Photochemical internalization of epidermal growth factor receptor-targeted drugs

by

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UNIVERSITAS OSLOENSIS MDCCCXI

Series of dissertations submitted to the
Faculty of Mathematics and Natural Sciences, University of Oslo
Nr. 839

ISSN 1501-7710

Cover: Inger Sandved Anfinsen.
Printed in Norway: AiT e-dit AS, Oslo, 2009.

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Acknowledgements

The work presented in this thesis was carried out at the department of Radiation Biology, Institute for Cancer Research-Norwegian Radium Hospital, Rikshospitalet University Hospital, Oslo, Norway during the years 2004-2008. The work has been supported by the Norwegian Radium Hospital Research Foundation.

I would like to thank Dr. Kristian Berg for his excellent supervision throughout the process. Unselfishly, he has shared his knowledge of cancer research and photo-medicine, for which I am very grateful. During international meetings and seminars, Dr. Berg has introduced me to his large international PDT "family", which has been very instructive and encouraging for the thesis. Working with the PCI group has combined challenges of a professional nature with social delight from day one, and I am grateful to Dr. Berg for giving me this opportunity.

Much of the presented lab work was performed with assistance from Marie-Therese Roppestad Strand, and I thank her for providing excellent laboratory skills and accuracy to my projects. Marie-Therese has been greatly supportive throughout the thesis, and our shared scientific (as well as non-scientific) experience in the office, has been highly appreciated.

The PCI group is very social, and I thank both former and present members of the group for creating this environment. It has been enjoyable spending time with the group, both at work and in private, and I hope we can keep this spirit alive.

My working days have been spiced up by my many colourful colleagues at the Department of Radiation Biology. Dr. Olav Kaalhus is due special thanks for his invaluable help with statistics and his patience with a confused student. Dr. Even Angell-Petersen's help with technical problems setting this document together is also much appreciated.

In private I have been supported by a caring family and close friends, and I wish to thank them for always helping me keep a foot (or at least a toe) outside the lab.

I have been extraordinary lucky to share my scientific interest with the love of my life and dear husband Dr. Pål Kristian Selbo, and I thank him for sharing his knowledge, for the productive scientific discussions, and his admirable patience. I will always appreciate our combination of striking synergistic uncleverness and productivity.

Oslo, August 2008

Anette Weyergang

Contents

Abbreviations

$AlPcS_{2a}$	aluminium phtalocyanine with two sulfonate groups on adjacent phthalates
5-ALA	5-aminolevulinic acid
AMD	age-related macular degeneration
BPD	benzoporphyrin-derivative monoacid ring A
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMEA	European Medicines Agency
ERK	extracellular signal regulated kinase
FD	fluorescence diagnosis
FDA	US Food and Drug Administration
IL-2	interleukin-2
ISC	intersystem crossing
i.p.	intraperitoneal
JNK	c-Jun NH_2 terminal kinase
HER2	human epidermal growth factor receptor 2
H_2O_2	hydrogen peroxide
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated ERK kinase
MTT	3-[4,5-demethylthiazol-2-y]-2,5 diphenyltetrazolium bromide
$^3O_2$	ground state oxygen
$^1O_2$	singlet oxygen
$O_2^{\bullet -}$	superoxide anion
$O_2^{\bullet}$	superoxide radical
$OH^{\bullet}$	hydroxyl radical
PpIX	protoporphyrin IX
PS	photosensitizer
PCI	photochemical internalization
PDT	photodynamic therapy

RIP	ribosome inactivation protein
ROS	reactive oxygen species
RB	Rose Bengal
$t_{1/2}$	half life
TKI	tyrosine kinase inhibitor
$TPPS_{2a}$	meso-tetraphenylporphine with 2 sulfonate groups on adjacent phenyl rings
tyrphostin	tyrphostin AG1478

List of publications

I Weyergang A., Selbo P.K., and Berg K. (2006): Photochemically stimulated drug delivery increases the cytotoxicity and specificity of EGF-saporin. *J.Control Release.* **111**: 165-173.

II Yip W.L., Weyergang A., Berg K., Tønnesen H.H., and Selbo P.K. (2007): Targeted delivery and enhanced cytotoxicity of cetuximab-saporin by photochemical internalization in EGFR-positive cancer cells. *Mol.Pharm.* **4**: 241-251.

III Weyergang A., Selbo P.K., and Berg K. (2007): Y1068 phosphorylation is the most sensitive target of disulfonated tetraphenylporphyrin-based photodynamic therapy on epidermal growth factor receptor. *Biochem.Pharmacol.* **74**: 226-235.

IV Weyergang A., Kaalhus O., and Berg K. (2008): Photodynamic therapy with an endocytically located photosensitizer cause a rapid activation of the mitogen-activated protein kinases extracellular signal-regulated kinase, p38 and c-Jun NH2 terminal kinase with opposing effects on cell survival. *Mol.Cancer Ther.* **7**: 1740-1750.

V Weyergang A., Kaalhus O., and Berg K. (2008): Photodynamic targeting of EGFR does not predict the treatment outcome in combination with the EGFR tyrosine kinase inhibitor Tyrphostin AG1478. *Photochem.Photobiol.Sci. In press*

VI Weyergang A. and Berg K. (2008): Photodynamic therapy in combination with Tyrphostin AG1478 and Cetuximab act distinctly on EGFR and downstream signalling causing opposite cytotoxic responses. *Submitted*

1. Introduction

1.1 Photodynamic therapy

Surgery, ionizing-radiation therapy and chemotherapy are still the most important treatment modalities against cancer. Despite their wide use, these methods have several limitations and the mortality is therefore still high for most cancer forms. Only in the US more than 550 000 people die from cancer every year (American Cancer Society, statistics for 2008). Damage to healthy tissue is one of the main limitations of today's cancer therapy and lack of specificity of the treatment and subsequent adverse effects often reduce both local and systemic control. Optimization of cancer treatment may therefore be exerted through improved treatment specificity.

Even though application of light-activated compounds, photosensitizers (PSs), were used for repigmentation of vitiligenous skin in India as early as 1400 BC (Daniell & Hill 1991), the utilization of photochemistry in therapy was not investigated scientifically until the beginning of the 20th century when von Tappeiner and co-workers found that the toxic effect of acridine was enhanced by light (Raab 1900) and then published the first results on photodynamic therapy (PDT) on skin cancer using eosin as a PS (Von Tappeiner & Jesionek 1903). In 1912, Dr. Meyer-Betz followed up the PDT research demonstrating the potency of PSs by injecting hematoporphyrin into himself before he was exposed to sun light (Meyer-Betz 1913). Throughout the first part of the 20th century some reports on PS accumulation in tumours were published (Daniell & Hill 1991, Macdonald & Dougherty 2001). However, it was not until the 1970s, when

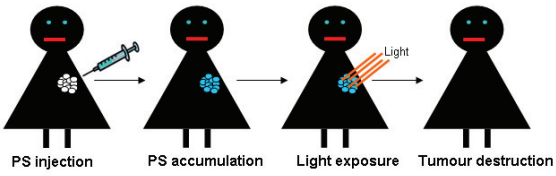

Fig.1: Photodynamic therapy against cancer. The PS is systemically injected and preferentially retained in the tumour tissue. Light exposure activates the PS which eventually kills the cancer cells.

haematoporphyrin derivate-induced PDT was shown to induce long-term cures of several cancers *in vivo* (Dougherty *et al.* 1975, Kelly *et al.* 1975), that the development of PDT was seriously escalated.

PDT is today a treatment modality for cancer (Dolmans *et al.* 2003) and age-related macular degeneration (AMD) (Mennel *et al.* 2007a, Mennel *et al.* 2007b). However, the method is also under evaluation for the treatment of psoriasis (Szeimies *et al.* 2002), rheumatoid arthritis (Hansch *et al.* 2008) and microbial infections (Jori *et al.* 2006). PDT is based on administration of a PS to the diseased area (Dougherty *et al.* 1998). Light exposure of the PS with appropriate wavelengths causes formation of reactive oxygen species (ROS), which eventually kill the target cells. The combination of PS and

Photosensitizer	Trade name	Producer	Indication	References
Hematoporphyrin derivative (HpD	Photofrin	Axcan Pharma	Barrett`s Oesophagus, Cervical dysplasia, Cervical cancer, Lung cancer, Oesophageal cancer, Gastric cancer, Bladder cancer	Nakamura *et al.* 2001, Dolmans *et al.* 2003, Yamaguchi *et al.* 2005, Juzeniene *et al.* 2007, Overholt *et al.* 2007, Corti *et al.* 2007
Benzoporphyrin-derivative monoacid ring A (BPD)	Visudyne	Novartis	Age-related macular degeneration	Mittra & Singerman 2002
Meta-tetra hydroxyphenyl chlorine (m-THPC), temoporfin	Foscan	Biolitec Pharma	Head and neck cancer	D'Cruz *et al.* 2004
5-Aminolevulinic acid (ALA)	Levulan	DUSA Pharmaceuticals.	Actinic keratosis Basal-cell carcinoma	Calzavara-Pinton 1995, Braathen *et al.* 2007
Methyl aminolevulinate (MAL)	Metvix	Photocure ASA	Actinic keratosis Basal-cell carcinoma	Pariser *et al.* 2003, Braathen *et al.* 2007
Hexyl aminolevulinate (HAL)	Hexvix	Photocure ASA	Diagnosis of bladder cancer	Witjes & Douglass 2007

Table 1: Photosensitizers with marketing authorization for clinical use

light is also used in cancer diagnosis, named fluorescence diagnosis (FD) (also known as photodynamic diagnosis or PDD), where fluorescence from the photo-activated PS is used to detect cancer tissue. A schematic illustration of the principle of PDT in cancer treatment is shown in Fig.1. Table 1 shows PSs with marketing authorization for PDT and FD.

1.1.1 The physical and chemical mechanisms of PDT

A PS is defined as a chemical entity, which upon absorption of energy from light, induces a chemical or physical alteration of another chemical entity (Dougherty *et al.* 1998). The absorbed energy excites electrons from the ground state to higher energy orbitals (Macdonald & Dougherty 2001). An excited electron usually has a very short lifetime (ps-ns) before the absorbed energy is released as heath or fluorescence and the molecule is transferred to its ground state. PSs, however, have the ability to undergo intersystem crossing (ISC), where the PS is transferred to a longer-lived excited triplet state (μs-ms) (Macdonald & Dougherty 2001) (Fig.2). The triplet state of the PS can also return to the ground state by emitting a photon (phosphorescence) or heat, or it can transfer its acquired energy to other molecules through Type I or Type II photochemical reactions (Kelly *et al.* 1975, Moan & Sommer 1985) (Fig.3). In PDT both Type I and Type II reactions take place, but the Type II reactions are regarded as the dominating. Type I reactions are, however, more frequent in hypoxic environments. In type I reactions, the PS in the triplet state reacts with another PS or with an organic substrate. Electron or hydrogen-atom transfer between the reacting molecules creates oxidized and reduced compounds that can react with molecular oxygen and produce oxygen radials as superoxide anions (O_2^-), hydroxyl radicals ($OH^\bullet$), hydrogen peroxide (H_2O_2) or other peroxides with the ability to induce oxidative damage (Ochsner 1997, Macdonald & Dougherty 2001, Castano *et al.* 2004). In oxygenated environments the type II reactions dominate. The energy in the triplet state of the PS is then transferred to ground state molecular oxygen (triplet state) that becomes excited to its singlet state (singlet oxygen ($^1O_2$)), which in turn can oxidize other compounds (Ochsner 1997, Macdonald & Dougherty 2001, Castano *et al.* 2004). $^1O_2$ is considered as the most important ROS formed during PDT (Weishaupt *et al.* 1976, Moan & Sommer 1985).

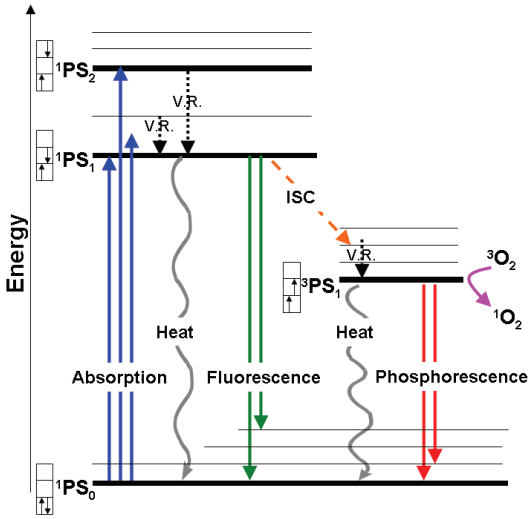

Fig.2: Jablonski diagram. The PS in its ground state ($^1PS_0$) absorbs energy from light and is excited to higher energy orbitals (^1PS_n) from which the energy can be released as heat or fluorescence after vibrational relaxation (V.R). An excited PS in the singlet state may also undergo ISC and transfer the PS to its triplet state ($^3PS_1$). $^3PS_1$ may release its energy as heat or phosphorescence, or react with other molecules. When the $^3PS_1$ reacts with molecular oxygen ($^3O_2$) the ROS singlet oxygen ($^1O_2$) is formed.

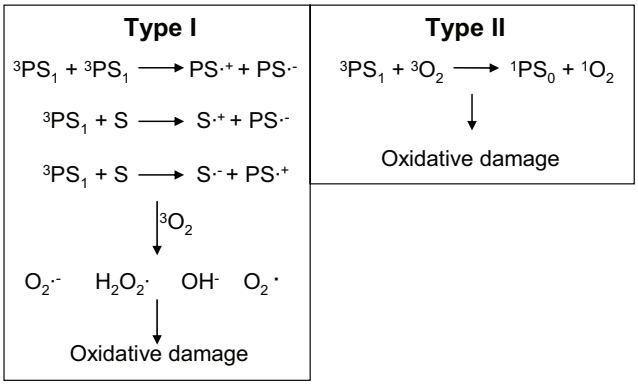

Fig.3: Type I and type II photochemical reactions.

Nearly all of the solid matter in cells consists of 4 forms of biomolecules; proteins, nucleic acids, polysaccharides and lipids. Polysaccharides seems not to suffer from photochemical oxidation, while unsaturated fatty acids (Doleiden *et al.* 1974, Bachowski *et al.* 1988), proteins (especially 5 amino acids (histidine, tryptophan, cystein, methionine and tyrosine)) (Jori *et al.* 1969, Jori *et al.* 1971, Doleiden *et al.* 1974, Das *et al.* 1985, Berg & Moan 1988, Berg *et al.* 1990a) and the nucleotide guanine (Gutter *et al.* 1977) are sensitive to PDT-mediated oxidation.

1.1.2 PDT mediated targeting of tumours

PDT causes tumour damage directly by inducing necrosis, apoptosis (Dougherty *et al.* 1998, Kessel & Luo 1998, Plaetzer *et al.* 2005) or autophagy (Kessel *et al.* 2006, Buytaert *et al.* 2006) in the tumour cells. PDT may also stiumulate shutdown of the tumour vasculature (Fingar *et al.* 1999, Engbrecht *et al.* 1999, Chen *et al.* 2002, Woodhams *et al.* 2006) and, in addition, PDT is shown preclinically to activate anti-tumour immunity (Castano *et al.* 2006, Kousis *et al.* 2007). Recently, photochemically-induced anti-tumour immunity was demonstrated in a patient with recurrent angiosarcoma, where distant non-treated tumours disappeared after PDT (Thong *et al.* 2007). The contribution of the different mechanisms to PDT-mediated tumour destruction depends on the photosensitizer, its formulation, the administration route, the time between PS administration and light exposure and the target tissue (Berg 2007). The present thesis focuses on the direct cytotoxic effect of PDT.

PDT is a selective treatment modality for cancer due to preferential accumulation of the PS in tumour tissue (Bossu *et al.* 1997) and the confined light exposure of the cancerous area. The mechanisms involved in the accumulation of PSs in cancer tissue are not fully understood, however, several properties of the tumour may contribute to the selection (Hamblin & Newman 1994). First, PSs tend to bind to LDL and are therefore facilitated for uptake in cancer cells, which often express elevated levels of LDL receptors (Kessel 1986, Maziere *et al.* 1991). Second, many of the clinical relevant PSs are weak bases and the acidic tumour environment therefore makes the PSs more lipophilic and consequently able to diffuse more easily into the tumour and adsorb to the tumour membranes (Friberg *et al.* 2003, Gerweck *et al.* 2006). Third, the leaky vasculature and poor lymphatic drainage also probably contributes to the retention of

the PS in the tumour (Bugelski *et al.* 1981). It is also proposed that the PS is easily taken up in macrophages, and that the elevated level of macrophages in tumours contribute to the tumour retention (Korbelik *et al.* 1991). The preferential retention of PSs in cancer cells is not just utilized in PDT, but also in FD of cancer as well as for fluorescence guided resection of tumour tissue (Zimmermann *et al.* 2001, Mayinger *et al.* 2008, Jocham *et al.* 2008).

1.1.3 Intracellular targets of PDT

Singlet oxygen has a short lifetime in organic tissue and its diffusion length in cells has been estimated to 10-20 nm (Moan & Berg 1991). The intracellular primary targets of PDT are therefore highly dependent on the localization of the PS at the time of light exposure. Intracellular distribution of the PS is dependent on the chemical properties of the compound, but also on the incubation time and cell type. PDT with different PSs has been shown to target both the plasma membrane (Kessel 1989), mitochondria (Ji *et al.* 2006, Saczko *et al.* 2007), Golgi apparatus (Rodal *et al.* 1998, Fabris *et al.* 2001), endoplasmic reticulum (Rodal *et al.* 1998, Uzdensky *et al.* 2001), endosomes and lysosomes (Roberts & Berns 1989, Berg *et al.* 1990b) and the microtubuli (Berg *et al.* 1990a). Clinically relevant PSs for cancer treatment do usually not localize to the nucleus because of their negative charge (Evensen & Moan 1982). The present thesis focus on amphiphilic PSs, that first bind to the plasma membrane before they are transported to the membranes of endosomes and lysosomes by endocytosis.

1.2 Photochemical internalization (PCI)

Macromolecular drugs based on proteins, DNA and RNA are becoming increasingly relevant in cancer therapy due to the potential high selectivity of the treatment. Such macromolecular drugs are often hydrophilic and lack an effective transport mechanism into the cell cytosol where their targets often are located, or from where they can easily be reached. These drugs are taken up by endocytosis (Mousavi *et al.* 2004, Mayor & Pagano 2007) and are transported to endosomes from where only a minor fraction is usually able to escape into the cytosol before the drug is degraded in lysosomes. Endo/lysosomal degradation contributes to reduced therapeutic effects and results in a

need for dose escalation, causing increased adverse effects of the treatment. The endo/lysosomal membrane permeability therefore limits the applicability of these drugs (Lloyd 2000). Many compounds have been used to increase the cytosolic release of endo/lysosomally trapped drugs, such as ammonium chloride, chloroquine, monensin and saponin (Casellas *et al.* 1984, Wu 1997, Heisler *et al.* 2005). These reagents are, however, not optimal for *in vivo* applications due to lack of selectivity towards target cells and high toxicity. Liposomes (Fretz *et al.* 2005), nanoparticles (Vasir & Labhasetwar 2007), polymers (Neu *et al.* 2005) and viruses (Pouton *et al.* 2007) are today investigated as formulation principles for intracellular delivery of macromolecular drugs. Endo/lysosomal sequestration and subsequent degradation is, however, also shown to be an obstacle for these delivery systems.

1.2.1 The principle of photochemical internalization

Photochemical internalization is a relative new method for cytosolic delivery of drugs that are trapped in endosomes and lysosomes (Berg *et al.* 1999, Hogset *et al.* 2004).

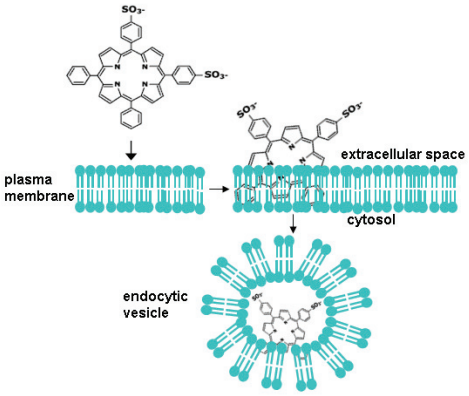

Fig.4: Cellular localization of the PCI PS TPPS$_{2a}$. TPPS$_{2a}$ first adsorbe to the plasma membrane. By endocytosis, the PS is transported into the cell and is kept localised in the membranes of the endocytic vesicles. (Fig. is out of scale.)

This drug delivery system is based on photosensitizers that localize to the cells endosomes and lysosomes such as meso-tetraphenylporphine with 2 sulfonate groups on adjacent phenyl rings ($TPPS_{2a}$) and aluminium phtalocyanine with two sulfonate groups on adjacent phthlates ($AlPcS_{2a}$) (Berg *et al.* 1990b, Berg & Moan 1994, Berg & Moan 1997, Selbo *et al.* 2001b) (Figs.9A and D). These PSs are amphiphilic and are, upon administration, first adsorbed to the plasma membrane (Berg *et al.* 1990b) before they are endocytosed into the membranes of the endocytic vesicles (Fig.4).

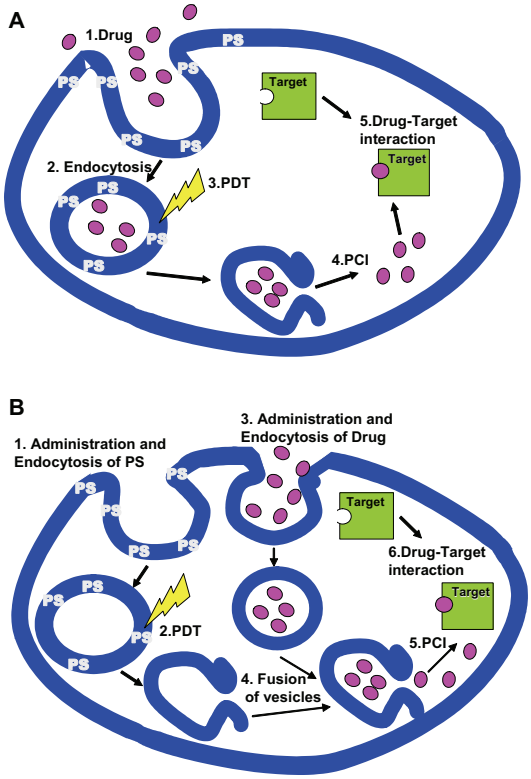

Fig.5: Schematic illustration of PCI. A: PCI with the "light after" procedure (paper IV). B: PCI with the "light first" procedure.

Illumination with an appropriate light source results in a photochemical reaction which causes rupture of the endo/lysosomal membranes so that the drugs that are trapped on the inside can escape into the cytosol and reach their target. This PCI practice, where the photochemical reaction is generated after administration of the macromolecular drug, is termed “light after” procedure (Fig.5A). Interestingly, PCI has also been shown effective for some macromolecules when the photochemical treatment is performed prior to the drug administration, a practice termed as “light fist procedure” (Prasmickaite *et al.* 2002, Berg *et al.* 2006) (Fig.5B). It has been hypothesised that the explanation for this is that the photochemically induced damaged endocytic vesicles fuse with drug-containing vesicles and that the drugs, in this way, are able to escape from endosomes and lysosomes into the cytosol before lysosomal degradation.

1.2.2 PCI of different classes of molecules

PCI has been shown to increase the cytosolic delivery and subsequent therapeutic effect *in vitro* of many macromolecules such as proteins (Selbo *et al.* 2000a, Dietze *et al.* 2003), immunotoxins (Selbo *et al.* 2000b, Selbo *et al.* 2001a) and DNA delivered by cationic polymers (Hogset *et al.* 2000, Prasmickaite *et al.* 2000, Prasmickaite *et al.* 2001, Prasmickaite *et al.* 2004), adenovirus (Hogset *et al.* 2002, Bonsted *et al.* 2004, Engesaeter *et al.* 2005, Engesaeter *et al.* 2006a, Engesaeter *et al.* 2006b) and adeno-associated virus (Bonsted *et al.* 2005). PCI *in vitro* has also been reported as an intracellular delivery system for peptides (Berg *et al.* 1996, Berg *et al.* 1999), PNAs (Shiraishi & Nielsen 2006, Berg *et al.* 2007), siRNA (Oliveira *et al.* 2007) and some chemotherapeutics, such as bleomycin (Berg *et al.* 2005), doxorubicin (Lou *et al.* 2006, Lai *et al.* 2007) and mitoxantrone (Adigbli *et al.* 2007). PCI has been demonstrated *in vivo* with the protein toxin gelonin (Selbo *et al.* 2001b, Dietze *et al.* 2005), a nonviral p53 gene (Ndoye *et al.* 2006), and has also been shown to increase the therapeutic effect of bleomycin (Berg *et al.* 2005). PCI of bleomycin is now approaching the first clinical trial with PCI.

1.2.3 PCI: a method for selective drug delivery to tumours

One of the main limitations of cancer therapeutics is poor selectivity towards target cells and chemotherapeutic treatment is often withdrawn due to adverse effects. PCI represents a method for selective drug delivery to the cancer cells. The reasons for this is that the PS is preferentially retained in tumour tissues and that the light is only applied to the desired area (see section 2.1.2). Since PCI releases drugs that would otherwise be degraded, the overall administrated dose, and also the adverse effects of the drug may be reduced without affecting the treatment outcome.

The selectivity of PCI towards cancer cells can be further increased by delivery of drugs that selectively targets the tumours. Indeed, this has been shown using targeted toxins such as MOC31-gelonin (Selbo *et al.* 2000b), EGF-saporin (paper I) and cetuximab-saporin (paper II), and targeted genes with both non-viral and viral vectors (Kloeckner *et al.* 2004, Bonsted *et al.* 2006, Bonsted *et al.* 2008).

The ideal drug for delivery by PCI has an intracellular target. The drug should not be able to penetrate the plasma membrane and must be taken up in cells by means of endocytosis and accumulate in endocytic vesicles. The drug should also ideally by it self be unable to escape from the endocytic vesicles into cytosol. In addition, the drug must be capable of diffusion from the blood vessels into the tumour-tissue.

1.3 Targeted protein-toxins

Targeted protein-toxins are molecules consisting of one cell binding moiety and one protein-toxin moiety (Vitetta *et al.* 1993, Pastan & Kreitman 1998, Kreitman 1999). The cell binding part is an antibody, an endogen ligand or a fragment of one of these two and recognizes only cells expressing a specific target antigen. The protein-toxin part is a toxin derivated from either plants or bacteria (Pastan & Kreitman 1998). Targeted protein-toxins in cancer treatment have been studied for several decades. The first and second generation targeted protein-toxins used in the beginning of these studies suffered from lack of specificity, heterogeneous composition due to the chemical methods for protein linkage and poor stability which made the clinical progress slow. Development of recombinant third generation targeted protein-toxins has, however, speeded up the process and the first targeted protein-toxin, denileukin, which consists of interleukin-2

(IL-2) and a trunced diphtheria toxin, was approved by the American Federal Drug Agency (FDA) in 1999 for cutaneous T cell lymphoma (Pastan *et al.* 2007). Several other targeted toxins are currently in clinical trials for both hematologic and solid tumours (Pastan *et al.* 2007).

1.3.1 Ribosome inactivating protein-toxins from plants

Some plants such as *Ricinus communis*, *Gelonium multiflorum* and *Saponaria officinalis* produce ribosome inactivating protein-toxins (RIPs) (Barbieri *et al.* 1993). These RIPs exerts N-glycosidase activity against the 28S RNA of the 60S ribosomal subunit, causing arrest of the protein synthesis which consequently induces cell death (Endo *et al.* 1987, Barbieri *et al.* 1992). RIPs can mainly be divided into 2 groups, type I and type II (Barbieri *et al.* 1993, Nielsen & Boston 2001). Type I RIPs, as gelonin, agrostin and saporin consist only of the cytotoxic chain with N-glycosidase activity (A-chain), while type II RIPs, as ricin, abrin and mistelthoe lectin, have a cell binding B chain in addition to the toxic A-chain. The toxic A-chain from the different RIPs is argued to use distinct mechanisms for cytosolic translocation (Vago *et al.* 2005, Sandvig & van 2005). However, once inside the cell cytosol, type I and II RIPs have similar potency (Barbieri *et al.* 1993). The lack of a cell binding B-chain in type I RIP causes, however, poor cellular uptake, and the cytotoxic effect of these RIPs is therefore often absent or very low (Barbieri *et al.* 1993). Type II RIPs are therefore more frequently utilized than type I RIPs in targeted protein-toxins. The type I RIPs saporin (Stirpe *et al.* 1983) and gelonin (Stirpe *et al.* 1980) are used in the present thesis. Both saporin and gelonin are mainly taken up in the cells passively by means of pinocytosis (Barbieri *et al.* 1993). Gelonin has in addition been shown to be taken up by endocytosis through the mannose receptor (Madan & Ghosh 1992).

1.4 Epidermal growth factor receptor (EGFR)

Several attempts have been made to find cancer cell specific targets that can be utilized in therapy. Conventional chemotherapeutic agents such as the alkylating agents (cyclophosphoamide), antimetabolites (metotrexate) and cytotoxic antibiotics (bleomycin) exert their effect mainly on frequently dividing cancer cells. Severe adverse

Tumour type	Tumours overexpressing EGFR	References
Colorectal	30% - 80%	McKay *et al.* 2002, Spano *et al.* 2005, Leung *et al.* 2008
Head and neck	80% - 100%	Herbst & Shin 2002, Zimmermann *et al.* 2006, Kalyankrishna & Grandis 2006
Pancreatic	32% - 69%	Thybusch-Bernhardt *et al.* 2001, Bloomston *et al.* 2006, Dancer *et al.* 2007
Nonsmall cell lung carcinoma	32% - 67%	Hirsch *et al.* 2003, Onn *et al.* 2005, Nakamura *et al.* 2006
Breast	7% -76%	Bhargava *et al.* 2005, Reis-Filho *et al.* 2005, van Diest *et al.* 2006
Renal cancinoma	76% - 93%	Yoshida *et al.* 1997, Langner *et al.* 2004
Ovarian	38% - 62%	Nielsen *et al.* 2004, Vermeij *et al.* 2008

Table 2: EGFR overexpression in tumours

effects are, however, observed in normal fast dividing cells and depression of the bone marrow as well as damage to the epithelium of the gastrointestinal tract are often experienced. In the last decades proteins as targets for cancer therapeutics has become attractive. Several proteins have been shown to be overexpressed in cancer cells compared to normal cells and protein-targeted cancer therapeutics, such as kinase inhibitors and antibodies, have obtained marketing authorisation by both FDA and the European Medicines Agency (EMEA). Epidermal growth factor receptor (EGFR) is one of the most studied protein targets for cancer therapy (Rowinsky 2004, Ciardiello & Tortora 2008). The receptor is overexpressed in several different cancers (Table 2), and activation of the receptor is associated with cancer cell related properties as increased proliferation (Perry *et al.* 1998), blocking of apoptosis (Kulik *et al.* 1997), migration (Woodburn 1999) and vascularisation (Schreiber *et al.* 1986, Gille *et al.* 1997) which make the receptor an interesting target for anti-cancer drugs. A recent report showed that EGFR also stimulate to survival of cancer cells independent of its kinase activity, by inhibiting autophagy (Weihua *et al.* 2008).

1.4.1 EGFR; physiology and activation

EGFR/ErBb1/HER1 is a 170 kDa transmembrane tyrosine kinase where the polypeptide chain crosses the plasma membrane once (Fig.6).

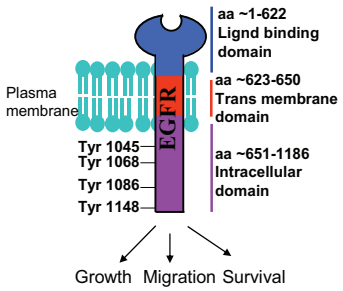

Fig.6: Illustration of EGFR and its localization in the plasma membrane

The receptor consists of a cysteine-rich extracellular ligand binding domain, a hydrophobic transmembrane chain and an intracellular domain where the kinase activity is located (Wells 1999, Rowinsky 2004, Normanno *et al.* 2006). Upon activation by one of its ligands the receptor undergoes homo- or hetero dimerization with another receptor in the EGFR family (Fig.7). Human epidermal growth factor receptor 2 (HER2) is regarded as the major partner in EGFR hetero-dimerization and EGFR-HER2 dimers are assumed to be more stable and generate a stronger and more prolonged activation signal compared to the EGFR homodimers (Tzahar *et al.* 1996, Lenferink *et al.* 1998). Dimerization of EGFR activates the kinase by inducing a conformational change of the receptor complex, causing exposure of the ATP-binding site and subsequent phosphorylation of tyrosines in the intracellular domain of the receptor (Klein *et al.* 2004, Mattoon *et al.* 2004, Gan *et al.* 2007). A cascade of phosphorylation reactions is initiated where the RAS-RAF-MEK-ERK pathway and the AKT pathway are common for all ligands (Normanno *et al.* 2006). The receptor dimerization also stimulates endocytosis of the receptor (Wang *et al.* 2005) after which EGFR is either recycled back to the plasma membrane or transported to the lysosomes where it is degraded, depending on its ubiquitinylation (Yarden 2001, Dikic 2003, Huang *et al.* 2006) (Fig.7).

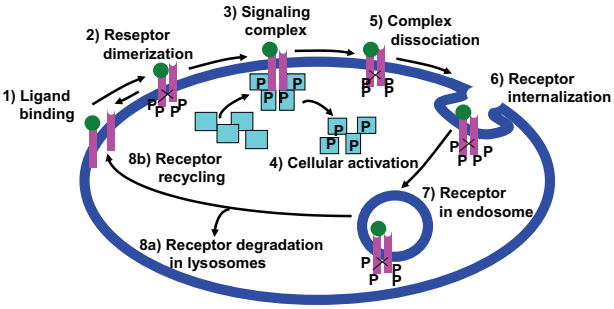

Fig.7: The EGFR signal transduction. p = phosphorylation

1.4.2 EGFR targeted drugs

Current EGFR targeted drugs can be divided in two groups (Castillo *et al.* 2004); specific tyrosine kinase inhibitors (TKIs) as erlotinib, lapatinib and gefitinib (Herbst *et al.* 2004, Bareschino *et al.* 2007, Ciardiello & Tortora 2008) and monoclonal antibodies (mAbs) as cetuximab and panitumumab (Baselga 2001, Cohenuram & Saif 2007). EGFR TKIs have the backbone structure of 4-anilinoquinazolins (Al-Obeidi & Lam 2000, Denny 2002, Yun *et al.* 2007). These drugs are small and lipophilic and diffuse across the plasma membrane where they function as competitive antagonists for the intracellular ATP-binding domain of EGFR (Denny 2002). The TKIs thereby inhibits ligand induced EGFR activation and shut down the growth and survival promoting signalling from the receptor (Fig.8a). EGFR targeted TKIs are also reported to induce inactive EGFR/HER2 heterodimers and inhibits in this way HER2 signalling as well as EGFR signalling (Matar *et al.* 2004, Gan *et al.* 2007). EGFR specific mAbs, on the other hand, recognizes and antagonize the ligand binding extracellular domain of the receptor and inhibits EGFR activation and subsequent growth and survival promoting activation (Li *et al.* 2005, Yoshida *et al.* 2008) (Fig.8b). Antibody dependent cellular cytotoxicity is, in addition, suggested to be an important mechanism for cetuximab-induced cytotoxicity *in vivo* (Naramura *et al.* 1993, Kurai *et al.* 2007). Other EGFR targeting strategies are under development for therapeutic use such as utilization of EGFR specific siRNA (Kang *et al.* 2006, Yamanaka *et al.* 2008), EGFR targeted

chemotherapeutics (Vega *et al.* 2003, Mamot *et al.* 2005) and EGFR targeted radioimmonotherapy (Li *et al.* 2004). EGFR targeted toxins is also an interesting approach in cancer therapy (Engebraaten *et al.* 2002, Sampson *et al.* 2008). RIPs exert their cytotoxicity intracellularly by inhibition of the ribosome activity (see 2.3.1). Selectivity towards EGFR expressing cancer cells may be achieved by linking RIPs to EGFR targeting moieties, subjecting the RIPs to EGFR mediated endocytosis (Fig.8c). Fig.8 shows EGFR targeted drugs used in the present thesis.

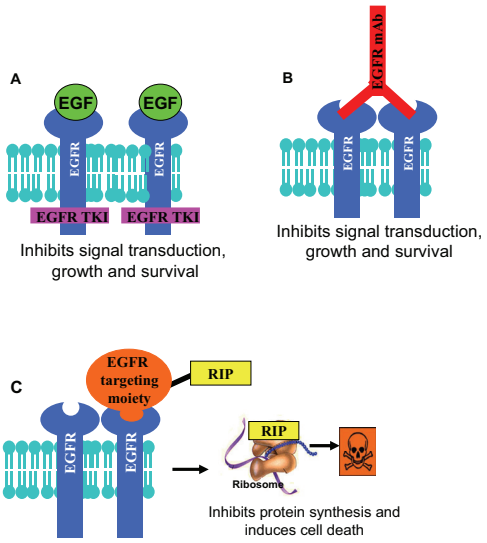

Fig.8: EGFR targeted drugs used in the present thesis. A: EGFR specific TKIs bind to the intracellular ATP binding domain of the receptor. B: EGFR specific mAbs bind to the extracellular ligand binding domain of the receptor. C: EGFR targeted RIPs are taken up in

2. Aims of the study

PCI of an EGFR targeted toxin was expected to exert a 3-fold selectivity for cancer cells, i.e utilization of a tumour targeting toxin, use of photosensitizers that accumulate preferentially in tumour tissue and exposure of light only to the tumour area. It was therefore hypothesized that PCI of EGFR targeted toxins was a promising modality for cancer therapy.

The specific aims of the investigations where:

- To evaluate EGFR as a target for PCI-delivered drugs, utilizing both an endogen ligand and a monoclonal antibody as EGFR targeted moieties.
- To study if PDT with PSs appropriate for use in PCI damage EGFR, and evaluate its impact on PCI of EGFR targeted drugs.
- To study the effect of PDT and PCI on EGFR and mitogen-activated protein kinase (MAPK) signal transduction, and evaluate its importance for treatment cytotoxicity.
- To study the treatment outcome after combination therapy with PDT and EGFR targeted drugs.
- To study how activation and inhibition of EGFR influence on PDT induced EGFR- and MAPK-signalling and evaluate the impact of such manipulations on the treatment outcome when PDT is combined with an EGFR targeted drug.

3. General experimental considerations

3.1 Cell lines

The PCI principle has been documented in more than 30 cell lines *in vitro* (Dietze *et al.* 2006). The present experiments are performed in cell lines with different expression levels of EGFR. The A-431 human epidermoid carcinoma cell line is one of the most used cell lines in EGFR targeted research *in vitro*, and was included in the present studies. The EGFR is highly expressed in A-431 cells (Wrann & Fox 1979) which are reported to contain 10^6 receptors per cell compared to 10^4-10^5 receptors/cell in other epidermal cells (Stryer 1975). The three human cell lines WiDr (colorectal adenocarcinoma), HCT-116 (colorectal carcinoma) and Du-145 (prostate carcinoma) has been used in previous PCI research (Dietze *et al.* 2006). These cell lines express EGFR (paper I-III), (Caceres *et al.* 2008) and where therefore included as EGFR positive models in the present work. The NuTu-19 rat ovarian cancer cell line (used in paper I, III, VI and V) is claimed to mimic human ovarian cancer and represent a good model for preclinical ovarian cancer research (Major *et al.* 1997). NuTu-19 cells may be established as an orthotopic model (Sloan Stakleff *et al.* 2005) and is hence an interesting cell line for future PCI experiments. NuTu-19 cells express EGFR, as do up to 62 % of ovarian cancers in humans (table 2). Since NuTu-19 cells are of rat origin, EGFR in this cell line is not recognized by the humanized murine antibody cetuximab (results not shown). Two EGFR negative cell lines, the human uterus sarcoma cell line MES-SA, and the human breast cancer MDA-MB435 (paper I), were used as negative controls in the present studies.

3.2 Photosensitizers and light sources

Two photosensitizers were used in the present thesis, $TPPS_{2a}$ and $AlPcS_{2a}$ (Fig 9). Both of these photosensitizers localize to endosomes and lysosomes in the cells, but their absorption spectra differ significantly (Fig. 9). $AlPcS_{2a}$ absorbs red light with a maximum at approximately 670 nm and an absorption coefficient (ε_{674}) of 190 000$M^{-1}cm^{-1}$ (as reported for $AlPcS_4$ in methanol (Brasseur *et al.* 1987). $TPPS_{2a}$ absorbs light in the blue region of the spectrum with a maximum at approximately 415 nm and ε_{413}=

500 000 $M^{-1}cm^{-1}$ (as reported for $TPPS_4$ in water (Rahman & Harmon 2006)). Red light penetrates more efficiently through tissues than blue light, and $AlPcS_{2a}$ is therefore the preferred photosensitizer for *in vivo* use. $TPPS_{2a}$ is, however, the most efficient photosensitizer for *in vitro* experiments in our laboratory due to the irradiance from our blue light source that is higher than the red light source (13.5 mW/cm^2 and 1.5 mW/cm^2 respectively). The emission spectra for the blue light source is presented in figure 9. A 300 mW 670 nm diode laser was used as a light source *in vivo*.

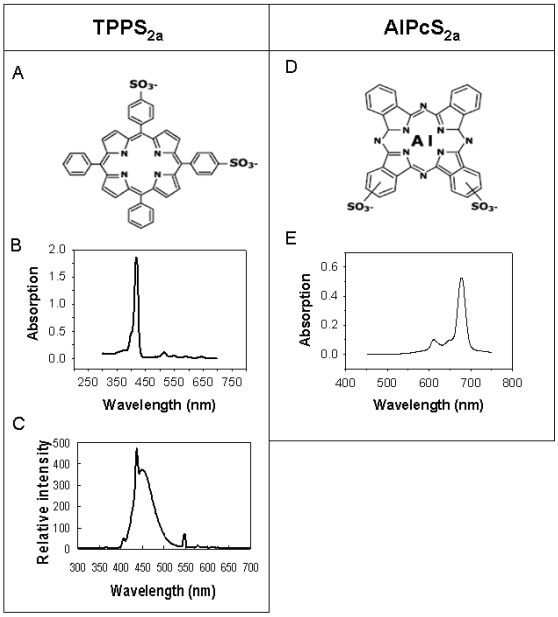

Fig.9: PSs and *in vitro* light source used in the present thesis. The molecular structure and absorption spectra for the PS used in experiments *in vitro*, $TPPS_{2a}$, is presented in A and B. The emission spectrum for the blue light source used *in vitro* is shown in C. The molecular structure and absorption spectra for the *in vivo* relevant PS $AlPcS_{2a}$ is presented in D and E.

3.3 PDT

Most of the photochemical reactions (PDT) in the present studies were performed with the PCI procedure where the PS is targeted to the endocytic vesicles before light exposure. This is achieved *in vitro* by an 18 hrs incubation of $TPPS_{2a}$ followed by a 4 hrs chase period in drug-free medium before light exposure (paper I-VI) (Fig.10A). In the *in vivo* experiments, $AlPcS_{2a}$ was injected *i.p.* 48 hrs prior to light exposure, a procedure which leads to PS accumulation in endosomes and lysosomes (Selbo *et al.* 2001b). The PS localisation in endosomes and lysosomes is caused by passive targeting due to the overall endocytosis of the cell (adsorptive endocytosis). Clinically, one may phase scenarios where some of the PS still is present on the plasma membrane at the time of light exposure. The present studies therefore includes PDT procedures where cells were exposed to light directly after the PS incubation (without the 4 hrs chase period) localizing the photosensitizer to the plasma membrane in addition to endo/lysosomal vesicles (paper III, IV and V) (Fig.10B). Experiments where cells were exposed to a 30 min incubation of $TPPS_{2a}$ directly followed by light exposure were also exerted to target most of the PS to the plasma membrane at the time of light exposure (paper V) (Fig.10C).

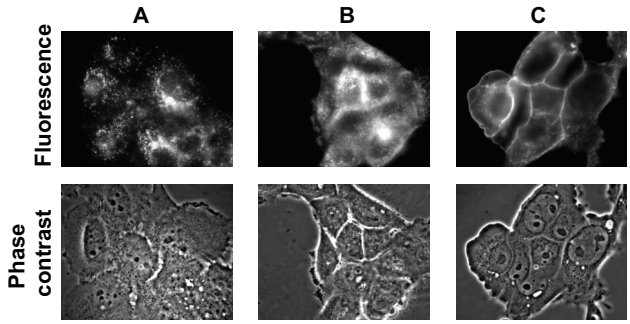

Fig.10: $TPPS_{2a}$ localization in A-431 cells. Fluorescence micrographs of A-431 cells after $TPPS_{2a}$ incubation procedures targeting the PS primary to the endo/lysosomal vesicles (A), to the plasma membrane in addition to the endo/lysosomal vesicles (B) or only to the plasma membrane (C).

3.4 PCI and PDT-drug combination therapy

The terminology PCI is in the present work used when the photochemical treatment, targeted to endocytic vesicles, is combined with drugs with intracellular targets that are taken up in cells by means of endocytosis and trapped in endocytic vesicles. When the photochemical treatment was combined with other drugs, as low molecular inhibitors that passively diffuses across the plasma membrane or the extracellular binding antibody cetuximab, the dual treatments were termed as PDT-drug combination therapy. PCI was performed with both the "light after" and "light first" procedure (paper I and II) (Fig 4A). The treatment regimen for the PDT-drug combination therapy was also varied, performing PDT prior to, after or during the drug incubation period (paper V and VI). The impact of the treatment schedule on the outcome of PCI as well as PDT-drug combination therapy was therefore investigated in paper I, II, V and VI.

3.5 Assays for measurements of cytotoxicity

In the present thesis, cytotoxicity was evaluated by three different methods. The MTT (3-[4,5-demethylthiazol-2-y]-2,5 diphenyltetrazolium bromide)-assay was used to measure viability 24-48 hrs after PDT and PCI treatments in all papers. In the MTT assay cells are incubated 2-4 hrs with the MTT-reagent. MTT is cleaved by succinate dehydrogenase and other dehydrogenases located in the mitochondria of the cells. Cleavage results in the formation of blue formazan crystals, which are dissolved in DMSO and measured colourimetrically at 570 nm. An advantage of the MTT assay is that it is fast and easy to perform. A limitation of the method is that this assay measures cell viability rather than direct cell survival. The time point at which the assay is performed is therefore of high importance to correlate the MTT data to cytotoxicity. The detection range for colourimetric measurements is also less than 2 log due to variable background absorption in combination with non-linear absorption at OD above ~1.2 with the instrument used in the present studies. Clonal cell survival experiments were used as control experiments to the MTT-assay in paper II. In the clonal cell survival assay the cells were incubated 7-10 days after treatment so that surviving cells could form colonies. The colonies were fixed in ethanol and stained with a saturated solution of methylene blue and dried before manual colony counting. Clonal cell survival as a

method for cytotoxicity measurements, do not suffer from problems with high background counts. The method also provides a broad dynamic range, which is an advantage of this method. Measuring the clonal cell survival after treatment indicates, however, the colony-forming ability of the cells, rather than toxicity. The assay is therefore not optimal evaluating treatments which induce growth arrest rather than cell death. Counting the actual number of viable cells by a Coulter counter was also used to measure the treatment effects in this thesis (paper V and VI). In the Coulter counter procedure, cells were trypsinated and resuspended in PBS before they were subjected to counting. This method provides the actual number of cells in the samples and may be used to study cytotoxic as well as cytostatic effects. The method also provides the cell size distribution in counted samples, which may indicate the cells position in the cell cycle. Disadvantages utilizing the Coulter counter for cytotoxic measurements are that the method is time consuming, and may provide large standard errors between parallels, due to loss of cells during centrifugation and resuspension.

3.6 Statistical analysis of synergistic and antagonistic effects

In paper V and VI a statistical method was used to determine synergistic and antagonistic effects of PDT-drug combination therapies. This statistical model is based on the assumption that PDT and the drug have distinct mechanisms of action. The expected survival fraction (SF) after an additive effect of the combined treatment will therefore be the product of the SFs of each treatment separately:

$SF_{add} = SF_{PDT} \times SF_{drug}$ or $\log SF_{add} = \log SF_{PDT} + \log SF_{drug}$

The SF_{PDT} and SF_{drug} were calculated dividing the surviving number of treated cells on the cell number in the untreated controls. SF_{add} was then compared to the observed SF of the combined treatments (SF_{comb}) using the synergy/antagonism parameter DL defined as the difference in logarithm between the observed SF_{comb} and the calculated SF_{add}:

$DL = -(\log SF_{comb} - \log SF_{add}) = \log (SF_{add} / SF_{comb}) = \log SF_{PDT} + \log SF_{drug} - \log SF_{comb}$

Positive DL values indicate synergistic effects, while negative DL values indicate antagonistic effects of the combined treatments. Additive effects of the treatments result in DL values close to zero. Significant differences of DL from zero were established through t-tests using a two-tailed significance level of $p \leq 0.05$. The treatment

modalities were accepted synergistic if the DL value was significant positive and antagonistic if the DL value was significant negative.

4. Summary of publications

4.1 Paper I

This publication is the first report on PCI of an EGFR targeted drug. One of the endogenous ligands of EGFR, EGF, was linked to saporin through the biotin-streptavidin bond. The EGFR targeted toxin was shown to be taken up specifically by EGFR receptor-mediated endocytosis. Saporin and EGF-saporin inhibited protein synthesis to a similar extent in a cell free reticulocyte lysate system. EGF-saporin was, however, much more toxic than saporin in NuTu-19 and A-431 cells. PCI of EGF-saporin was approximately 1000-fold more cytotoxic than PCI of saporin in NuTu-19. PCI of EGF-saporin was more effective when the drug was administered before light exposure ("light after" strategy) than when the photochemical treatment was performed prior to the EGF-saporin incubation ("light first" strategy). In order to verify selective toxicity of the PCI treatment in EGFR positive cells, the EGFR negative cell lines MES-SA and MDA-MB435, receptor blocking with an EGFR antibody and incubation with an excess of free EGF were utilized. It was concluded that PCI of EGFR targeted toxins is a promising method to increase the specificity and toxicity of protein-toxins.

4.2 Paper II

This paper present PCI of an EGFR targeted immunotoxin, cetuximab-saporin. The EGFR targeted mAb cetuximab and saporin were linked by the biotin-streptavidin bond and specific binding of cetuximab-saporin to EGFR in HCT-116 cells was shown by fluorescence microscopy with competing excess of free cetuximab. It was reported that free cetuximab attenuated EGF-induced EGFR phosphorylation in HCT-116 cells, but induced only a minor reduction of cell viability in agreement with other reports. Cetuximab-saporin alone was, however, much more toxic in HCT-116 cells with an LD_{50} of ~100 pM and binding of cetuximab to saporin increased the toxicity of saporin about 10-fold. Treatment of HCT-116 cells with 3 pM cetuximab-saporin did not cause any cytotoxicity. However, PCI of 3 pM cetuximab-saporin increased the cytotoxicity 20-fold compared to the photochemical reaction with photosensitizer and light as measured by clonal cell survival. The cetuximab-saporin-induced toxicity was

completely reversed in the presence of a 200 fold excess of free cetuximab. Similar effects were obtained in the EGFR positive DU-145 and A-431 cell lines. Experiments with the EGFR negative MES-SA cell line showed no difference in cytotoxicity between saporin and cetuximab-saporin independent on delivery with PCI. PCI of cetuximab-saporin was only effective when the targeted toxin was administered prior to the photochemical treatment ("light after" strategy) in HCT-116 cells and had no effect when PDT was performed first ("light first" strategy) compared to the photochemical treatment alone. It was concluded that PCI of cetuximab-saporin is an unique method for cancer treatment that specifically kills target cells by three different mechanisms; (i) Blocking of EGFR signalling by a monoclonal antibody, (ii) the photochemical reaction generated by a tumour accumulating photosensitizer and tumour directed light and (iii) the ribosome inactivation activity of saporin after PCI induced cytoplasmic release in EGFR positive cells.

4.3 Paper III

The aim of this study was to investigate photochemical targeting of EGFR *in vivo* and *in vitro* using PSs utilized in PCI. Two different protocols were used for the photodynamic treatment of NuTu-19 cells *in vitro*, one in which the treatment with the PS was optimized for accumulation in endocytic vesicles prior to light exposure and one where the PS was located on the plasma membrane in addition to the endocytic vesicles. It was shown that ~LD_{50} PDT immediately inhibited the ability of EGFR to phosphorylate upon EGF stimulation with both protocols. Fluorescence microscopy of Alexa488 labelled EGF showed that PDT inhibited EGF binding when the photosensitizer was located to both the plasma membrane and the endocytic vesicles, but not when primarily present in the endocytic vesicles. The decreased EGF-induced EGFR phosphorylation after endo/lysosomally targeted PDT can therefore not be explained by reduced EGF binding. Total EGFR was attenuated by PDT only when the photosensitizer was located on the plasma membrane in addition to the endocytic vesicles. The mechanism behind the photochemical targeting of total EGFR was studied. Degradation in endocytic vesicles did not contribute to the photochemical damage of total EGFR, as shown in experiments where cells were kept on ice, inhibiting overall endocytosis as well as in experiments in the presence of the cathepsin inhibitor E-64. It was observed on Western

blots that only the intracellular part and not the extracellular part of total EGFR was subjected to photochemical degradation, indicating a direct photochemical damage of specific sites of the receptor rather than degradation of the whole protein. Y1068 was indicated as the most sensitive site for photochemical oxidation. The mechanisms behind $TPPS_{2a}$-PDT mediated EGFR damage in NuTu-19 cells *in vitro* was concluded to be dependent on the amount of photosensitizer present on the plasma membrane at the time of light exposure. The photochemically induced EGFR damage *in vitro* was shown to be cell line dependent since no effects on EGF-induced EGFR phosphorylation was observed in WiDr cells after endo/lysosomal targeted PDT. PDT with the *in vivo* relevant PCI photosensitizer $AlPcS_{2a}$ resulted in a decrease in total EGFR in WiDr tumours growing subcutaneously in Balb C (nu/nu) mice. No decrease in total EGFR was detected until 24 hrs after light exposure *in vivo*, and inhibition of EGFR translation and/or transcription was suggested as a possible mechanism for the total EGFR reduction observed *in vivo*.

4.4 Paper IV

This paper reports on MAPK signalling after LD_{50} $TPPS_{2a}$-PDT and the impact of this signalling on the treatment outcome after both PDT and PCI in two different cell lines. It was found that $TPPS_{2a}$-PDT immediately activated both extracellular signal regulated kinase (ERK) and p38 in a transient manner in both NuTu-19 and WiDr cells. The activation of ERK observed after PDT was stronger than that obtainable with EGF incubation alone and the absolute EGF-mediated activation of ERK was the same in PDT-treated and untreated cells. The subsequent deactivation of ERK after 2 hrs seemed, in contrast to other reports, not to depend on activation of phosphatases as shown in the presence of the phosphatase inhibitors okadaic acid and vanadate. Activation of c-Jun NH_2 terminal kinase (JNK) was also observed after $TPPS_{2a}$-PDT, but only in NuTu-19 cells at doses reducing the cell viability by 50% or more. Experiments with suitable inhibitors revolved that p38 is an immediate death signal, while JNK rescues cells after PDT and PCI. Activation of ERK seemed, however, not to influence on PDT or PCI mediated cell death in NuTu-19 or WiDr cells.

4.5 Paper V

The aim of this study was to evaluate the impact of photochemically induced EGFR damage when $TPPS_{2a}$-PDT was combined with an EGFR targeted drug. PDT was performed with two different protocols, one targeting the photosensitizer to the endocytic vesicles and one where the photosensitizer was targeted to the plasma membrane at the time of light exposure. PDT, when targeted to the endocytic vesicles, did not induce EGFR damage at doses killing up to 90 % of A-431 cells. However, the EGFR specific TKI, tyrphostin AG1478 (tyrphostin), caused an immediate inhibition of EGF-stimulated EGFR activation lasting for ~24 hrs. Tyrphostin, when administered directly after endo/lysosomal PDT in A-431 cells, resulted in a synergistic cytotoxic effect as measured by cell counting 72 hrs after tyrphostin incubation. A photochemical damage of EGFR was observed after plasma membrane targeted PDT (LD_{50}) in A-431 cells. Surprisingly, an even stronger synergistic effect on cytotoxicity was observed when tyrphostin was administered directly after plasma membrane targeted PDT compared to endo/lysosomal targeted PDT in A-431 cells, indicating that the outcome of PDT and EGFR-targeting drug combination therapy was not dependent on photochemical EGFR damage. It was studied whether the synergistic effect between PDT and tyrphostin was cell line dependent. Hence, the PDT-tyrphostin combination treatment was also performed in NuTu-19 cells. Endo/lysosomal targeted PDT in NuTu-19 cells reduced the ability of EGFR to phosphorylate upon EGF-stimulation 5 min-4 hrs after light exposure. Tyrphostin also reduced EGF-induced EGFR phosphorylation, but in a more sustained manner lasting for at least 48 hrs. Cytotoxic evaluation after the PDT-tyrphostin combination treatment in NuTu-19 cells showed, surprisingly, an antagonistic effect when tyrphostin was administered directly after endo/lysosomal targeted PDT. The antagonistic effect observed after treatment with the PDT-tyrphostin combination was dependent on the timing of drug incubation as shown by the additive effect on cytotoxicity observed when PDT was exerted after tyrphostin incubation as well as during the tyrphostin incubation period. It was concluded that the outcome of PDT-tyrphostin combination treatment is not correlated to photochemical EGFR damage and that the therapy require further evaluation.

4.6 Paper VI

The molecular mechanisms of cell signalling when PDT is combined with EGFR targeted drugs were studied in this paper. Two different drugs, the EGFR specific TKI, tyrphostin, and the EGFR mAb cetuximab, were administered directly after endo/lysosomal targeted $TPPS_{2a}$-PDT in A-431 cells. Surprisingly, an antagonistic effect on cytotoxicity was observed after the PDT-cetuximab treatment compared to the synergistic outcome when PDT and tyrphostin were combined. Fluorescence microscopy of Alexa488-labelled cetuximab revealed no decreased cetuximab binding to EGFR after PDT. Western blot experiments showed that tyrphostin and cetuximab induced distinct EGFR-, ERK- and p38- signalling and the impact of this on the toxicity of the combination treatments was evaluated. PDT induced a prolongation of tyrphostin induced EGFR inhibition, but had no effect on cetuximab induced EGFR signal transduction. The PDT-tyrphostin combination treatment also induced a sustained inhibition of phospho-ERK that was not observed after the PDT-cetuximab combination treatment. Using the MEK inhibitor PD98059, ERK was found to be an important mediator of tyrphostin- and PDT-induced cytotoxicity as well as for the synergistic outcome of the PDT-tyrphostin combination treatment, in contrast to the cetuximab monotherapy and PDT-cetuximab combination treatment. It was concluded that the synergistic cytotoxic effect observed after the PDT-tyrphostin combination treatment was caused by a prolonged inhibition of EGFR and ERK, not detected after the antagonistic PDT-cetuximab treatment.

5. Discussion

PCI has been shown to be an effective drug delivery system for protein-toxins, genes and some conventional chemotherapeutics *in vivo* and *in vitro* (see Introduction). Photosensitizers used in PCI are preferentially retained in tumour tissue and with the possibility to direct light only to the diseased area, PCI can exert a two fold targeting of cancer cells. The selectivity of PCI towards cancer cells may be increased by delivering drugs which themselves target cancer cells. The search for tumour specific antigens that can be utilized in cancer therapy has been, and is still, a large field in cancer research and EGFR is one of the most investigated proteins for cancer targeted therapeutics. EGFR is endocytosed as a part of the regulation of its activity and the receptor was therefore anticipated as a suitable target for PCI-delivered drugs.

5.1 The efficacy of PCI of EGFR targeted protein-toxins

An advantage of using RIP based targeted toxins in cancer therapy is that they are highly toxic when they enter the cell cytosol. A problem with targeted toxins in the treatment of solid tumours is, however, poor penetration through the malignant tissue due to their large molecular size in addition to the poor convection in solid tumours (Fukumura & Jain 2007a, Fukumura & Jain 2007b). PCI is expected to enhance the effect of targeted toxins which have reached the tumour cells, and will in this way reduce the treatment obstacle made by poor tumour delivery. Another limitation in the use of therapeutic targeted toxins is the formation of neutralizing antibodies due to repeated injections of the drug. Since PCI may enhance the therapeutic effect of a targeted toxin up to 1000 fold (paper I), the clinical number of treatments is likely to be highly reduced compared to treatments with the targeted toxins alone, and formation of neutralizing antibodies will thereby be of less importance. Another major limitation for the clinical use of targeted toxins is their uptake in normal cells. This may lead to vascular leak syndrome (VLS), haemolytic uremic syndrome and damage to healthy organs which express the target antigen on the cell surface (Pastan *et al.* 2007). These adverse effects are highly dose-dependent, and since PCI is expected to increase the treatment specificity and thereby probably reduce the necessary dosage of the targeted

toxin, it is to be expected that these adverse effects can be reduced by introduction of the PCI technology.

The present thesis includes two reports on PCI of EGFR targeted toxins. In the first study one of the endogen ligands of EGFR, EGF, was linked to the protein-toxin saporin to form an affinity-toxin (paper I) while in the second study cetuximab, an EGFR monoclonal antibody, was linked to saporin to form an immunotoxin (paper II). EGF-saporin and cetuximab-saporin were specifically taken up by EGFR indicating that utilization of EGFR targeted toxins can improve the specificity of PCI towards cancer cells compared to delivery of untargeted toxins. Accordingly, PCI of EGF-saporin and cetuximab-saporin was much more cytotoxic than PCI of saporin, indicating that EGFR is a promising candidate for PCI mediated enhancement of the cytotoxicity of targeted protein-toxins.

5.1.1 Bioconjugation of the targeting ligand and the protein-toxin

The biotin-streptavidin binding was used to link saporin to both EGF and cetuximab. The binding is non-covalent with an association constant of $10^{15}M^{-1}$ (Diamandis & Christopoulos 1991), and is therefore stronger than the association constant when an antibody binds to its receptor, reported to be in the range of 10^{8}-$10^{12}M^{-1}$ (Siiman & Burshteyn 2000, Xie *et al.* 2005). The binding forms rapidly and is convenient for proof-of-concept studies as those performed in paper I and II. One of the obstacles of conjugating a targeting ligand to a protein-toxin is loss of effect of the toxic moiety (Atkinson *et al.* 2001). Linking of EGF to saporin through the biotin-streptavidin binding did not influence the RIP activity of saporin (paper I), and this binding seems therefore promising for screening and evaluation of targeting ligands for PCI-mediated delivery of protein toxins. There are, however, several disadvantages of using the streptavidin-biotin bond in formation of targeted toxins. Streptavidin is a tetramer of 60 kDa where every monomer has a biotin binding site. This gives the possibility of 4 biotinylated compounds to bind to each streptavidin tetramer. The binding reaction of biotinylated proteins to streptavidin labelled saporin can be difficult to control due to steric hindrance, which gives the possibility of heterogeneity in reaction products. The ability of streptavidin to bind to 4 biotinylated proteins can also result in very large products. Four molecules of biotinylated cetuximab bound to streptavidin-saporin may

form an immunotoxin of ~700 kDa which may be to large for efficient delivery to solid tumours. The diffusion of macromolecules through tissues is dependent on their size and weight and must be kept relatively low to ensure sufficient diffusion (Jain & Baxter 1988, Jain 1990). On the other hand, hydrophilic drugs with low molecular size are subjected to high renal clearance. Hence, the molecular size of such drugs must be high enough to avoid immediate excretion (Brenner *et al.* 1978, Maack *et al.* 1979). If EGF is linked to saporin (1:1) through a simple disulfide bridge, this would result in a ~40 kDa affinity-toxin that may be subjected to a rapid plasma clearance. However, EGF linked to saporin (1:1) through the streptavdin-biotin bond would form an affinity toxin with a MW of ~100 kDa, more suitable for *in vivo* use. The biotin-streptavidin bond has been clinically used (Knox *et al.* 2000, Weiden 2002, Forero *et al.* 2004, Gruaz-Guyon *et al.* 2005), however, immunogenicity of streptavdin is a major disadvantage of this conjugation strategy (Meredith & Buchsbaum 2006). As described above, PCI will probably be clinically applied in a limited number of times (1-2), and therefore streptavidin-induced immunogenicity may not be a treatment obstacle. The biotin-streptavidin system in the present thesis is used to demonstrate proof –of-principles of PCI of EGFR targeted toxins. Other methods for conjugation of toxin and targeting moieties should therefore be explored in future studies. In production of type I RIP based targeting toxins the targeting ligand and toxin have traditionally been chemically conjugated through disulfide bridges (Stirpe *et al.* 1980, Hirota *et al.* 1989, Kreitman 1999, Selbo *et al.* 2000b, Polito *et al.* 2004). These chemical reactions are difficult to control and suffer from heterogeneity in the reaction products, which in turn require several separation and purification steps. The last decades, research on recombinant technologies have made it possible to synthesize immunotoxins in transfected *E.coli.* Recombinant synthesis of an immunotoxins offers high control of the product, and the technology makes it possible to induce a peptide linker in the product to obtain distance between the moieties and a desired molecular size of the fusion toxin. The only targeted toxin with a marketing authorisation on today's market, Ontak^{TM}, consists of IL-2 and truncated diphtheria toxin made recombinantly. Paper I and II are both *in vitro* studies where the streptavidin-biotin linkage has been shown useful to demonstrate proofe-of-concept of PCI of EGFR targeted drugs. Preclinical and clinical models would, however, probably benefit from recombinant made EGFR targeted toxins.

5.1.2 EGF versus cetuximab as a targeting ligand

Both EGF-saporin and cetuximab-saporin were taken up selectively by the EGFR. The affinity of EGF towards EGFR is lower than that of cetuximab (Huether *et al.* 2005) and higher doses of the EGF-saporin complex compared to cetuximab-saporin may be required to induce comparable cytotoxicity. No direct comparison has been made between EGF-saporin and cetuximab-saporin in the present studies, and which of the targeting ligands that is most suitable for PCI delivered EGFR targeted toxins is difficult to determine. Both targeted toxins were, however, studied in the A-431 cell line and the results indicate that PCI increases the cytotoxicity of EGF-saporin to a larger extent than PCI of cetuximab-saporin. A LD_{99} dose of PCI of the two toxins were observed at 5 pM and 100 pM for EGF-saporin and cetuximab-saporin respectively, using a photochemical dose which reduced the viability by ~50% (Paper I and II). It can therefore be estimated that EGF-saporin is approximately 25 times more effective in combination with PCI than cetuximab-saporin. EGF stimulates to a more rapid endocytosis of EGFR than cetuximab (Friedman *et al.* 2005, Jaramillo *et al.* 2006). The increased cytotoxicity after PCI of EGF-saporin compared to cetuximab-saporin could therefore be explained by a more effective endocytosis of the former, accumulating more toxin in the endo/lysosomal vesicles at the time of light exposure. The endocytic trafficking of EGFR has also been shown to differ dependent on EGF- or cetuximab-stimulation (Roepstorff *et al.* 2008). This could also influence on the PCI induced toxicity of the two targeting toxins as the effect of PCI may be dependent on the type of endocytic vesicle from which the toxin is to be released (Selbo *et al.* 2000a). The incubation time for the two toxin conjugates were, however, different in the two papers (4 hrs for EGF-saporin and 18 hrs for cetuximab-saporin), and the two targeted toxins must be evaluated under the same experimental conditions to confirm the difference in PCI mediated toxicity.

EGF is an endogen ligand for EGFR and binding of EGF-saporin to a cell may induce growth and survival signalling. Since the protein-toxin will kill the cell after light exposure, this growth stimuli may not be important in PCI treated cells. However, tumour cells that suffer from poor light delivery, i.e. distant metastasis or EGFR expressing normal cells, may be subjected to growth and survival signals. However, growth stimulating effects have not been reported using other ligands, as VEGF (Veenendaal *et al.* 2002) and IL-2 (Foss 2006), as targeting moieties in targeted toxins,

and EGF fused to DAB389 has been investigated in a clinical phase I/II trial against lung cancer (Kreitman 1999). Using cetuximab as the targeting ligand in EGFR targeted toxins will, on the other hand, block EGFR signalling and reduce growth and survival of the cells. PCI of cetuximab-saporin could therefore probably exert a three-fold directed toxicity towards tumour cells (Fig.11); (i) the photochemical reaction, (ii) cetuximab induced toxicity and (iii) saporin induced toxicity. In addition, cetuximab may stimulate to antibody dependent cellular cytotoxicity *in vivo*, which is claimed to be its main mechanism of action (Naramura *et al.* 1993, Kurai *et al.* 2007). All together, these factors may favour cetuximab as the most suited targeting ligand for PCI-delivered EGFR- targeted toxins. On the other hand, it was shown in paper VI that cetuximab acts antagonistic in combination with the photochemical reaction utilized in PCI, indicating that cetuximab is not the optimal targeting ligand for EGFR targeted protein-toxins delivered by PCI.

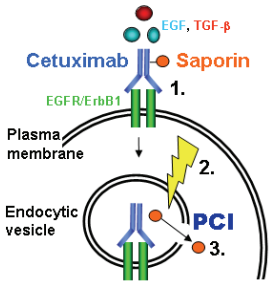

Fig.11: Three postulated mechanisms for PCI of cetuximab-saporin induced cytotoxicity. (1) Cetuximab blocks growth and surviving promoting signalling. (2) PDT induced cell death. (3) Inhibition of protein synthesis by saporin (paper II).

Since cetuximab (150 kDa) is approximately 25 times larger than EGF (6.3 kDa), targeted-toxins using cetuximab as a ligand will be larger and consequently probably more immunogenic compared to when EGF is utilized as the targeting moiety. This may cause an increased formation of neutralizing antibodies, inhibiting the effect of the targeted toxins when cetuximab is used as the targeting moiety compared to EGF. The smaller size of EGF compared to cetuximab may be an advantage for tissue penetration

of the targeted toxin. Even though not evaluated in the present thesis, it is possible to make targeted toxins where the targeting ligand contains only the Fv fragment of the monoclonal antibody. Targeted toxins containing Fv fragments are shown to be less immunogenic than whole antibodies (Reiter & Pastan 1998), and an Fv fragment against EGFR could provide a targeting moiety with the preferred properties of cetuximab together with a more practical size. Fv-based EGFR targeted protein-toxins should therefore be included in future work on PCI of EGFR targeted drugs.

5.2 Photodynamic targeting of EGFR

PDT, utilizing several photosensitizers, has been shown to reduce total EGFR as well as the activation ability of the receptor (de Witte *et al.* 1993, Ahmad *et al.* 2001, Wong *et al.* 2003, Zhuang *et al.* 2003, Schieke *et al.* 2004) . PCI of EGFR targeted toxins is dependent on a functional plasma membrane-bound EGFR that binds and internalizes the drug. Photochemically induced damage of EGFR should therefore be expected to attenuate PCI of EGFR-targeted toxins if any of the PS is present at the plasma membrane at the time of light exposure and the EGFR targeted toxin is delivered after the photochemical reaction (PCI with the "light first" procedure).

5.2.1 $TPPS_{2a}$-PDT induced damage to EGFR

The photosensitizers for use in PCI are first adsorbed to the plasma membrane upon administration and are then localised to endosomes and lysosomes at the time of light exposure. It is, however, not unlikely that a minor fraction of the PS can be retained on the plasma membrane during the PCI procedure and this may cause EGFR damage upon light exposure. This was indicated in paper III showing that $TPPS_{2a}$-PDT, as performed with the PCI procedure, inhibited EGF-induced EGFR activation in NuTu-19 cells 5 min after exposure to light, even though no photosensitizer was detected on the plasma membrane as measured by fluorescence microscopy. Little is known about the molecular mechanisms behind PDT-mediated EGFR targeting. However, Zhuang *et al.* reported that attenuation of EGFR after Rose Bengal(RB)-PDT was dependent on activation of both caspase-3 and protein phosphatases (Zhuang *et al.* 2003). The decreased EGFR activation after endo/lysosomal PDT observed in paper III can also be

caused by activation of phosphatases, and experiments in the presence of caspase- and phosphatase-inhibitors should be performed to study this hypothesis. Experiments in paper III with a significant amount of $TPPS_{2a}$ present on the plasma membrane in addition to the endocytic vesicles also inhibited EGFR activation, and in addition, total EGFR was reduced immediately after light exposure. The results on total EGFR attenuation after PDT with plasma membrane bound $TPPS_{2a}$ indicated a direct oxidation of the receptor in the NuTu-19 cells. Amphiphilic photosensitizers such as $TPPS_{2a}$ will generally be localized in the outer leaflet of the plasma membrane with the hydrophilic part in the extracellular space and the hydrophobic part inside the membrane. Moan and Berg estimated in 1991 diffusion length of $^1O_2$ in cellular membranes to 10-20 nm corresponding to a lifetime of 10-40 ns (Moan & Berg 1991). However, more recent studies have indicated longer lifetimes for $^1O_2$ (Skovsen *et al.* 2005, Hatz *et al.* 2007) and it is not unlikely that this ROS can diffuse across the 10-20 nm tick plasma membrane. On the other hand, singlet oxygen will be easily quenched by biomolecules in the plasma membrane (Bronshtein *et al.* 2004) and more research is required to conclude on the mechanisms causing the damage of EGFR in $TPPS_{2a}$-treated cells.

The susceptibility of $TPPS_{2a}$-PDT mediated EGFR damage seems to be cell line dependent. In contrast to the NuTu-19 cells, no damage of EGFR was observed after a LD_{50} dose of endo/lysosomal targeted $TPPS_{2a}$-PDT in WiDr (paper III) nor in A-431 cells (Paper V). When the photosensitizer was targeted only to the plasma membrane in A-431 cells, a reduction in EGF-stimulated EGFR phosphorylation was observed (paper V. This reduction was comparable to that reported after PDT in the NuTu-19 cells when the PS was targeted to endosomes and lysosomes (paper III). We can therefore conclude that photochemical damage of EGFR by $TPPS_{2a}$-PDT is dependent on the amount of photosensitizer present at the plasma membrane at the time of light exposure. The susceptibility towards $TPPS_{2a}$-induced photochemical damage in different cell lines therefore probably depends on the ratios of endocytosis and exocytosis in the cells. If the EGFR damage observed in the present thesis is caused by $^1O_2$ that diffuses through the plasma membrane and directly oxidize the intracellular domain of the receptor, the composition of the plasma membrane and presence of quenching biomolecules should also be considered as determinant factors for the photochemical damage of EGFR. Even though not discussed in the relevant publications, PDT induced EGFR damage with Photofrin (Wong *et al.* 2003), 5-ALA induced protoporphyrin IX (PpIX) (Wong *et al.*

2003), Hypericin (de Witte *et al.* 1993) and RB (Zhuang *et al.* 2003, Schieke *et al.* 2004) as PSs may be caused by a direct oxidation of the receptor at light exposure time, as all of these PSs has been shown to localize to the plasma membrane (Thomas & Pardini 1992, Lin *et al.* 2000, Selbo *et al.* 2001a, Hsieh *et al.* 2003b). In addition, the lipophilicity of these PSs indicate that they can freely diffuse through the plasma membrane, and thereby easily target the intracellular domain of EGFR upon light exposure.

EGFR damage has been claimed important for PDT-mediated toxicity (Ahmad *et al.* 2001, Schieke *et al.* 2004). Administration of EGFR inhibitors before PDT may be utilized to evaluate the importance of photochemical receptor damage for cytotoxicity. If photochemical EGFR targeting represents an important signal for $TPPS_{2a}$-PDT induced cell death, PDT-mediated cytotoxicity should be expected to decrease in the presence of an EGFR inhibitor. This was observed when PDT was combined with the TKI tyrphostin and the mAb cetuximab in the NuTu-19- and A-431-cell line respectively (paper V and VI). An increase in PDT induced toxicity was, however, observed after the PDT-Tyrphostin combination treatment in the A-431 cell line (paper VI), but as this synergistic effect seemed to be correlated to an enhanced inhibition of ERK as well as EGFR (for further discussion see section 5.3.1), the present results together indicate that photochemical induced damage of EGFR may contribute to $TPPS_{2a}$-PDT mediated cell death.

It has also been argued that the effect of PDT may be enhanced by addition of an EGFR targeted drug (Ahmad *et al.* 2001). The results obtained in the present studies shows that the outcome of such combination therapies are highly dependent on the cell type as well as the mechanism of action of the EGFR targeted drug and its interaction with the photochemical treatment. In general, data obtained in the present studies indicate that combinations of PDT and EGFR targeted drugs must be strictly evaluated before they are introduced to the clinic to avoid antagonistic effects of the treatments.

5.2.2 Influence of photochemical-induced EGFR damage on PCI of EGFR targeted toxins

PCI of a drug, as described in details in the introduction chapter, can be performed with two different treatment procedures; either with the drug for delivery administered

before (“light after” strategy) or after (“light first” strategy) the photochemical treatment. PCI of EGF-saporin was much more effective with the “light after” strategy compared to the “light first” strategy in NuTu-19 cells, even though no differences were found between the “light first” and “light after” strategy when PCI of streptavidin-saporin was performed (paper I). The same observation was made by PCI of cetuximab-saporin in HCT-116 cells, where no PCI effect was observed when the “light first” strategy was applied compared to the “light after” strategy that induced a synergistic effect on cell killing (paper II). $TPPS_{2a}$-PDT mediated EGFR damage were, in the present thesis, not studied in HCT-116 cells. However, the photochemical damage of EGFR in NuTu-19 cells (paper III) correlates well with the decreased effect of PCI of EGF-saporin with the “light first” strategy in this cell line. If PCI of EGFR targeted toxins with the “light first” procedure is inhibited by photochemical induced EGFR damage, the “light first” and “light after” strategy should be expected to have similar efficacy in A-431 cells where no photochemical damage of EGFR was observed after endo/lysosomal targeted PDT (paper V). Fig.12 presents PCI of EGF-saporin with both the “light after” and “light first” strategy in A-431 and NuTu-19 cells. PCI of EGF-saporin in A-431 cells seems just as effective with the “light first” strategy as with the “light after” strategy compared to the NuTu-19 cells where the “light first” strategy is less efficient. Consequently, these results, together with the data from the studies on PDT-mediated EGFR damage, indicate that the photochemical treatment may inactivate the target receptor and should be taken into account when designing treatment protocols for PCI of EGFR targeted drugs. As PCI of EGFR targeted toxins seems to depend on photochemically induced EGFR damage and because these effects tend to be cell line dependent, it will be important to find factors predicting the EGFR damage and consequently responsiveness to the PCI treatment in a specific cell type.

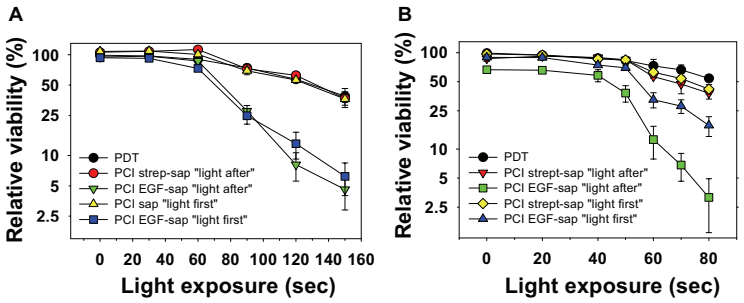

Fig.12: PCI of EGF-saporin with the light first and light after strategy. PCI of streptavidin-saporin and EGF-saporin was performed with both the light first and light after strategy in A-431 cells (A) and NuTu-19 cells (B) (paper I).

5.3 EGFR targeted drugs; effects on PDT and PCI induced protein signalling and subsequent toxicity

Combination therapy is becoming increasingly relevant in cancer therapy. Even though PCI mainly has been used as a drug delivery system for large hydrophilic drugs with intracellular targets, the photochemical treatment (PDT) may be combined with other drugs. Increased cytotoxicity is reported after dual therapy with PDT and different chemotherapeutic drugs such as cisplatin (Nonaka *et al.* 2002) cyclophosphamide (Casas *et al.* 1998), 5-fluoro-2-deoxyuridine (5FdUr) (Zimmermann *et al.* 2003), metotrexate (Sinha *et al.* 2006) and doxorubicin (Kirveliene *et al.* 2006). Recently, novel anticancer drugs as TKIs (Dimitroff *et al.* 1999, Liu *et al.* 2007), mAbs (del Carmen *et al.* 2005, Ferrario & Gomer 2006) and COX-2 inhibitors (Ferrario *et al.* 2005) have been reported to enhance PDT-mediated toxicity. However, antagonistic responses have also been reported with PDT in combination with doxorubicin and 5FdUr (Zimmermann *et al.* 2003, Kirveliene *et al.* 2006). Multimodality therapy is generally considered most effective when the different monotherapies have distinct mechanisms of action (del Carmen *et al.* 2005, Zhang *et al.* 2005, Soffietti *et al.* 2007). Knowledge of the molecular mechanisms of action following PDT, as well as the drug intended as an adjuvant, is therefore of importance to predict the outcome when the treatments are combined and to avoid antagonistic cytotoxicity. This information will

also have value when evaluating PDT for incorporation into established treatment modalities.

5.3.1 The impact of MAPK signalling

The events leading to cell death or survival after PDT can be evaluated by studying the protein signalling after the treatment. Even though the signal transduction after PDT mainly is triggered by the formation of singlet oxygen, cellular signalling after PDT is dependent on the photosensitizer, its localization, PDT-dose and also on the cell type used (Moor 2000, Piette *et al.* 2003, Almeida *et al.* 2004, Buytaert *et al.* 2007, Uzdensky 2008). Several groups have shown an increasing interest in this field, and many different proteins and protein cascades have been investigated. The PDT-induced mitochondrial release of cytochrome C (Vantieghem *et al.* 1998, Kim *et al.* 1999, Reiners, Jr. *et al.* 2002), caspases (Granville *et al.* 1998, Zhuang *et al.* 2001) and MAPKs (for references see below) are, however, of the most studied, and the present work has revealed the MAPK signalling after $TPPS_{2a}$-mediated PDT. The three most investigated proteins in the MAPK family are the extracellular signal regulated kinase (ERK), p38 and JNK. These three MAPKs are all parts of protein phosphorylation cascades where the end points are post transcriptional modification and activation of different transcription factors regulating growth, differentiation, apoptosis and inflammation (Bonni *et al.* 1999, Chang & Karin 2001, Hazzalin & Mahadevan 2002).

ERK is a protein in the RAS-RAF-MEK cascade downstream of EGFR associated with growth and cell survival (Zebisch *et al.* 2007). $TPPS_{2a}$-PDT induced ERK signalling was found to be cell line dependent in the present studies. ERK was activated 5 min post LD_{50} $TPPS_{2a}$-PDT in both NuTu-19 and WiDr cells (paper IV). This is in agreement with Tong *et al.* who reported on a similar ERK-activation after Photofrin-induced PDT (Tong *et al.* 2002). However, in that study the observed ERK activation was correlated to PDT-resistance (Tong *et al.* 2002), which is in contrast to our results showing no direct connection between ERK activation and PDT-induced cell death in neither NuTu-19 nor WiDr cells (paper IV). Interestingly, PDT in NuTu-19 cells inhibited EGF-induced EGFR phosphorylation by 50 % (paper III), while the EGF-stimulated ERK activation seemed not to be influenced by the photochemical reaction (paper IV). Since PDT inhibits EGF-induced EGFR activation without affecting EGF-induced ERK activation, these data indicate that the rate limiting step in the EGFR-ERK

pathway in NuTu-19 cells is located downstream of EGFR. We have suggested RAF activation as a possible rate limiting step in this pathway in agreement with an other report (Dougherty *et al.* 2005) (paper IV). In contrast to the $TPPS_{2a}$-PDT induced activation of ERK in NuTu-19 cells, PDT using 5-ALA induced PpIX, Photofrin, RB, and hypericin as photosensitizers has been reported to attenuate ERK activity, suggested to be important for PDT-mediated cytotoxicity (Assefa *et al.* 1999, Wong *et al.* 2003, Schieke *et al.* 2004). These reports are, however, in agreement with the results obtained in paper VI, where attenuation of phosphorylated ERK was indicated as a death signal after $TPPS_{2a}$-PDT in A-431 cells.

The present studies indicate that $TPPS_{2a}$-PDT induced ERK signalling may have impact on the treatment outcome when PDT is combined with EGFR targeted drugs (paper IV, VI and VI). An antagonistic effect on cytotoxicity was observed when endo/lysosomal targeted PDT, resulting in activation of ERK, was combined with tyrphostin in the NuTu-19 cells compared to the synergistic effect obtained after the combination in A-431 cells where PDT attenuated ERK activation. The antagonistic effect observed after the PDT-tyrphostin combination treatment in the NuTu-19 cells may be explained by PDT-induced EGFR damage which in turn inhibits the tyrphostin-induced toxicity (paper V) (Fig 13.A). It remains to be studied if tyrphostin inhibits the ERK activation observed immediately after PDT in NuTu-19 cells, but this will probably have no effect on PDT-induced cytotoxicity since the MEK inhibitor PD98059 have no effect on survival of NuTu-19 cells after PDT (paper IV). In the A-431 cell line, endo/lysosomal targeted PDT does not target EGFR (paper VI). The synergistic effect observed when this PDT treatment is combined with tyrphostin may be due to a prolonged inhibition of EGFR as well as ERK compared to the mono-therapies (paper V, VI and Fig 13B). Plasma membrane targeted $TPPS_{2a}$-PDT in A-431 cells also causes synergistic cytotoxic effects on cell survival in combination with tyrphostin even though this photochemical regimen damage EGFR (paper V) (Fig 13.C). PDT induced EGFR damage in combination with Tyrphostin therefore causes synergistic toxicity in A-431

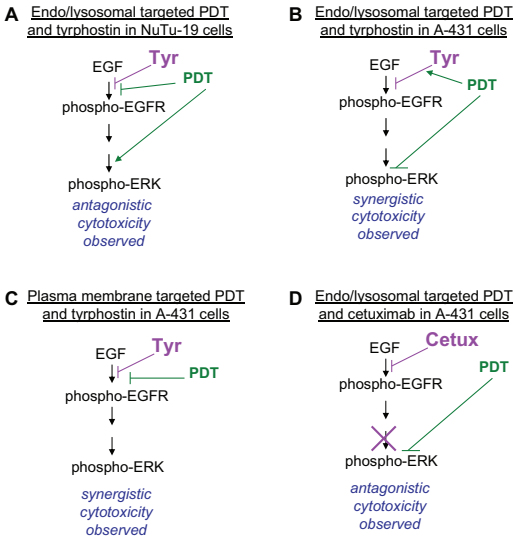

Fig.13: Protein signaling and cytotoxic outcome when TPPS2a-PDT is combined with EGFR targeting drugs in the present thesis.

cells while antagonistic effects are observed after the combination in NuTu-19 cells. Paper V therefore concluded that the outcome of PDT–tyrphostin combination treatment is not correlated to EGFR damage. Neither EGFR nor ERK was investigated after PDT-tyrphostin treatment when the PS was targeted to the plasma membrane in A-431 cells. The importance of prolonged ERK inhibition for a synergistic outcome when endo/lysosomal targeted PDT is combined with an EGFR targeting drug in A-431 cells was, however, indicated in the experiments on PDT-cetuximab combination therapy in paper VI. In contrast to the tyrphostin treatment, cetuximab-treatment alone as well as the PDT-cetuximab combination treatment induced only a 2 hrs incomplete inhibition of ERK in the A-431 cells and an antagonistic effect was observed after the PDT-cetuximab combination treatment. Compared to the tyrphostin treatment, cetuximab alone induced a more prolonged inhibition of EGFR and PDT did not increase cetuximab-induced EGFR inhibition. This can explain the antagonistic cytotoxicity after the PDT-cetuximab treatment compared to the PDT-tyrphostin treatment in the A-431 cells (Fig.13D). As discussed above, combination therapy is generally considered most effective when the different modalities have distinct action mechanisms. The results in

paper VI indicate different modes of action between PDT and cetuximab concerning EGFR and p38, but mutual effects on ERK. To explain the antagonistic cytotoxicity of PDT and cetuximab, other effectors downstream of EGFR should be investigated to reveal if the two treatments have common action mechanisms on other proteins.

ERK signalling may influence on the outcome of PCI induced toxicity. The antagonistic effect observed when cetuximab is combined with PDT indicates that this mAb may not be optimal as a targeting moiety for PCI delivered EGFR targeted toxins, even though PCI clearly enhanced cetuximab-saporin induced cytotoxicity in paper II. The results also indicate than PCI can be optimized by delivering drugs which inhibits ERK as a part of their mechanism of action, although the effect seems to be cell line dependent.

The JNK proteins are stress induced kinases (Vlahopoulos & Zoumpourlis 2004) shown to be activated by PDT with different photosensitizers (Almeida *et al.* 2004). Assefa *et al.* reported that JNK activation rescues cells from hypericin mediated PDT (Assefa *et al.* 1999) while JNK activation after Photofrin-PDT has been shown not to influence on cytotoxicity (Hsieh *et al.* 2003a). The impact of JNK activation after PDT seems therefore to depend on the photosensitizer and/or on the cell line. In the present studies increased phosphorylation of JNK was observed in NuTu-19 cells 1 hr after $TPPS_{2a}$-PDT at doses reducing the viability to 50% or less (paper IV). Here it was demonstrated that in presence of the JNK inhibitor SP125600, the cytotoxic effects of PDT were significantly increased, suggesting that JNK activation rescues cells from PDT-mediated death in this cell line (paper IV). No activation of JNK was observed neither in WiDr nor in A-431 cells after LD_{50}-$TPPS_{2a}$-PDT (paper IV, results not published), but activation of JNK at higher doses cannot be excluded. Activation of JNK may inhibit PCI induced cell death at higher doses than applied in paper IV. The results on JNK obtained in the present studies may therefore indicate that macromolecular drugs, that inhibit JNK, may be suitable for PCI delivery in cases where JNK is activated after PDT.

P38 is also a stress induced kinase (Zarubin & Han 2005) shown to be activated after PDT with different photosensitizers. PDT-induced p38 activation has been reported as a death mechanism as well as a rescuing signal and the impact of p38 signalling on cell death after PDT seems to depend on both the photosensitizer, PDT-dose and the cell line (Klotz *et al.* 1999, Assefa *et al.* 1999, Xue *et al.* 1999, Zhuang *et al.* 2000, Tong *et*

al. 2003, Kralova *et al.* 2007, Buytaert *et al.* 2008). Activation of p38 was observed 5 min after LD_{50} $TPPS_{2a}$-PDT in all investigated cell lines (NuTu-19, WiDr and A-431) (paper IV and VI). The impact of this PDT-induced p38 activation was studied in the presence of the p38 inhibitor SB203580. It was found that p38 activation is an immediate death signal after $TPPS_{2a}$-induced PDT (paper IV and VI). The two EGFR targeted drugs tyrphostin and cetuximab was in paper VI shown to have distinct effects on p38 signalling: p38 was shown as a death signal after PDT-cetuximab combination treatment, however, not after PDT-tyrphostin treatment (paper VI). The p38 inhibitor was also included in experiments with PCI of gelonin. The results indicated that the p38 signal transduction observed after $TPPS_{2a}$-PDT also occurred after PCI of gelonin, and influenced on the PCI mediated cell death to a similar extent as observed for PDT induced toxicity (paper IV), but at lower photochemical cytotoxic doses. The results on PCI of gelonin in the presence of the p38 inhibitor therefore indicate that p38 activation influence on the effect of gelonin in addition to the photochemical-induced toxicity. $TPPS_{2a}$-PDT, as performed during these works, exerts its effect mainly by endo/lysosomal rupture, relocalization of the PS and subsequent photochemical effects on different membrane bound organelles. If SB203580 inhibits the photochemical rupture of the endo/lysomal vesicles, this may explain the decreased PCI effect in the presence of the inhibitor, since less gelonin will be able to escape into the cytosol. Another possibility is that the p38 inhibitor attenuates gelonin-induced toxicity in the cytosol. The present thesis focuses on PCI of EGFR-targeted toxins, where the overall aim is to improve the targeting and enhance the killing of cancer cells. PCI is also used for delivery of genes where the aim is not necessarily to kill the target cell, but rather to deliver the gene of interest. If p38 inhibition does not influence on photochemically-induced rupture of endosomes and lysosomes, inhibition of photochemical induced death signals, as the p38 activation, in PCI of genes is likely to increase the fraction of surviving transducable cells and may in this way increase the spesificity of the treatment. PDT induced p38 activation has by others also been associated with induction of VEGF using both BPD and Hypericin as photosensitizers (Hendrickx *et al.* 2005, Solban *et al.* 2006) and activation of p38 after hypericin PDT is in addition shown to upregulate cyclooxygenase-2 (Hendrickx *et al.* 2003) and heme-oxygenase 1 (Kocanova *et al.* 2007). Induction of both VEGF (Ferrara & Gerber 2001, Kowanetz & Ferrara 2006), COX-2 (Bakhle 2001) and heme-oxygenase (Jozkowicz *et al.* 2007) may

stimulate to angiogenesis and tumour promoting survival *in vivo*. If PDT with PCI relevant PSs induces the same signalling as observed with BPD and hypericin, introduction of a p38 inhibitor to the PCI procedure may optimize the treatment. A summary of the $TPPS_{2a}$-PDT induced MAPK signalling data of the present thesis is presented in table 3.

A

Protein	NuTu-19	WiDr	A-431
p-EGFR	decrease	no effect	no effect
EGFR	no effect	no effect	no effect
p-ERK	increase	increase	decrease
ERK	no effect	no effect	no effect
p-JNK	increase	not detected	not detected
JNK	no effect	no effect	no effect
p-p38	increase	increase	increase
P38	no effect	no effect	no effect

B

Protein	NuTu-19	A-431
p-EGFR	decrease	decrease
EGFR	decrease	no effect
p-ERK	increase	-
ERK	no effect	-
p-JNK	-	-
JNK	no effect	-
p-p38	increase	-
P38	no effect	-

Table 3: Protein signaling detected in the present thesis after LD_{50} $TPPS_{2a}$-PDT with the PS targeted primary to endocytic vesicles (A) and to the plasma membrane in addition to the endocytic vesicles.

6. Conclusions

Specificity and efficacy of PCI can be optimized by delivering drugs which accumulate in cancer cells. EGFR is a suitable target for such drugs for two main reasons. First, the receptor is overexpressed in several different cancers and second, EGFR is internalized as a part of its physiology and is therefore able to transport attached drugs to endosomes and lysosomes, the primary targets for PCI.

PCI of EGFR targeted drugs exerts a three-fold selectivity for cancer cells: (i) the photosensitizers is preferentially retained in tumour tissues, (ii) the light is directed only to the tumour area and (iii) utilization of an anticancer drug which targets cancer cells.

Photodynamic targeting of EGFR is cell line dependent and is correlated to the amount of photosensitizer present on the plasma membrane at the time of light exposure. However, other cell specific properties seems also important in PDT induced EGFR damage.

PCI of EGFR targeted drugs may be influenced by the treatment procedure, i.e the use of “light first” or “light after” strategy. Photodynamic damage of EGFR seems to decrease the efficacy of the PCI when the photochemical treatment is performed prior to administration of the EGFR targeted drug (“light first” procedure). Hence, the timing of drug administration and light activation in cancer patients may be critical to obtain optimal effects with PCI of EGFR-targeted drugs.

Both the endogen ligand of EGFR, EGF, and the EGFR mAb cetuximab may be suitable as the EGFR targeting moiety for PCI delivered drugs. The protein signal transduction induced by the EGFR targeted drug and the photodynamic reaction can, however, interact which in turn may influence on the cytotoxic effect of the treatment.

The present thesis demonstrates the proof-of-concept of PCI of EGFR targeted drugs and pinpoints the importance of studying the intracellular signalling post endo/lysosomal targeting PDT paving the way for further improvements of the PCI technology and possible future clinical applications.

7. Future perspectives

The therapeutic applicability of anticancer drugs is to a large extent limited by the adverse effects of the treatment. PCI of EGFR targeted protein-toxins is in the present thesis shown as an effective anticancer treatment that exerts a high specificity for cancer cells *in vitro* and the results warrant further work with *in vivo* preclinical models. These studies may be initiated using EGFR expressing tumour models in mice and measure the tumour growth after the treatment. The impact of receptor status and malignancy for the treatment outcome after PCI of EGFR targeted drugs should be addressed in these studies. Future work on the PCI technology also includes development of the method towards specific cancer indications.

The two papers on PCI of EGFR targeted toxins in the present thesis where proof-of-principle studies where EGF-saporin and cetuximab-saporin were made without any optimalization concerning PCI delivery. Future work should therefore include studies evaluating the optimal composition of the targeted toxins such as the ratio of toxin to targeting moiety. Recombinant targeted toxins are assumed to be advantageous over chemical conjugates due to increased specificity, higher stability and the homogeneity of reaction products, and future work should include establishment of recombinant EGFR targeted toxins. The preclinical evaluation of recombinant EGFR targeted toxins should include fragments of antibodies, but also EGF and other natural ligands since EGF was found to be a promising ligand for protein-toxin delivery by PCI. Saporin was used as the toxic moiety in both EGFR targeted toxins used in this thesis. Gelonin is another type I RIP shown to work very efficient when delivered with PCI. Gelonin alone is less toxic to whole cells than saporin and may increase the specific toxicity when utilized in PCI delivered EGFR targeted toxins compared to saporin. PCI of EGFR targeted toxins based on gelonin should therefore be considered.

The PCI technology is highly dependent on effective endocytosis of the delivered drug. The present thesis has, however, not evaluated the endocytosis ratio of the different EGFR targeted toxins. Endocytosis of EGFR is dependent on the ligand attached and also on whether the receptor is homodimerized, heterodimerized or not dimerized. Future work should therefore include experiments on EGFR targeted drug- and toxin mediated endocytosis and its impact on the efficacy of PCI.

The present thesis reports on MAPK signal transduction after photochemical treatment as performed by PCI. Knowledge about death and survival mechanisms induced by this form of photochemical treatment can improve the PCI technology and also predict suitable drugs for PCI delivery. The work on mapping protein signal transduction with impact on cell death and survival after the photodynamic treatment should therefore be continued. This should include investigations of protein pathways involved in apoptosis, autophagy and necrosis.

EGFR-mediated signal transduction and its impact on PDT- induced protein signalling has also been investigated in the present work. It was shown that interactions between EGFR targeted drugs and the photochemical treatment had an impact on the treatment outcome when the two modalities were combined, causing both synergistic and antagonistic effects on cytotoxiciy of the treatment. Interactions between PDT and EGFR targeting on the level of signal transduction should be further explored in future studies to avoid antagonistic toxicity and optimize the modality of PCI of EGFR targeting drugs.

8. References

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