Photochemical internalization of recombinant toxins targeting EGFR and HER2 for treatment of aggressive and resistant cancers

by

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"Sometimes you succeed… and other times you learn."

(Robert Kiyosaki)

Oslo, January 2015

Maria Elisabeth Brandal Berstad

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ABBREVIATIONS

ADCC	antibody-dependent cellular cytotoxicity
ALA	aminolevulic acid
$AlPcS_{2a}$	aluminium phtalocyanine with two sulfonate groups on adjacent phthalates
AMD	age-related macular degeneration
ATP	adenosine triphosphate
CDC	complement-dependent cytotoxicity
DMSO	dimethyl sulfoxide
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMA	european medicines agency
ERK	extracellular signal regulated kinase
Fc	fragment crystallizable
FDA	food and drug administration
HER2	human epidermal growth factor receptor 2
HER3	human epidermal growth factor receptor 3
HER4	human epidermal growth factor receptor 4
HNSCC	head and neck squamous cell carcinoma
IC50	median inhibition concentration (inhibit cellular proliferation by 50 %)
IL-2	interleukin-2
IT	immunotoxin
JNK	c-Jun NH_2 terminal kinase
LC3	microtubule-associated protein 1A/1B-light chain 3
mAb	monoclonal antibody

MAPK	mitogen-activated protein kinase
MβCD	methyl-β-cyclodextrin
mTOR	mammalian target of rapamycin
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NSCLC	non-small cell lung cancer
$^1O_2$	singlet oxygen
PARP	Poly (ADP-ribose) polymerase
PDT	photodynamic therapy
PCI	photochemical internalization
PCR	polymerase chain reaction
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PpIX	protoporphyrin IX
rGel	recombinant gelonin
RIP	ribosome inactivating protein
ROS	reactive oxygen species
SAPK	stress-activated protein kinase
scFv	single-chain variable fragment
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
siRNA	small interfering RNA
TKI	tyrosine kinase inhibitor
$TPCS_{2a}$	meso-tetraphenyl chlorin with two sulphonate groups on adjacent phenyl rings
$TPPS_{2a}$	meso-tetraphenyl porphine with 2 sulfonate groups on adjacent phenyl rings
$TPPS_4$	tetrasulfonated tetraphenyl porphine
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

LIST OF PUBLICATIONS

I Berstad M.B., Weyergang A. and Berg K. (2012): Photochemical internalization (PCI) of HER2-targeted toxins: synergy is dependent on the treatment sequence. *Biochim Biophys Acta.* **1820**: 1849-1858.

II Bull-Hansen B., Berstad M.B., Berg K., Cao Y., Skarpen E., Rosenblum M.G. and Weyergang A. (2015): Photochemical activation of MH3-B1/rGel; a HER2-targeted treatment approach for ovarian cancer. *Oncotarget, in press.*

III Berstad M.B., Cheung L.H., Berg K., Peng Q., Fremstedal A.S.V., Patzke P., Rosenblum M.G. and Weyergang A. (2015): Design of an EGFR-targeting toxin for photochemical delivery; *in vitro* and *in vivo* selectivity and efficacy. *Oncogene, in press.*

1. AIMS OF THE STUDY

The principal aim of this study was to investigate photochemical internalization (PCI) as a means to activate the therapeutic potential of type I ribosome-inactivating proteins (RIPs) targeted to cancer cells by EGFR or HER2 binding; i.e. PCI of EGFR- and HER2-targeted toxins. It was hypothesized that PCI of EGFR- and HER2-targeted toxins could represent an alternative treatment in cancers with limited sensitivity to currently available EGFR- and HER2-targeted therapies. PCI has previously been shown to potentiate the efficacy of EGFR-targeted toxins based on the biotin-streptavidin binding between the targeting and toxin moieties. These previous studies were, however, proof-of-concept studies and the targeted toxins utilized had little potential for clinical use. PCI of HER2-targeted toxins had not previously been investigated.

The present thesis had two overall aims:

A) To demonstrate the principle of PCI of HER2-targeted toxins.

- First, as a proof-of-principle study utilizing an antibody-toxin conjugate based on the streptavidin-saporin binding.
- Secondly, to investigate the potential of PCI in improving the efficacy of an already established recombinant HER2-targeted toxin.
- To study PCI of HER2-targeted toxins in relevant cancers with poor responsiveness towards already available HER2-targeted therapies.

B) To further develop the concept of PCI of EGFR-targeted toxins.

- To design and produce a recombinant EGFR-targeted fusion toxin based on the type I RIP gelonin for PCI-mediated administration.
- Document the effect of PCI of an EGFR-targeting recombinant toxin both *in vivo* and *in vitro* with respect to mechanisms of cytotoxicity, specificity, antitumor effects and potential side effects.
- To study PCI of the established EGFR-targeted fusion toxin in HNSCC cell lines and in a clinically relevant HNSCC tumor xenograft.

2. INTRODUCTION

2.1 Résumé; Rationale for PCI of toxins targeting the EGFR family

Cancer represents a major global health problem causing over 8 million deaths annually. The global cancer burden is increasing and is expected to nearly double by 2030 (American Cancer Society). Surgery, chemotherapy and radiotherapy are still the three cornerstones of cancer therapy. Major efforts are, however, being put into developing new cancer treatments that more precisely identify and attack cancer cells in order to improve disease control while reducing damage to healthy tissue. Epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) are two of the most utilized cancer-associated proteins for targeted therapy of solid cancers. However, currently available EGFR- and HER2-targeted monoclonal antibodies (mAbs) and tyrosine kinase inhibitors (TKIs) have clear limitations related to induction of drug resistance and treatment specificity (Chong and Janne, 2013; Nahta et al., 2006). The ability of EGFR and HER2 to undergo receptor-mediated endocytosis combined with their overexpression in several cancers makes them interesting candidates for targeted delivery of protein toxins to cancer cells. EGFR- and HER2-targeted toxins use these receptors as delivery portals of potent cytotoxic agents into the cytosol. The mechanistic targets of action for these targeted toxins are ribosomes where the toxin inhibits translation. Targeted toxins are, therefore, clinically not necessarily limited by the same mechanisms as mAbs and TKIs (Lewis Phillips et al., 2008). An extensive number of fusion toxins targeting EGFR and HER2 have been described in the literature and many of them have shown anti-tumor effects in human xenograft models, including breast cancer (Cao et al., 2012), ovarian cancer (Cao et al., 2009), head and neck cancer (Thomas et al., 2004; Barnea et al., 2013; Engebraaten et al., 2002), brain tumors (Liu et al., 2005; Phillips et al., 1994) and pancreatic cancer (Bruell et al., 2005). Dose-limiting toxicity as well as immunogenicity have been demonstrated as limitations for clinical use (Pai-Scherf et al., 1999; Azemar et al., 2000; Azemar et al., 2003) and no EGFR- or HER2-targeted toxin has so far gained clinical approval from the US Food and Drug Administration (FDA) or the European Medicines Agency (EMA). There is, therefore, a need for clinically relevant strategies to reduce off-target toxicity and immunogenicity provided by such targeted toxins.

PCI is a modality for site-specific cytosolic release of drugs entrapped in cellular endosomes and lysosomes (Berg et al., 1999; Selbo et al., 2010). Type I RIPs linked to targeting moieties are ideal candidates for delivery by PCI, since these are specifically taken up in cancer cells, but have severely limited efficacy due to endosomal entrapment (Pirie et al., 2011; Yazdi and Murphy, 1994). However, once translocated to the cytosol, these toxins are equally toxic to type II toxins (Vago et al., 2005). PCI may increase the tumor-specific potential of type I RIP-based targeted toxins by promoting cytosolic translocation only in tissue simultaneously targeted by photosensitizer and light (Fig. 1) (Weyergang et al., 2011). Hence, the toxin dosage and, subsequently, the adverse effects, may be reduced by PCI. PCI may further combat problems with immunogenicity associated with iterative administration of fusion toxins, since PCI in clinical studies has been shown effective with only one single treatment cycle. In this thesis, we hypothesized that PCI may have great potential in improving the efficacy and specificity of type I RIP-based EGFR- and HER2-targeted toxins and, hence, may represent an interesting alternative for treatment of solid cancers overexpressing EGFR and HER2.

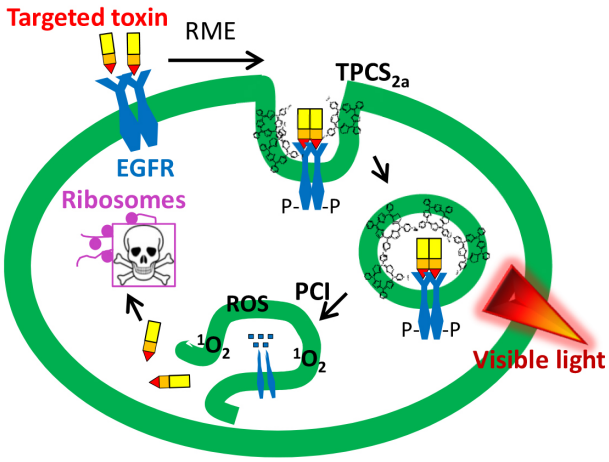

Figure 1: The concept of PCI of type I RIP-based targeted toxins (with targeting of EGFR as an example). The targeted toxin is taken into the cell by receptor-mediated endocytosis and localizes to endocytic vesicles with the photosensitizer ($TPCS_{2a}$) in the membrane compartment. Visible light at appropriate wavelengths activates the photosensitizer and induces oxidative damage to the membrane. The toxin is then released into the cytosol where it may induce potent cytotoxic effects by targeting the ribosomes.

2.2 Photochemical internalization (PCI)

Hydrophilic macromolecular drugs with intracellular action points hold great promise as novel therapeutics, but their use is often limited by the lack of safe, efficient and specific delivery strategies. The plasma membrane act as a barrier to cellular entry of such drugs and these are, therefore, taken up by different mechanisms of endocytosis (Alberts et al., 2002). The targets of these macromolecular drugs are often located in the cytoplasm or nucleoplasm, but usually only a minor fraction of the drug molecules are able to escape endosomes before they are degraded in lysosomes (Lloyd, 2000; Pirie et al., 2011). This prevents the macromolecular drugs from reaching their therapeutic potential and results in the need for dose escalation, which increases the risk of adverse effects.

Photochemical internalization (PCI) is a relatively new treatment modality for release of drugs that accumulate in endosomes and lysosomes (Berg et al., 1999; Selbo et al., 2002). PCI may be used to potentiate the effect of drugs that do not reach their full potential due to lysosomal degradation. PCI utilizes two individually nontoxic components; a photosensitive compound (photosensitizer) (Berg et al., 2011) that accumulates in the membrane of endocytic vesicles and visible light at specific wavelengths (Norum et al., 2009b). In combination, these two components induce oxidative damage to the vesicle membrane and subsequently, the drug trapped inside the endocytic vesicles is released into the cytosol where it may reach its target. It was initially thought that the photosensitizer and macromolecule to be released had to be localized in the same endocytic vesicles at the time of light exposure ("Light after" principle) (Fig. 2). Later, it was discovered that the endocytic vesicles could very well be permeabilized up to 6-8 hrs before delivery of the macromolecule of interest, with the advantage of avoiding a prolonged and potential detrimental stay of the molecules in the endocytic vesicles (Prasmickaite et al., 2002). The likely mechanism behind this effect (called the "Light first" principle) is the fusion of photochemically ruptured vesicles with intact vesicles carrying the macromolecules, leading to endosomal release of the macromolecules. Which approach leads to the better result seems to depend on the macromolecule to be delivered. The two strategies have been shown equally effective for delivery of bleomycin and reporter transgenes (Prasmickaite et al., 2002; Berg et al., 2005a; Berg et al., 2011), while "Light first" PCI has been found superior

for delivery of the protein toxin gelonin (Prasmickaite et al., 2002; Berg et al., 2006). In contrast, PCI of targeted protein toxins has only been proven effective when using the "Light after" principle (Yip et al., 2007; Selbo et al., 2009; Berstad et al., 2012). Photochemical damage to the targeting receptor has been proposed as a mechanism behind these findings (Weyergang et al., 2007; Berstad et al., 2012). PCI has been shown to stimulate cytosolic delivery of a large variety of drugs, including proteins, protein toxins, adenovirus, nucleic acids and chemotherapeutic drugs, reviewed in (Selbo et al., 2010) and (Weyergang et al., 2011).

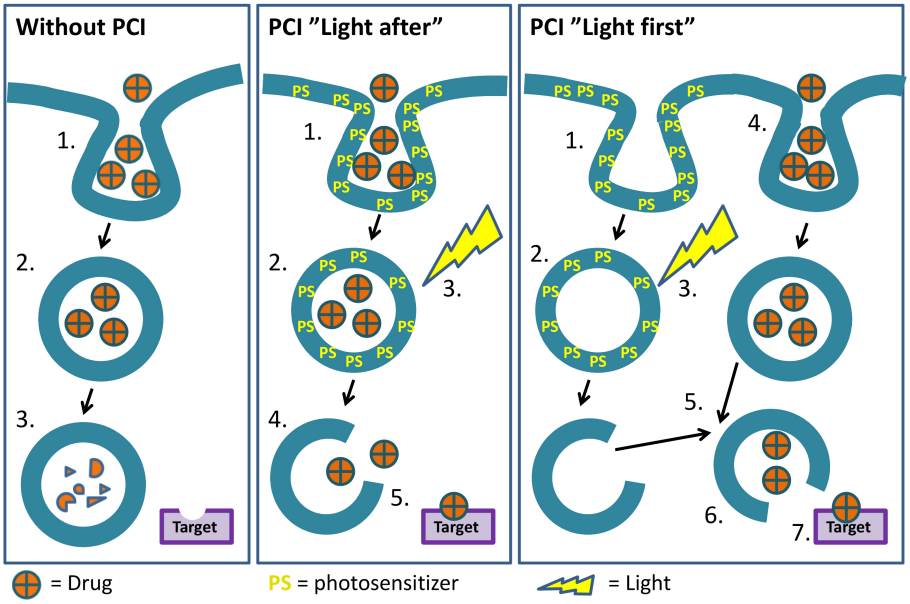

Figure 2: Schematic illustration of PCI. Without PCI: The drug is taken up by endocytosis and transported via endosomes to lysosomes for degradation. PCI "Light after": The drug is localized in endocytic vesicles with photosensitizer (PS) in the vesicle membrane. Light-induced activation of the photosensitizer causes disruption of the membrane and the drug escapes to interact with its target in the cytosol. PCI "Light first": The photosensitizer is first taken up in endocytic vesicles and activated by light. Further, the drug is administered and internalized into the cell. Drug-containing vesicles fuse with the ruptured vesicles, leading to drug release and interaction with target.

2.2.1 Photochemical reaction mechanisms

A photosensitizer is defined as a chemical entity that upon light absorption initiates a photochemical or photophysical alteration of another chemical entity (Dougherty et al., 1998). The first scientific reports on the use of photosensitizers in combination with light date back to the early 20th century when Raab, von Tappeiner and Jesionek showed that light could potentiate the cytotoxic effects of acridine, eosin and hematoporphyrin (Von Tappeiner and Jesionek, 1903), a phenomenon described as "Photodynamic action". These findings marked the beginning of what we today know as photodynamic therapy (PDT), described in Section 2.2.5 (Agostinis et al., 2011).

Following the absorption of light (photons) at the right wavelength, a photosensitizer is excited from its ground state (^{0}p) to a short-lived singlet state (^{1}p, Fig. 3) (MacDonald and Dougherty, 2001). From the singlet state, the absorbed energy may be emitted as heat (internal conversion), fluorescence or alternatively, the photosensitizer can enter a more stable triplet state (^{3}p) through a process termed intersystem crossing (ISC). From the triplet state, the energy can be emitted as heat, phosphorescence or the photosensitizer can react with molecular oxygen, forming singlet oxygen ($^1O_2$) (MacDonald and Dougherty, 2001). $^1O_2$ is assumed to be the most important ROS in therapeutic utilization of photosensitizers, and $^1O_2$ is the favored photoreaction product in an oxygenated environment (type II reaction) (Weishaupt et al., 1976). Alternatively, the photosensitizer in its triplet state might exchange an electron or a hydrogen atom with a substrate, forming a reactive free radical (Geiger et al., 1997; Price et al., 2009). This radical can then react with oxygen, forming oxygen radicals, such as superoxide anion, hydroxyl radical and hydrogen peroxide. In areas with insufficient oxygen levels, type I reactions might dominate (Ferraudi et al., 1988; Moan and Sommer, 1985). However, other factors than O_2 also influence on the ratio between type I and II reactions, e.g. type of photosensitizer, substrate concentration and the binding affinity for the substrate (Foote, 1968). Both $^1O_2$ and type I photoreaction products are highly reactive and induce oxidative cellular damage. $^1O_2$ oxidizes several types of biomolecules, such as unsaturated fatty acids (Doleiden et al., 1974; Sakharov et al., 2005), cholesterol (Geiger et al., 1997), amino acids (tryptophan, histidine, cysteine, tyrosine and methionine) (Verweij et al., 1981; Spikes and Straight, 1967) and guanine (Simon and Van Vunakis, 1962).

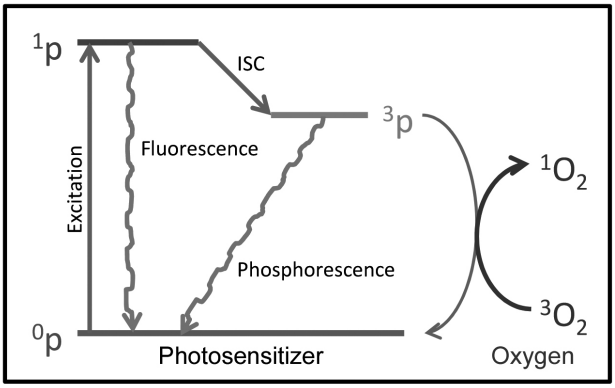

Figure 3: Simplified Jablonski diagram of the type II photoreaction leading to production of $^1O_2$.

2.2.2 The photochemistry of PCI

An optimal photosensitizer for PCI localizes to the membrane of endocytic vesicles. To be clinically useful, the photosensitizer should also accumulate selectively in tumor tissue, absorb light at preferential wavelengths (600-800 nm) to facilitate tissue penetration (Zonios et al., 2001), exert minimal toxicity when not subjected to light, produce reactive oxygen species (ROS) efficiently, be chemically without impurities and stable in solution, and allow preparation in a clinically useful formulation (Hamblin and Mroz, 2008). The best studied and most utilized photosensitizers for PCI are disulfonated compounds based on the tetrapyrrole macrocycle (Fig. 4) (Berg et al., 2011; Selbo et al., 2010; Dietze et al., 2006). This tetrapyrrole ring structure is named porphine and comprises four pyrrole subunits connected by methine bridges. Derivatives of porphin are termed porphyrins. Substituting porphyrins with sulphonate groups alters their hydrophilicity, as well as their cell and tissue distribution (Chan et al., 1990; Berg et al., 1990). Photosensitizers used for PCI have two sulphonate groups on adjacent phenyl rings, which give the amphiphilicity necessary for PCI. The hydrophobic part of the photosensitizer integrates into the plasma membrane and upon adsorptive endocytosis, the photosensitizer localizes to the membrane of endocytic vesicles with the hydrophilic part facing towards the lumen (Fig. 4E) (Berg et al., 2006). This is in contrast to tetrasulfonated hydrophilic photosensitizers (such as $TPPS_4$), which are taken up by pinocytosis and localize in the matrix of the endocytic vesicles, rendering them inefficient as PCI-photosensitizers (Berg and Moan, 1994). In naturally occurring tetrapyrroles, a metal ion is coordinated in the middle of the porphyrin

structures (e.g. Fe^{2+} in Heme). Porphyrin-based photosensitizers are generally devoid of paramagnetic ions, since these are known to shorten the lifetime of the triplet state lifetime and, consequently, reduce the ability to produce $^1O_2$ (Jori, 2004). Introduction of diamagnetic metals (e.g. Al^{3+}, Zn^{2+}) have, however, been shown to improve solubility and stability, making them interesting for therapeutic purposes (Berg et al., 2005b). Research on PCI has mainly been focused on three photosensitizers, $TPPS_{2a}$, $AlPcS_{2a}$ and $TPCS_{2a}$ (Fig. 4B, C, D), although PCI has also been shown effective with other photosensitizers, such as ALA-induced PpIX (Selbo et al., 2001a) and hypericin (Adigbli et al., 2007).

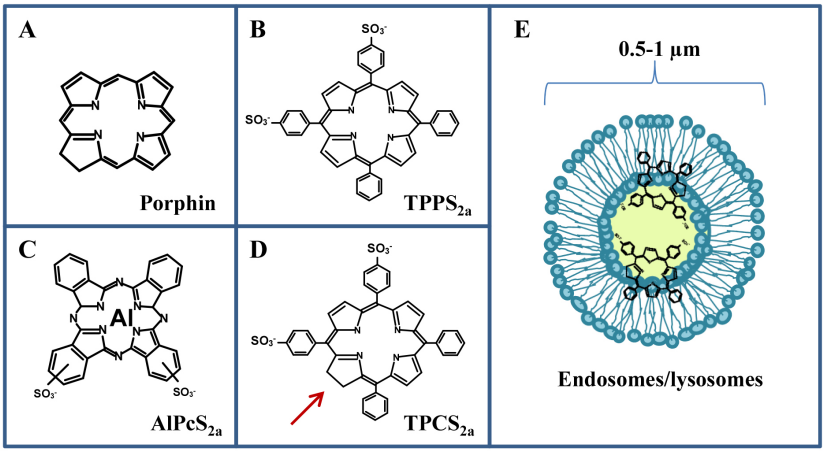

Figure 4: PCI photosensitizers based on the chemical structure of porphin (A); disulfonated meso-tetraphenylporphin, $TPPS_{2a}$ (B), aluminum phthalocyanine disulfonate, $AlPcS_{2a}$ (C) and meso-tetraphenyl chlorin disulfonate, $TPCS_{2a}$ (D). Arrow indicates reduced double bond. E: Intracellular localization of the photosensitizers in endosomes/lysosomes. NB! Figure is out of scale.

The photosensitizers used in PCI have been shown to retain preferentially in malignant tissue, usually with a tumor-to-normal-tissue ratio in the range 2-3:1 (Dougherty et al., 1998; Sheng et al., 2004; Berg et al., 2011). The biodistribution after systemic administration is, however, dynamic (Hamblin et al., 1999) and the localization of the photosensitizer at the time of irradiation will determine the sites of photodamage (Chen et al., 2005). The drug-light interval is therefore crucial for the therapeutic outcome (Castano et al., 2005). The photosensitizer localization is also important at an intracellular level due to the extremely short lifetime of $^1O_2$ in biological systems (estimated to <0.04 µs by Moan and Berg), which results in a radius

of action of less than 0.02 μm (Moan and Berg, 1991). The physiochemical properties of a photosensitizer determines the intracellular localization of the photosensitizer, e.g. plasma membrane, mitochondria, endocytic vesicles (Berg et al., 1990), and subsequently, the intracellular site of photochemical damage. The short action radius of the photochemical reaction is an advantage for PCI at the intracellular level since activation of photosensitizer localized in the membranes of endocytic vesicles is able to induce rupture of the endocytic vesicles without inactivating the drug located in the lumen. Substantial amounts of active lysosomal enzymes have been found in the cytosol after subtoxic doses of $TPPS_{2a}$-PCI, in contrast to cells treated with $TPPS_4$ and light where no enzyme activities were released from the lysosomes (Berg and Moan, 1994). Studies have, however, shown that photosensitizers might be re-localized after light exposure (Berg et al., 1991; Wood et al., 1997), and photodamage might, therefore, also be induced at distant subcellular sites (Rodal et al., 1998).

2.2.3 Photochemically-induced toxicity

Mechanisms of action following PCI treatment is dependent on the drug which is released as well as the photosensitized reaction. Photochemical reactions as induced by PCI have been shown to target several proteins involved in cellular growth and survival, including EGFR (Weyergang et al., 2007; Weyergang et al., 2008a; Yang et al., 2013; Ahmad et al., 2001; Tsai et al., 2009), HER2 (Koval et al., 2009; Berstad et al., 2012), mTOR (Weyergang et al., 2009), ERK, p38 and JNK (Weyergang et al., 2008b), and to cause direct tumor damage by inducing necrosis (Piette et al., 2003), apoptosis (Oleinick et al., 2002; Reiners, Jr. et al., 2002) or autophagy (Reiners, Jr. et al., 2010) in the tumor cells. The balance between these different cell death mechanisms depends not only on the cell type (Srivastava et al., 2001), tissue oxygenation (Golab et al., 2002) and localization and nature of the photosensitizer (Hsieh et al., 2003; Kessel and Luo, 1998; Moor, 2000), but also on the degree of photosensitization (photosensitizer dose, light dose/fluence and intensity/fluence rate), with necrosis as the principal mode of cell death in strongly photosensitized cells, while apoptosis (and also autophagy) predominate when photosensitization is not as extensive (Piette et al., 2003; Mroz et al., 2011; Vantieghem et al., 1998). Autophagy may, dependent on the circumstances, contribute both in a pro-death and pro-survival manner (Reiners, Jr. et

al., 2010; Kessel et al., 2012). In addition to direct tumor cell damage, the photochemical treatment has also been shown to induce indirect tumor damage by targeting the tumor-associated vasculature, depriving the tumor of oxygen and nutrients (Norum et al., 2009a), and to induce inflammatory and immunological responses (Castano et al., 2006; Korbelik, 2006). Tumor cells surviving the initial direct cytotoxic insult may still be eradicated by these indirect effects, which probably are highly important for a long-term tumor control (Castano et al., 2006).

2.2.4 The therapeutic potential of PCI

PCI is dependent on localization of the drug of interest in endocytic vesicles at some stage in the process. All mammalian cells, except mature erythrocytes, exert endocytic activity and PCI should therefore be effective in all other types of cells. Indeed, the principle of PCI has been documented in >80 cell lines and >12 different xenograft models (Selbo et al., 2010). PCI has been shown to stimulate the cytosolic delivery and, hence, the potency of a wide range of macromolecules and small drugs (Table 1). By activating the potential of endo/lysosomally entrapped drugs only in tissue simultaneously targeted by photosensitizer and light, PCI has been indicated to reduce the total therapeutic drug dose and PCI should, therefore, represent a strategy to reduce the dose-dependent adverse effects of a drug without reducing the clinical efficacy. PCI is currently undergoing clinical evaluation for delivery of the chemotherapeutic drug bleomycin (www.clinicaltrials.gov). In the two completed clinical trials (NCT00993512, NCT01872923), PCI of bleomycin demonstrated highly promising results in patients with head and neck neoplasms and a phase II study on recurrent head and neck squamous cell carcinomas is currently recruiting patients (NCT01606566). PCI of gemcitabine followed by gemcitabine/cisplatin chemotherapy is currently assessed in patients with locally advanced inoperable cholangiocarcinomas (phase I/II study, NCT01900158). PCI is, however, not only applicable to treatment of the tumor parenchyma. Recent publications have investigated the use of PCI targeted to the tumor vasculature (Vikdal et al., 2013b; Weyergang et al., 2014) and PCI has also been used in autologous vaccination for stimulation of CD8+ T-cell responses (Waeckerle-Men et al., 2013; Hakerud et al., 2013).

Macromolecules	Documented	References
Non-targeted proteins/polymers	*In vitro, in vivo*	(Berg et al., 1999; Selbo et al., 2000a; Selbo et al., 2001b; Prasmickaite et al., 2002; Dietze et al., 2003; Selbo et al., 2006; Berg et al., 2006; Fretz et al., 2007; Lai et al., 2008)
Targeted proteins/polymers	*In vitro, in vivo*	(Selbo et al., 2000b; Weyergang et al., 2006; Yip et al., 2007; Selbo et al., 2009; Berstad et al., 2012; Bostad et al., 2013; Lund et al., 2014; Bull-Hansen et al., 2014; Weyergang et al., 2014)
Nonvirus-mediated genes	*In vitro, in vivo*	(Berg et al., 1999; Prasmickaite et al., 2002; Hellum et al., 2003; Kloeckner et al., 2004; Ndoye et al., 2006)
Virus-mediated genes	*In vitro*	(Prasmickaite et al., 2002; Dietze et al., 2003; Bonsted et al., 2004; Dietze et al., 2005; Bonsted et al., 2006)
Nucleic Acids	*In vitro, in vivo*	(Folini et al., 2003; Oliveira et al., 2007; Oliveira et al., 2008; Raemdonck et al., 2010)
Chemotherapeutic drugs	*In vitro, in vivo*	(Berg et al., 2005a; Lou et al., 2006; Norum et al., 2009a; Weyergang et al., 2014; Adigbli et al., 2007)
Vaccine antigens	*In vitro, in vivo*	(Waeckerle-Men et al., 2013; Hakerud et al., 2013)

Table 1: Macromolecules and small drugs delivered by PCI.

2.2.5 Photodynamic therapy (PDT)

Another treatment modality exploiting the cytotoxic combination of photosensitizer, light and oxygen is photodynamic therapy (PDT) (Agostinis et al., 2011). Contrary to PCI, which primarily utilizes this combination for drug delivery, PDT relies solely on ROS-induced cytotoxicity to eradicate the target cells. The development of modern PDT began in the 1960s with the work of Lipson and Schwartz and truly accelerated in the 1970s with the work of Dougherty et al. (Dolmans et al., 2003). The first controlled clinical PDT study conducted in 1978 reported on successful treatment of skin cancer (Dougherty et al., 1978) and since then more than 200 clinical studies have been reported. PDT is today primarily used to eradicate premalignant lesions or early-stage cancers with little or no metastatic potential and to reduce the size of end-stage tumors (Allison, 2014). The most utilized photosensitizer in clinical PDT is porfimer sodium (Photofrin®), which is approved for treatment of several cancer indications, including cervical cancer, lung cancer, oesophageal cancer and bladder cancer (Brown et al., 2004; Godoy et al., 2013). Other photosensitizers with marketing authorization for PDT include temoporfin (mTHPC/Foscan®) for treatment of head and

neck cancer (Green et al., 2013; Bredell et al., 2010; Hopper, 2000), benzoporphyrin derivate (Visudyne®) for treatment of age-related macular degeneration (Sickenberg, 2001) and topically administered 5-aminolevulinate acid (Levulan®) and methyl-aminolevulinate (Metvix®) for treatment of actinic keratosis and basal-cell carcinomas (Kormeili et al., 2004; Braathen et al., 2007). Hexyl aminolevulinate (Hexvix®) is used for detection of bladder cancer (based on fluorescence from the photoactivated photosensitizer) (Witjes and Douglass, 2007; Lange et al., 1999). PDT is today regarded as standard therapy within certain subtypes of cancer; however, the method has room for improvements with respect to prolonged photosensitivity, tumor selectivity, depth of necrosis and light exposure times (Allison, 2014). Investigational approaches within the PDT field include combination therapies (Bhuvaneswari et al., 2009), utilization of PDT-induced vascular leakiness to facilitate drug delivery (Snyder et al., 2003), conjugation of the photosensitizer to targeting moieties, such as mAbs or peptides (Master et al., 2012; van Dongen et al., 2004; Bhatti et al., 2008), and encapsulation of the photosensitizer in nanoparticle formulations to facilitate drug delivery and improve tumor selectivity of the treatment (Solban et al., 2006).

2.3 Ribosome-inactivating protein toxins from plants

Ribosome-inactivating proteins (RIPs) produced by plants exert *N*-glycosidase activity against the 60S ribosomal subunit in eukaryotic cells (Barbieri et al., 1993). Removal of a specific adenine residue from 28S RNA leads to inhibition of protein synthesis and, consequently, cell death (Endo et al., 1987). RIPs from plants are divided into type I and type II toxins depending on their chemical structure (Stirpe and Battelli, 2006). Type I RIPs consist only of the enzymatically active A-chain responsible for ribosomal inactivation (Stirpe et al., 1980; Rosenblum et al., 1995; Bergamaschi et al., 1996), while type II RIPs consist of the A-chain linked to a cell-binding B-chain through a disulfide bridge (Nielsen and Boston, 2001). The B-chain mediates cellular uptake by binding to glycolipids or glycoproteins with terminal galactose (Sandvig and van Deurs, 2005), after which the toxin A-chain is translocated to the cytosol via the Golgi complex and endoplasmic reticulum (ER) (Rapak et al., 1997). Unlike bacterial toxins, no distinct translocation domain responsible for ER retrieval has been identified for type II RIPs (Wesche et al., 1999; Chaudhary et al., 1990). The characteristics of type I versus type II toxins are summarized in Table 2.

	Type I RIPs	Type II RIPs
Chemical structure	A (Active) domain	A (Active) domain - S – S - B (binding) domain
Molecular size	~30 kDa	~65 kDa
Internalization mechanism	Mainly pinocytosis	Adsorptive endocytosis
Principal intracellular fate	Transport to lysosomes	Retrograde transport via Golgi to ER and translocation to the cytosol
Ribosome-inactivating activity	High	High
Cytotoxicity to intact cells	Low	High
Suitable for delivery by PCI	Yes	No
Examples	Gelonin, Saporin	Abrin, Ricin

Table 2: Characteristics of type I and type II RIPs from plants.

It has been indicated that as little as 1-10 RIP molecules in the cytosol are sufficient to kill one cell (Eiklid et al., 1980). Once translocated to the cytosol, type I and type II RIPs have similar ribosome-inactivating activity. However, due to different intracellular transport mechanisms, they differ substantially in their toxicity towards intact cells (Barbieri et al., 1993; Vago et al., 2005). To enhance their potency in cancerous tissue and minimize toxicity in normal tissue, RIPs have been evaluated as part of targeted toxin formulations (Laske et al., 1997; Hirota et al., 1989; Lyu et al., 2010). The escape from endo/lysosomal compartments has, however, been shown to be a bottleneck for successful delivery of type I RIP-based fusion toxins (Pirie et al., 2011; Yazdi and Murphy, 1994). Several attempts have been made in order to utilize endosomal acidification and lysosomal processing for cytosolic release of type I RIP-based targeted toxins. These include incorporation of cleavable peptides (Heisler et al., 2003), reducible disulfide or thioether linkers (Lewis Phillips et al., 2008; Erickson et al., 2006) and combination strategies with saponins (Weng et al., 2012) or cytolysins (Pirie et al., 2013). The two type I RIPs used in the present study, saporin and gelonin, are mainly taken up by the passive mechanism of fluid-phase pinocytosis (Barbieri et al., 1993). Glycosylated natural saporin and gelonin have, however, also been shown to be taken up by receptor-mediated endocytosis through the α_2-macroglobulin (Cavallaro et al., 1995) and mannose receptor (Madan and Ghosh, 1992), respectively. The

recombinant version of gelonin is devoid of the carbohydrate groups present in native gelonin that promote mannose-specific uptake (Rosenblum et al., 1995) and is therefore taken up exclusively by pinocytosis.

2.3.1 Targeted protein toxins

Targeted protein toxins are composed of one cell-binding moiety (antibody, endogen ligand or a fragment of one of these) and one toxin moiety (derived from bacteria or plant) (Pastan et al., 2007; FitzGerald et al., 2004). The cell-binding moiety binds to a surface antigen expressed by cancer cells and triggers receptor-mediated endocytosis of the toxin. The toxin moiety must be translocated from endocytic vesicles to the cytosol in order to kill the cell by inhibiting protein synthesis (Sandvig and van Deurs, 2005; Pastan et al., 1986). The term immunotoxin describes antibodies or antibody fragments linked to toxins, while endogenous ligands linked to toxins may be termed affinity toxins. Previously, immunotoxins were produced by chemically conjugating complete structures of antibodies (fully/partly murine/human) to native toxins, resulting in large and heterogeneous products with high immunogenicity (Pastan et al., 2007). However, with recent recombinant technology it is now possible to fuse smaller DNA fragments coding only the antibody domain responsible for receptor binding and the enzymatically active domain of toxins, with the advantages of homogenous and less immunogenic products with desired size and reduced production costs (Fig. 5) (Kreitman, 2006; Potala et al., 2008).

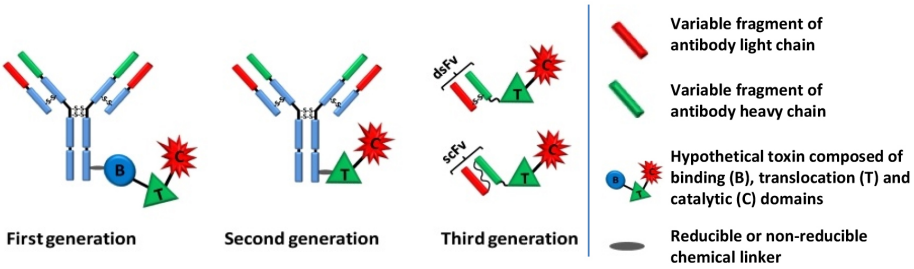

Figure 5: Three generations of immunotoxins (Adapted from (Shapira and Benhar, 2010) with modifications).

Off-target toxicity, usually characterized by hepatotoxicity and vascular injury, in addition to immunogenicity after prolonged administration have been identified as major limitations for clinical use of both bacterial and plant toxin-based immunotoxins (Pai-Scherf et al., 1999; von Minckwitz et al., 2005; Cao et al., 2012; Schmidt et al., 1999). Several approaches have been investigated in order to reduce nonspecific tissue and cellular uptake by reducing aggregation in serum and preventing association with negatively charged cellular membranes and receptors specific for protein toxins in normal tissues such as blood vessels. These include modifications to mask or remove positive surface charge and immunogenic groups such as B-cell epitopes, as well as the use of biodegradable carriers containing environmentally (e.g. pH, enzyme) sensitive linkages to promote site-specific activity of the toxin moiety (Chen et al., 2008; Onda et al., 2001; Onda et al., 2011; Heisler et al., 2003; Erickson et al., 2006). Immunogenicity may also be overcome by co-administration with immunosuppressive agents (Siegall et al., 1997).

Only one targeted fusion toxin has so far been approved by the FDA for clinical use, namely the IL-2 truncated diphtheria toxin fusion protein, denileukin diftitox (Ontak®) for treatment of cutaneous T-cell lymphoma (Kaminetzky and Hymes, 2008). The use of fusion toxins to treat nonhematological cancers has, however, not been very successful. The distribution to and retention of targeted toxins in solid tumors after systemic administration are obtained by a combination of passive and active targeting. The leaky and torturous vessels surrounding solid tumors and poor lymphatic drainage of the tumor tissue facilitates passive targeting by the enhanced permeation and retention (EPR) effect, while active targeting is mediated by interaction of the targeting moiety with receptors on the cancer cells (Jain, 2012). The distribution within the tumor has been shown to be influenced by the receptor affinity, as well as the internalization and dissociation rate of the targeted toxins (Adams et al., 2001; Rudnick et al., 2011; Cao et al., 2012). Due to high interstitial pressure in solid tumors rendering convection inefficient, tumor penetration relies on the slow mechanism of diffusion (Jain, 1989). Long distances from the tumor vasculature to the target cells and heterogeneous blood supply combined with short half-lives of 3rd generation immunotoxins may consequently serve as potential barriers for homogenous distribution of fusion toxins into solid tumors (Jain, 1989).

2.4 The epidermal growth factor receptor (EGFR/ErbB) family

The epidermal growth factor receptor (EGFR/ErbB) family comprises four receptor tyrosine kinases (RTKs): EGFR (ErbB1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). These receptors are expressed in a variety of normal tissues (predominantly cells of mesodermal and ectodermal origins) where they control key cellular processes, such as proliferation, differentiation, adhesion, migration and survival (Yarden and Sliwkowski, 2001). The EGF receptors consist of an extracellular region, a transmembrane helix and a cytoplasmic region (Bublil and Yarden, 2007). All EGF receptors except HER2 are activated by binding of endogenous soluble ligands to their extracellular region. Ligand binding stimulates ErbB receptor homo- or heterodimerization and autophosphorylation of tyrosine residues at the cytoplasmic domain (Fig. 6) (Olayioye et al., 2000; Rowinsky, 2004; Lemmon, 2008). The cytoplasmic domain of HER3 is, however, catalytically inactive; therefore, HER3 is dependent on interaction with the other EGF receptors for signal transduction (Jura et al., 2009). Phosphotyrosine-binding proteins associate with the cytoplasmic tail of the catalytically active EGF receptors and initiate a complex system of signaling cascades, including the mitogen-activated protein kinase (MAPK), phosphoinositol-3-kinase (PI3K)/Akt and protein kinase C (PKC) pathways and also stress-activated protein kinase (SAPK) cascades, eventually leading to transcriptional events in the nucleus (Fig. 5) (Yarden and Sliwkowski, 2001). The receptors are internalized and, depending on the receptor ubiquitinylation, the ErbB receptors either follow the endocytic pathway to lysosomes for degradation or they are recycled back to the plasma membrane (Wiley, 2003; Austin et al., 2004). Although HER2 itself cannot bind any endogenous ligands, it is the preferred dimerization partner for kinase-active EGFR, HER3 and HER4 (Graus-Porta et al., 1997). HER2 promotes stabilization of the receptor dimers at the plasma membrane and increases recycling of internalized receptors to the cell surface, leading to prolonged signaling and reduced receptor downregulation (Hendriks et al., 2003; Citri and Yarden, 2006).

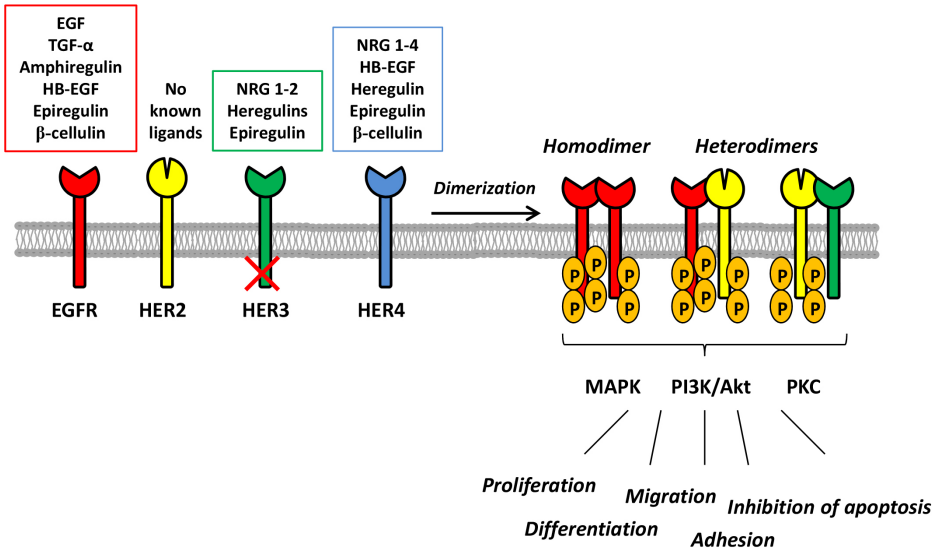

Figure 6: Simplified overview of ErbB receptor activation, dimerization and signaling, as well as some of the affected biological processes.

2.4.1 EGFR and HER2 in cancer

EGF receptors, particularly EGFR and HER2, have been implicated in the development of many types of solid tumors (Yarden and Sliwkowski, 2001; Rowinsky, 2004). Alterations, including receptor overexpression, mutations or in-frame deletions, as well as upregulation of ligands, lead to constitutive receptor activation and have been shown to drive cancer development (Slamon et al., 1987; Sok et al., 2006; Grandis and Tweardy, 1993). A brief overview of EGFR and HER2 overexpression in cancer is presented in Table 3. Large variation in overexpression frequency reported for some of the cancers is ascribed to differences in the study methodology and source of material. Although overexpression and/or mutation of EGFR or HER2 is correlated with poor prognosis in several cancers, including breast (Slamon et al., 1987; Rimawi et al., 2010), ovary (Verri et al., 2005), non-small cell lung carcinoma (NSCLC) (Hirsch et al., 2003; Brabender et al., 2001) and head and neck squamous carcinoma (HNSCC) (Grandis and Tweardy, 1993; Sok et al., 2006), the relationship between ErbB receptor overexpression and patient survival is not straightforward in all cancer types (Rowinsky, 2004); neither is the correlation between receptor expression and clinical response to ErbB-targeted therapies (Dua et al., 2010).

Cancer type	Tumors overexpressing		References
	EGFR	HER2	
Head and neck	Up to 90%	Rarely detectable	(Grandis and Tweardy, 1993; Hanken et al., 2014)
Non-small cell lung carcinoma	34-62%	16-35%	(Hirsch et al., 2003; Hirsch et al., 2002; Brabender et al., 2001)
Colorectal	43-80%	6-77%	(Spano et al., 2005; Seo et al., 2014; Khelwatty et al., 2014)
Breast	6-18%	20-30%	(Sorlie et al., 2001; Slamon et al., 1987; Rimawi et al., 2010; van, V et al., 1988; Bhargava et al., 2005; Lazaridis et al., 2014)
Ovarian	48-62%	11-40%	(Verri et al., 2005; Tuefferd et al., 2007; Nielsen et al., 2004; Lafky et al., 2008)
Pancreatic	42-69%	61%	(Uegaki et al., 1997; Tobita et al., 2003; Bloomston et al., 2006; Komoto et al., 2009)
Glioma	40-50%	Rarely detectable	(Shinojima et al., 2003; Faulkner et al., 2014; Haynik et al., 2007)
Cervical	33-73%	19-42%	(Kim et al., 1996; Oka et al., 1994; Tangjitgamol et al., 2005)

Table 3: EGFR and HER2 overexpression in selected human solid tumors.

It was originally thought that EGFR amplification promoted tumor growth exclusively by enhancing signaling through wild-type EGFR. Since then, several EGFR mutations in both the intracellular and extracellular domain have been identified as predictors for response to EGFR-targeted therapies (Sok et al., 2006; Kobayashi et al., 2005). Interestingly, EGFR has been found upregulated not only in the tumor parenchyma, but also in tumor-associated endothelial cells compared to normal vasculature, suggesting EGFR as a contributor in tumor angiogenesis and as a potential target in antiangiogenic therapy (Amin et al., 2006). EGFR is associated with radioresistance (Akimoto et al., 1999) and has also been implicated in epithelial-to-mesenchymal transition (EMT), which contributes to therapeutic resistance (Thomson et al., 2005; Ren et al., 2014).

HER2 is recognized as a more potent oncoprotein than other members of the EGFR family and its overexpression has been shown to result in constitutive ligand-independent receptor signaling (Ghosh et al., 2011). HER2-HER3 dimers have been identified as a particularly strong oncogenic unit activating both MAPK and PI3K/Akt signaling (Amin et al., 2010; Lee-Hoeflich et al., 2008). The role of HER2 as a

prognostic marker is highly recognized in breast cancer, where HER2 is associated with poor overall survival and short time to relapse (Slamon et al., 1987). HER2 has also been recognized as a predictive marker for response to HER2-targeted therapies (Ross and Fletcher, 1998). An increasing number of publications suggest, however, that also other factors influence on the therapeutic response to HER2-directed therapies (Esteva et al., 2010).

2.4.2 EGFR- and HER2-targeting cancer therapeutics

An enormous effort has been put into developing therapies that target the EGF receptors and their signaling pathways. Successful approaches used in the clinic include monoclonal antibodies (mAbs) and tyrosine kinase inhibitors (TKIs) (Imai and Takaoka, 2006), and recently also an antibody-drug conjugate (Lewis Phillips et al., 2008). MAbs are ~150 kDa proteins that bind to the extracellular domain of the receptor and thereby block ligand-induced downstream signaling (Schaefer et al., 2006; Li et al., 2005). Antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) induced through the Fc domain of mAbs have in addition been implied as important mechanisms of action *in vivo* (Clynes et al., 2000; Cooley et al., 1999). TKIs are small lipophilic drugs that diffuse through the plasma membrane and bind to the intracellular ATP binding site of the kinase, thereby blocking downstream signaling of the ligand-receptor complexes (Grunwald and Hidalgo, 2003; Herbst et al., 2004). Antibody-drug conjugates combine an antibody as the targeting molecule with a cytotoxic agent (Carter and Senter, 2008). In addition to inhibition of receptor signal transduction by the antibody, this treatment approach takes advantage of receptor-mediated endocytosis to deliver a cytotoxic agent into the cell. Currently approved mAbs, antibody-drug conjugates and TKIs targeting EGFR and HER2 are listed in Table 4. To this date, the most utilized EGFR family-targeted drugs in the clinic are the humanized IgG_1 anti-HER2 mAb trastuzumab (Herceptin®), which received FDA-approval for treatment of HER2+ metastatic breast cancer already in 1998 (Vogel et al., 2002; Baselga et al., 2006; Tokuda et al., 2009) and the chimeric IgG_1 anti-EGFR mAb cetuximab (Erbitux®), which was approved by the FDA for treatment of metastatic colorectal cancer in 2004 (Baselga, 2001; Vincenzi et al., 2008).

Table 2: EGFR- and HER2- targeting therapies approved for cancer treatment		
	Targeting receptor	**Clinical use**
Monoclonal antibodies		
Cetuximab (Erbitux®)	EGFR	Head and neck cancer, Colorectal cancer
Trastuzumab (Herceptin®)	HER2	Breast cancer (HER2+), Gastric cancer
Panitumumab (Vectibix®)	EGFR	Colorectal cancer
Pertuzumab (Perjeta®)	HER2	Breast cancer (HER2+)
Nimotuzumab (BIOMAb EGFR®*)	EGFR	HNSCC
Antibody-drug conjugates		
Ado-trastuzumab emtansine (Kadcyla®)	HER2	Breast cancer (HER2+)
Tyrosine kinase inhibitors		
Erlotinib (Tarceva®)	EGFR	NSCLC, Pancreatic cancer
Gefitinib (Iressa®)	EGFR	NSCLC
Afatinib (Gilotrif®)	EGFR/HER2	NSCLC
Lapatinib (Tykerb®)	EGFR/HER2	Breast cancer (HER2+)
Vandetanib (Caprelsa®)	EGFR (RET, VEGFR2)	Medullary thyroid cancer

*Only approved in certain countries (not by the FDA or EMEA).

Table 4: Currently approved EGFR and HER2 targeting therapeutics.

The introduction of mAbs and TKIs targeting EGFR and HER2 have revolutionized cancer management and opened up for more individualized treatment regimens. However, *de novo* or acquired resistance in a significant number of patients is a major clinical limitation for both mAbs and TKIs (Nahta et al., 2006; Lu et al., 2007; Chong and Janne, 2013; Kim et al., 2010) and this have stimulated the development of alternative ErbB-targeted treatment approaches, including dual receptor targeting (Konecny et al., 2006; Waldron et al., 2012), irreversible or mutant-specific inhibitors (Chandramohan et al., 2013), antisense oligonucleotides and siRNA, immunoconjugates (Gelardi et al., 2010), ligand or antibody-cytotoxic drug conjugates (Lewis Phillips et al., 2008) and recombinant toxins (Azemar et al., 2000; Thomas et al., 2004; Cao et al., 2009). Ado-trastuzumab emtansine (T-DM1) received FDA approval for treatment of HER2-positive breast cancer in 2013 (Table 3). This is an antibody-drug conjugate consisting of trastuzumab linked via a thioether linkage to the microtubule-depolymerizing maytansinoid, mertansine (Lewis Phillips et al., 2008).

3. GENERAL EXPERIMENTAL CONDITIONS

3.1 Cell lines

The 15 cell lines used in this thesis was mainly chosen based on their expression of the targeting receptors, EGFR and/or HER2. The cell lines selected to prove the principle of PCI of HER2-targeted trastuzumab-saporin (Paper I; ZR-75-1, MDA-MB231) or MH3-B1/rGel (Paper II; SK-BR-3) were of breast cancer origin, since HER2-targeted therapeutics are approved for this indication (Table 3). PCI of MH3-B1/rGel (Paper II) was also evaluated in three ovarian cancer cell lines, SKOV-3, MOC-7 and Nu-Tu19, to explore if PCI could overcome the resistance towards traditional HER2-targeted therapies in HER2+ ovarian cancers.

Of the >80 cancer cell lines that have been demonstrated sensitive to PCI (Selbo et al., 2010), A-431 epidermoid carcinoma cells are among the most utilized (Weyergang et al., 2006; Yip et al., 2007; Oliveira et al., 2007; Kloeckner et al., 2004). This cell line is also among the most frequently used in EGFR research (Merlino et al., 1984; Aerts et al., 2009) and was therefore chosen as an EGFR-positive model for characterization of the rGel/EGF construct and for evaluation of PCI of rGel/EGF (Paper III). WiDr (colorectal adenocarcinoma) was included as a control with more moderate EGFR expression, since the A-431 cells are debated for their remarkably high EGFR expression (Haigler et al., 1978; Fabricant et al., 1977). PCI has been shown efficient in WiDr cells for delivery of MOC31-gelonin (Selbo et al., 2000b) and free gelonin, both with the "light first" and "light after" strategy (Selbo et al., 2001b; Berg et al., 2006), and the WiDr cells were therefore also used as a positive control in Paper I for demonstration of the "light first" PCI principle. Two cell lines, MES-SA human uterus sarcoma and MDA-MB-435 melanoma were used as EGFR-negative controls in Paper III.

HNSCCs were chosen as clinically relevant models for PCI of rGel/EGF based on the presence of EGFR as a validated target and indicator of poor prognosis in ~90% of HNSCC patients (Grandis and Tweardy, 1993; Ang et al., 2002), as well as the promising preliminary results on PCI of bleomycin in head and neck cancer in clinical studies (NCT00993512, NCT1872923). Also, the overall survival of HNSCC patients

has not changed much the last 50 years, emphasizing the need for new and improved treatment strategies (Gregoire et al., 2010). Four HNSCC cells lines derived from tumors with diversities in site of origin, stage and differentiation (Martin et al., 2008), as well as EGFR expression levels, were used for *in vitro* studies on PCI of rGel/EGF.

3.2 Tumor xenograft models

HER2-targeted therapies have to this date yielded disappointing clinical results in HER2-positive ovarian cancer (Bookman et al., 2003; Sheng and Liu, 2011). The exploration of novel HER2-targeted approaches in this type of cancer is therefore of clinical interest. To investigate the *in vivo* potential of PCI of MH3-B1/rGel in ovarian cancer, human SKOV-3 tumor xenografts were selected. The growth of SKOV-3 tumors have previously been shown to be inhibited by repeated administration of HER2-targeted immunotoxins consisting of Herceptin or its derived scFv, 4D5, fused to recombinant gelonin (Cao et al., 2014).

To demonstrate the EGFR-specific toxicity of PCI of rGel/EGF *in vivo,* human A-431 tumor xenografts were selected. This is, as already stated, a well-known EGFR-overexpressing model extensively investigated both *in vitro* and *in vivo* (Hirota et al., 1989; Phillips et al., 1994).

The rationale for choosing HNSCC as a clinically relevant model for evaluation of PCI of rGel/EGF is already discussed in the previous section (3.1). The literature on HNSCC xenografts is modest. Effort was therefore put into establishing new HNSCC xenograft models. Of the four HNSCC cell lines used *in vitro,* two of these (SCC-026 and SCC-040) formed tumors after s.c. inoculation in athymic nude mice. Both SCC-026 and SCC-040 tumor xenografts developed as fluid-filled cystic structures, which was clearly evident after reaching a tumor size of approx. 900 and 400 mm^3, respectively (3-4 weeks after injection) (Fig. 7). These findings are in agreement with previous reports describing cystic metastasis from HNSCC in patients (Goldenberg et al., 2006). The SCC-026 xenograft model was selected for *in vivo* evaluation of PCI of rGel/EGF. The SCC-026 cells express EGFR at moderate levels compared to the A-431 cells, which has been estimated to express up to 25-30 times more EGFR compared to

other epidermal cells (Haigler et al., 1978; Fabricant et al., 1977; Merlino et al., 1984). This further emphasizes the clinical relevance of the SCC-026 HNSCC model.

All tumor xenografts were grown s.c. in the left thigh of athymic nude mice.

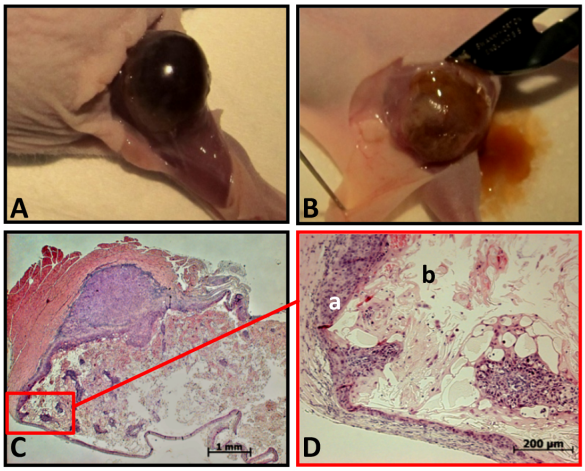

Figure 7: Characterization of HNSCC xenografts. A, B: Photos of ~1000 mm^3 SCC-040 tumors 38 days after inoculation. C, D: H&E staining of SCC-040 tumor harvested 38 days after inoculation. a; rim of viable tumor cells; b, areas of drained fluid.

3.3 Photosensitizer and light source

$TPCS_{2a}$ (Fig. 4D) was applied as the photosensitizer in the present studies. Dependent on the incubation procedure, $TPCS_{2a}$ will be integrated in the plasma membrane or in the membrane of endosomes and lysosomes (Fig.8).

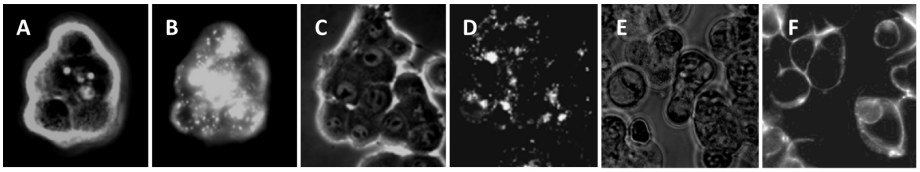

Figure 8: $TPCS_{2a}$ localization in Zr-75-1 cells. Phase contrast pictures (A, C, E) and fluorescence micrographs (B, D, F). 18 hrs incubation (37ºC) targets $TPCS_{2a}$ to the plasma membrane in addition to endocytic vesicles (A, B). 18 hrs incubation + 4 hrs chase in drug free medium (both at 37ºC) targets $TPCS_{2a}$ primarily to the endocytic vesicles (C, D). 30 min incubation at 4ºC targets the photosensitizer only to the plasma membrane (E, F).

The chemical structure of $TPCS_{2a}$ is virtually identical to that of $TPPS_{2a}$, which has been used in previous studies on PCI of EGFR-targeted toxins (Weyergang et al., 2006; Yip et al., 2007). However, a reduced double bond in the porphyrin backbone (red arrow, Fig. 4) characterizes $TPCS_{2a}$ as a chlorin and results in an absorption peak in the red part of the absorption spectrum (650 nm), rendering $TPCS_{2a}$ advantageous for clinical use (Fig. 9). $TPCS_{2a}$ is patented for use in combination with PCI and is currently undergoing clinical evaluation for PCI of the chemotherapeutic drug bleomycin (www.clinicaltrials.gov). $TPCS_{2a}$ is present as three isomers dependent on the arrangement of the sulfonate groups relative to the reduced double bond.

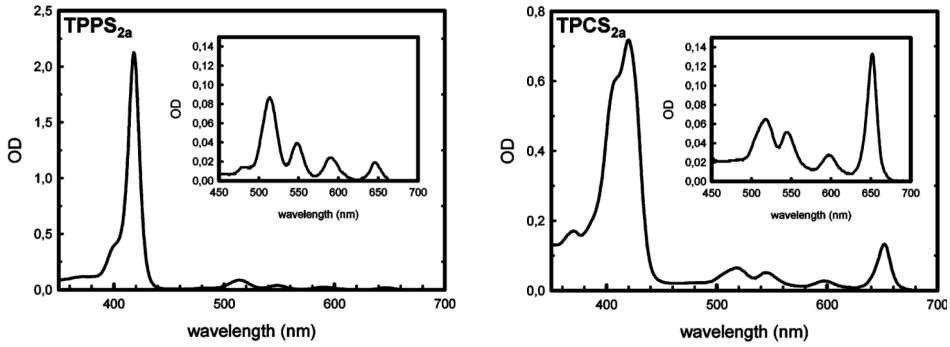

Figure 9: Absorption spectrum for $TPPS_{2a}$ (left) and $TPCS_{2a}$ (right).

The absorption spectrum of $TPCS_{2a}$ allows photosensitizer activation by both blue light and red light. LumiSource® (PCI Biotech), equipped with four light tubes (18W/tube, Osram L 18/67) emitting blue light with a peak wavelength at approximately 435 nm and an irradiance of 11.7 mW/cm^2, was used for illumination of cells *in vitro*. Red light penetrates skin more efficiently than blue light (Zonios et al., 2001). Therefore, a 652 nm diode laser (Ceramoptec GmbH, Bonn, Germany) equipped with a laser fiber (Med*light* SA, Eclubens, Switzerland) with an irradiance of 90 mW/cm^2 was used for illumination of tumors *in vivo.* The tumors were irradiated 72 hrs after injection of 5 mg/kg $TPCS_{2a}$ via the tale vein. The animals were covered with aluminum foil with a margin of about 2 mm to the visible tumor.

3.4 Assays for cytotoxicity measurements

In the present study, cellular viability was mainly assessed using the MTT assay, which is based on cleavage and conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to blue formazan crystals by cellular dehydrogenases. The cells were incubated 2-4 hrs with the MTT reagent, after which the formazan crystals were dissolved in DMSO and measured colorimetrically at 570 nm. Viability was expressed as percentage of controls. The MTT assay is fast, cheap and easy to carry out. However, an important limitation of the MTT assay is that it measures enzymatic activity as a surrogate viability marker rather than direct cell survival, and therefore, dying cells with some remaining mitochondrial activity might be identified as viable cells. The potential of underestimating dying cells is therefore substantial at low time intervals after treatment (Komissarova et al., 2005). Cellular viability was evaluated 48 hrs after the photochemical treatment for all cell lines except the slowly proliferating SKOV-3 cells, for which viability was measured 96 hrs after treatment. The MTT assay was, however, also performed at 72 hrs to exclude the possibility that altering the time before MTT assessment would significantly influence on the viability results. The clonogenic cell survival assay has been suggested as the gold standard among *in vitro* cytotoxicity assays (Roper and Drewinko, 1976; Sumantran, 2011). This assay measures the ability of a single cell to form a viable colony, thus estimating the long-term effects of a drug, including the sum of all cell death modes and delayed growth arrest. No significant difference has, however, been observed in previous PCI studies when comparing cytotoxicity data measured by MTT 2-5 days after treatment to clonogenic cell survival assessed 7-14 days after treatment (Yip et al., 2007; Bostad et al., 2014; Bostad et al., 2013). MTT can be assessed in 96 well plates, while the clonogenic assay is usually performed in larger wells to achieve a sufficient sample size for statistical significance. The MTT assay thus demands much less material and is also less time consuming compared to clonogenic cell survival. Considering the limited amount of targeted toxins available for the present studies, the MTT assay was chosen as the standard assay. The results obtained by MTT on MH3-B1/rGel and rGel/EGF with and without PCI were, however, confirmed by the crystal violet staining method (Paper II and III). The dye in this assay, crystal violet, binds to sugar type molecules, such as DNA. Upon solubilization of the dye, the color intensity will be proportional to the cell number (Vega-Avila and Pugsley, 2011). After removing

the culture medium, the remaining adherent cells were incubated 30 min with the crystal violet solution (0.5% in 20% methanol), and after a thorough washing and drying procedure, the stained cells were solubilized with Sørenson's buffer (0.1 mol/L sodium citrate, pH 4.2, in 50% ethanol). Absorbance was measured at 595 nm. As an alternative to the clonogenic assay, long-term effects of the antibody cetuximab (Paper II) were assessed using the Incucyte kinetic imaging system (Essen BioScience, Hertfordshire, UK), which measures well confluence as an estimate for cellular proliferation.

Neither the MTT nor crystal violet assay is able to discriminate between different cell death modes. Molecular characteristics of autophagy, apoptosis and necrosis were therefore investigated in order to obtain information on the mechanisms of cell death (Kepp et al., 2011). Autophagic cells were identified by detecting the conformation of cytosolic LC3-I into membrane-bound LC3-II associated with autophagosomes (Kabeya et al., 2000). Apoptotic cells were identified by detecting cleavage of the caspase substrate PARP in addition to a TUNEL assay identifying DNA fragmentation. Unlike apoptosis, where the integrity of the plasma membrane is retained until secondary necrosis intervenes or apoptotic bodies are cleared, primary necrosis is characterized by an early membrane collapse. Necrotic cells were therefore identified by measuring uptake of the exclusion dye propidium iodide (PI) 6 hrs after treatment.

3.5 Quantification of PCI targeting efficacy

The *in vitro* potency of a drug is commonly measured by its 50% inhibitory concentration. When comparing the efficacy of a targeted toxin among different cell lines as a result of receptor targeting (i.e. targeting efficacy), the cell line sensitivity to the non-targeted toxin should, however, be correlated for. The relationship between efficacy of the targeted toxin and the non-targeted toxin in a specific cell line may be expressed as the targeting index (TI):

$$Targeting\ index = \frac{IC_{50}\,(non-targeted\ toxin)}{IC_{50}\,(targeted\ toxin)}$$

Accordingly, when comparing the efficacy of PCI of targeted toxins among different cell lines, the cell line sensitivity towards the non-targeted toxin should be correlated for. In Paper II, the PCI efficacy of MH3-B1/rGel was corrected for rGel sensitivity according to this formula:

$$rGel-corrected\ PCI\ efficacy = \frac{LD_{50}\,(Photochemical\ treatment)\,x\,IC_{50}\,(rGel)}{LD_{50}\,(PCI)}\ x\ 100$$

where the photochemical treatment is the combination of photosensitizer and light without any protein toxin added, and LD_{50} is the light dose where PCI or the photochemical treatment induces a 50% reduction in cell viability.

The rGel-corrected PCI efficacy estimated in Paper II correlates the efficacy of PCI of the targeted toxin for sensitivity to the photochemical treatment (photosensitizer + light) and non-targeted rGel as monotherapies. In Paper III, a PCI targeting index (TI_{PCI}) was established which corrected the reduced viability obtained by PCI of the targeted toxin for the cell line sensitivity to PCI of non-targeted toxin (i.e. photosensitizer + light + rGel). The TI_{PCI} was assessed at IC_{90} (90 % reduction in cell viability) in experiments where the photochemical treatment itself reduced the viability by 30-60%.

$$PCI\ Targeting\ index = \frac{IC_{90}\,(PCI\ of\ non-targeted\ toxin)}{IC_{90}\,(PCI\ of\ targeted\ toxin)}$$

Low toxicity of the targeted toxin against cells not simultaneously targeted by photosensitizer and light contributes to the specificity induced by PCI and, consequently, a large gap between the TI and TI_{PCI} is expected to be advantageous in the clinical setting. The ratio between the TI with and without PCI was therefore used as an estimate of the potential of PCI to augment the cytotoxic effect of the targeted toxin in target cells.

4. SUMMARY OF PUBLICATIONS

4.1 Paper I

This is the first publication on PCI of a HER2-targeted toxin. The HER2-targeted mAb trastuzumab was linked to saporin through a streptavidin-biotin linker. HER2-selective uptake and cytotoxicity of PCI of trastuzumab-saporin was demonstrated by increased cytotoxicity of PCI of trastuzumab-saporin compared to PCI of saporin in HER2-positive Zr-75-1 cells, while the difference was much smaller in the HER2 low-expressing MDA-MB231 cells. HER2-specificity of PCI of trastuzumab-saporin was also confirmed by the ability to reduce the cytotoxic effect by blocking HER2 with an excess of free trastuzumab. PCI of trastuzumab-saporin was much more efficient when using the "light after" procedure compared to the "light first" procedure. This could be correlated to photochemically-induced HER2 damage, which inhibited internalization of trastuzumab-saporin. The photochemical HER2 damage was shown to be dependent on the photosensitizer localization, with $TPCS_{2a}$ inducing more severe effects on total HER2 when targeted to the plasma membrane as compared to endocytic vesicle membranes. The ability of HER2 to become phosphorylated at Y1221/1222 was, however, reduced to the same extent using both targeting procedures. The data obtained with trastuzumab-saporin suggest that, in principle, PCI of HER2-targeted immunotoxins should be a valid approach for treatment of HER2-overexpressing cancers in general, such as breast, ovary, stomach, colon and esophagus. The present results emphasize, however, that the order of the different steps in the PCI protocol is of vital importance, with administration of the HER2-targeted immunotoxin prior to light exposure (i.e. "light after" procedure) as a prerequisite for successful PCI therapy.

4.2 Paper II

This paper explores the *in vitro* and *in vivo* potential of PCI of a recombinant HER2-targeted immunotoxin for treatment of ovarian cancer, a type of cancer where HER2-targeted therapy generally has demonstrated limited efficacy. The immunotoxin comprise a single-chain variable fragment (scFv) of an anti-HER2 mAb (MH3-B1) fused to recombinant gelonin (rGel) through a GGGGS linker. *In vitro* efficacy of PCI of MH3-B1/rGel was demonstrated in three HER2-expressing ovarian cancer cell lines, SKOV-3, HOC-7 and NuTu-19. The SKOV-3 cells, although found relatively resistant to both trastuzumab and MH3-B1/rGel, responded strongly to PCI of MH3-B1/rGel and were found equally sensitive to this treatment as the SK-BR-3 breast cancer cells, although the SK-BR-3 cells were 10-fold more sensitive to MH3-B1/rGel monotherapy (when correlating for rGel sensitivity). Lysosomal degradation was indicated as the mechanism of resistance to MH3-B1/rGel monotherapy in the SKOV-3 cells, as shown by the positive correlation between localization of MH3-B1/rGel and Lysotracker in these cells. Significant growth inhibitory effects of PCI of MH3-B1/rGel were demonstrated *in vivo* on SKOV-3 xenografts as measured by tumor size 16 days post treatment. The *in vivo* effect induced by PCI of 2 mg/kg MH3-B1/rGel was, however, smaller than expected based on the presented *in vitro* results and it was concluded that the *in vivo* protocol must be optimized with respect to MH3-B1/rGel dose. In conclusion, the efficiency of PCI of MH3-B1/rGel in SKOV-3, HOC-7 and NuTu-19 cells supports general applicability of this PCI approach in ovarian cancer and indicates PCI of MH3-B1/rGel as a possible HER2-targeted treatment approach for ovarian cancers resistant to HER2-targeted therapeutics.

4.3 Paper III

This paper is the first report on the development and production of an EGFR-targeting recombinant fusion toxin specifically designed for cytosolic delivery by PCI. It is also the first study showing antitumor effects of a gelonin-based EGFR-targeted toxin in head and neck tumors. Recombinant gelonin (rGel) was fused to the epidermal growth factor (EGF) through a flexible GGGGS linker. The fusion protein was expressed in *Escherichia coli* and purified by immobilized metal affinity chromatography (IMAC). Despite significantly reduced ribosome-inactivating activity of rGel/EGF compared to that of rGel in a cell-free reticulocyte lysate system, rGel/EGF exerted increased cytotoxicity against EGFR-expressing A-431 and HNSCC cell lines. PCI further increased the cytotoxicity of rGel/EGF in EGFR-expressing cells and was shown to increase the EGFR-targeting index of rGel/EGF up to 40-fold in HNSCC cell lines resistant to cetuximab (Erbitux®). EGFR-specific cytotoxicity PCI of rGel/EGF was verified by control experiments on EGFR-negative MES-SA and MDA-MB435 cells, in addition to receptor blocking with an excess of free cetuximab. LC3 lipidation, PI uptake, PARP cleavage and DNA fragmentation (TUNEL) was demonstrated following PCI of rGel/EGF in A-431 cells *in vitro*. Control experiments suggested that apoptosis and necrosis were induced by rGel/EGF after photochemical release, while the photochemical treatment ($TPCS_{2a}$ + light, no drug) induced autophagy. *In vivo,* PCI of rGel/EGF induced antitumor effects at an rGel/EGF dosage of only 0.1 mg/kg, as shown by a significant growth delay of A-431 tumor xenografts and a reduction of tumor perfusion and necrosis induction in SCC-026 HNSCC tumors. A dose-dependent effect was, however, seen in the A-431 tumors as doubling the rGel/EGF dose resulted in synergistic effects. The data obtained suggest that there is three tumor-specific aspects of PCI of rGel/EGF; i) preferential retention of the photosensitizer in tumor tissue, ii) light exposure confined to the cancerous area, and iii) EGFR-targeting properties of rGel/EGF. Tumor specificity is further supported by minimal toxicity of rGel/EGF in off-target cells due to lysosomal degradation. It was concluded that PCI of rGel/EGF has potential for further preclinical development towards treatment of patients with HNSCC, although with optimization of the rGel/EGF product as a prerequisite for further preclinical evaluation.

5. GENERAL DISCUSSION

The molecular pathways of EGFR and HER2 have attracted considerable attention as therapeutic targets for solid tumors. To this date, the most successful EGFR- and HER2-targeted therapeutic strategies (i.e. mAbs and TKIs) exert their pharmacologic action via inhibition of receptor activation (Ciardiello and Tortora, 2008; Nielsen et al., 2009). EGFR and HER2 are, however, also investigated as delivery portals for cytotoxic agents into the cells (Pastan et al., 2007). EGFR- and HER2-targeted toxins based on the bacterial toxins, Pseudomonas exotoxin (PE) or Diphteria toxin (DT) have demonstrated *in vivo* antitumor effects in several cancers, including brain cancer (Phillips et al., 1994; Engebraaten et al., 2002; Chandramohan et al., 2013; Liu et al., 2005), pancreatic cancer (Bruell et al., 2005), gastric cancer (Batra et al., 1992) and head and neck cancer (Thomas et al., 2004; Waldron et al., 2012; Barnea et al., 2013; Azemar et al., 2000). Off-target effects with these highly potent targeted toxins, as well as formation of neutralizing antibodies have, however, been demonstrated as obstacles for clinical use (Pai-Scherf et al., 1999; von Minckwitz et al., 2005; Cao et al., 2012; Azemar et al., 2003) and, so far, no targeted toxin has been approved for treatment of nonhematological cancers. In order to obtain toxin-based treatment strategies that are clinically viable, it is necessary to preserve the potency of the toxins in tumor cells while sparing normal cells.

The present study explores PCI as a method to increase the tumor-specific toxicity of EGFR- and HER2-targeted toxins. PCI induces site-specific cytosolic release of drugs that are entrapped in endocytic vesicles. PCI of targeted toxins based on PE and DT is little effective since these bacterial toxins have translocation domains for endosomal escape (Weyergang et al., 2011). Type I RIP-based targeted toxins are, however, ideal for tumor-selective activation by PCI, since these are equally potent as PE and DT once entering the cytosol (Eiklid et al., 1980), but lack an effective mechanism for endosomal escape (Barbieri et al., 1993; Vago et al., 2005). Prior to this thesis, PCI was shown to increase the EGFR-specific cytotoxicity of the type I RIP-based targeted toxins EGF-saporin and cetuximab-saporin based on streptavidin-saporin (Weyergang et al., 2011; Yip et al., 2007; Weyergang et al., 2006). These studies formed a strong rationale for optimizing PCI of EGFR-targeted toxins for *in vivo*

applications. The overexpression of HER2 in several cancers and its ability to undergo internalization was the reason why also this receptor was included as a potential target for PCI-induced toxin delivery in the present project.

5.1 Construction and characterization of the targeted toxins

The present thesis includes three reports on PCI of type I RIP-based targeted toxins. The first report proves the principle of PCI of a HER2-targeted toxin, a concept which is further explored both *in vitro* and *in vivo* in the second report using a more clinically relevant recombinant toxin. The third report describes the design, production and preclinical evaluation of an EGFR-targeted toxin custom-made for delivery by PCI. The targeted toxins used in the present study were constructed and prepared using different techniques. Their characteristics are summarized in Table 5.

	Paper I	**Paper II**	**Paper III**
Targeting receptor	HER2	HER2	EGFR
Targeted toxin	Trastuzumab-saporin	MH3-B1/rGel	rGel/EGF
Production method	Noncovalent bioconjugation	Recombinant DNA technology	Recombinant DNA technology
Targeting moiety	Humanized IgG1 mAb	scFv	Endogenous ligand
Toxin moiety	Natural saporin	Recombinant gelonin	Recombinant gelonin
Linker	Biotin-streptavidin	GGGGS	GGGGS
Size	280 – 730 kDa	55 kDa	42 kDa

Table 5: Targeted toxins used in the present thesis.

The HER2-targeted immunotoxin described in Paper I was established by linking trastuzumab to saporin through the biotin-streptavidin binding. This binding is essentially the strongest noncovalent biological interaction known. The extraordinarily

high affinity between streptavidin and biotin (K_a = 10^{15} L/mol) ensures rapid and essentially non-reversible formation of trastuzumab-biotin-streptavidin-saporin complexes (Diamandis and Christopoulos, 1991). Each streptavidin tetramer has four binding sites for biotin, and streptavidin-saporin was therefore combined with biotin–trastuzumab at a biotin:streptavidin ratio of 4:1. This method offers an easy and convenient way to establish targeted toxins for proof-of-principle studies. However, with two saporin per streptavidin molecule and the ability to bind four biotinylated compounds, steric hindrance and, consequently, formation of heterogeneous products with different ratios between trastuzumab and saporin is likely. In theory, the conjugate size may exceed 700 kDa and, thus, these products have little potential for clinical utilization.

MH3-B1/rGel (Paper II) and rGel/EGF (Paper III) were prepared by recombinant DNA technology, a technique that allows joining of DNA fragments from different species (recombination) and insertion into a host organism to produce large quantities of the encoded protein. The technique makes it possible to introduce a peptide linker to obtain distance between the targeting and toxin moiety and also enables genetic alterations such as insertions and deletions. The carbohydrate groups responsible for mannose-specific uptake of natural gelonin (Madan and Ghosh, 1992) are not a part of recombinant gelonin, reducing the unspecific uptake of the EGFR- and HER2-targeted toxins (Rosenblum et al., 1995). MH3-B1 and EGF were fused to opposite ends of rGel, the N- and C-terminal, respectively, through a flexible GGGGS linker. The sequences were constructed by overlapping PCR, inserted into a plasmid vector and transformed into *Escherichia Coli* for expression. Recombinant production allows strict control of the size, homogeneity and purity of the products and gives great opportunities for optimizing and tailoring targeted toxins for clinical utilization (Kreitman, 2006; Potala et al., 2008).

The MH3-B1/rGel fusion protein has been described previously and has been shown to have high purity in solution (Cao et al., 2012). The enzymatic activity of MH3-B1/rGel has not been reported, although similar IC_{50} values of rGel and MH3-B1/rGel in HER2-negative cells suggest that the ribosome-inactivating ability of rGel and MH3-B1/rGel is comparable (Cao et al., 2012). The rGel/EGF product described in Paper I was the result of a preliminary production and this fusion protein was upon

characterization found to have low purity and considerably reduced *N*-glycosidic activity compared to recombinant gelonin (rGel). Several approaches must be considered in future studies to optimize the rGel/EGF production, such as re-optimizing the codon choice to improve protein expression in *E. coli* and modifying the experimental conditions during protein expression (e.g. temperature, OD and IPTG concentration) to increase the rGel/EGF yield. Secondly, considering the low enzymatic activity of the toxin moiety, the current configuration of rGel/EGF may not be the optimal one. The presence of inclusion bodies in the rGel/EGF solution and the disability to form trimers after incorporating an isoleucine-zipper trimer indicated misfolding of the protein. Comparisons should therefore be made with EGF fused to the N-terminal of rGel (EGF/rGel). Of note, effort was also put into developing a EGFR-targeting fusion toxin based on the scFv425 (Bruell et al., 2003; Bruell et al., 2005). Although the *N*-glycosidic activity of the rGel/scFv425 fusion toxin was found virtually identical to that of rGel, the uptake was found to be EGFR-nonspecific. The rGel/scFv425 fusion toxin product was therefore discarded, but should certainly be reconsidered as a modified version, e.g. reoriented with rGel positioned in the C-terminal.

Nonetheless, PCI potentiated the cytotoxic effect of rGel/EGF in an EGFR-specific manner and was found 70-fold more effective compared to PCI of rGel in A-431 cells (Paper III). The promising results demonstrated by PCI of rGel/EGF despite reduced ribosome-inactivating activity indicate that rGel/EGF has great potential for further optimization. Considering the already demonstrated EGFR specificity of PCI of rGel/EGF, increasing the ribosome-inactivating ability of rGel/EGF 65-fold (i.e. to the level of rGel) should result in an EGFR-specific treatment alternative with striking potency in targeted cells. The low molecular weight of the 42 kDa monomer might, however, present an obstacle for systemic delivery due to renal clearance. Although not absolute, the threshold for renal clearance as described in the literature ranges from approximately 40-65 kDa (Noguchi et al., 1998; Schneider et al., 2009). Thus, also MH3-B1/rGel (55 kDa) might be susceptible to rapid excretion through the kidneys. Indeed, MH3-B1/rGel has been found to accumulate in the kidneys after systemic administration (Cao et al., 2012). Future work should include attempts to optimize the size (e.g. by incorporating a peptide/protein linker), as well as to increase the purity, ribosome inactivating activity and stock concentration of the final rGel/EGF product.

5.2 PCI of targeted toxins; impact of the toxin moiety

Several targeted toxins based on the type I RIPs saporin and gelonin have been evaluated for PCI administration (Weyergang et al., 2011). These toxins are highly potent as it has been estimated that only 1-10 type I RIP molecules in the cytosol may be sufficient to kill a cell (Eiklid et al., 1980). PCI has been shown to greatly enhance the efficacy of saporin-containing targeted toxins *in vitro*, as shown for EGF-saporin (Weyergang et al., 2006), cetuximab-saporin (Yip et al., 2007) and trastuzumab-saporin (Paper I). Up to 1000-fold enhanced therapeutic effect of EGF-saporin was demonstrated upon activation by PCI (Weyergang et al., 2006). Saporin has, however, been shown to have approximately 10 times higher ribosome-inactivating activity compared to gelonin and correspondingly higher toxicity to mice (LD_{50} = 4.0 vs. 40.0) (Battelli et al., 1990; Barbieri et al., 1993). Saporin is therefore likely to induce more off-target effects *in vivo* compared to gelonin.

The targeted toxins evaluated in the present study contain either natural saporin or the recombinant, unglycosylated version of gelonin (Rosenblum et al., 1995). Recombinant gelonin (rGel) is less toxic to intact mammalian cells compared to natural gelonin due to lack of mannose-specific uptake (Rosenblum et al., 1999). However, immunoconjugates comprising rGel has been shown to demonstrate identical *N*-glycosidic activity compared to immunoconjugates produced with natural gelonin, as well as improved *in vivo* pharmacodynamics and tissue distribution (Rosenblum et al., 1999). Studies have indicated that for every 10^7 gelonin-based immunotoxin molecules that are internalized, only one reaches the cytoplasm (Yazdi and Murphy, 1994). In another study, it was estimated that ~5 x 10^6 rGel molecules must be internalized in order to kill a cell (Pirie et al., 2011). The high ribosome-inactivating ability of rGel combined with its low toxicity to normal cells, as well as improved *in vivo* performance compared to natural gelonin, suggest rGel as an ideal candidate for design of targeted toxins custom-made for PCI application.

PCI of rGel-based targeted toxins is believed to exert superior tumor selectivity through the tumor-targeting potential of the targeted toxin, the tumor-selective accumulation of the photosensitizer (as shown in Paper III), as well as light exposure confined to the tumor area. By controlled cytosolic release, PCI may reduce the toxin dosage necessary to kill the targeted cells. PCI-enhanced delivery of rGel-based targeted

toxins has been shown to induce significant antitumor effects in several tumor xenograft models using fusion toxin and photochemical treatment dosages that did not induce significant effects as monotherapies. This was demonstrated with scFvMEL/rGel in A-375 non-pigmented skin melanoma (Selbo et al., 2009), $VEGF_{121}$/rGel in CT26.CL25 murine colon carcinoma (Weyergang et al., 2014), MH3-B1/rGel in SKOV-3 ovarian carcinoma (Paper II) and rGel/EGF in A-431 epidermoid carcinoma and SCC-026 HNSCC (Paper III). The fusion toxins were administered i.v. at 2 mg/kg (scFvMEL/rGel, MH3-B1/rGel) or 4 mg/kg ($VEGF_{121}$/rGel) or intratumorally at 0.1 mg/kg (rGel/EGF). Tumor growth suppression has previously been demonstrated by several gelonin-based fusion toxins with different targeting moieties at a total dose of 20 mg/kg or higher administered as multiple i.v. injections (Cao et al., 2009; Zhou et al., 2011; Cao et al., 2012; Cao et al., 2012; Zhou et al., 2012). Altogether, these results indicate that PCI may reduce the effective dose and thereby increase the therapeutic window of rGel-based targeted toxins. In all the mentioned *in vivo* PCI studies, the photosensitizer and targeted toxin was administered as single injections and light was applied only once. Also in clinical studies, PCI has been shown effective using only one treatment cycle (NCT00993512, NCT01872923). These findings, therefore, suggest that PCI may abrogate immunogenicity problems related to prolonged treatment with targeted toxins, such as formation of neutralizing antibodies (Pai-Scherf et al., 1999; Azemar et al., 2003).

5.3 EGFR and HER2 as targets for PCI-induced toxin delivery

Both EGFR and HER2 are endocytosed as a part of their physiological mechanism. This, combined with their high expression in several cancer types, makes them interesting targets for delivery of toxins to cancer cells. Although EGFR and HER2 share common characteristics, they differ in their trafficking patterns (Wiley, 2003) and in their distribution and function in normal tissue (Yarden and Sliwkowski, 2001), which is of relevance when evaluating their potential as candidates for efficient and selective delivery of toxins to cancer cells.

Upon ligand stimulation, EGFR alters its trafficking pattern towards accelerated internalization and enhanced lysosomal targeting (Wiley, 2003; Baulida et al., 1996).

EGFR has been found in early endosomes as soon as 2-5 minutes after EGF stimulation (Haigler et al., 1979; Miller et al., 1986) and its first appearance in lysosomes has been detected already after 20 minutes (Beguinot et al., 1984). The internalization rate of HER2 appears slower than that of EGFR and is, on the contrary, similar in empty and occupied states (Sorkin et al., 1993; Baulida et al., 1996). It is therefore reasonable to believe that rGel/EGF is more rapidly internalized into the cells compared to trastuzumab-saporin and MH3-B1/rGel. rGel/EGF may, however, also be more rapidly degraded due to increased lysosomal accumulation. Unlike EGFR, HER2 is not efficiently sorted to lysosomes, but rather recycled back to the plasma membrane. Trastuzumab has been found to efficiently recycle with HER2 after endocytosis (Austin et al., 2004), suggesting that trastuzumab-saporin follows the same route. The lysosomal localization of MH3-B1/rGel demonstrated in SKOV-3 cells (Paper II) suggests, however, that MH3-B1/rGel might be able to dissociate from HER2 after entering endocytic vesicles. Thus, the amount of targeted toxin localized in endocytic vesicles at the time of light exposure is not only determined by the internalization rate of the receptor, but also on the intracellular trafficking route of the toxins. As a consequence, the amount of toxins available for PCI-induced cytosolic release is not necessarily higher after EGFR-targeting compared to HER2-targeting. The picture is also further complicated by the fact that homodimers and heterodimers of receptors in the EGFR family appear to have different trafficking patterns (Shankaran et al., 2008; Lenferink et al., 1998). Dependent on the relative expression of EGFR and HER2, the intracellular fate of EGFR-HER2 dimers may be shifted in favor of lysosomal targeting or recycling, respectively (Worthylake and Wiley, 1997; Worthylake et al., 1999; Hendriks et al., 2003; Hartman et al., 2013). A rationale for the increased lysosomal localization of MH3-B1/rGel in the SKOV-3 cells compared to the SK-BR-3 cells (Paper II) may therefore be the higher EGFR:HER2 ratio documented in the SKOV-3 cells.

PCI of trastuzumab-saporin (Paper I) was found to induce cytotoxic effects comparable to that demonstrated with PCI of cetuximab-saporin (Yip et al., 2007). A higher concentration of trastuzumab-saporin compared to cetuximab-saporin was, however, required to achieve similar effects with PCI (100 pM and 3 pM, respectively). These findings are in agreement with the lower rate reported for trastuzumab-induced HER2 endocytosis (Zhu et al., 2007) compared to cetuximab-induced EGFR endocytosis (Bhattacharyya et al., 2010). PCI was found to enhance the effect of MH3-

B1/rGel in SKOV-3 cells with higher lysosomal targeting of MH3-B1/rGel compared to SK-BR-3 cells (Paper II) and has also been found highly effective in HER2-low expressing MDA-MB-231 cells (Bull-Hansen et al., 2014). Altogether, these results indicate that PCI can potentiate the cytotoxicity of type I RIP-based targeted toxins over a wide range of internalization rates and that even a low number of toxins present in endo/lysosomal vesicles at the time of light exposure may be sufficient to induce cytotoxicity with PCI. PCI of MH3-B1/rGel (Paper II) and PCI of rGel/EGF (Paper III) were found to induce similar cytotoxic effects at a comparable toxin dose (2 nM and 1 nM, respectively). Presuming that the enzymatic activity of MH3-B1/rGel is similar to rGel itself, these results are in agreement with the lower *N*-glycosidic activity of rGel/EGF compared to rGel, which is believed to counteract a possibly higher uptake of rGel/EGF by EGFR-mediated compared to MH3-B1/rGel by HER2-mediated endocytosis. The higher concentrations of MH3-B1/rGel and rGel/EGF compared to trastuzumab-saporin and cetuximab-saporin required to induce cytotoxicity with PCI are in agreement with the 10-fold higher ribosome-inactivating activity of saporin compared to gelonin (Battelli et al., 1990). Although the results of the present study indicate that EGFR-targeting may be more effective compared to HER2-targeting for receptor-mediated uptake of toxins, both receptors seem to have sufficient endocytosis for targeted PCI-induced delivery.

Expression of EGFR and HER2 in normal tissue constitutes a potential risk for adverse effects of targeted toxins directed against these receptors. Cutaneous toxicity, such as acne and rash, a common adverse effect of EGFR-targeted mAbs and TKIs, has been linked to EGFR expression in the epidermis (Liu et al., 2013; Petrelli et al., 2013) and hepatotoxicity, which has limited the clinical dose-escalation of several HER2-targeted toxins, is presumably related to the presence of HER2 on hepatocytes (Pai-Scherf et al., 1999; Cao et al., 2012). The correlation between cardiotoxicity induced by trastuzumab and HER2 expression is not well defined, although HER2 has been implicated in cardiac development and function. However, trastuzumab-induced cardiotoxicity is largely reversible in the majority of cases (Ewer et al., 2005) and adverse effects resulting from EGFR inhibition are generally mild and not clinically limiting. Both EGFR- and HER2-related adverse effects have been shown to correlate with the administered dose and the duration of treatment (Grunwald and Hidalgo, 2003; Herbst et al., 2004; Azemar et al., 2003; Pai-Scherf et al., 1999). PCI is expected to

increase the specificity and reduce the therapeutic dose of type I RIP-based EGFR- and HER2-targeted toxins by activating their effect only in illuminated areas of the body. Existing data suggest that PCI may be efficient with only one or very few administrations of the targeted toxin (Berg et al., 2005a; Selbo et al., 2009; Weyergang et al., 2014). Consequently, it is expected that off-target toxicity due to the presence of EGFR and HER2 in normal tissue should be reduced compared to prolonged targeted toxin monotherapy. The accumulation of PCI-photosensitizers in the skin (Berg et al., 2011) constitutes, however, a potential challenge for delivery of EGFR-targeted toxins, since surface illumination may activate EGFR-targeted toxins accumulated in the overlying epidermis. This problem may, however, be resolved by delivering the light directly into the tumor through interstitial laser fibers. Interstitial laser light application is currently explored in patients with recurrent head and neck squamous cell carcinoma (NCT01606566).

EGFR, but not HER2, has been shown to be more highly expressed in tumor endothelial cells compared to skin endothelial cells (Amin et al., 2006) and amphiphilic PCI-photosensitizers have been demonstrated to accumulate in cells of the tumor vasculature (Vikdal et al., 2013b; Weyergang et al., 2014). The DCE-MRI results in Paper III indicated that also EGFR expressed by the tumor vasculature may be targeted by rGel/EGF, which may contribute to the tumor inhibiting effect of the treatment by depriving the tumor of oxygen and nutrients. Such tumor-specific vascular effects may represent an advantage with EGFR-targeted toxins compared to HER2-targeted toxins.

5.4 Inhibition of endocytic processes by the photochemical treatment; implications on the PCI protocol

PCI induces cytosolic release of drugs that are entrapped in endocytic vesicles. The two protocols developed for PCI are based on photochemical disruption of endocytic vesicles either before (“light first”) or after (“light after”) administration of the drug to be delivered (Prasmickaite et al., 2002). The “light first” procedure may be advantageous in that it reduces the risk of potential harmful effects induced by the photochemical treatment (photosensitizer and light) or by lysosomal enzymes on the drug. It has, however, previously been shown that photodamage targeted primarily to

endosomes and lysosomes may suppress plasma membrane endocytosis (Kessel et al., 2011). Any such interference of the photochemical treatment with endocytic processes is likely to interfere with the PCI "light first" procedure.

5.4.1 PCI of non-targeted toxins

Type I RIPs like gelonin and saporin are predominantly taken up by fluid-phase endocytosis and localize to the lumen of endocytic vesicles. The amphiphilic PCI-photosensitizers are taken up by adsorptive endocytosis with the hydrophobic part integrated into the outer leaflet of the plasma membrane. PCI of gelonin with $AlPcS_{2a}$ as the photosensitizer has been shown substantially more efficient using the "light first" compared to the "light after" procedure both *in vitro* (Prasmickaite et al., 2002) and *in vivo* (Berg et al., 2006). These studies indicated that the photochemical treatment directed against endosomes and lysosomes does not inhibit subsequent fluid-phase endocytosis. However, the fact that PCI was found superior with gelonin administered after the photochemical treatment indicated that co-administration of gelonin with $AlPcS_{2a}$ may reduce uptake, alter trafficking or partially inactivate gelonin. Using dextran as a surrogate marker for non-specific endocytosis, it was, indeed, confirmed that $AlPcS_{2a}$ in a dose-dependent manner strongly reduced uptake and altered trafficking of fluid-phase endocytosed drugs in both HT1080 tumor cells and vascular endothelial cells (HUVECs) (Vikdal et al., 2013a). Co-incubation with $TPPS_{2a}$ did, however, not significantly attenuate the dextran uptake. Hence, the interference of PCI-photosensitizers with fluid-phase endocytosed drugs seems to vary with the type of photosensitizer and photosensitizer dose.

PCI of saporin with $TPCS_{2a}$ as the photosensitizer was found less efficient using the "light first" compared to the "light after" procedure in Zr-75-1 cells (Paper I). This has also previously been shown in NuTu-19 cells with $TPPS_{2a}$ as the photosensitizer. Studies on the uptake of Alexa488-dextran indicated that fluid-phase endocytosis in Zr-75-1 cells was inhibited by the $TPCS_{2a}$-based photochemical treatment (Paper I). No inhibition of dextran uptake could, however, be seen following the photochemical treatment in WiDr cells where "light first" PCI of gelonin has previously been shown highly efficient (Berg et al., 2006). These findings indicate that inhibition of non-

targeted endocytic processes by the endo/lysosomally targeted photochemical treatment is not only determined by the photosensitizer, but is also cell line dependent.

5.4.2 PCI of targeted toxins with the "light first" procedure

PCI of targeted toxins is highly dependent on functional binding of the targeting moiety to the receptor followed by receptor-mediated endocytosis. The PCI "light first" procedure has repeatedly been found inefficient for delivery of targeted toxins (Weyergang et al., 2006; Yip et al., 2007; Selbo et al., 2009; Berstad et al., 2012). Photochemical damage to the targeting receptor or nearby mediators has been proposed as a rationale behind this observation, likely caused by remaining photosensitizer in the plasma membrane at the time of light exposure (Weyergang et al., 2007; Weyergang et al., 2008a). Receptor damage to HER2 was therefore studied following photochemical treatment with $TPCS_{2a}$ targeted either primarily to endocytic vesicles or to the plasma membrane (Paper I). Activated $TPCS_{2a}$ was shown to reduce the phosphorylation of HER2 at Y1221/1222 irrespective of the amount of photosensitizer on the plasma membrane, while photochemical damage of total HER2 was most severe when $TPCS_{2a}$ was localized to the plasma membrane. These findings are in agreement with studies by Weyergang et al. on the effects of $TPPS_{2a}$-induced photodamage on total and phospho-EGFR (Y1068) in NuTu-19 cells (Weyergang et al., 2007). The observed photodamage on EGFR in NuTu-19 cells and HER2 in Zr-75-1 cells correlate positively with the lack of effect following "light first" PCI of EGF-saporin (Weyergang et al., 2006) and trastuzumab-saporin (Paper I) in these cell lines. In A-431 cells, PCI of EGF-saporin was, however, shown equally efficient with the "light after" and "light first" strategy, in agreement with the lack of photodamage on EGFR observed in this cell line (Weyergang et al., 2008a). Altogether, these findings suggest that photochemical receptor damage is a predictive factor for the responsiveness towards PCI of targeted toxins applied with the "light first" procedure.

The results in Paper I suggest that the photochemical HER2 damage abrogates the "light first" PCI effect by inhibiting uptake by receptor-mediated endocytosis. However, whether the observed effects on HER2 (Paper I) and EGFR phosphorylation (Weyergang et al., 2007; Weyergang et al., 2008a) are due to direct receptor

photodamage is still unknown. The impairment of the photochemical treatment on EGFR and HER2 phosphorylation could also be a result of photodamage to nearby mediators in the plasma membrane, such as cholesterol, which is a known substrate for singlet oxygen (Geiger et al., 1997). Both EGFR and HER2 have been shown to reside in cholesterol-rich, low-density domains called lipid rafts (Pike, 2005; Orr et al., 2005).

5.4.3 PCI of targeted toxins with the "light after" procedure

Although photochemical receptor damage presumably does not inhibit PCI-induced release of targeted toxins administered prior to light ("light after" strategy), it is likely to contribute to the mechanistic action following the PCI treatment if the targeting receptors are involved in cellular growth and survival. The differential photodamage to EGFR observed in NuTu-19 and A-431 cells indicates that the susceptibility to receptor damage induced by endo/lysosomally targeted photochemical treatment is cell line dependent (Weyergang et al., 2007; Weyergang et al., 2008a). In Paper III, A-431 was, despite its high EGFR expression, found to be less sensitive to PCI of rGel/EGF than what could be expected based on a linear correlation. It was therefore argued that the efficacy of PCI of rGel/EGF may not only be determined by the amount of receptor available for binding to the targeting moiety, but also on the cell line sensitivity to photochemical damage to EGFR and the dependency on EGFR for growth and survival. The lack of photochemical damage to EGFR seen in A-431 cells after $TPPS_{2a}$-based photochemical treatment (Weyergang et al., 2008a) suggest that the effects on EGFR induced by the $TPCS_{2a}$-based photochemical treatment might be correspondingly. Lack of photochemical EGFR-targeting in A-431 cells therefore represents a plausible explanation for the low efficacy of PCI of rGel/EGF in these cells despite their high EGFR expression. Accordingly, the remarkably high PCI efficacy in HNSCC SCC-026 cells may indicate strong dependency on EGFR signaling in these cells, and hence, the photodamage on EGFR in these cells should be explored in future studies.

In conclusion, the general trend has been that drugs taken up by fluid-phase endocytosis can be successfully delivered by PCI using both protocols (Dietze et al., 2003; Berg et al., 2005a), while PCI of targeted therapeutics is most effective using the "light after" strategy (Selbo et al., 2010; Weyergang et al., 2011). However, the

efficiency of “light after” versus “light first” PCI seems to depend not only on the mechanism of drug-induced endocytosis, but also on the photosensitizer and the cells susceptibility to photodamage.

5.5 The *in vivo* protocol for PCI of targeted toxins

The ability to administrate the targeted toxin right before or right after illumination would be advantageous in a clinical setting, since this reduces the number of treatment steps to two, i.e. injection of photosensitizer (step 1) and injection of targeted toxin + illumination (step 2), and thereby simplifies the PCI treatment protocol. However, current knowledge obtained from *in vitro* studies indicates that in most cases, targeted toxins must be given prior to light exposure in order to obtain successful PCI effects. The drug-light interval should be determined based on the biodistribution of each targeted toxin and the trafficking pattern of the targeting receptor. An interval of 24 hrs from drug administration to illumination has previously been proposed as suitable for i.v. injection of toxins targeting gp240 or VEGFR (Selbo et al., 2009; Weyergang et al., 2014). Although maximal tumor concentration of MH3-B1/rGel has been demonstrated 48 hrs post injection (Cao et al., 2012), PCI of MH3-B1/rGel was found equally effective using a 24 or 48 hrs drug-light interval (Paper II). The antitumor effects induced by PCI of MH3-B1/rGel were, however, disappointing compared to the promising effects demonstrated *in vitro*.

Tumors that are easily accessible, e.g. in the head and neck region, may be injected directly with the targeted toxin. A drug-light interval of 6 hrs has been indicated as proper for intratumoral injection of gelonin (Selbo et al., 2001b) or rGel/EGF (Paper III) for delivery by PCI. The ideal drug-light interval for intratumoral delivery is, however, likely to vary with the molecular weight and affinity of the targeted toxin, as well as the trafficking pattern of the receptor (Adams et al., 2001; Rudnick et al., 2011). The intratumoral administration route offers potential advantages compared with the systemic route, such as high local drug concentration and minimal systemic drug levels (Lammers et al., 2006). Homogenous distribution throughout the tumor remains, however, a substantial challenge as the targeted toxin might be quickly cleared into the blood supply of the tumor interstitial space (Goldberg et al., 2002).

6. CONCLUSIONS

PCI of targeted protein toxins exploits tumor-associated proteins for delivery of highly cytotoxic payloads into target cells. The surface protein targets are merely utilized as delivery portals providing receptor-mediated endocytosis of the toxin moiety, while the pharmacological target is the ribosomes localized in the cytosol. Targeted toxin treatment approaches exert different mechanisms of action compared to currently approved mAbs and TKIs and may therefore not be restricted by resistance mechanisms associated with these therapies. Hence, PCI of EGFR- and HER2-targeted toxins may potentially represent an alternative treatment in cancers resistant to EGFR- and HER2-targeted mAbs and TKIs.

PCI was in the present study shown to strongly enhance the efficacy of type I RIP toxins taken up by both EGFR- and HER2-mediated endocytosis. PCI of HER2-targeted toxins was demonstrated in breast cancer and in ovarian cancer with low sensitivity to trastuzumab (Herceptin®). PCI of EGFR-targeting rGel/EGF was demonstrated in several EGFR-expressing solid cancers from different origins, including HNSCC that were resistant to cetuximab (Erbitux®). However, while the *in vivo* efficacy of PCI of 2 mg/kg MH3-B1/rGel was concluded as low compared to promising *in vitro* results, the antitumor effects of PCI of 0.1 mg/kg rGel/EGF were surprisingly effective considering the low activity and purity of the preliminary rGel/EGF product. Both previous and present studies suggest EGFR as a more efficient delivery portal for PCI-mediated toxin delivery compared to HER2. This is likely due to differences in ligand-induced endocytosis rate, since EGFR is internalized at a much higher rate compared to HER2, and intracellular trafficking (i.e. recycling vs. lysosomal targeting) of these two receptors.

The photochemical treatment utilized to induce endosomal rupture with PCI was found to attenuate HER2 and, consequently, HER2-mediated endocytosis. These findings stress the influence of the treatment protocol on the therapeutic outcome. In agreement with previous studies on PCI of EGFR-targeted toxins, it was concluded that PCI of HER2-targeted toxins should be applied using the “light after” strategy where the targeted toxin is administered prior to the photochemical treatment.

7. FUTURE PERSPECTIVES

This thesis has contributed to broader insight into how the treatment protocol and target expression may influence on the therapeutic outcome of PCI of targeted toxins. There are, however, several unanswered questions regarding the photochemical treatment responsible for the cytosolic drug release, such as which specific targets are subject to oxidation by the photochemical treatment and what determines the sensitivity among different cell lines to the ROS-induced damage. The observed attenuation of HER2 may be an indirect effect of photodamage to nearby mediators, such as cholesterol. The same holds true for previously demonstrated damage on EGFR (Weyergang et al., 2007; Weyergang et al., 2008a). The outcome of PCI is not a result of two separate treatments (photochemical treatment and drug), but rather an interaction between these two. Hence, future studies are warranted to determine the contribution of the photochemical treatment to the overall therapeutic effect of PCI.

The recombinant fusion toxins utilized in this study were produced in Dr. Rosenblums lab at MD Anderson, Houston, Texas. In order to improve and expand the development drugs for PCI-mediated delivery, it would, however, be highly beneficial to establish similar production facilities at the Institute for Cancer Research, Oslo University Hospital. The ability to produce and purify fusion proteins in our own facilities would allow us to more heavily invest in tailoring drugs that do not exert toxicity without PCI. Both the present and previous studies suggest EGFR as a more efficient candidate for targeted PCI compared to HER2 and the main focus of following studies should, therefore, be on the optimization of EGFR-targeting protein toxins for PCI application. Firstly, the rGel/EGF fusion product and its production process should be optimized to allow i.v. administration and large scale testing. Secondly, rGel/EGF should be compared with a redesigned version of the rGel/scFv425 fusion toxin mentioned in Section 5.1. Both rGel/EGF and rGel/scFv425 should be reoriented with rGel placed in the C-terminal to see if this may improve the *N*-glycosidic activity and EGFR specificity of the two fusion proteins, respectively. Thirdly, to decrease the cytotoxicity of gelonin-based targeted toxins without PCI, the recombinant gelonin sequence should be optimized with the aim of completely inhibiting its cytosolic translocation. PCI of fusion toxins based on the new and optimized version of gelonin

should be evaluated in relevant tumor xenografts models with the aim of development towards clinical trials.

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