Pierce, Erembodegem-Aalst, Belgium). Samples of 10 μg of total protein were prepared and loaded on a NuPAGE Novex Bis-Tris mini gel (4-12% gradient, 1.0 mm) (Invitrogen). Proteins were size separated and subsequently electrotransferred to a nitrocellulose membrane. The non-specific binding to the membrane was blocked overnight with 1% BSA in PBS-T buffer (PBS with 0.05% Tween-20) at 4 °C on a roller bench. Thereafter, the membrane was split into two parts. One was incubated with a rabbit polyclonal anti-EGFR antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in 0.1% BSA in PBS-T buffer, for 1 h at RT on a roller bench. The lower part was incubated with a rabbit anti-β actin antibody (Abcam, Cambridge, UK) as loading control. Both membranes were then incubated with the secondary antibody, goat anti-rabbit antibody labelled with Cy5 dye (Amersham Pharmacia Biotech, Little Chalfont, UK) in 0.1% BSA in PBS-T buffer. For fluorescence visualisation, a Typhoon 9400 scanner (Amersham Biosciences, Buckinghamshire, UK) was used. The intensity of the bands was quantified with the Totallab software, version 2003 (nonlinear dynamics, Newcastle upon Tyne, UK).

Assessment of EGFR kinase activity

In 6-well tissue culture plates (Greiner Bio-One, Frickenhausen, Germany), $2x10^5$ cells were seeded per well. The next day, cells were serum starved for 5h. Next, EGF (4nM), liposomes (0.5 mM TL), or EGa1-liposomes (equivalent to 200 and 400 nM EGa1) were added to the cells and incubated for 10min at 37 °C. After this incubation, cells were washed with PBS and cell lysates were prepared similarly to the above description for total EGFR, except for the further addition of phosphatase inhibitors to the RIPA buffer. The cell lysates were collected and after determination of the total protein content (Micro BCA assay, Pierce, Erembodegem-Aalst, Belgium) samples of 10 μg of total protein were prepared. Gel electrophoresis and blotting of the proteins to the nitrocellulose membrane were performed as described above for total EGFR detection. The non-specific binding to the membrane was blocked for 1 h at RT with 5% milk-powder in TBS-T buffer (TBS with 0.1% Tween-20). Thereafter, the membrane was split into two parts, one was incubated with a rabbit anti-pY1068 EGFR (Cell Signaling Technology, Inc., Danvers, MA, USA) and the second part was incubated with a rabbit anti-β actin antibody (Cell Signaling Technology, Inc.) for the loading control, both in 5% BSA in TBS-T buffer, overnight at 4 °C on a roller bench. The following day, both membranes were incubated with the secondary antibody, goat anti-rabbit antibody coupled to HRP (Cell Signaling Technology, Inc.) in 5% milk-powder TBS-T buffer for 1 h at RT. For detection and visualisation a chemiluminescence-based reagent (Supersignal West Femto, Pierce, Rockford, IL, USA) was used together with a Gel Doc Imaging system equipped with a XRS camera and Quantity One analysis software (Bio-Rad, Hercules, CA, USA).

Investigation of epitope dependency

The 7D12 nanobody, previously described in [11], is an anti-EGFR nanobody binding to domain III (formerly named L2) of the EGFR and was selected from phage nanobody

libraries (Vosjan *et al*, manuscript in preparation). The 7D12 nanobody recognises a different EGFR epitope than the EGa1 nanobody. The 425 single chain antibody fragment (425scFv) binds to domain III of EGFR and competes for binding to EGFR with the EGa1 nanobody. The cDNA encoding scFv 425 [27]**,** cloned as bispecific single-chain Fv antibody fragment in pSecTag [28] was a kind gift of Dr. Van Beusechem (Division of Gene Therapy, Department of Medical Oncology, VU Medical Center, Amsterdam, the Netherlands). The cDNA was excised from pSecTag with the restriction enzymes *Sfi*1, *Not*1 and *Xho*1 and the 750 bp band corresponding to the *Sfi*1-*Not*1 fragment encoding scFv 425 was purified from gel. The cDNA was then cloned using these enzymes in an expression vector for bacterial expression almost identical to pUR5850 [29], only lacking the C-terminal biotinylation sequence (LRSIFEAQKMEW). Induction of protein expression and purification of scFv from the periplasmic space were performed as previously described by Roovers *et al* [30]. Both the 7D12 nanobody and 425scFv were chemically modified with the SATA reagent and coupled to liposomes, as described for EGa1 nanobody. For binding capacity assessment, these compounds were tested in similar settings as described for EGa1, i.e. competition assay in ELISA setup, except that CetuxiMab (Erbitux, ImClone Systems, Inc.) was employed as a positive control for 7D12 nanobody, and EGa1 employed as a control for 425scFv.

Cell proliferation assays

Two assays were performed, one to determine the total cell number and the second to assess the number of dividing cells. For both assays, three thousand cells were seeded in 96-wells tissue culture plates (Greiner Bio-One, Frickenhausen, Germany). One day after, medium was replaced by EGa1 nanobody, EGa1-liposomes or liposomes, covering a concentration range from 0.02 to 2 mM TL in complete culture medium. These were added to the cells in hexa-duplicate.

After two days of growth, total cellular protein was precipitated by addition of 5% (w/v) of trichloro-acetic acid (TCA) and stained with sulpho-rhodamine B (SRB) as described by Skehan *et al* [31]. OD was read at 540 nm and the total cell number was measured relative to the number at day 0, i.e. one day after seeding the cells.

For the second assay, BrdU reagent was added to the cells after two days of incubation with the nanobody, nanobody-liposomes or liposomes. One day later, the ELISA BrdU-colorimetic immunoassay (Roche Applied Science, Penzberg, Germany) was performed, as described by the manufacturer. After stopping the colorimetric reaction with H_2SO_4, the absorbance at 450 nm was measured, with the reference wavelength set to 655 nm, using a Bio-Rad Novapath Microplate Reader (Bio-Rad Laboratories, Veenendaal, The Netherlands).

Statistical analysis

Statistical analysis were performed using GraphPad Prism version 4.02 for Windows, GraphPad Software (San Diego, CA) using analyses based one-way ANOVA with Bonferroni correction for multiple comparisons. A p-value < 0.05 was considered to be significant.

RESULTS

Nanobody-liposome preparation and characterisation

Liposomes were prepared by lipid film rehydration and extrusion. The liposomes presented an average size of 120-130 nm with a PDI of 0.05-0.1, indicating small variations in particle size, and a near neutral surface charge (5-10 mV). Subsequently, EGa1 nanobody-liposomes (EGa1-L) were prepared and these nanobody-liposomes presented no significant variation in either size or surface charge, compared to liposomes devoid of nanobody.

The presence of the coupled EGa1 nanobody on the liposomes was assessed by western blot (Figure 1A). The coupling-reaction led to the formation of several adducts which corresponded to coupling of one nanobody to one or more mal-PEG-DSPE molecules, as indicated by the 3 kDa increase in molecular weight of the bands (Mw mal-PEG-DSPE = 2941.6 g/mol). This is possible, as nanobody modification with the SATA reagent was performed via primary amines and nanobodies mostly contain more than one solvent-accessible primary amine group. The introduction of protected sulfhydryl groups was confirmed by MALDI-TOF mass spectrometry, indicating that indeed one or more groups were introduced per nanobody molecule. Nevertheless, the band with highest intensity corresponds to approximately 18 kDa which is expected for one nanobody molecule (15 kDa) coupled to one mal-PEG-DSPE molecule. A small fraction (< 10%) of unbound nanobody was also detected.

To determine whether the nanobody-coupled liposomes preserved binding capacity to EGFR, ELISAs were performed. In these assays, filamentous phage expressing EGa1 were pre-mixed with a large excess of chemically modified nanobody (mEGa1) or nanobody coupled to liposomes (EGa1-L) and incubated with immobilised EGFR. Figure 1B clearly shows that the SATA-modification of the nanobody did not compromise the binding capacity of the EGa1 nanobody, as the mEGa1 shows competition with the EGa1-phages used in the assay. The EGa1-L show a reduction in binding capacity of approximately 20-30%, likely related to the multivalency of the nanobody-liposomes and the resulting steric hindrance of the colloidal system for binding to immobilised EGFR.

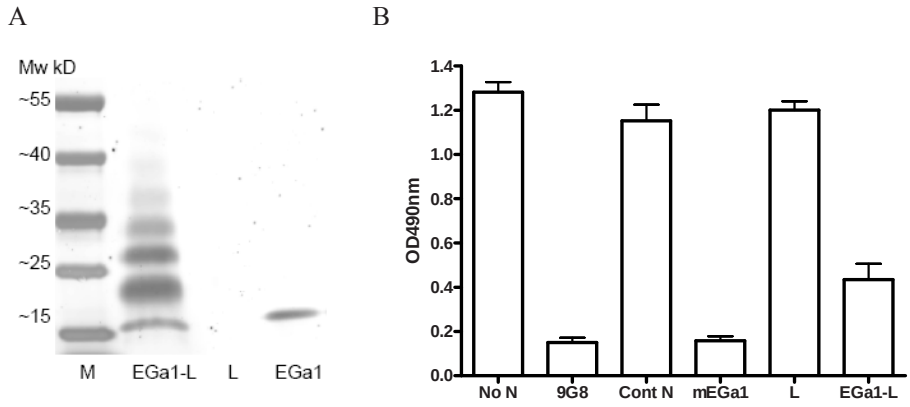


Figure 1. Characterisation of EGa1 nanobody-liposomes.
A. Nanobody coupled to liposomes is detected by means of western blot. Samples of 0.25 μg of nanobody and lipids equivalent to 1.0 μg of nanobody for the nanobody-liposomes (considering 0.4 nmol/μmol TL) were loaded on a 4-12% gradient polyacrylamide gel. Proteins were size separated and then blotted to a nitrocellulose membrane. Nanobody was then detected with a primary rabbit polyclonal antibody directed against nanobodies, followed by a secondary antibody coupled with Cy5 fluorophore for detection. M – marker for molecular weight, EGa1-L – EGa1-nanobody-liposomes, L – liposomes, EGa1 – free EGa1 nanobody. **B.** Modified EGa1 and EGa1-liposomes retain binding capacity and the original EGa1 specificity. ELISA plates were coated with EGFR. The binding of EGa1, expressed on the tip of filamentous phage, to EGFR was then tested in the presence of no competing nanobody - no N, of the 9G8 nanobody - 9G8 (Vosjan *et al*, manuscript in preparation), of a control (anti-RR6) nanobody - Cont N, of SATA modified EGa1 - mEGa1, of liposomes – L, and of EGa1-coupled liposomes - EGa1-L. Bound phage was detected with a HRP-coupled anti-M13 monoclonal antibody and staining with OPD/H2O.

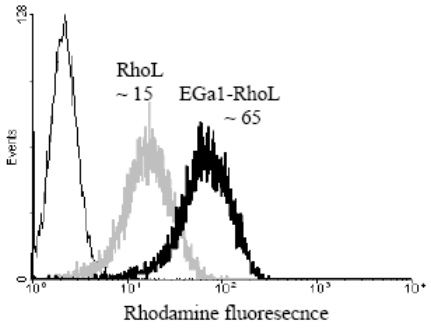
Nanobody-liposomes display increased cell association and cellular uptake

To determine the added value of EGa1 nanobodies coupled to the surface of liposomes for targeting purposes, cell association studies were performed. A fluorescently labelled phospholipid, Rhodamine-PE, was incorporated in the lipid mixture during preparation of the liposomes. The characteristics of the fluorescently labelled liposomes (RhoL) were comparable to the non-fluorescent liposomes (L). Cell association of nanobody coupled RhoL (EGa1-RhoL) and RhoL was determined by flow cytometry.

As expected, incubation of the 14C cells with increasing concentrations of liposomes resulted in an increase in mean fluorescence intensity (MFI) for both formulations which was 4-fold higher for the EGa1-RhoL than RhoL (Figure 2A). These MFI values were obtained when cells were incubated with liposomes at a 0.125 mM total lipid (TL) concentration. This concentration corresponded to a *plateau* value. For higher concentrations, the MFI increased proportionally for both EGa1-RhoL and RhoL, indicating non-specific interactions. These experiments were also performed with the A431 human cervix carcinoma cell line, which has approximately 3 to 4 times more EGFR per cell when compared to 14C cells. As expected, the *plateau* of MFI was reached at higher concentrations of TL. Additionally, cell lines expressing no and very low levels of EGFR showed no detectable differences between the two formulations (EGa1-RhoL and RhoL) (data not shown).

The observed enhancement of cell association of EGa1-coupled liposomes was also assessed by confocal microscopy. In these experiments, fluorescent liposomes were used at 0.5 mM TL concentration and cells were incubated with the liposomes for 1 h at 4 °C (i.) in order to examine the difference between specific cell and non-specific cell association of liposomes. Furthermore, cellular uptake was investigated. For that, incubation was performed at 37 °C for 4 h (ii.), to determine whether differences in cellular uptake could be observed for the different liposome formulations.
Figure 2B shows the results of the microscopy analysis. It is clearly visible that the incubation at 4 °C shows the specific cell association of the nanobody-liposomes, whereas non-specific interactions caused far less cell association (Figure 2Bi.). The nanobody-liposomes were located on the cell surface, all around the cell membrane, whereas interactions of plain liposomes were much weaker. Incubation for 4 h at 37 °C showed that both types of liposomes were taken up by the cells (Figure 2Bii.). Nevertheless, the fluorescence intensity of the cells incubated with nanobody-liposomes was higher and more distributed over the cells than for the liposomes devoid of nanobody. These images confirm that the presence of the EGa1 nanobody on the surface of the liposomes enhances cell association, causing increased cell uptake.

A



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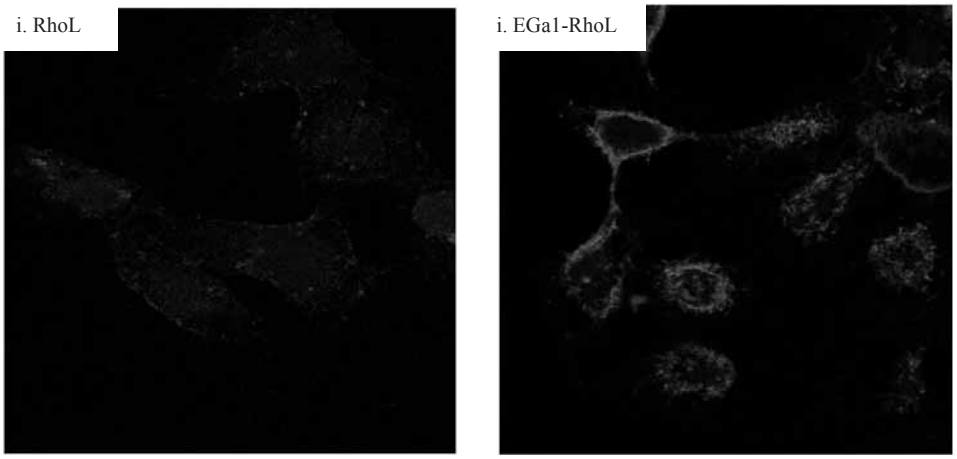


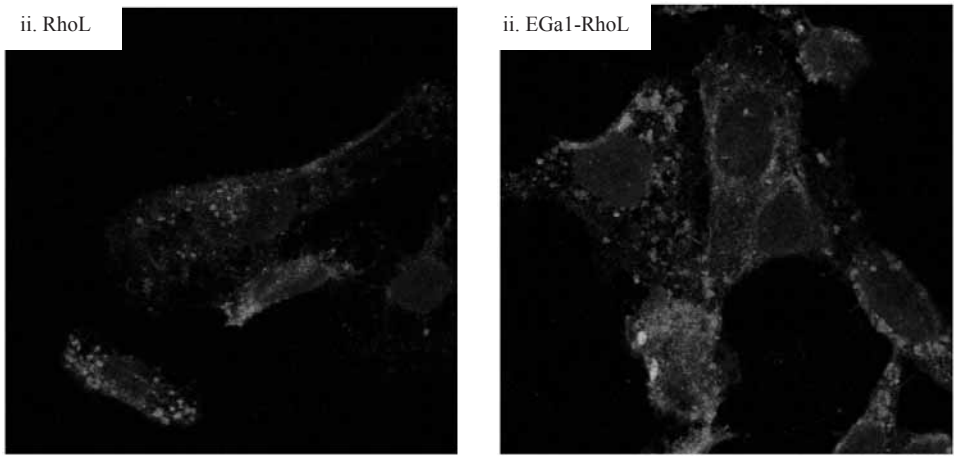


Figure 2. EGa1-liposomes display increased cell association compared with non-conjugated liposomes that results in enhanced cellular uptake.
A. Cells in suspension were incubated for 1 h at 4 °C with fluorescently labelled liposomes at 0.125 mM TL concentration. After washing, the mean fluorescence intensity (MFI) was determined. Thin black line control (non-stained) cells, thick grey line – cells incubated with rhodamine-liposomes (RhoL), thick black line – cells incubated with EGa1-nanobody-rhodamine-liposomes (EGa1-RhoL). Values depicted in the figure correspond to the average MFI of triplicates. **B.** Cells were incubated with fluorescently labelled liposomes (red colour) for 1 h at 4 °C (i.) or 4 h at 37 °C (ii.). After incubation, cells were washed, fixed, incubated with Draq5 (blue colour) for nuclear staining and finally mounted for visualisation. Left - RhoL – cells incubated with rhodamine-liposomes, right - EGa1-RhoL – cells incubated with EGa1-nanobody-rhodamine-liposomes.

Nanobody-liposomes reduce cell surface EGFR levels

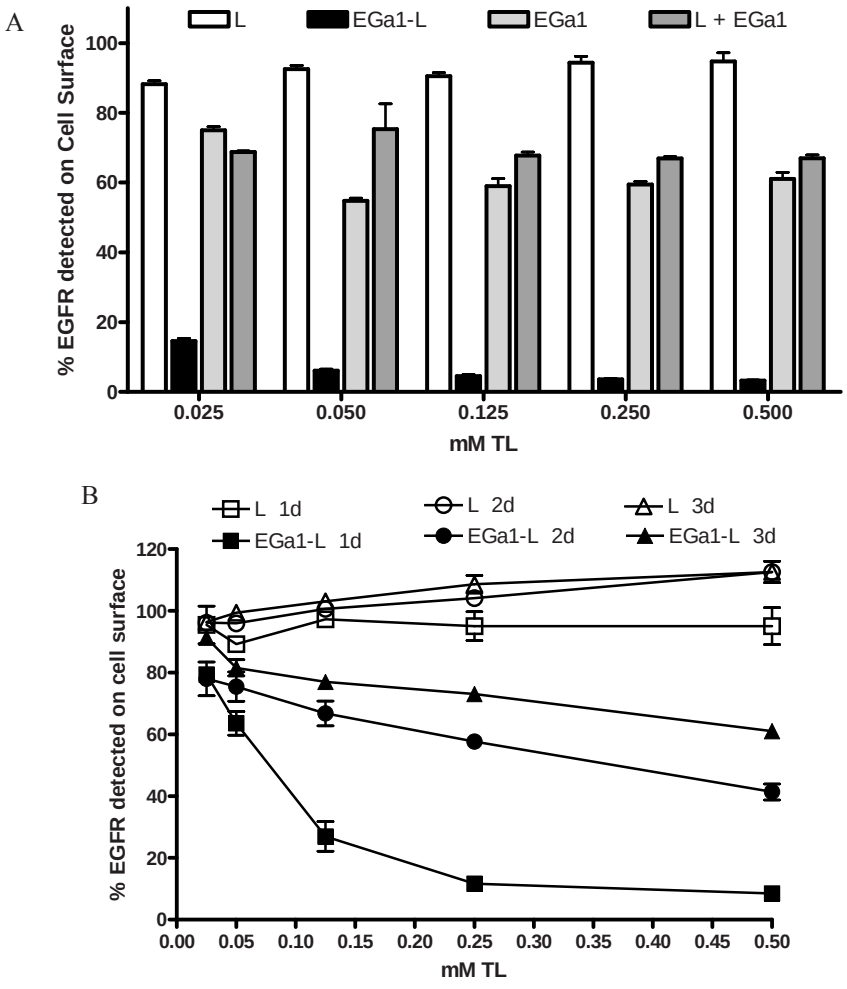
The pronounced cellular uptake of EGa1-liposomes raised the question to which degree the EGFR was internalised together with EGa1-liposomes. In order to investigate the reduction of EGFR levels from the cell surface, an anti-EGFR antibody coupled to the fluorescent molecule Alexa488 was used to stain cells right after the 4 h incubation with the liposomes. The incubation with Alexa488-anti-EGFR antibody was performed at 4 °C with living cells ensuring that the antibody would only bind to receptors available at the cell surface. As controls, free EGa1 nanobody, liposomes and free EGa1 nanobody were used.

Figure 3A shows a remarkable reduction of EGFR level from the cell surface after incubation of the cells with the EGa1-liposomes. More than 90% of the cell surface EGFR was no longer detectable by the anti-EGFR-Alexa488 immediately after the 4 h incubation. Both the free EGa1 and the combination of liposomes with uncoupled EGa1, had a significantly ($p<0.001$) weaker effect on reducing the EGFR from the cell surface (approximately 30-40%). These results are surprising and, to our knowledge, similar reductions of EGFR from the cell surface have not been reported before. Importantly, the 95% reduction observed here was obtained after a *pulse* incubation of 4 h. To investigate the duration of the effect, we decided to perform *pulse-chase* experiments, where cells were followed for longer times after refreshing the medium, immediately after the *pulse* incubation period with the liposome formulations.

The reduction of EGFR-cell surface levels by nanobody-liposomes was detectable up to three days after the *pulse* incubation (Figure 3B). Even after three days EGFR-levels were still reduced by 30%. As EGFR is detected by antibody labelling, steric hindrance by liposomal receptor binding may prevent the complete detection of cell surface EGFR. However, the disappearance of EGFR is further supported by the high number of fluorescent vesicles, indicating a substantial uptake of liposomes in Figure 2Bii. Furthermore, strong reductions were still measured after several days of *chase*, when it is very unlikely that liposomes remain attached to the cell surface.

To determine whether the effect described here could be further enhanced, experiments were performed with liposomes containing twice the amount of nanobody coupled to the liposomes (EGa1-0.8-L).

Figure 3C shows the cell surface level of EGFR of cells treated with EGa1-0.4-L and EGa1-0.8-L liposomes, in % of the level of non-treated cells. Overall, the EGa1-0.8-L formulation prolonged the reduction of EGFR cell surface level, being therefore more efficient than the original formulation.



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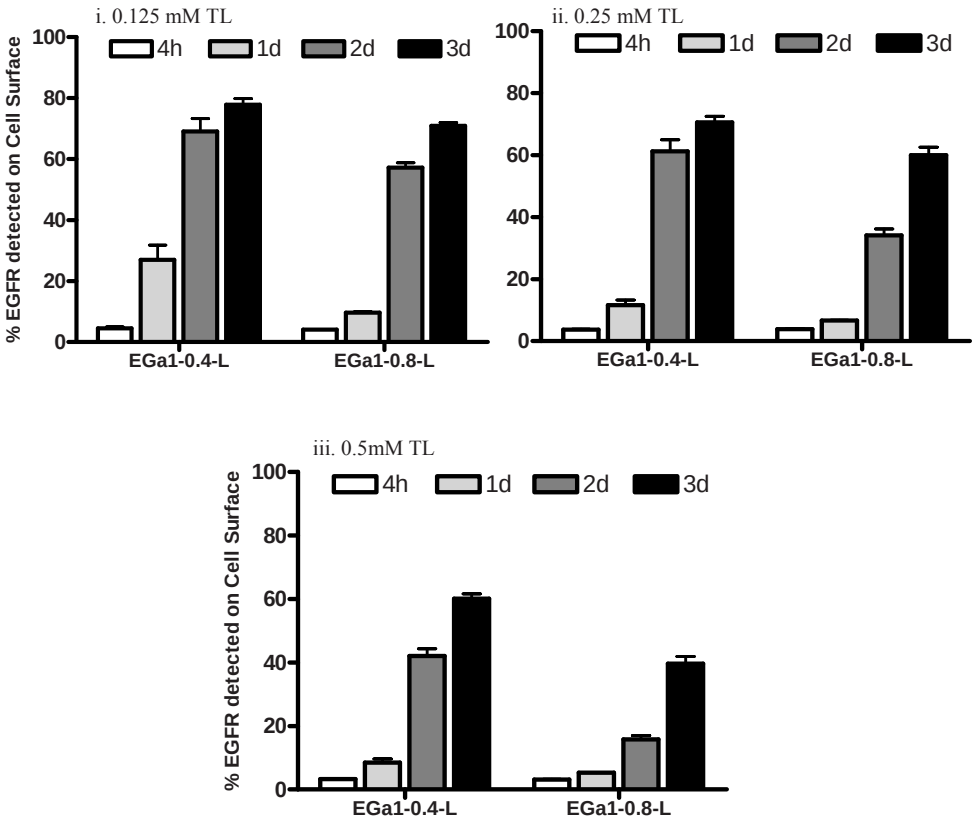


Figure 3. EGa1-liposomes cause a massive sequestration of cell-surface EGFR.
Cells were incubated for 4 h at 37 °C with EGa1-liposomes, or liposomes and/or free EGa1 nanobody at concentrations ranging from 0.025 to 0.5 mM TL. **A.** Right after the 4 h *pulse* incubation, cells were collected and stained for cell surface EGFR. Graph bars show average % of EGFR cell surface levels obtained from three independent experiments, in which samples were prepared in triplicates, and error bars show the SEM. ($p<0.001$ for EGa1-L and EGa1; $p<0.001$ for EGa1-L and EGa1 + L; $p>0.05$ for EGa1 and EGa1 + L).
B. After the *pulse* incubation, medium was refreshed and cells were further incubated for 1, 2 and 3 days period.
C. After *pulse* incubation with liposomes of 0.4 and 0.8 nmol/μmol TL nanobody content, at (i.) 0.125, (ii.) 0.25 and (iii.) 0.5 mM TL concentrations, medium was refreshed and samples were collected over time. The mean fluorescence intensity (MFI) was determined and the MFI of non-treated samples was set to 100% of EGFR cell surface level for relative quantification. L – liposomes, EGa1-L – EGa1 nanobody-liposomes, EGa1 – free EGa1 nanobody, L+EGa1 – liposomes and uncoupled EGa1 nanobody, EGa1-0.4-L – EGa1-nanobody-liposomes at 0.4 nmol/μmol TL; EGa1-0.8-L – EGa1-nanobody-liposomes at 0.8 nmol/μmol TL. Values plotted were obtained from repeated experiments, in which samples were prepared in triplicates, and the error bars show SEM.

The unexpected effect here reported, i.e. the decrease in cell surface EGFR induced by a *pulse* incubation of cells with the EGa1-liposomes, encouraged further experiments to investigate whether the EGFR removal from cell surface was dependent on kinase activity.

EGa1-mediated EGFR sequestration from the cell surface is independent of kinase activity

In order to elucidate the mechanism involved in the decrease of EGFR from the cell surface, the involvement of receptor activation was assessed by measuring the phosphorylation of EGFR 1068 tyrosine residue (Y1068). These experiments were performed to determine whether, in absence of EGF, the EGa1 nanobody could induce phosphorylation of EGFR tyrosine residues, and furthermore, to determine if the previously reported antagonism of this EGa1 nanobody was retained when EGa1 was coupled to the liposomes [15, 23].

Figure 4 shows that, in absence of EGF, none of the formulations induced detectable phosphorylation of the Y1068 residue of the EGFR. Furthermore, when EGF was present together with free EGa1 or EGa1-L there was a clear inhibition of phosphorylation of Y1068 compared with stimulation by EGF alone. Controls of EGF alone and combined with liposomes devoid of nanobody induced a strong phosphorylation of this tyrosine residue, indicating kinase activity. These results confirm that no kinase activity is involved in the internalisation of the EGFR by nanobody-liposomes, and that EGa1 nanobody has antagonistic properties to EGF, also when coupled to liposomes.

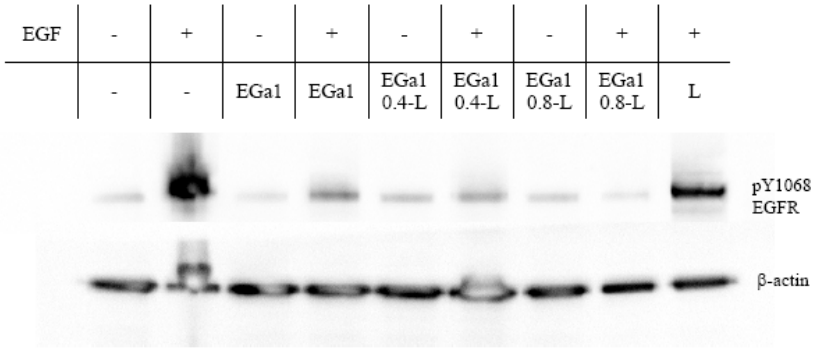


Figure 4. EGa1-liposomes do not activate the EGFR, but inhibit EGF-induced EGFR phosphorylation. Cells were serum starved for 5 h prior to the addition of the liposomes and/or EGF. Cell lysates were prepared after 10 min incubation with the liposomes and/or EGF. EGF was added at 4 nM, liposomes at 0.5 mM TL concentration, and EGa1-liposomes at 200 nM and 400 nM (EGa1 equivalent). Proteins were size-separated and blotted to nitrocellulose membranes. The membrane was split into two parts: one for detection of the phosphorylated 1068 tyrosine residue (upper) and the second for detection of β-actin (lower), used as a loading control. EGa1 – free nanobody, EGa1-0.4-L – EGa1-nanobody-liposomes at 0.4 nmol/μmol TL nanobody to lipid ratio, EGa1-0.8-L – EGa1-nanobody-liposomes at 0.8 nmol/μmol TL nanobody to lipid ratio, L – liposomes.

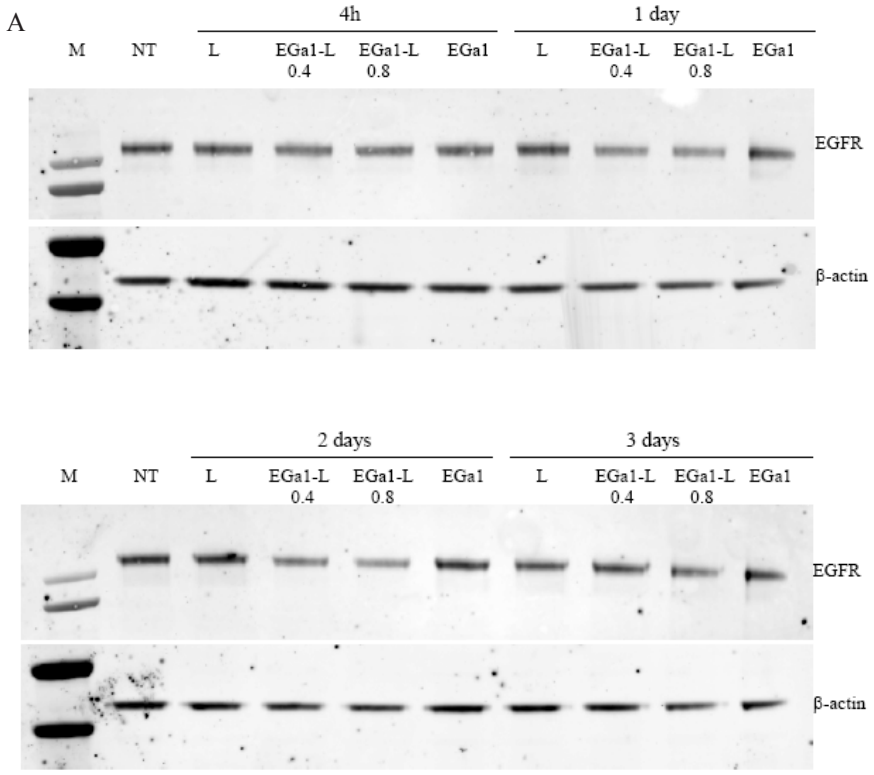
Induction of EGFR downregulation by EGa1 nanobody-liposomes

To determine whether the observed disappearance of EGFR from the cell surface, which is independent of receptor activation, resulted in a downregulation at the total protein level, the total EGFR protein content was assessed by western blot (WB).

Figure 5 presents the total amount of cellular EGFR measured in time by WB, after a *pulse* incubation of 4 h with EGa1-liposomes. Besides the two nanobody-liposome

formulations (0.4 and 0.8), free EGA1 nanobody and liposomes devoid of nanobody were also tested. Figure 5B corresponds to the analysis of three independent WB experiments, in which lysates were prepared 2 days after the *pulse* incubation.

Unlike the extreme reduction of cell surface EGFR level detected immediately after the *pulse* incubation, the total content of EGFR is less affected by the nanobody-liposomes. However, EGFR levels decreased and two days after the *pulse* incubation EGFR levels are diminished by 40% and 55% for EGa1-0.4-L and EGa1-0.8-L, respectively (Figure 5B). The samples prepared 3 days after the incubation showed higher EGFR content, though still below the regular level (NT). Clearly, the downregulation of total EGFR levels was most pronounced two days after the *pulse* incubation with EGa1-liposomes. In addition, no detectable effect on EGFR content was observed for cells incubated with liposomes devoid of nanobody and cells incubated with free EGa1 nanobody.



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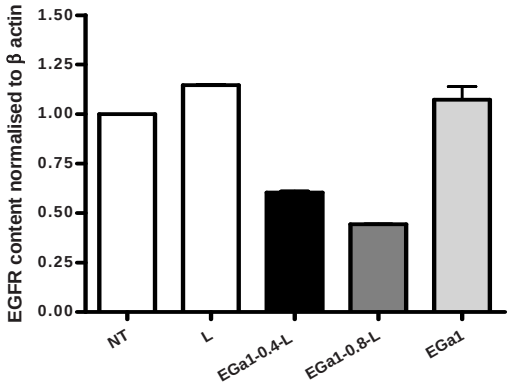


Figure 5. EGa1-liposomes induce downregulation of total EGFR protein levels.
Cells were incubated for 4 h at 37 °C with liposomes containing two different ratios of nanobody to TL (0.4 and 0.8 nmol/μmol TL) at 0.5 mM TL concentration. After the 4 h incubation, medium was refreshed and samples were collected over time. **A.** Cell lysates analysed by WB, where both EGFR and β-actin (as loading control) were detected. **B.** Ratio's of the intensity of EGFR bands normalised with β-actin bands (non-treated set to 100%), from 3 independent experiments, of lysates prepared 2 days of *chase* after the 4 h *pulse* incubation. M – marker for molecular weight, NT – non-treated samples, L – liposomes, EGa1-0.4-L – EGa1-nanobody-liposomes at 0.4 nmol/μmol TL nanobody to lipid ratio; EGa1-0.8-L – EGa1-nanobody-liposomes at 0.8 nmol/μmol TL nanobody to lipid ratio; EGa1 – free nanobody.

EGFR downregulation induced by nanobody-liposomes is epitope-independent

In order to investigate whether the observed EGa1-induced downregulatory effect was dependent on the epitope on EGFR recognised, two other targeting molecules were coupled to liposomes and tested in similar experiments. These molecules were the 7D12 nanobody [11], an anti-EGFR nanobody which binds to an epitope on domain III of the EGFR (Vosjan *et al*, unpublished results), and 425scFv, a single chain antibody fragment which binds to an overlapping epitope of that recognised by nanobody EGa1.

As performed for the EGa1-liposomes, these two formulations were also characterised for their binding capacity to EGFR. Figure 6 presents the results of ELISA's showing the binding of the 7D12 nanobody (A) and the EGa1 nanobody (B), expressed on filamentous phage, in the presence of a large molar excess of the different liposome formulations. As can be concluded from Figure 6A, the binding of 7D12-phage is inhibited in the presence of the whole mAb Erbitux, of the 7D12 nanobody and of 7D12-liposomes, but not by the presence of EGa1 or EGa1-liposomes. On the other hand, as shown in Figure 6B, EGa1-phage is unable to bind EGFR in the presence of EGa1 itself, of EGa1-liposomes and of scFv 425 and scFv452-liposomes. These results confirm the binding properties mentioned above, i.e. EGa1 and 425scFv bind to overlapping epitopes on EGFR, whereas 7D12 binds to a different one, overlapping with that recognised by Erbitux. Importantly, Figure 6 shows that liposome coupling does not compromise the binding capacity of neither the 7D12 nanobody, nor 425scFv.

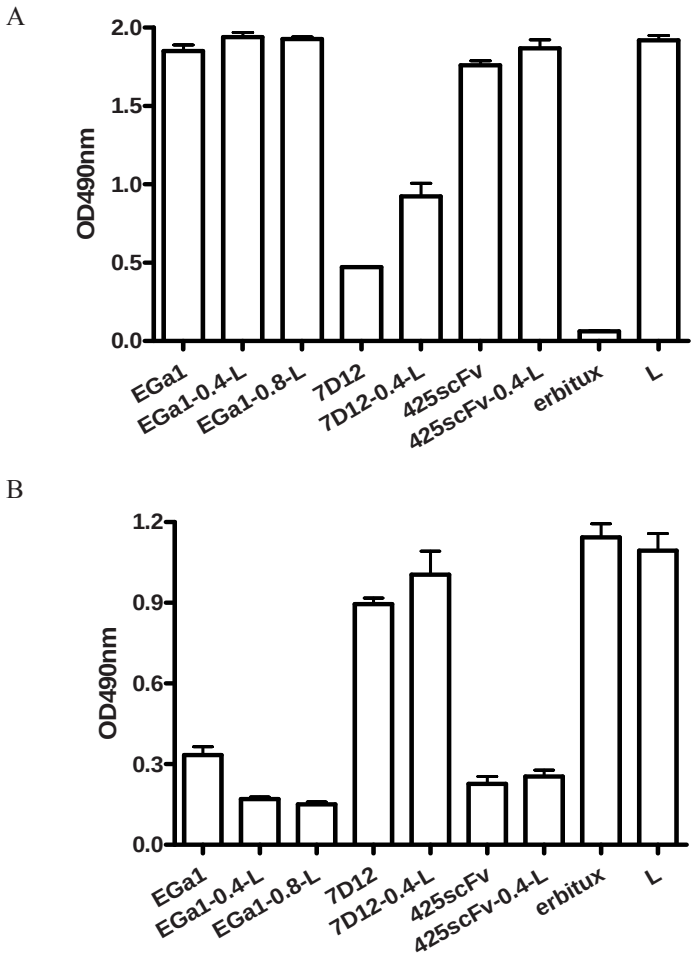


Figure 6. 7D12-liposomes and 425scFv-liposomes retain binding capacity to EGFR.
ELISA plates were coated with EGFR. The binding of 7D12 (A) or EGa1 (B) expressed on the tip of filamentous phage to EGFR was tested in the presence of: EGa1 nanobody (EGa1), EGa1-nanobody-liposomes at 0.4 and 0.8 nmol/μmol TL (EGa1-0.4-L and EGa1-0.8-L), 7D12 nanobody (7D12), 7D12-nanobody-liposomes at 0.4 nmol/μmol TL (7D12-0.4-L), 425 single chain antibody fragment (425scFv), 425scFv-liposomes at 0.4 nmol/μmol TL (425scFv-0.4-L), Erbitux, and liposomes (L). Bound phage was detected with a HRP-coupled anti-M13 monoclonal antibody and staining with OPD/H2O.

To determine whether the observed downregulatory effect was either dependent on the epitope to which nanobody-liposome bound EGFR, or was a specific feature of the nanobodies when coupled to liposomes, the level of EGFR on cell surface was determined two days after a *pulse* incubation of 14C cells with the different liposomes formulations.
As depicted in Figure 7, both the cell surface level of EGFR and total EGFR content were reduced when cells were treated with 7D12-nanobody-liposomes. In fact, the observed effect was similar to the downregulation induced by the EGa1-0.4-L formulation.

However, this was not observed for 425scFv-liposomes, suggesting that the pronounced downregulatory effect here reported is a feature exclusive to the nanobody-liposomes. Statistical analysis revealed no significant differences for EGa1-0.4-L and 7D12-0.4-L ($p>0.05$) but significant differences for EGa1-0.4-L and 425scFv-0.4-L ($p<0.05$), EGa1-0.8-L and 425scFv-0.4-L ($p<0.01$), and as expected for L and EGa1-0.4-L ($p<0.01$), and L and EGa1-0.8-L ($p<0.001$).

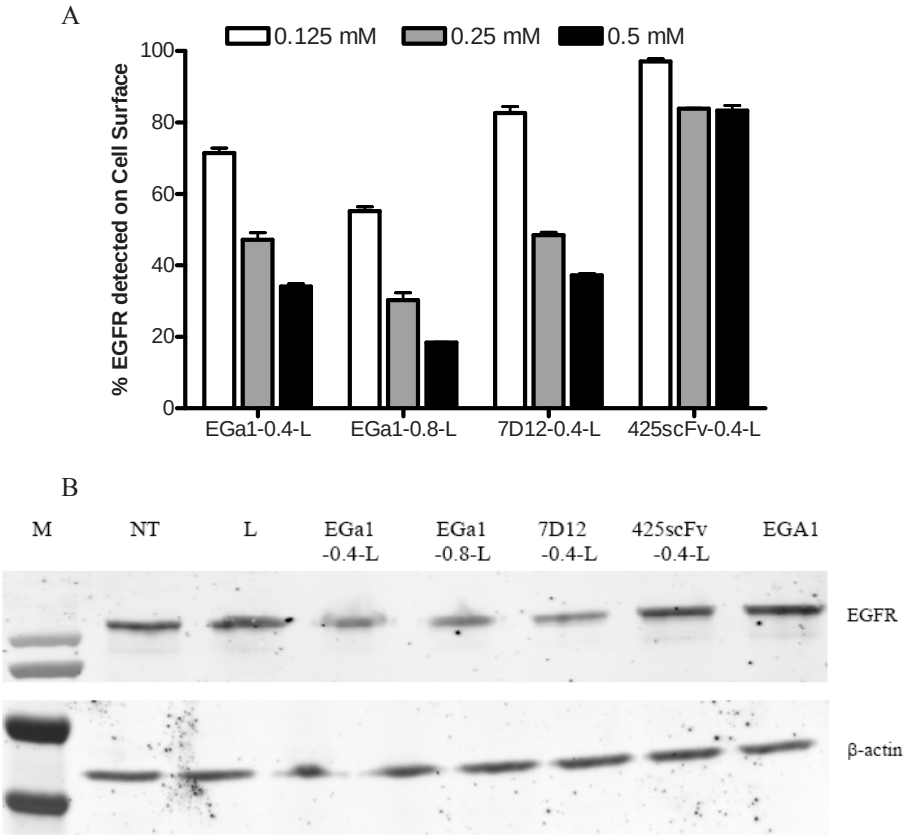


Figure 7. EGFR downregulation is independent of the EGFR epitope recognised but a specific feature of nanobody-liposomes.
Cells were incubated for 4 h at 37 °C with different liposome formulations: EGa1 nanobody liposomes, 7D12 nanobody liposomes and 425 single chain fragment (scFv) liposomes. **A.** After the incubation with liposomes at 0.5, 0.25 and 0.125 mM TL concentrations, cells were further incubated for 2 days. Thereafter, cells were incubated with an alexa-488-anti-EGFR antibody for 1 h at 4 °C to determine the cell surface EGFR. **B.** Cell lysates were prepared from samples incubated with 0.5 mM TL concentration. Western blots were prepared and both the total EGFR level (upper panel) and β-actin level (lower panel, loading control), were determined. EGa1-0.4-L – EGa1-nanobody-liposomes at 0.4 nmol/μmol TL; EGa1-0.8-L – EGa1-nanobody-liposomes at 0.8 nmol/μmol TL, 7D12-0.4-L – 7D12-nanobody-liposomes at 0.4 nmol/μmol TL, 425scFv-0.4-L – 425 single chain fragment antibody-liposomes at 0.4 nmol/μmol TL, M – marker for molecular weight, NT – non-treated, L – liposomes, EGa1 – free EGa1 nanobody.

EGa1-liposomes inhibit cell proliferation of 14C cells in a dose-dependent manner

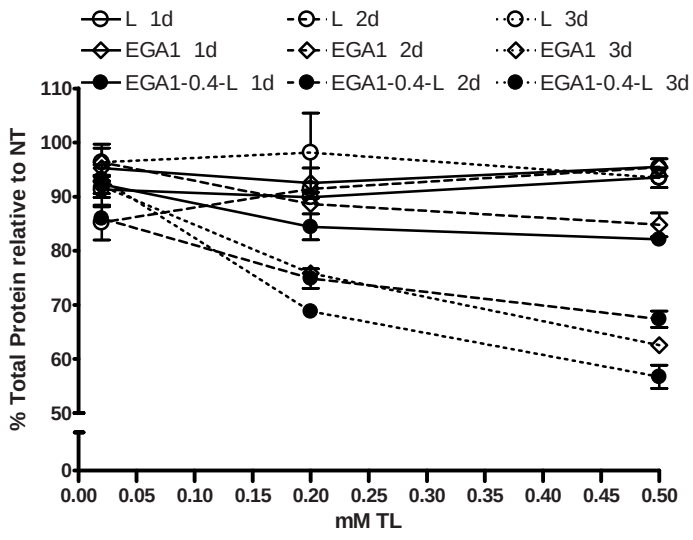
In order to investigate the potential therapeutic effect of this new targeted drug delivery system, empty nanobody-liposomes (EGa1-L) were tested for their effect on tumour cell proliferation.

The detection of total cellular protein was used as a marker for cell growth and total cell number determination [31]. Figure 8A shows increasing reduction of cell growth with prolonged incubation with the nanobody-liposomes: starting from 15% reduction after 1 day, to 30% and 45% after 2 and 3 days, respectively at 0.5 mM TL. EGa1-liposomes were slightly more active than EGa1 alone. However, at higher concentrations (2.0 mM TL, Figure 8Aii.) the effects were much stronger. Reduction of cell growth varied from 40, to 70 and 90% for nanobody-liposomes, and from 10, 20 and 40% for EGa1 nanobody alone, after 1, 2 and 3 days of incubation, respectively (after 1 day $p>0.05$ for L and EGa1; for each day $p<0.001$ for EGa1-L and EGa1).

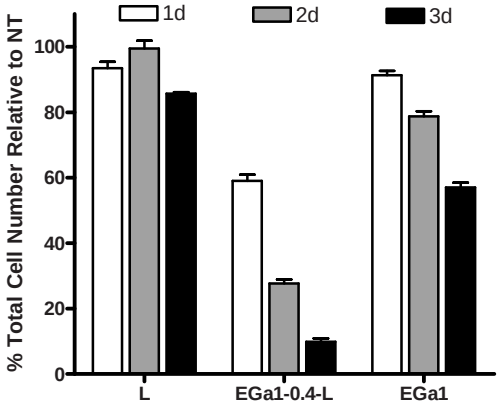
To support these results, cell proliferation based on BrdU-incorporation was tested. In this assay, the number of dividing cells is assessed as an alternative to total protein determination. Figure 8B shows a reduction of cell proliferation by the EGa1-0.4-L formulation from 20 to 55% for 0.5 and 2.0 mM TL, respectively, which is consistent with the effect observed in the previous assay.

Overall, these results clearly show that a significant inhibition of tumour cell proliferation was induced by nanobody-liposomes and that the effect is concentration dependent.

Ai.



Aii. 2.0 mM TL



B

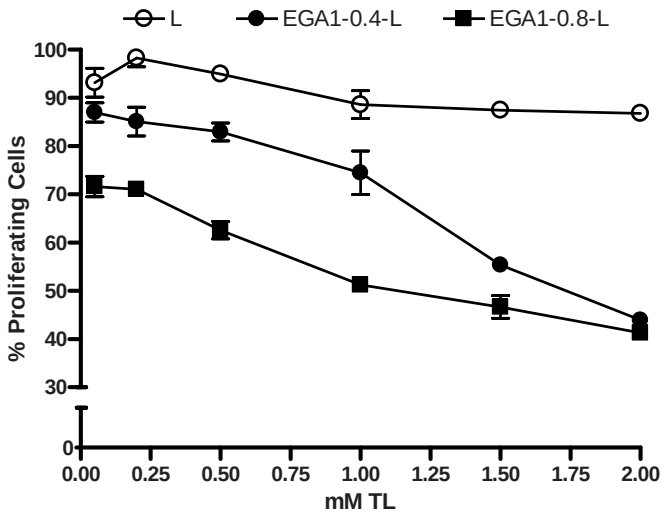


Figure 8. EGa1-liposomes inhibit cell proliferation of 14C cells
A. Total cell number: cells were incubated with liposomes for 1, 2 and 3 days at concentrations ranging from 0.02 to 2 mM TL. After the incubation, total cellular protein was precipitated by TCA and SRB used for detection. Values plotted were determined relative to non-treated samples (100% total cell number). **B.** Assay by incorporation of BrdU: cells were incubated for 2 days with liposomes of 0.02 – 2 mM TL concentrations. After 2 days, medium and cells were incubated one more day with BrdU. Values plotted were determined relatively to non-treated samples (100% proliferation). L – liposomes, EGa1 – free EGa1 nanobody, EGa1-0.4-L – EGa1-nanobody-liposomes at 0.4 nmol/μmol TL, EGa1-0.8-L – EGa1-nanobody-liposomes at 0.8 nmol/μmol TL.

DISCUSSION

In this manuscript, a new targeted drug delivery system is presented: anti-EGFR nanobodies coupled to the surface of liposomes. The coupling of the nanobody to the liposome surface induced EGFR-mediated cell association and cellular uptake of the liposomes. Surprisingly, nanobody-liposomes caused a significant loss of cell surface EGFR, which was maintained for several days. This effect was dependent on the amount of nanobody coupled to the liposomes. The remarkable sequestration of cell surface EGFR, induced by nanobody-liposomes, was found to be independent of receptor activation and to cause EGFR downregulation. The observed inhibition of tumour cell proliferation, which is induced by empty EGa1-liposomes, shows great promise for the therapeutic application of this new and active drug delivery system.

The remarkable sequestration of cell surface EGFR and consequent downregulation, mediated by nanobody-liposomes, is the most striking observation in this study. Even though the possibility of steric hindrance may be considered when EGFR was detected right after the *pulse* incubation, the disappearance of EGFR is likely not overestimated. In fact, similar reductions of cell surface EGFR (90%, Figure 3C) were detected after two days of *chase*, when it is very unlikely that liposomes remain attached to the cell surface. Furthermore, these results are supported by the distinctive downregulatory effect induced by the nanobody-liposomes, clearly observed in Figure 5. The effect here reported was not observed for uncoupled nanobody. EGa1 alone only induced a small reduction of cell surface EGFR of 30-40% right after the *pulse* incubation, which was further minimised to 20 and 10% after two and three days of *chase*, respectively. As total cellular EGFR content was not reduced, the downregulatory effect observed for nanobody-liposomes seems to be absent. These effects are likely related to the multivalency of the nanobody-liposomes, which probably allows cellular association mediated by several receptors together and, subsequently, induces a major decrease of surface EGFR upon liposome internalisation. This possibility is in agreement with the study by Friedman *et al* in which the formation of large lattices, by combination of different monoclonal antibodies, is suggested to induce a faster removal of cell surface EGFR and consequent degradation [32]. Also, a study by Jaramillo *et al* suggests that a fast removal of cell surface EGFR that alters EGFR cellular distribution increases the chance of EGFR degradation [33].

Furthermore, this observation is suggested to be specific for nanobody-liposomes as a similar approach with scFv coupled to liposomes showed no downregulatory effect (Figure 7). A possible explanation is based on the strength of the association between ligand and receptor. Generally, when the ligand remains bound to the receptor at the slightly acidic pH in the late endosomes, the complex is directed to the lysosomes where both are degraded. Nanobodies are generally known to bind very tightly to their antigens and even at low pH (unpublished data). This aspect may well be involved in the downregulatory effect induced by nanobody-liposomes. A less stable association of scFv with the receptors in the endosomal compartment could then explain the observed difference between nanobodies and scFv. In this sense, the differences between nanobody coupled liposomes and nanobodies alone could be explained by the multivalency of the

liposomal system that likely favours receptor dimerisation. In a similar context, Perez-Torres *et al* reported that antibody-mediated downregulation of EGFR requires receptor dimerisation, and this is not observed for monovalent Fabs of cetuximab [34].
The mechanism by which nanobody-liposomes induce EGFR downregulation needs further clarification. In addition, the lipid composition of the liposomes, their fate inside the cell after the probable redistribution to cellular organelles, and their influence in the mechanism here involved, are part of another investigation.
Even though uncoupled EGa1 nanobody was not able to induce a detectable EGFR downregulation, it functioned as an antagonist to EGFR, preventing ligand binding and receptor activation (Figure 4). This is in agreement with previous studies [15]. The antagonism translated into 40% inhibition of tumour cell proliferation after three days of incubation at 2 mM TL. However, nanobody-liposomes under the same conditions induced a 90% inhibition of cell proliferation (Figure 8A). Again, the downregulation of EGFR expression by the multivalent systems seems to offer substantial benefits over the monovalent nanobody.

We have established the potential therapeutic effect of the empty nanobody-liposomes *in vitro*. This encourages studies to determine the therapeutic effect of this formulation *in vivo*. As the nanobody-liposomes can be loaded with different types of therapeutic molecules, a wide range of opportunities for this new drug delivery system is opened ranging from conventional chemotherapeutic agents to nucleic acids [17]. Also, recent studies have employed small molecular tyrosine kinase inhibitors encapsulated in targeted liposomes [35]. EGFR inhibition has been reported as more effective when different inhibitors of EGFR are combined, or when different growth factors and respective receptors are inhibited [36]. As an example, the simultaneous inhibition of EGFR and insulin-like growth factor receptor (IGFR) has already been explored [37]. This new carrier system shows great potential for such combinations through direct downregulation of EGFR mediated by the coupled nanobody and by inhibition of other signalling pathways by incorporation of effective and specific drugs in the liposomes. In all these cases, synergistic effects are expected.

In conclusion, we describe a new and attractive targeted drug delivery system. The observed EGFR downregulation induced by empty nanobody-liposomes proved to have therapeutic potential and, together with the variety of antitumour agents that can be incorporated in this system, anti-EGFR nanobody-liposomes promise to be exceptional potent drug carriers for targeted tumour therapy.

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CHAPTER 8

SUMMARISING DISCUSSION

EPIDERMAL GROWTH FACTOR RECEPTOR: A RATIONAL TARGET

Cancer therapy is shifting from the non-specific cytostatic activity of conventional chemotherapy, to a target oriented therapy. This is enabled by recent advances in cancer research that have indentified cancer-specific molecular targets. These molecular targets, involved in either the initiation or the progression of cancer, have been used for the development of new *smart cancer drugs* [1, 2]. A recent review by Yasui *et al* discusses approximately 30 new rationally designed anticancer drugs and divides those into three categories: targeting cell surface receptors, targeting intracellular pathways, and targeting cell maintenance processes. At present, 20 out of the 30 target-specific drugs described have already been approved by the responsible authorities and are currently employed in the clinic [3].

The epidermal growth factor receptor (EGFR) is an example of an extensively investigated molecular target and, as described in the introductory **Chapter 1**, it is also the target in this thesis**.** In healthy cells, the activation of this protein kinase receptor, after ligand binding, initiates several signalling pathways that reach the nucleus and consequently stimulate cell proliferation, differentiation, survival, adhesion and migration [4]. Many human cancers, such as cancers of the head and neck, breast, lung, pancreas, and colon, overexpress the EGFR and are characterised by dysregulated EGFR signalling. In these cases, EGFR activation is associated with uncontrolled cell proliferation, inhibition of apoptosis, stimulation of angiogenesis, tissue invasion and metastasis [5].

EGFR-targeted molecules have been developed to interfere with EGFR activation and related signalling pathways. The main classes of EGFR inhibitors are monoclonal antibodies (mAbs) and tyrosine kinase inhibitors (TKIs). At present, two mAbs (cetuximab and panitumumab) and two TKIs (gefitinib and erlotinib) are approved for clinical use [6]. mAbs block ligand from binding to the receptor and prevent receptor dimerisation, whereas TKIs bind to the ATP-binding site preventing receptor phosphorylation and activation. In **Chapter 2**, these and other strategies for EGFR inhibition are discussed, underlying that EGFR inhibition can be beneficial in cancer therapy.

TARGET EGFR FOR SILENCING

The main approach followed in this thesis to interfere with EGFR is to silence the expression of the EGFR protein, by use of small interfering RNA (siRNA). Theoretically, siRNA can be used to interrupt the expression of any protein, when the corresponding mRNA sequence is used as a template to design target-specific siRNA molecules.
siRNA specifically targeting the EGFR was first reported in 2003 by Nagy *et al,* who showed extensive knockdown of EGFR expression on A431 human epidermoid carcinoma cells [7]. Other *in vitro* studies followed [8, 9] in general showing EGFR inhibition leading to cell cycle arrest, induction of apoptosis, and inhibition of cell proliferation and migration. These reports added a new class of EGFR inhibitors. Since then, the therapeutic potential of EGFR silencing has now also been demonstrated *in vivo*. A recent study by Li *et al* has reported tumour growth inhibition in human lung cancer xenografts, after three daily i.v. injections of anti-EGFR siRNA formulated in a self-assembling nanoparticle [10].

Endosomal escape: a limiting factor for silencing

For efficient silencing, siRNAs need to be present in the cytoplasm of the target cells, where the RNAi machinery exists. This can be achieved by the intracellular production of siRNA, through expression of genes encoding for short-hairpin RNA (shRNA), or by direct intracellular delivery of chemically synthesised siRNA. The general preference for chemically synthesised siRNA is certainly due to the fact that these siRNAs do not need to pass the nuclear membrane to induce RNAi, contrary to shRNA-encoding DNA, which circumvents a significant barrier.
In this thesis, a lipid-based carrier was used to deliver anti-EGFR siRNA into cells. Lipofectamine is a commercially available lipid carrier (from Invitrogen, [11]) widely used in nucleic acid delivery. The cationic lipid mixture forms charge interactions with the negative siRNA, resulting in complexes (lipoplexes) with a net positive charge. This positive charge on the lipoplex surface promotes interaction with the negatively charged cell surface, allowing internalisation of the lipoplexes mainly by endocytosis. However, before reaching the cell cytoplasm, siRNAs need to escape from endosomes.
Many lipid- and polymer-based carrier systems possess endosomal escape properties. For example, liposomes containing the neutral helper lipid dioleoyl phosphatidyl-ethanolamine (DOPE) have shown improved endosomal escape [12] and the activity of the polymer polyethylenimine (PEI) has been attributed to its buffering capacity allowing swelling of endosomes and membrane permeabilisation (i.e. proton sponge effect) [13]. New polyamidoamines with disulfide bonds (SS-PAA) have been reported to have even higher buffering capacities than PEI [14] which could result in very efficient endosomal escape.
Lipofectamine contains DOPE, thus suggesting endosomal escape properties. However, our studies resulted in relatively inefficient silencing efficiencies (in general below 20% knockdown). Therefore, the possibility to enhance endosomal escape, and consequent silencing efficiency, was investigated. Two methods were employed to facilitate the endosomal escape of siRNA-Lipofectamine (LF) lipoplexes.

In **Chapter 3** a fusogenic peptide, diINF-7, synthesised based on the fusion domain of the influenza virus, is utilised in association with the lipoplexes. This peptide has previously been employed showing effective permeabilisation of endosomes [15] and, coupled to certain polymers (such as pDMAEMA, poly(2-(dimethylamino)ethyl methacrylate), it enhances transfection efficiencies [16].
To test the fusogenic capacity of diINF-7 associated with siRNA lipoplexes, three cell lines were used: C26 murine colon carcinoma, A431 human epidermoid carcinoma, and 14C human head and neck squamous cell carcinoma. In the first, K-ras was the target protein. Results showed a 4-fold enhancement of the silencing efficiency for lipoplexes when combined with the fusogenic peptide. For the other two cell lines, EGFR was the target. In these cells, the silencing efficiency was enhanced 2-fold in A431 cells and 1.2-fold in 14C cells. This study shows that endosomal escape is a limiting factor for siRNA silencing efficiency.
Chapter 4 describes, for the first time, the use of photochemical internalisation (PCI) to trigger the endosomal release of entrapped siRNA lipoplexes *in vitro*. PCI was first described in 1999 [17]. The technique makes use of a photosensitiser that accumulates at the endosomal membranes. Upon light exposure, the photosensitiser is excited and subsequently participates in the formation of reactive oxygen species that are responsible for the destabilisation of the endosomal membranes, enabling the release of the entrapped molecules into the cell cytoplasm.
In our studies, the PCI principle was confirmed by confocal microscopy images. Cells on which PCI was applied presented a diffuse siRNA-fluorescence, suggesting endosomal escape, as compared to a punctuated pattern when cells were not exposed to light. This evidence of light-triggered endosomal escape was supported by the enhanced EGFR silencing in A431 cells when anti-EGFR siRNA-Lipofectamine lipoplexes were combined with PCI. Overall, PCI enhanced the silencing efficiency of lipoplexes 10-fold at conditions that did not compromise cell viability. Again, these results show that Lipofectamine-mediated siRNA delivery can be improved by methods that promote endosomal escape and give supporting evidence for endosomal escape limiting siRNA silencing efficiency.
Recently, other studies have also combined carriers possessing endosomal escape properties with PCI, showing improvements of silencing efficiencies. Bøe *et al* reported on the benefit of combining PCI with the commercial carrier JetSI (Polyplustransfection) [18]. This cationic lipid carrier was designed with a proton sponge mechanism similar to PEI. Nevertheless, the carrier has relatively low silencing efficiencies. The combination with PCI dramatically increased the efficiency. The authors also tested Lipofectamine, although, in their case no enhancement of the silencing efficiency was obtained when siRNA-Lipofectamine lipoplexes were combined with PCI. This is most likely related to the high silencing efficiency obtained by the lipoplexes alone (90% knockdown). A different study showed that the silencing efficiency of PEI polyplexes could also be improved with PCI, depending on the structure of the polymer, the ratio of siRNA:polymer used, and the settings in which PCI was applied [19].
Our results highlight the importance of endosomal escape efficiency for functional siRNA delivery. Taking into account the side effects associated with administration of

high doses of siRNA, the use of lower concentrations of siRNA enabled by improved endosomal escape is very appealing [20-22]. The two methods employed in this thesis are examples of efficient endosomal escape enhancers that ensure effective delivery of siRNA into the cell cytoplasm.

Photochemical internalisation: feasible *in vivo*

PCI was first employed *in vivo* in 2001 for the cytosolic delivery of gelonin, a ribosome-inactivating protein that is toxic to cells when present in their cytoplasm [23]. However, this protein is unable to cross cell membranes. Therefore, PCI was employed to promote endosomal escape. The authors showed absence of growth inhibition in human adenocarcinoma xenografts for gelonin alone, whereas the combination with PCI produced strong inhibitory effects. Later, Berg *et al* reported the use of PCI for delivery of the chemotherapeutic agent bleomycin showing a synergistic inhibition of tumour growth in three different tumour models for the PCI bleomycin combination [24]. PCI was also used for gene delivery: Ndoyed *et al* reported in 2006 a successful study where PCI was used to enhance endosomal escape of polyplexes transporting p53 gene, administered intratumorally, in mice bearing head and neck squamous cell carcinoma xenografts [25].

As described in **Chapter 5**, PCI can also be successfully employed *in vivo*, for intratumoral delivery of siRNA. EGFR expression was reduced 80% in A431 tumour xenografts when lipoplexes were administered with PCI, compared to 30% knockdown induced by the lipoplexes alone. The 80% knockdown of EGFR only translated into a small tumour growth delay of approximately 3-4 days. The small effect on tumour growth suggests a rapid recovery of tumour cells after silencing of EGFR expression. Indeed, siRNA is known to induce a transitory effect [26]. In rapidly dividing cells, such as A431 tumour cells, a single dose of siRNA is quickly diluted resulting in short term effects. Consecutive treatments would probably prolong efficacy. Taken together, in this chapter PCI was successfully employed to enhance the silencing efficiency of locally injected siRNA lipoplexes, inducing a strong reduction of EGFR expression.

Altogether, these PCI studies demonstrate the applicability of this technique *in vivo*. In fact, a Phase I clinical trial employing PCI for delivery of bleomycin is currently in preparation (sponsored by PCI Biotech).

The application of PCI *in vivo* involves special concerns. The formation of reactive oxygen species that destabilise the endocytic vesicles is similar to photodynamic therapy (PDT). Therefore, a careful determination of the conditions under which PCI is employed is necessary. The main differences between PCI and PDT are in the intensity of the photochemical reactions and nature of the photosensitiser molecule employed. Regarding the intensity, PCI should confine damage to endosomal membranes, whereas PDT aims at a more general cellular damage leading to cell death (e.g. mitochondria, Golgi apparatus, endoplasmic reticulum, or plasma membrane) [27].

Singlet oxygen is the main reactive compound formed after the photosensitiser is excited by light exposure and it is known to damage mainly cellular membranes by oxidising amino acids, unsaturated fatty acids and cholesterol. Because singlet oxygen has a short lifetime (0.01-0.04 μs) and a short diffusion range (10-20 nm), the damage induced is

generally restricted to the area where the photosensitiser is localised. PCI usually involves a photosensitiser that is amphiphilic and consequently accumulates in endosomal membranes. Side effects of PCI can occur, for instance, when insufficient time is given for the photosensitiser to localise at the endosomal membranes, or when the photochemical reactions induced produce too many reactive oxygen species. In this sense, it is important to include control groups in the studies so that the effect of the application of PCI alone is determined. The study by Selbo *et al* and our study showed the abovementioned side effects that can be referred to as PDT. However, a clear benefit on inhibition of tumour growth was observed when this was combined with the PCI-mediated cytosolic delivery of gelonin and anti-EGFR siRNA, respectively. In cancer, a PDT effect may actually be of advantage to the treatment. Naturally, in situations where delivery of the therapeutic molecules is not intended for cell proliferation arrest or cell death, a PDT effect is undesirable. For this reason, parameters such as photosensitiser concentration, light dose, and time interval between the applications of these two need to be carefully optimised according to the particular application.

Our *in vivo* PCI studies were performed based on the gene delivery study by Ndoyed *et al* and the siRNA-Lipofectamine lipoplexes were administered intratumorally. Intravenous administration of lipoplexes is likely compatible with PCI applications, as long as the lipoplexes efficiently accumulate at the tumour site. Furthermore, photosensitiser molecules could also be administered systemically [28, 29]. Potentially, photosensitiser and siRNA, or other therapeutic molecules, could be incorporated in one carrier system [30]. This would facilitate PCI-mediated therapy, provided that the therapeutic molecules accumulate inside endosomes and the photosensitiser at their membranes.

Because the photosensitiser depends on light exposure to be activated, PCI will only occur at the illuminated areas, which limits the possible side effects. Light exposure of the target tissue or organ can be a limiting step for PCI efficiency. The wavelengths (600-800 nm) employed for this purpose usually penetrate skin to a maximum of 2 cm depth [31]. At the same time, the treatment of internal organs has become possible due to the development of light sources and fibre optics allowing light exposure of virtually any region of the human body. These advances have increased the possible applications of PCI as a tool for drug delivery.

Delivery of siRNA: local and systemically

Direct delivery of siRNA into the target organ/tissue appears to be a promising approach used by many researchers in preclinical studies and also in clinical trials. Three of the clinically most advanced siRNAs have been developed for age-related macular degeneration and are directly injected into the vitreous compartment. Another siRNA-based therapy is developed for treating respiratory syncytial virus and is delivered locally through inhalation. Local delivery of siRNA appears to be the most attractive route of administration for delivering RNAi therapeutics as it circumvents many of the hurdles encountered during the transport phase of siRNA delivery. However, local administration is not always possible and a number of delivery systems are being developed pre-clinically to achieve systemic delivery of siRNA [32, 33].

Chapter 6 discusses *in vivo* targeted delivery of siRNA after intravenous administration. For this administration route, delivery systems are necessary to transport the siRNA to the target tissue. To prevent rapid clearance of these systems by macrophages of the mononuclear phagocyte system (MPS), shielded carrier systems have been designed. Especially, polyethylene glycol (PEG) is a commonly employed shielding molecule that enhances colloidal stability and reduces surface charge, thereby preventing interactions with proteins and cells. The delivery systems also need to assure penetration into the target tissue. For this, ligands are usually coupled to the delivery systems that allow target-cell association. In this chapter, chemical modifications of siRNA molecules are also discussed. They are introduced to improve siRNA stability and capacity to cross membranes.

These and other advances have contributed to the recent commencement of clinical trials employing intravenous administration of siRNA [32]. The first systemic administration of siRNA occurred in November 2007, in a Phase I clinical trial of a chemically modified siRNA, AKIi-5, developed for the treatment of acute renal failure (Quark Pharmaceuticals / Silence Therapeutics). This siRNA is designed to inhibit the expression of the transcription factor p53, which is involved in apoptosis generally associated with tubular cell death in acute kidney injury. The i.v. administration of a nanoparticle containing siRNA occurred for the first time in June 2008, in a Phase I trial using CALAA-01 (Calando Pharmaceuticals). The siRNA is formulated in a nanoparticle named RONDEL (RNAi-oligonucleotide nanoparticle delivery). CALAA-01 is a non-chemically modified siRNA against the M2 subunit of ribonucleotide reductase and RONDEL is a three-part delivery system: the first is composed of cationic and linear cyclodextrin, that forms complexes with anionic siRNA; the second is the surface modification for increased stability and serum half-life, which involves PEG attached to adamantane groups that form inclusion complexes with cyclodextrin; and the third is the incorporation of targeting ligands, such as transferrin, that assure targeting of the nanoparticles to the tissues of interest.

Even though the current state-of-the-art in clinical delivery of siRNA is local administration, clinical trials now in progress reflect efforts made towards systemic application.

TARGET EGFR FOR DELIVERY

A different line of research followed in this thesis is concerned with improving the therapeutic efficacy of anticancer drugs by using the cancer cell-specific expression of EGFR to develop targeted drug delivery systems that could specifically deliver anticancer drugs to tumour cells.

Liposomes are important drug delivery systems in which chemotherapeutic agents (e.g. doxorubicin, lurtotecan, vincristine) and other molecules have been incorporated (e.g. peptides, siRNA, plasmid DNA) [34]. For successful delivery, the liposomal surface has usually been modified with polymers and targeting ligands in order to prolong circulation time and improve target-cell association.

The first report on liposomes targeting EGFR was published in 1989 and EGF was employed as the targeting ligand [35]. However, the use of EGF as a targeting ligand likely induces EGFR activation. Therefore, different ligands have been employed. Monoclonal antibodies (mAbs), fragments of antibodies (Fabs), or single chain variable fragments (scFv) recognising the EGFR, have been coupled to the surface of liposomes. These liposome formulations are generally known as immunoliposomes.

Nanobody-liposomes

In **Chapter 7** the preparation and characterisation of anti-EGFR nanobody-liposomes as a new drug delivery systems is described. This targeted system employs anti-EGFR nanobodies as targeting ligands, coupled to the surface of PEGylated cationic liposomes. Nanobodies are the smallest functional fragments of heavy-chain only antibodies. These are antibodies devoid of light chains, first identified in camelids [36]. The size of nanobodies is in the nanometre range, and their affinity and specificity to antigens is similar to that of mAbs. In addition, nanobodies are more hydrophilic than Fabs and scFv, and also re-fold very efficiently upon thermal denaturation. These characteristics seemed favourable for the application of nanobodies as targeting moieties on the surface of liposomes.

Other applications of nanobodies have been reported, such as for tumour imaging. Their small size proved to be ideal for tumour penetration and rapid clearance of unbound molecules [37, 38]. Furthermore, nanobodies have demonstrated therapeutic efficacy. Roovers *et al* employed trivalent, bispecific anti-EGFR nanobodies that induced tumour growth inhibition in A431 human epidermoid xenografts [39].

The anti-EGFR nanobody employed in this thesis, EGa1, was previously described as an antagonist for EGFR, i.e. EGa1 competes with EGF for EGFR binding without activation of the receptor [40]. Therefore, coupling of the EGa1 nanobody to long-circulating PEG-liposomes seemed to be an attractive strategy to develop a targeted drug delivery system that could also possess a certain biological activity due to the presence of the EGa1 nanobody.

As expected, the presence of EGa1 on the surface of liposomes resulted in an increased cell association and cellular uptake of liposomes by the 14C head and neck squamous carcinoma cells. The efficient uptake of EGa1-liposomes by the 14C cells induced a remarkable decrease of EGFR cell surface level (95% reduction), an effect not induced to the same extent by uncoupled anti-EGFR nanobody (only 30 to 40% reduction).

The sequestration of cell surface EGFR was determined to be independent of kinase activity and dependent on the amount of nanobody coupled to the liposomes. In addition, we observed that the cell surface EGFR recovered only slowly after its disappearance: a remarkable 50% reduction was still detected three days after incubation with nanobody-liposomes. Furthermore, the disappearance of cell surface EGFR was associated with downregulation of EGFR at the protein level, an effect not observed for free EGa1. The observed inhibition of tumour cell proliferation, induced by empty nanobody-liposomes, indicates therapeutic potential. Altogether, the novel nanobody-coupled liposomes show considerable promise as a targeted system with intrinsic therapeutic potential.

The mechanism of EGFR downregulation induced by nanobody-liposomes is not completely understood, but our studies suggest that this effect is exclusive to nanobodies coupled to liposomes. We observed that the nanobody alone is clearly not capable of inducing EGFR sequestration as significantly as nanobody-liposomes. This could be explained by the multivalency of the nanobody-liposomes that probably allows liposome interaction with several receptors. These receptors are subsequently removed from the cell surface upon liposome internalisation. Furthermore, the observed degradation of EGFR (downregulation) is possibly related to the high affinity nanobodies bind to antigens. Nanobodies appeared remarkably resistant to acid washes in experiments where cell surface removal of nanobodies was required. Inside endosomes, the stable bonds between receptor and nanobody-liposomes may direct the complex towards degradation in lysosomes.

For future studies on the therapeutic effect of these targeted liposomes, encapsulation of additional therapeutic molecules can be envisioned. This could be a combination with chemotherapeutic agents, kinase inhibitors or siRNAs, considering the variety of molecules that can be incorporated into liposomes.

PERSPECTIVES ON ANTI-EGFR THERAPIES

Over the years, many anti-EGFR therapies have been proposed and some of them are currently used in the clinic [6]. These are generally not given as monotherapy, but combined with chemotherapeutic agents or radiotherapy. Combinations are beneficial as tumour growth is determined by many signalling pathways in a variety of cell types present in the tumour microenvironment. Some of these signalling pathways are common to different growth factors, thus cancer cells may overcome the blockade of one particular receptor quite easily.

Some examples of combination therapies are provided in Chapter 2, principally the combination of anti-EGFR therapies (mAbs or TKIs) with traditional chemotherapy or radiotherapy; combinations of anti-EGFR therapies with therapies that inhibit other growth factors (such as VEGF or IGF); and combinations with anti-angiogenic therapies. These combinations are generally beneficial, inducing stronger antitumour effects.

Similarly, the approach described in this thesis of silencing the EGFR by use of siRNA may be insufficient. Others have reported on beneficial combinations of anti-EGFR siRNA and chemotherapeutic drugs [10, 41], and also on a combination of two siRNAs targeting different molecules, such as EGFR and COX-2 [42]. The combination of EGFR silencing with siRNA and TKI targeting proteins downstream of the EGFR signalling cascade has also shown promising results [43]. In this respect, the novel targeted drug delivery system described in Chapter 7 gives a wide range of opportunities to combine different anti-EGFR therapies or to combine one anti-EGFR therapy with another targeted-oriented strategy.

In conclusion, this thesis has addressed the inhibition of EGFR as therapeutic strategy. EGFR was inhibited by silencing of protein expression with siRNA and by downregulation induced by nanobody-liposomes. For the siRNA approach, this thesis addressed the delivery of siRNA *in vitro* and *in vivo*. The importance of endosomal escape properties of siRNA carriers was highlighted. Two methods improved endosomal escape of siRNA lipoplexes, photochemical internalisation and a fusogenic peptide, and consequently enhanced silencing efficiencies. For the downregulatory effect, anti-EGFR nanobody-liposomes revealed to be a novel and efficient targeted drug delivery system that holds intrinsic therapeutic potential and offers attractive properties for use in combination strategies.

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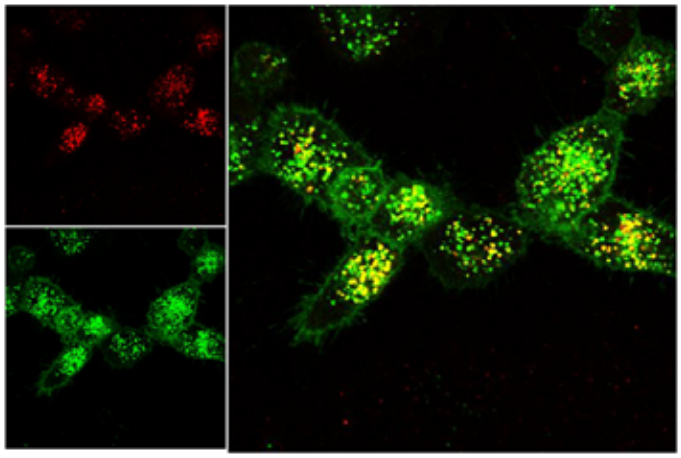
Appendix

COLOUR FIGURES

CHAPTER 4
Figure 1 (page 64)

A



B

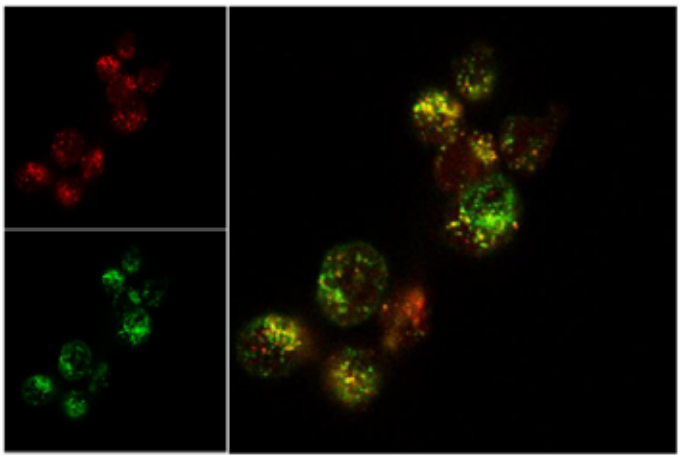


Figure 1. Release of siRNA in the cytoplasm after PCI.
Confocal Microscopy Images show cells incubated for 4 h with siRNA-Alexa633/LF complexes (red) and EGF-Alexa488 (green) (A), and cells which were illuminated after the 4 h-incubation (B). The punctuated pattern (A), most likely indicating localisation in endocytic vesicles, as indicated by co-localisation (yellow, in the overlay image) of complexes and EGF-Alexa488, gives place to a less punctuated and more diffuse pattern (B) where the fluorescence is spread throughout the cytoplasm of the cells. Cells were previously incubated with PS, as described in Materials and Methods.

CHAPTER 5
Figure 3 (page 84)

Panel 1 - Figure 3. Characteristics of performed treatments.

IHC	PS dose	siRNA 7.5μg	Light dose
A	0.1 μg/mm^3	EGFR	-
B	0.1 μg/mm^3	EGFR	75 J/cm^2
C	0.1 μg/mm^3	NS	90 J/cm^2
D	0.1 μg/mm^3	EGFR	90 J/cm^2

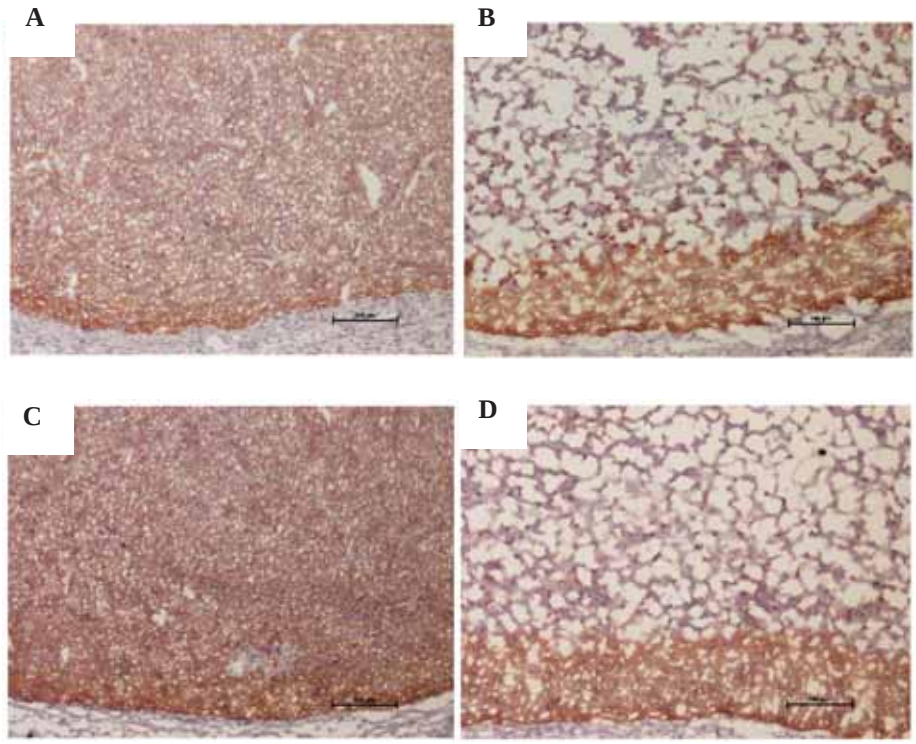


Figure 3. Detection of EGFR expression on tumour tissue.
Tumours harvested two (A, B) and three days (C, D) after the treatment (i.e. days 4 and 5) according to Figure 2. Panel summarising the characteristics of the treatments performed. Sections of snap frozen tumours were subjected to IHC staining where brown colour indicates EGFR protein.

CHAPTER 5
Figure 5 (page 87)

Panel 2 - Figure 5. Characteristics of performed treatments.

IHCi / IHCii / photo	PS dose	siRNA 7.5μg	Light dose
A / C / 1	0.1 μg/mm^3	NS	90 J/cm^2
B / D / 2	0.1 μg/mm^3	EGFR	90 J/cm^2

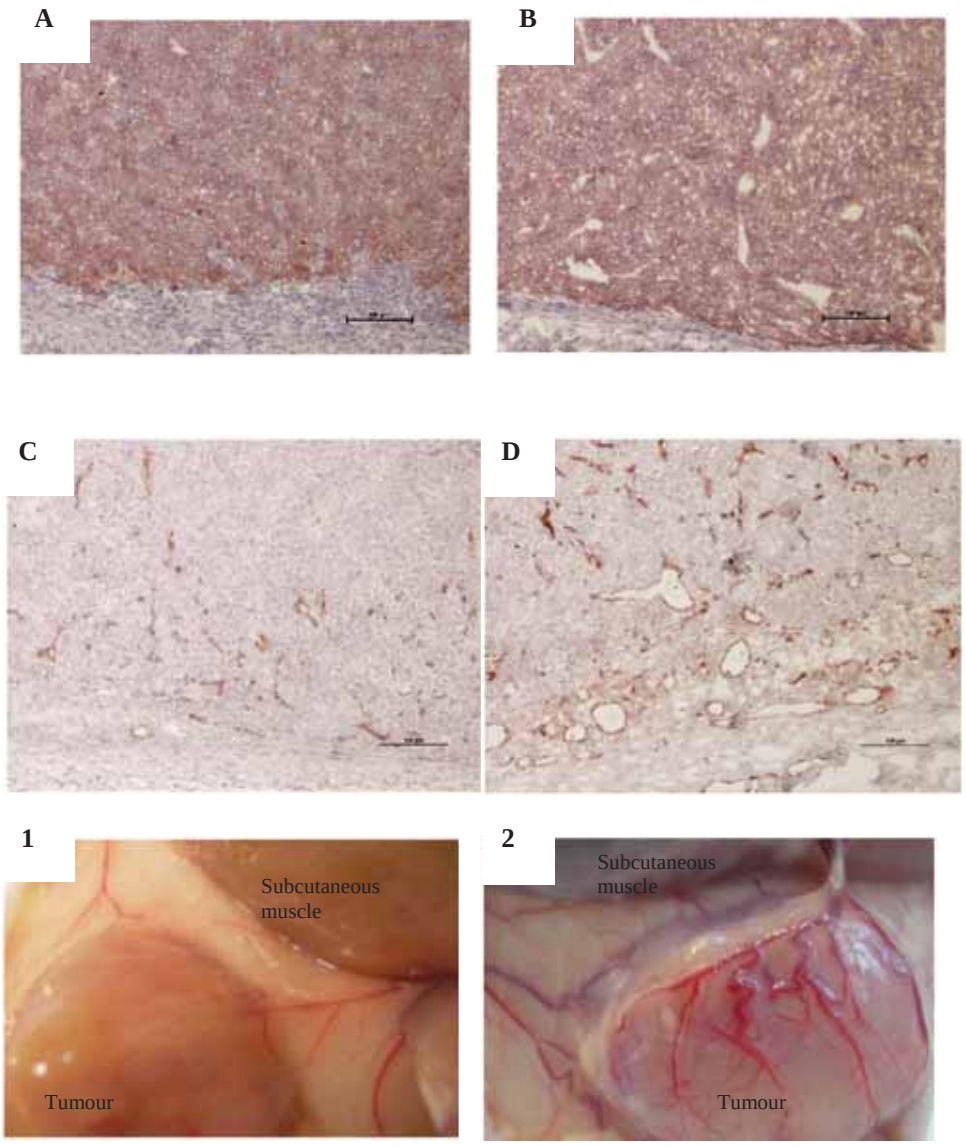


Figure 5. Detection of EGFR expression and blood vessels on tumour sections.
Tumours harvested at the last days of the experiment, when tumour size approached 2 cm^3, according to Figure 2. Panel summarising treatment settings. Sections of snap frozen tumours were subjected to IHC staining where brown colour indicates EGFR protein (A, B). IHC staining for CD31, marker of endothelial cells, indicating blood vessels in brown (C, D). Pictures (1, 2) taken when tumours were harvested.

CHAPTER 7
Figure 2B (page 127-128)

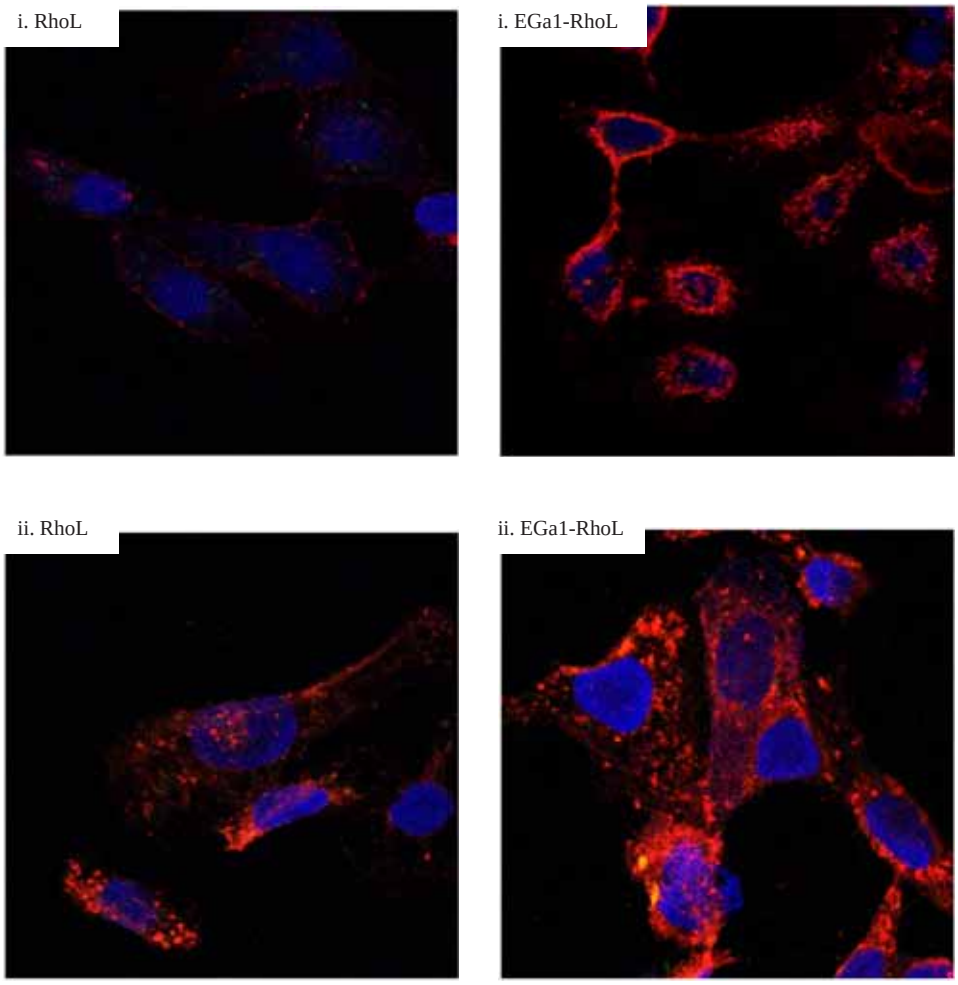


Figure 2. EGa1-liposomes display increased cell association compared with non-conjugated liposomes that results in enhanced cellular uptake.
B. Cells were incubated with fluorescently labelled liposomes (red colour) for 1 h at 4 °C (i.) or 4 h at 37 °C (ii.). After incubation, cells were washed, fixed, incubated with Draq5 (blue colour) for nuclear staining and finally mounted for visualisation. Left - RhoL – cells incubated with rhodamine-liposomes, right - EGa1-RhoL – cells incubated with EGa1-nanobody-rhodamine-liposomes.

SAMENVATTING IN HET NEDERLANDS

KANKERTHERAPIE

Het belangrijkste doel van kankertherapie is het stoppen van de proliferatie en de migratie van kwaadaardige cellen, liefst zonder andere cellen te beïnvloeden. Conventionele chemotherapie valt delende kankercellen aan door de celcyclus te blokkeren. Omdat kankercellen ten opzichte van gezonde cellen snel delen is chemotherapie tot op zekere hoogte selectief toxisch voor tumorweefsel. Maar niet alleen kankercellen delen snel. Hematopoietische cellen en haarfollikels kennen ook een snelle celcyclus. Daarom heeft chemotherapie veel bijwerkingen in sneldelende weefsels.

De laatste jaren zien we een verschuiving in de kankertherapie van de niet-specifieke cytostatische chemotherapie naar een doelgerichte, meer selectieve therapie. Dit is mogelijk door recente ontwikkelingen in het kankeronderzoek waarbij tumorspecifieke eiwitten zijn geïdentificeerd. Deze eiwitten, die zijn betrokken bij het ontstaan of de uitgroei van kanker, kunnen worden gebruikt voor de ontwikkeling van selectievere geneesmiddelen. Ook kunnen ze worden gebruikt om geneesmiddelen te verbeteren doordat deze eiwitten kunnen fungeren als doel voor gerichte aflevering van geneesmiddelen, het concept van *drug targeting*. Een toepassing is bijvoorbeeld het plaatsen van antilichamen tegen tumor-specifieke eiwitten op het oppervlak van liposomen. Deze zogeheten immunoliposomen kunnen zo een conventionele cytotoxische vracht specifieker afleveren in de tumor wat de effectiviteit ten goede komt en bijwerkingen reduceert.

DE EPIDERMALE GROEIFACTOR RECEPTOR IN TUMOR THERAPIE

De epidermale groeifactor receptor (EGFR) is een eiwit dat betrokken is bij kanker en in verhoogde mate op kankercellen tot expressie komt. Het eiwit is uitgebreid onderzocht als aangrijpingspunt voor therapie en als doelmolecuul voor *drug targeting* en wordt beschreven in **Hoofdstuk 1**. Het onderzoek vastgelegd in dit proefschrift richtte zich op deze receptor.

In gezonde cellen leidt activatie van de EGFR, na binding van een ligand, tot activatie van verschillende signaaltransductieroutes in de cel. Deze routes activeren cellulaire processen die celproliferatie, -differentiatie, -overleving, -adhesie en -migratie bevorderen. In vele humane tumoren, zoals kanker van het hoofd en halsgebied, borst, long, alvleesklier, en dikke darm, komt de EGFR verhoogd tot expressie. Deze tumoren worden gekenmerkt door ongereguleerde activatie van de receptor, dat op zijn beurt weer leidt tot ongecontroleerde celproliferatie, tot remming van apoptose en tot stimulatie van angiogenese en metastasering. De belangrijkste klassen van EGFR-remmers blokkeren binding van de ligand en/of activatie van de signaaltransductie. In **Hoofdstuk 2** zijn deze en andere strategieën voor remming van de EGFR besproken.

EGFR-INHIBITIE

De belangrijkste methode die in dit proefschrift is onderzocht om EGFR te remmen is het verminderen van EGFR-expressie met behulp van *small interfering RNA* (siRNA). Therapie met siRNA biedt, in theorie, vele aangrijpingspunten omdat het gebruikt zou kunnen worden om de expressie van elk eiwit dat bijdraagt aan een ziekte te verminderen door het coderende mRNA af te brekente.

Ontsnapping uit het endosoom: een beperkende factor voor siRNA

Voor efficiënte remming van EGFR-expressie met deze methode moeten de EGFR-specifieke siRNAs aanwezig zijn in het cytoplasma van de kankercellen. Omdat siRNA vrijwel niet spontaan de celmembraan kan passeren, zijn dragersystemen (*drug delivery* systemen) noodzakelijk die het siRNA kunnen afleveren in het cytoplasma. Deze *drug delivery* systemen worden na binding aan de cel over het algemeen opgenomen via endocytose, en dus is ontsnapping van de siRNA-*delivery* systemen uit het endosoom nodig om het cytoplasma te bereiken. Sommige *delivery* systemen zijn tamelijk efficiënt wat betreft deze ontsnappingsstap. Bij Lipofectamine bijvoorbeeld, zorgt het helperlipide DOPE voor fusie met de endosoommembraan waardoor siRNA het cytoplasma zou kunnen bereiken. Onze resultaten toonden echter aan dat Lipofectamine beperkt actief was. In het algemeen werd de expressie van EGFR met minder dan 20% gereduceerd. Daarom werd onderzocht of deze ontsnapping uit het endosoom verder verbeterd kon worden. Twee methodes werden gebruikt.

In **Hoofdstuk 3** is een, van het griepvirus afgeleid, fusogeen peptide getest in combinatie met lipoplexen van siRNA en Lipofectamine. De resultaten toonden aan dat de aanwezigheid van het peptide leidt tot een verbeterde reductie van eiwitexpressie.

Hoofdstuk 4 beschrijft, voor het eerst, het gebruik van fotochemische internalisatie (PCI) om siRNA-lipoplexen uit het endosoom te laten ontsnappen. De PCI-techniek maakt gebruik van een fotosensitizer die in de endosomale membraan accumuleert. Na blootstelling aan licht wordt de fotosensitizer aangeslagen wat leidt tot vorming van reactieve zuurstofradicalen die de endosomale membraan kunnen destabiliseren.

In onze studies werd het principe van PCI bevestigd met confocale microscopie-opnamen die de verbeterde ontsnapping van siRNA-lipoplexen naar het cytoplasma lieten zien. Bovendien leidde het toepassen van PCI tot een 10-voudige verbetering van de EGFR-expressie inhibitie vergeleken met de lipoplexen alleen.

Beide studies tonen aan dat de ontsnapping uit het endosoom een belangrijke beperkende factor voor siRNA-efficiëntie is en dat de beschreven strategieën de effectiviteit van siRNA-lipoplexen kunnen verbeteren. Omdat zo lagere concentraties siRNA gebruikt kunnen worden, zullen bijwerkingen veroorzaakt door hoge siRNA spiegels aanzienlijk verminderd kunnen worden.

PCI *in vivo*

Hoofdstuk 5 laat zien dat PCI ook de efficiëntie van siRNA-lipopexen *in vivo* kan verbeteren. EGFR-expressie werd met 80% gereduceerd in A431 tumoren na intratumorale injectie van lipoplexen en het toepassen van PCI. Dit in vergelijking met 30% reductie door lipoplexen alleen. Desalniettemin vertaalde deze 80% EGFR reductie zich slechts in een marginale vertraging van de tumorgroei van ongeveer 3-4 dagen. Dit kleine effect op de tumorgroei is waarschijnlijk te wijten aan een herstel van EGFR-expressie in de tumorcellen. A431 tumorcellen delen snel waardoor de siRNA concentratie wordt verdund. Verlenging van de behandeling lijkt logisch om het therapeutische effect uit te breiden.

Ondanks deze gunstige resultaten is de toepassing van PCI *in vivo* niet eenvoudig. De vorming van reactieve radicalen die de endosomale membraan moeten destabiliseren is, in velerlei opzicht, gelijksoortig aan fotodynamische therapie (PDT). Het doel van PDT is echter het vernietigen van de cel door de radicaalvorming. In kanker kan dit PDT-effect voordelig zijn, zoals aangetoond in **Hoofdstuk 5**. Echter, in sommige situaties is dit effect ongewenst. Om de toxische effecten van de fotosensitizer te controleren is een zorgvuldige evaluatie noodzakelijk van de condities waaronder PCI toegepast wordt. Om deze reden moeten allerlei parameters zoals fotosensitizer concentratie, lichtdosis en tijdsinterval tussen de toediening van fotosensitizer en licht zorgvuldig worden geoptimaliseerd.

Afleveren van siRNA: lokaal en systemisch

Lokale toediening van siRNA in het doelorgaan/weefsel is op dit moment de meest toegepaste methode in pre-klinische en klinische studies. Voorbeelden hiervan uit klinische studies zijn inhalatie in de longen (voor het bestrijden van respiratoir syncytiëel virus) en intravitreale injectie in het oog (voor het bestrijden van ‘natte’ macula degeneratie). Maar lokale toediening is niet altijd mogelijk en daarom worden ook *drug delivery* systemen ontwikkeld voor het afleveren van siRNA via de systemische route.

Hoofdstuk 6 richtte zich op het ontwerp van *drug delivery* systemen voor intraveneuze toediening van siRNA *in vivo*. Om gebruik te kunnen maken van deze toedieningsroute, moet het *drug delivery* systeem in staat zijn het siRNA via de bloedbaan te vervoeren naar de plaats van de ziekte en het siRNA in staat stellen om het cytoplasma te bereiken van de cellen die het ongewenste eiwit tot expressie brengen. Het blijkt lastig om systemen te ontwikkelen die op beide fronten goed presteren. Op dit moment is slechts één siRNA-formulering in een vroege fase van klinisch onderzoek voor systemische toediening.

EGFR VOOR DRUG TARGETING

Een tweede onderzoekslijn in dit proefschrift is het gebruik van de tumor-specifieke expressie van EGFR voor *targeting* van liposomen. Liposomen zijn belangrijke *drug delivery* systemen bestaande uit een lipiden bilaag die een waterige ruimte omsluit. Voor succesvolle toepassing *in vivo* wordt het oppervlak van liposomen vaak gemodificeerd met polymeren (om de circulatietijd te verlengen) en *targeting* liganden (om binding aan het zieke weefsel te verbeteren). Populaire liganden zijn antilichamen of de antigeen-bindende fragmenten van antilichamen.

Nanobody-liposomen

In **Hoofdstuk 7** worden *nanobodies* tegen EGFR toegepast als *targeting* ligand. *Nanobodies* zijn de kleinste antigeen-bindende fragmenten die uit de antilichamen van lama's en kamelen kunnen worden geïsoleerd. Een *nanobody* is 10 keer kleiner dan conventionele antilichamen maar bindt aan een antigeen met dezelfde hoge affiniteit. Eén type *nanobody*, EGa1, gericht tegen EGFR werd op het oppervlak van gepegyleerde positief geladen liposomen geplaatst.

Zoals verwacht, resulteerde de aanwezigheid van EGa1 op het liposoomoppervlak in een verhoogde binding en internalisatie. Deze verhoogde cellulaire internalisatie van EGa1-liposomen veroorzaakte een verbluffende reductie van 95% van EGFR expressie op het celoppervlak. Dit effect werd niet gezien als het anti-EGFR *nanobody* niet gekoppeld was aan het liposoom. De verlaging van EGFR niveau was het gevolg van verhoogde intracellulaire degradatie van EGFR wat zich uiteindelijk vertaalde in remming van celdeling. Deze remming van proliferatie van de tumorcel geeft aan dat deze liposomen intrinsiek therapeutisch actief zouden kunnen zijn. Bovendien kunnen de liposomen worden beladen met allerlei farmaca, waarmee synergistische combinaties gevormd zouden kunnen worden. In dit verband kan, voor toekomstige therapeutische studies met EGFR-gerichte liposomen, gedacht worden aan chemotherapeutica, kinase-inhibitors, of siRNAs.

Samenvattend zijn in dit proefschrift verschillende therapeutische strategieën tegen de EGFR gepresenteerd voor de behandeling van kanker. Enerzijds de remming van EGFR-expressie met behulp van siRNA, anderzijds de *targeting* van EGFR met *nanobody*-liposomen. Voor de siRNA-benadering, richtte dit proefschrift zich op de aflevering van siRNA *in vitro* en *in vivo*. Het belang van ontsnapping uit het endosoom voor siRNA *delivery* systemen werd onderzocht. Twee methodes verbeterden het vrijkomen van siRNA-lipoplexen uit het endosoom: PCI en fusogene peptiden. De verbeterde entree in het cytoplasma verhoogde ook de efficiëntie waarmee EGFR-expressie kon worden gereduceerd. De *nanobody*-liposomen zijn een nieuw systeem voor *targeting* van farmaca. Opmerkelijk genoeg vertoonde het systeem een intrinsiek therapeutische effect doordat binding leidde tot een versterkte degradatie van EGFR. Deze eigenschappen maken *nanobody*-liposomen bijzonder aantrekkelijk voor gebruik in combinatie-strategieën.

SUMÁRIO EM PORTUGUÊS

TERAPIA ONCOLÓGICA

O principal objectivo das terapias em oncologia consiste em travar a divisão e migração das células malignas, sem afectar as células saudáveis. A quimioterapia convencional apresenta a capacidade de eliminar células que se dividem rapidamente, através do bloqueio do seu ciclo celular. Uma vez que as células cancerígenas apresentam uma rápida capacidade de divisão, esta terapia é relativamente selectiva e eficaz no tratamento do cancro. No entanto, existem outros tipos celulares que apresentam um *turnover* elevado, nomeadamente os folículos capilares e as células hematopoiéticas (que originam as células presentes no sangue) e, como tal, esta terapia induz efeitos secundários severos. Recentemente, a terapia anti-tumoral tem sido alvo de várias inovações, de modo a passar de uma estratégia relativamente inespecífica para uma terapia direccionada para determinado alvo molecular (conceito de *targeting*). Neste contexto, os avanços da investigação científica na área da biologia molecular do cancro têm sido fundamentais na identificação desses alvos moleculares. Deste modo, tem sido possível desenvolver novas moléculas, e um novo conceito de medicamentos, designados *inteligentes*, pela forma específica e direccionada como actuam. Adicionalmente, a identificação de novos alvos moleculares tem contribuído para optimizar a quimioterapia convencional, ao permitir o direccionamento e transporte selectivo dos fármacos para as células cancerígenas sem afectar, portanto, as células saudáveis (conceito de *targeted delivery*). Na área do transporte e direccionamento de medicamentos no organismo, os imunolipossomas (pequenas vesículas de lípidos que transportam no seu interior o medicamento, apresentando no exterior anticorpos que permitem o direccionamento para o alvo) têm sido uma estratégia importante no redireccionamento, no âmbito da terapia oncológica.

RECEPTOR DO FACTOR DE CRESCIMENTO EPIDÉRMICO: UM ALVO RACIONAL

O receptor do factor de crescimento epidérmico (EGFR) é um exemplo de um alvo molecular extensivamente investigado e, como foi referido no **Capítulo 1**, é o centro-alvo desta tese. Em células saudáveis, a activação do EGFR induz divisão, diferenciação e migração celular. Em muitos dos cancros, como o da mama, do pulmão e cólon, este receptor encontra-se sobre-expresso e a sua sobre-activação contribui para o crescimento, desenvolvimento e alastramento do cancro. Existem já novos medicamentos *inteligentes* que se baseiam na inibição do receptor do EGF, de modo a travar a progressão do cancro. De um modo geral, estes medicamentos actuam inibindo a activação do EGFR e/ou as acções que resultam dessa activação. No **Capítulo 2** foram apresentadas diferentes estratégias que permitem a inibição do EGFR, assim como exemplos de estudos onde se demonstra que a inibição do EGFR pode ser benéfica na terapia do cancro.

SILENCIAMENTO DO EGFR

A principal estratégia utilizada nesta tese, com o intuito de inibir o EGFR, consiste no silenciamento da expressão desta proteína, usando RNA de interferência (RNAi), um mecanismo que permite degradar o mRNA da proteína EGFR, impedindo que esta seja produzida pelas células. O mecanismo de RNAi utilizando o siRNA, pequeno RNA de interferência, permite teóricamente silenciar qualquer proteína cuja presença nas células possa ser causa ou promover o desenvolvimento de uma doença.

Escape do endossoma: um factor limitante para o silenciamento

Para que uma proteína seja eficazmente silenciada, o siRNA tem de ser transportado até ao citoplasma das células alvo. Contudo, os transportadores geralmente utilizados para que o siRNA seja entregue às células, são frequentemente internalizados por endocitose, de tal modo que as partículas (siRNA+transportador) necessitam de escapar dos endossomas a fim de se acumularem no citoplasma celular. Vários transportadores de siRNA possuem capacidade de induzir o escape do endossoma, como é o caso da Lipofectamine (agente de transfecção comercial) que possui um lípido adjuvante para esse efeito, o DOPE. Porém, os nossos estudos demonstraram que, apesar da presença do DOPE, o silenciamento do EGFR obtido mediado pela Lipofectamine era relativamente baixo (geralmente inferior a 20%). Tal facto, levou-nos a investigar se o escape do endossoma poderia ser optimizado e se tal modificação iria originar um silenciamento mais eficaz do EGFR. No **Capítulo 3** são descritas experiências onde utilizámos um peptídeo derivado do vírus influenza (geralmente causador de gripes), o qual apresenta a capacidade de provocar a destabilização dos endossomas e, como tal, libertar o seu conteúdo para o citoplasma celular. De acordo com o esperado, os resultados apresentados demonstram que os níveis do silenciamento do EGFR são superiores quando o siRNA é transportado por Lipofectamine conjuntamente com o referido peptídeo. No **Capítulo 4** é apresentado pela primeira vez o processo de internalização fotoquímica (PCI) aplicado à entrega do siRNA no citoplasma celular, *in vitro*. Este processo baseia-se na utilização de um composto fotossensível que se localiza a nível das membranas dos endossomas, o qual quando sujeito a uma radiação de determinada frequência, desencadeia reacções fotoquímicas que, por conseguinte, induzem a destabilização dos endossomas e a libertação do seu conteúdo no citoplasma das células. Nestes estudos, imagens de miscroscopia confocal permitiram confirmar o mecanismo descrito, tendo-se observado um acréscimo de 10 vezes nos níveis de silenciamento do EGFR, provando que a técnica do PCI é eficaz na promoção do escape de partículas do endossoma.

Em conclusão, ambos os estudos demonstram que o escape do endossoma é um factor limitante para o sucesso do silenciamento mediado pelo siRNA, e ambas as técnicas aqui apresentadas foram utilizadas com sucesso no melhoramento desse parâmetro. Por conseguinte, estes estudos salientam a importância da ocorrência do escape do endossoma para que o silenciamento seja eficaz. Considerando os efeitos secundários resultantes da administração de doses elevadas de siRNA, qualquer contribuição no sentido de diminuir a dose de siRNA administrada, poderá ter um impacto significativo na implementação de novas estratégias terapêuticas.

Internalização fotoquímica: aplicação *in vivo*

A técnica do PCI, para além de aplicável *in vitro* (Capítulo 4), também é aplicável *in vivo*, tal como descrito no **Capítulo 5**. Neste estudo, procedeu-se à entrega de siRNA no citoplasma celular por injecção intra-tumoral combinada com a aplicação do PCI. A expressão do EGFR em tumores desenvolvidos em ratinhos foi silenciada em 80%, após tratamento com siRNA transportado pela Lipofectamine em conjunto com PCI, comparando com apenas 30% quando a técnica de PCI não foi utilizada. Contudo, a percentagem de inibição de 80% traduziu-se apenas em 3 a 4 dias de atraso no crescimento dos tumores, relativamente ao controlo. Este curto efeito está relacionado com o carácter transitório do mecanismo de RNAi pelo siRNA e pela rapidez com que as células tumorais se dividem, fazendo com que geralmente os tratamentos repetidos apresentem efeitos de maior duração. No entanto, os resultados obtidos demonstram que a técnica de PCI pode ser utilizada com sucesso *in vivo*. É ainda importante realçar que, dada a natureza desta técnica, a aplicação do PCI requer que os parâmetros que determinam a ocorrência das reacções fotoquímicas sejam adequadamente determinados. Tal ajuste prende-se com o facto de que as próprias reacções fotoquímicas também poderão danificar estruturas essenciais ao funcionamento da célula e, por conseguinte, induzir a morte celular (à semelhança do que sucede na terapia fotodinâmica, PDT). No tratamento do cancro, a indução de morte celular por aplicação de PCI poderá até ser benéfica e desejada, contudo, em casos em que a entrega de partículas a nível celular não tem o intuito de danificar ou matar as células, esta estratégia tem de ser aplicada com redobrada precaução.

Entrega do siRNA: administração local e sistémica

Actualmente, a maioria dos ensaios clínicos com siRNA envolvem a aplicação directa destas moléculas no local/tecido a tratar: por inalação para tratamentos a nível dos pulmões (infecção pelo vírus sincicial respiratório) e por injecção intraocular para tratamentos a nível do olho (degeneração macular relacionada à idade). Contudo, a aplicação directa nem sempre é possível e, como tal, têm sido feitos esforços a nível pre-clínico para desenvolver transportadores eficazes para aplicação intravascular de siRNA, de forma a permitir que este percorra a corrente sanguínea e seja entregue no citoplasma das células alvo. No **Capítulo 6** são apresentados alguns exemplos destes esforços e discutidas as estratégias utilizadas. É de salientar que em Junho de 2008 teve início um ensaio clínico com o objectivo de estudar o primeiro siRNA integrado num transportador selectivamente direccionado para o cancro, um reflexo do esforço que tem vindo a ser efectuado na implementação da administração sistémica de siRNA.

EGFR PARA DIRECCIONAMENTO DE FÁRMACOS

Outra vertente de investigação apresentada nesta tese relaciona-se com o desenvolvimento de transportadores que permitam a entrega selectiva de medicamentos a células cancerígenas, sem que estes medicamentos danifiquem células saudáveis. Uma vez que muitas células tumorais sobre-expressam o EGFR, este receptor pode ser usado como alvo na entrega de princípios activos que sejam incorporados em transportadores específicos. Os lipossomas são um sistema de transporte muito versátil, que pode incorporar diversos tipos de moléculas, como por exemplo os convencionais agentes quimioterapêuticos. Adicionalmente, a superfície dos lipossomas pode ser modificada por associação de anticorpos e outro tipo de moléculas que promovam a interacção de lipossomas com células alvo, permitindo o sucesso do *targeting*.

Nanobody-lipossomas

No **Capítulo 7** é descrita a preparação e caracterização de um novo sistema de entrega, direccionado para o EGFR. Este sistema consiste em lipossomas, cuja superfície foi modificada com uma nova variante de anticorpos, os *nanobodies*, os quais desempenham funções de *targeting*. Os *nanobodies* são pequenos fragmentos derivados de anticorpos que se encontram em lamas e camelos. Apresentam um tamanho 10 vezes inferior aos anticorpos comuns, mas possuem o mesmo tipo de afinidade de ligação aos respectivos antigénios, possuindo portanto um grande potencial para diversas aplicações.

Estudos efectuados *in vitro* demonstraram que os lipossomas com superfície modificada com *nanobodies* anti-EGFR apresentaram maior facilidade para interagir com células cancerígenas, e níveis mais elevados de internalização celular. Esta eficiência de internalização observada com os *nanobody*-lipossomas induziu um decréscimo drástico e inesperado da do EGFR a nível da superfície celular (95% de redução), efeito não observado pelo *nanobody*, quando utilizado isoladamente (não associado a lipossomas).

Estudos subsequentes permitiram determinar que o desaparecimento do EGFR a nível da superfície celular está associado à degradação do EGFR a nível celular, o que revelou ter um efeito de inibição da proliferação celular. Por conseguinte, este novo sistema constitui não só uma nova forma de entrega direccionada para o EGFR, mas também apresenta considerável potencial terapêutico pela forma como induz a degradação do EGFR a nível intracelular. Estudos futuros investigarão as propriedades dos *nanobody*-lipossomas desenvolvidos contendo agentes quimioterapêuticos, assim como outro tipo de moléculas, no seu interior, de forma a avaliar o respectivo efeito terapêutico.

Em conclusão, esta tese é centrada na inibição do EGFR como estratégia terapêutica. O EGFR foi inibido pelo silenciamento da sua expressão, por meio de siRNA, e através do efeito induzido pelos *nanobody*-lipossomas. Quanto à estratégia de silenciamento promovido pelo siRNA, esta tese foca o transporte/entrega do siRNA *in vitro* e *in vivo*. Neste contexto, foi salientada a importância da existência de mecanismos de escape do endossoma por parte do transportador do siRNA. Ambos os métodos utilizados para promover o escape do endossoma, o peptídeo derivado do vírus influenza e a internalização fotoquímica, tiveram sucesso na promoção da eficiência do silenciamento. Relativamente aos *nanobody*-lipossomas, este novo sistema de entrega direccionada ao EGFR revelou possuir também um efeito inibitório do EGFR e, portanto, possui um potencial terapêutico intrínseco que, tendo em conta a variedade de moléculas terapêuticas que podem ser incorporadas nos lipossomas, promete ser um óptimo sistema para estratégias de terapia combinada contra o cancro.

RESUME EN FRANÇAIS

THERAPIE CONTRE LE CANCER

Le principal but de la thérapie contre le cancer est d'arrêter la prolifération et la migration des cellules malignes sans affecter les cellules saines. La chimiothérapie conventionnelle agit contre les cellules cancéreuses par blocage du cycle cellulaire. Pour cette raison, la chimiothérapie est relativement sélective aux tissus malins qui présentent une prolifération rapide. Cependant, d'autres types de cellules se divisent également rapidement, comme les cellules hématopoïétiques et les follicules pileux, engendrant de ce fait des effets secondaires sévères liés à cette thérapie. Ces dernières années, les thérapies utilisées dans le traitement contre le cancer se sont plus orientées vers des stratégies ciblées, et ce grâce à l'identification de cibles moléculaires spécifiques. Ces cibles moléculaires, impliquées dans l'initiation ou la progression du cancer, ont permis le développement de principes actifs *intelligents.* Les cibles moléculaires spécifiques sont également utilisées pour améliorer l'efficacité des thérapies conventionnelles en favorisant une délivrance ciblée de substances actives. L'utilisation de vecteurs ciblés permet la délivrance sélective vers les cellules malignes, comme par exemple, l'emploi d'immunoliposomes est une stratégie largement utilisée.

RECEPTEUR DU FACTEUR DE CROISSANCE EPIDERMIQUE: UNE CIBLE RATIONNELLE

Le récepteur du facteur de croissance épidermique (EGFR) est un exemple de cibles moléculaires particulièrement étudiés et, comme le décrit le **Chapitre 1**, constitue le thème de ces travaux de doctorat. Dans les cellules saines, l'activation de ce récepteur stimule la prolifération, la différentiation, la migration et l'adhésion cellulaire. Dans beaucoup de cancers humains, comme les cancers du sein, du poumon, du pancréas et du colon, l'EGFR est surexprimé et cela se caractérise par une prolifération cellulaire incontrôlée, l'inhibition de l'apoptose, la stimulation de l'angiogenèse, et le développement de métastases. Des molécules spécifiques ont été développées pour inhiber le fonctionnement du récepteur à l'EGF par blocage de la liaison des ligands ou de son activation. Dans le **Chapitre 2**, les stratégies pour l'inhibition de l'EGFR sont exposées démontrant que cette inhibition peut être particulièrement efficace comme stratégie anticancéreuse.

INHIBITION DU RECEPTEUR A L'EGF

La principale approche suivie dans ces travaux de recherche, est l'inhibition du récepteur par blocage de l'expression de la protéine utilisant le mécanisme d'ARN interférence. Ce mécanisme permet la dégradation de l'ARN messager de la protéine EGFR et donc prévient la production cellulaire du récepteur à l'EGF. Théoriquement, les petits ARN interférant (siARN) peuvent être employés pour interrompre l'expression de toute protéine qui initie ou contribue au développement de maladies.

L'échappement de l'endosome: un facteur limitant pour l'inhibition

Pour une inhibition efficace, les molécules de siARN doivent être présentes dans le cytoplasme des cellules cibles. Cependant, les vecteurs généralement employés pour leur transport induisent l'internalisation par endocitose, et donc les particules (siRNA et vecteur) doivent quitter l'endosome pour atteindre le cytoplasme cellulaire. Certains vecteurs, comme la Lipofectamine qui contient le lipide fusiogène DOPE, possèdent des propriétés particulières qui permettent l'induction de la rupture de l'endosome avant la fusion avec les lysosomes, et de ce fait préviennent la dégradation enzymatique des siARN. Nos résultats montrent cependant une inhibition relativement peu efficace (en générale moins de 20%). Pour cette raison, nous avons étudié la possibilité d'améliorer l'efficacité de la sortie des siARN de l'endosome, et donc l'effet sur l'efficacité de l'inhibition du récepteur à l'EGF. Deux méthodes ont été employées pour faciliter la sortie des particules de siARN et de Lipofectamine de l'endosome.

Le **Chapitre 3** décrit l'utilisation d'un peptide fusogénique dérivé du virus de la grippe en association avec les particules. Les résultats montrent que la présence de ce peptide induit une inhibition plus importante du récepteur à l'EGF.

Le **Chapitre 4** montre dans une étude *in vitro,* pour la première fois, l'emploi de l'internalisation photochimique (PCI) pour aider la sortie des particules de siARN de l'endosome. Cette technique utilise un photosensibilisateur, localisé au niveau de la membrane de l'endosome. L'exposition à une source lumineuse, active le photo-sensibilisateur et initie des réactions photochimiques, déstabilisant la membrane de l'endosome et libérant son contenu dans le cytoplasme. Dans notre étude, le principe de la PCI a été confirmé par microscopie confocale. L'inhibition, du récepteur à l'EGF, a été augmentée d'un facteur 10 par rapport à l'efficacité des particules de siARN et de Lipofectamine.

Les deux études soulignent que l'échappement de l'endosome est un facteur limitant pour l'inhibition dirigée par le siARN et que la délivrance par la Lipofectamine peut être améliorée par des méthodes qui aident la rupture ou la déstabilisation de l'endosome. Une vectorisation efficace du siARN permet d'administrer des doses moins importantes limitant ainsi les effets secondaires.

Internalisation photochimique: application *in vivo*

Comme le décrit le **Chapitre 5**, la PCI peut être employée avec succès *in vivo,* pour la délivrance intratumoral de siARN. L'expression du récepteur à l'EGF a été réduite de 80% dans un modèle murin de xénogreffes A431 lorsque les particules de siARN et de Lipofectamine ont été administrées avec la PCI, en comparaison avec les 30%

d'inhibition induit par le traitement seul. Les 80% d'inhibition du récepteur se sont traduits par un ralentissement de la croissance de la tumeur de 3 à 4 jours. Malgré les 80% d'inhibition, l'observation du faible ralentissement de la croissance de la tumeur suggère un rétablissement de l'activité tumorale. En effet, les siARN induisent un effet transitoire, et dans des cellules qui se divisent rapidement, comme les cellules de type A431, une seule dose de siARN est rapidement diluée et suboptimale, résultant à un effet à court terme. Des traitements répétitifs pourraient prolonger l'efficacité de cette thérapie. Il est important de souligner que l'application de la PCI *in vivo* implique des recommandations spéciales à cause des réactions photochimiques induites pendant l'utilisation de cette technique. Les paramètres qui caractérisent les réactions photochimiques, comme la dose de photosensibilisateur et le temps d'exposition à la source lumineuse, doivent êtres rigoureusement déterminés et optimisés pour chaque application. Le mécanisme de réactions photochimiques est commun à la thérapie photodynamique (PDT) mais la PCI doit seulement induire la déstabilisation des membranes de l'endosome et non la mort cellulaire. Un effet comparable à la PDT peut être un avantage pour le traitement antitumoral, comme le décrit le Chapitre 5. Naturellement dans les cas ou la délivrance de principes actifs n'a pas pour but l'arrêt de la prolifération cellulaire ou la mort cellulaire, il est de tout intérêt d'appliquer la PCI avec précaution.

Délivrance de siARN : local ou systémique

Actuellement, la majorité des essais cliniques sur des molécules de siARN se font avec l'application locale de siARN: par inhalation (infection pulmonaire du virus respiratoire syncytial) ou par injection intraoculaire (dégénérescence maculaire liée à l'âge). Par contre, l'administration directe aux tissus malades n'est pas toujours possible et de nombreuses études précliniques tentent de développer de nouveaux vecteurs pour l'administration systémique de siARN. Le **Chapitre 6** présente quelques exemples de stratégies en développement pour une délivrance ciblée de siARN par injection intraveineuse. Pour cette voie d'administration, le vecteur doit protéger et transporter le siARN jusqu'aux tissus où les cellules cibles sont présentes. Ce vecteur doit aussi permettre l'internalisation cellulaire et l'échappement de l'endosome pour permettre une délivrance optimale du siARN au cytoplasme des cellules cibles. En fait, depuis Juin 2008, la première molécule de siARN transportée par un vecteur ciblé est administrée systémiquement dans un essai clinique de phase I.

DELIVRANCE CIBLEE AU RECEPTEUR DE L'EGF

Une deuxième ligne de recherche a été employée pour ces travaux de doctorat, celle-ci concerne le développement d'un nouveau vecteur pour la délivrance ciblée de molécules anticancéreuses. Dans de nombreux cancers humains le récepteur à l'EGF est surexprimé. Par conséquent, des vecteurs qui guident des principes actifs vers ce récepteur seront certainement utiles pour le développement de nouvelles thérapies spécifiques contre le cancer.

Les liposomes représentent un système de délivrance particulièrement intéressant qui peut contenir une variété de molécules thérapeutiques. Leur surface peut être modifiée avec des polymères qui augmentent leur période de demi-vie dans la circulation sanguine après une administration systémique, comme le polyéthylène glycol (PEG). Les liposomes peuvent être également modifiés avec des molécules spécifiques à certains types de cellules qui permettent le ciblage, comme des anticorps ou fragments d'anticorps.

Nanobody-liposomes

Le **Chapitre 7** décrit la préparation et la caractérisation d'un nouveau vecteur pour la délivrance ciblée de médicaments: anti-EGFR *nanobody*-liposomes. La surface de ces liposomes a été modifiée avec une nouvelle variété d'anticorps, les *nanobodies*, qui reconnaissent le récepteur à l'EGF. Les *nanobodies* ont été développés à partir d'anticorps de chameaux. Ces *nanobobies* sont 10 fois plus petits que les anticorps conventionnels et ont la même affinité pour ces antigènes. Comme prévu, la présence du *nanobody*, EGa1, à la surface des liposomes a augmenté l'association et l'internalisation des liposomes par les cellules. Cette internalisation efficace a induit une diminution drastique de l'EGFR au niveau de la surface cellulaire (95% de réduction). Cet effet n'a pas été induit par le *nanobody* seul. Nos études ont permis de déterminer que la disparition du récepteur est associée à la dégradation du récepteur au niveau cellulaire, et ceci engendre un effet d'inhibition de la prolifération cellulaire. L'observation de cette inhibition de la prolifération cellulaire indique que ce vecteur possède un potentiel thérapeutique intrinsèque. De plus, par association des liposomes avec différentes molécules thérapeutiques, transportées par ce vecteur, ce système présente un important potentiel de stratégies combinées anticancéreuses. De prochaines études investiguerons l'activité thérapeutique de ce vecteur afin d'explorer le potentiel très prometteur de ces *nanobody*-liposomes.

En conclusion, ces travaux de doctorat ont porté sur l'inhibition du récepteur à l'EGF de deux façons, par blocage de l'expression de la protéine en employant le siARN et par l'emploi des nanobody-liposomes. La première stratégie a exploré la délivrance de siARN *in vitro* et *in vivo*. L'importance de l'échappement de l'endosome a été soulignée. Le peptide fusogénique et l'internalisation photochimique, deux méthodes employées pour faciliter la sortie des particules de siARN de l'endosome, ont été employés avec succès. De cette façon, l'efficacité de l'inhibition du récepteur a été augmentée. La deuxième stratégie a permis le développement d'un nouveau système pour la délivrance ciblée au récepteur à l'EGF qui offre d'avantage de possibilités pour des thérapies combiner contre le cancer.

ABBREVIATIONS

$AlPcS_{2a}$	Aluminium phtalocyanine
AMD	Age-related macular degeneration
ANOVA	Analysis of variance
ATCOL	Atelocollagen
bFGF	Basic fibroblast growth factor
CR	Cystein-rich
CT	Carboxyl terminal
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle's medium
DOPE	Dioleoylphosphatidylethanolamine
DOTAP	Dioleoyltrimethylammonium propane
dsRNA	Double-stranded RNA
DTA	Diphtheria toxin A chain
EC	Extracellular
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EPR	Enhanced permeability and retention
Fabs	Antibody Fragments
FACS	Fluorescence activated cell sorter
HNSCC	Head and neck squamous cell carcinoma
HRP	Horseradish peroxidase
IC	Intracellular
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IL-8	Interleukin-8
i.m.	Intramuscular
IP3	Inositol triphosphate
i.t.	Intratumoral
i.v.	Intravascular
JM	Juxtamembrane
K_d	Dissociation constant
L	Light
LF	Lipofectamine
mAbs	Monoclonal antibodies
Mal-PEG-DSPE	Maleimide-polyethyleneglycol-distearoylphosphatidylethanolamine
MAPK	Mitogen-activated protein kinase
MEK	MAPK-kinase
MFI	Mean fluorescence intensity
MPS	Mononuclear phagocytic system
mRNA	Messenger RNA
NSCLC	Non-small cell lung carcinoma
NT	Non-treated

PAR-2	Proteinase-activated receptor-2
PCI	Photochemical internalisation
PDGF	Platelet-derived growth factor
PDI	Polydispersity index
PEG	Polyethylene glycol
PEI	Polyethylenimine
PI3K	Phosphatidylinositol-3-OH kinase
PKC	Protein kinase C
PLCγ	Phospholipase Cγ
PS	Photosensitiser
RISC	RNA-induced silencing complex
Rho-PE	Rhodamine-phosphatidylethanolamine
RNAi	RNA interference
RSV	Respiratory syncytial virus
RTK	Receptor tyrosine kinase
SATA	*N*-succinimidyl-S-acetylthioacetate
s.c.	Subcutaneous
scFv	Single chain variable fragments
shRNA	Short hairpin RNA
siEGFR	Anti-EGFR siRNA
siNS	Non-specific siRNA
siRNA	Small interfering RNA
SRB	Sulpho-rhodamine B
STAT	Signal transducer and activator of transcription
TCA	Trichloro-acetic acid
TGF-α	Transforming growth factor-alpha
TKI	Tyrosine kinase inhibitors
TL	Total lipid
TM	Transmembrane
TNF-α	Tumour necrosis factor-alpha
$TPPS_{2a}$	Meso-tetraphenylporphine
VEGF	Vascular endothelial growth factor
VHH	Variable domains of heavy chain-only antibodies
WB	Western blot

CURRICULUM VITAE

Sabrina Oliveira was born on April the 7th 1980, in Saint Germain-en-Laye, France. In 1988 she moved to Portugal together with her family. From 1995 to 1998 she attended the secondary school Francisco Rodrigues Lobo in Leiria, Portugal, following studies in Science and Nature. In October 2004 she graduated as a Pharmacist after her studies in Pharmaceutical Sciences at the Faculty of Pharmacy, University of Coimbra, Portugal. In January 2004 she came for the first time to the division of Advanced Drug Delivery and Drug Targeting of the Utrecht Institute for Pharmaceutical Sciences, Department of Pharmaceutics, Utrecht University. This was within the framework of the Erasmus program. She worked for half a year on a project concerning the stability of therapeutic proteins in liquid formulations. In December 2004 she started her PhD research project, within the same department, under the supervision of Prof. Dr. Gert Storm and Dr. Raymond Schiffelers. Her project was focused on the epidermal growth factor receptor and its inhibition for cancer therapy. This research was funded by the Portuguese foundation: Fundação para a Ciência e a Tecnologia. The results of her PhD research are described in this thesis.

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S. Oliveira, P.M.P. van Bergen en Henegouwen, G. Storm, R.M. Schiffelers, Molecular biology of epidermal growth factor receptor inhibition for cancer therapy. Expert Opinion on Biological Therapy 6: 605-617 (2006)

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S. Oliveira, G. Storm, R.M. Schiffelers, Enhanced knockdown of EGFR by an improved endosomal escape of siRNA. Oral presentation at the spring meeting of the Belgian-Dutch Biopharmaceutical Society, Beerse, Belgium, May 2006

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S. Oliveira, G. Storm, R.M. Schiffelers, Strategies to improve the endosomal escape of siRNA and enhance the knockdown of EGFR. Poster presentation at the 3rd Pharmaceutical Sciences World Congress, Amsterdam, The Netherlands, April 2007

S. Oliveira, G. Storm, R.M. Schiffelers, siRNA-mediated knockdown of EGFR is improved by strategies which facilitate siRNA-endosomal escape. Poster presentation at the 34th Annual Meeting and Exposition of the Controlled Release Society, Long Beach, California, USA, July 2007

S. Oliveira, A. Høgset, G. Storm, R.M. Schiffelers, Enhanced silencing of EGFR by photochemical internalisation: a strategy to facilitate the endosomal escape of siRNA. Oral presentation at the GTRV Summer School on Oligonucleotide-based strategies to control gene expression – delivery issues, La Grande-Motte, France, September 2007

S. Oliveira, A. Høgset, G. Storm, R.M. Schiffelers, Photochemical internalisation: a strategy to facilitate the endosomal escape of siRNA thereby enhancing EGFR silencing i*n vitro* and i*n vivo*. Poster presentation at the 2nd International Symposium on Cellular delivery of therapeutic macromolecules, Cardiff, UK, June 2008

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That is why there are unforgettable moments and incomparable people…

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