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Targeted Photodynamic Therapy and Photochemical Internalization of Human Head and Neck Cancer

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Targeted Photodynamic Therapy and Photochemical Internalization of Human Head and Neck Cancer

A preclinical study *in vitro* and *in vivo*

Wei Peng

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Targeted Photodynamic Therapy and Photochemical Internalization of Human Head and Neck Cancer

A preclinical study *in vitro* and *in vivo*

PhD thesis

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Abbreviations

BLM	Bleomycin
DLI	Drug-light interval
EGFR	Epidermal growth factor receptor
EMA	European Medicines Agency
FDA	Food and Drug Administration in USA
FFPE	Formalin-fixed paraffin-embedded
Foscan [®]	mTHPC in the formulation of ethanol, propylene glycol and water
H&E	Hematoxylin and eosin
Hp	Hematoporphyrin
HpD	Hematoporphyrin derivative
IC	Internal conversion
ICD	Immunogenic cell death
IR700DX	Phthalocyanine dye IRDye 700DX
LDL	Low density lipoprotein
LED	Light-emitting diode
mTHPC	meta-tetrahydroxyphenyl chlorin
¹ O ₂	Singlet oxygen
PCI	Photochemical internalisation
PDT	Photodynamic therapy
ROS	Reactive oxygen species
TPCS2a	Disulfonated tetraphenyl chlorin

Chapter 1

General introduction with aims and outline of the thesis

Head and neck cancer is a group of tumours in the oral cavity, pharynx, larynx, nasal and sinus cavities, orbit and other surrounding tissue structures. According to the report by Ferlay et al., the worldwide incidence of Head and Neck cancer was estimated to be more than 680,000 each year with the mortality rate of about 370,000 (Ferlay et al., 2015). The major risk factors are the use of tobacco and consume alcohol (Hashibe et al., 2009). In the past decades an increasing incidence of Human Papilloma Virus induced oropharyngeal cancers occurred. The tumours mainly arise from the squamous cell linings with more than 90% squamous cell carcinoma (Vigneswaran and Williams, 2014). Because of the complexity of the head and neck region with its critical structures the treatment options do not only depend on type and stage, but also anatomic location of tumour. The conventional treatment includes surgery or radiotherapy for early stage (stage I/II) cancer (Wolfensberger et al., 2001; Argiris et al., 2008; Bhalavat et al., 2009) and combinations of surgery, radiotherapy and chemotherapy for advanced stage III/IV cancer (Cohen et al., 2004; Haddad RI et al., 2008; Pignon et al., 2009). However, both surgery and radiotherapy often cause severe damage to surrounding normal tissues with a loss of their functions (Finlay et al., 1992; Bundgaard et al., 1993). Such morbidities have encouraged one to search for new treatment alternatives for this disease.

1.1. Photodynamic therapy

Photodynamic therapy (PDT) uses a photosensitising agent accumulating in tumor tissue and light irradiation to induce photochemical and photobiological reactions in the presence of oxygen. These reactions lead to irreversible photodamage to the tumor. During this dynamic process the absorbed light energy by the photosensitiser can be transferred to molecular oxygen to generate reactive oxygen species (ROS) including singlet oxygen ($^1\text{O}_2$). These species react further with cellular components to cause cell death. Thus, as shown in Figure 1, PDT effectiveness is dependent upon the presence of a photosensitiser, light delivery and the amount of oxygen in tumour tissue

(Pass 1993; Dolmans et al., 2003; Castano et al., 2006; Agostinis et al., 2011; van Straten et al., 2017).

Figure 1. Principle of Photodynamic Therapy

PDT is an established cancer treatment modality with several FDA- and/or EMA-approved photosensitisers. Patients with a variety of different types of tumours have worldwide been treated with PDT in the locations that can be reached by light (with or without an optic fiber) such as skin, head & neck, bronchus, brain, digestive tract, female reproductive tract, bladder, prostate, etc. (van Straten et al., 2017). Almost all of these tumours respond to PDT including those advanced cancers that do not respond to conventional treatments. PDT can also be applied repeatedly since photosensitisers often have a short-lived pharmacokinetic pattern in the body.

1.1.1. History

The first use of light in human medicine can be traced back at least 3000 years in the ancient cultures of Egypt, Greece, China and India for the treatment of patients with a skin condition of vitiligo (Daniell and Hill, 1991; Dolmans et al., 2003). In principle, there

are two forms of light-mediated medicine: phototherapy and photochemotherapy. In phototherapy light is absorbed by endogenous chromophores and is, for example, employed in the treatment of infants with neonatal jaundice (Daniell and Hill, 1991). In photochemotherapy light is used after administration of light-absorbing exogenous photosensitisers. If $^1\text{O}_2$ is involved in the action of photochemotherapy it is often referred to as PDT.

Based on the finding by Oscar Raab in Munich that some 'dyes' killed paramecium cells when illuminated (Raab, 1900), Herman von Tappeiner first proposed PDT as a possible treatment modality in 1900 (von Tappeiner, 1900). Three years later von Tappeiner and Jesionek reported the first clinical PDT results of skin cancer (von Tappeiner and Jesionek, 1903). In 1904 von Tappeiner and Jodbauer found that the photosensitisation required the presence of oxygen (von Tappeiner and Jodbauer, 1904), and subsequently von Tappeiner coined the term of 'Photodynamic Therapy' to describe the phenomenon of oxygen-dependent photosensitisation in 1907 (von Tappeiner and Jodbauer, 1907; Daniell and Hill, 1991). Later, Auler and Banzer in Berlin and also Figge in USA reported that injected hematoporphyrin (Hp) preferentially localised in the animal tumour tissues (Auler and Banzer, 1942; Figge, 1942). In 1955 Schwartz et al. found that Hp was a mixture of various porphyrins with different properties and the more purified Hp component was actually the poorest tumour localiser; while the non-Hp fraction showed a greater affinity for the tumours (Schwartz et al., 1955). In order to purify more active components they then mixed Hp with acetic- and sulfuric acids to produce hematoporphyrin derivative (HpD). Lipson et al. reported in 1960s that HpD was better than Hp for the detection and treatment of tumours (Lipson and Baldes 1960; Lipson et al., 1961; 1967). The modern era of PDT started in the late 1970s by Thomas Dougherty and his co-workers at Roswell Park Comprehensive Cancer Center (Dougherty et al., 1978; Dougherty et al., 1979). From then on, clinical PDT treatments and mechanistic studies have extensively been explored worldwide to establish this modality of cancer (Ackroyd et al., 2001; Castano et al., 2006; Agostinis et al., 2011; van Straten et al., 2017).

1.1.2. Photosensitisers

A photosensitiser is defined as a chemical entity which can induce a chemical and/or physical change of another chemical entity after its absorption of energy from a photon. The chemical structure of most photosensitisers used in PDT is the tetrapyrrole macrocycle. As shown in Figure 2 porphyrins are composed of four pyrrol subunits linked with four methine bridges. Chlorins are a class of porphyrins in which one of the double bonds in a pyrrol subunit is reduced by hydrogen (arrow). This leads to a strong absorption at 652 nm (Berebaum et al., 1986; Bonnett et al., 1989). Phthalocyanines are azaporphyrins made up with a ring of four isoindoles linked by nitrogen atoms (Ben-Hur and Rosenthal, 1985; Rosenthal, 1991). In addition, some substituents can be added to the peripheral positions of the pyrrol rings of porphyrins and porphyrin-related dyes to improve chemical properties (stability, water/lipid solubility, etc.) and biological properties (favourable pharmacokinetics, cell/tissue distribution, etc.) (Abrahamse and Hamblin, 2016).

Figure 2. Chemical Structures of Photosensitisers

An ideal photosensitiser should meet several requirements: (1) high purity with no dark toxicity; (2) strong absorbance in the 600-800 nm region where tissue penetration of light is at maximum and where the wavelengths of light are still energetic enough to produce $^1\text{O}_2$ (Hill et al., 2014); (3) preferential distribution in a target tissue; (4) rapid elimination from the body with low systemic toxicity; and (5) easy administration through various routes (Castano et al., 2004).

HpD is a complex of Hp monomers, dimers and oligomers. Removal of the monomer fraction leads to the commercial compound, porfimer sodium (Photofrin[®]) with a mixture of oligomers by ethers and esters (Figure 2). This was the first photosensitiser to receive an official approval for PDT of recurrent superficial papillary bladder cancer by the Canadian Health Protection Branch in April, 1993, and later for palliative PDT of oesophageal cancer by FDA in USA in September, 1994. Porfimer sodium is now used worldwide for a number of indications including tumors of cervix, bladder, lung, stomach and brain (van Straten et al., 2017). Despite great success, porfimer sodium still suffers from several drawbacks such as: (i) mixtures of several porphyrins that have not fully been characterised (Dougherty 1987); (ii) relatively low absorption coefficient in the 600-800 nm red regions where light penetration into tissue is optimal (Wilson et al., 1985); (iii) relatively low tumor selectivity; and (iv) induction of prolonged skin photosensitivity for at least 4-6 weeks (Dougherty et al., 1990). These shortcomings of porfimer sodium have stimulated a search for second-generation of photosensitisers of single substances of known chemical structures with more suitable properties of large extinction coefficients at the wavelengths of 600-800 nm, favourable pharmacokinetics (high and preferential distribution in tumour tissue) with short-term retention in normal tissues (minimal or no skin photosensitization) and a low systemic toxicity.

The second-generation of photosensitising compounds used in clinical PDT today mainly includes three families: porphyrin precursors (e.g. 5-aminolevulinic acid and its ester derivatives), chlorins (e.g. meta-tetrahydroxyphenyl chlorin, mTHPC) and phthalocyanines (e.g. aluminium phthalocyanine tetrasulfonate) (Brown et al., 2004;

Abrahamse and Hamblin, 2016). 5-aminolevulinic acid and its methylester are approved in USA and Europe for the 'topical' PDT of skin premalignant and malignant disorders (Wen et al., 2017); while mTHPC (Figure 2) in the formulation of ethanol and propylene glycol (temoporphin) is approved in Europe for the 'systemic' PDT of the palliative treatment of advanced head and neck cancer (de Visscher et al., 2013). In addition, tetrasulfonated aluminum phthalocyanine has been used in human patients in Russia for the PDT treatment of various types of cancer (Ormond and Freeman, 2013). Further, the phthalocyanine dye IR700DX (Figure 2) with its strong photostable property has been conjugated to receptor-binding antibodies, ligands, etc. as a third-generation photosensitiser for fluorescence-guided surgical resection of cancer (DeLong et al., 2016; Nagaya et al., 2017) as well as targeted PDT (or photoimmunotherapy, PIT) (Mitsunaga et al., 2011; Moore et al., 2016).

1.1.3. Photochemistry and photophysics

Photons have energy given as $E=hc/\lambda$, where h is the Planck constant, c is the vacuum velocity of light and λ is the wavelength. If the photon energy equals the energy gap between two molecular energy levels, absorption may occur, ie. the photon disappears and the molecule is excited to a higher energy level. Photons excite electrons to higher orbitals and also vibrational and rotational molecular energy levels (Turro, 1991). The excited condition is not stable and the molecule usually loses first its vibrational and rotational energy via internal conversion (IC) down to the first excited orbital level. From this excited orbital level further relaxation through IC may dispose of the remaining excitation energy, but fluorescence may also occur. Fluorescence is the emission of a photon with energy equal to the energy gap between the excited orbital state and the relaxed ground state. Because of IC the emitted light has normally lower energy with longer wavelengths than excitation light.

Figure 3. Modified Jablonski diagram of type II photochemical (photodynamic) reaction

(Thick dark horizontal lines represent electron orbital energy levels; thin light lines illustrate vibrational levels. Abbreviations: S_0 : ground state, S_1 : excited singlet state, S_2 : excited triplet state, T_1 : first excited triplet state; 3O_2 : triplet state, 1O_2 : singlet state).

On absorption of light of an appropriate wavelength a photosensitiser is excited from its ground state (S_0) to a higher excited triplet state (S_2), and relaxed down to the short-lived first excited singlet state (S_1) as illustrated in Figure 3 of a modified Jablonski diagram. The S_1 photosensitiser can further be deactivated by emitting fluorescence or releasing heat. Alternatively, it can undergo intersystem crossing to the long-lived first excited triplet state (T_1). The photosensitiser at the triplet state may then go to its ground state via phosphorescence.

The T_1 -state photosensitiser is crucial for PDT, as its lifetime is long enough to react with biomolecules. This interaction can take place through two different forms, usually referred to as type I and type II photochemical reactions. The Type I reaction is the direct interaction between the excited photosensitiser and macromolecules in cells, involving a transfer of an electron or a hydrogen atom. The Type II reaction, however, is the transfer of excited energy to oxygen molecules. As the ground state of oxygen is triplet, it may easily quench the triplet condition of a photosensitiser via photochemical energy transfer by triplet-triplet annihilation, leaving the oxygen in an excited singlet state to generate ROS including 1O_2 . The photosensitiser is then back to its ground state. 1O_2 is a powerful oxidant able to react with many kinds of

membranous biomolecules of amino acids, unsaturated fatty acids and cholesterol; and believed to be the key agent of cellular damage in PDT (Weishaup et al., 1976; Vrouenraets et al., 2003). Since it has a lifetime shorter than 40 ns with a radius of action less than 20 nm (Moan and Berg, 1991), only targets close to the location of the photosensitiser and oxygen molecule are damaged upon light exposure, leaving distant molecules and organelles unharmed.

Photobleaching of a photosensitiser occurs when the dye is unable to fluoresce because of photon-mediated cleavage of covalent bonds, or non-specific reactions between the dye and its surrounding molecules. The photobleaching of the dye with such photochemical change (damage) is measurable by its fluorescent signals.

In PDT excitation light with above 600 nm wavelengths is desirable, since they penetrate deeper into tissue (Anderson and Parrish, 1981; Wilson et al., 1985; Ash et al., 2017). This is due to the fact that molecules like melanin in the skin and hemoglobin in blood absorb less with longer wavelengths above 600 nm; while water absorption is significant only above 1200 nm. The choice of an optimal light wavelength for PDT must be balanced between photosensitisers that absorb light with a good tissue penetration depth and desirable production of $^1\text{O}_2$. Although porphyrins absorb up to 635 nm with a tissue penetration depth of 1-3 mm (Anderson and Parrish, 1981) and fulfil well both criteria, several other photosensitisers including chlorins and phthalocyanines absorb more strongly at longer wavelengths (650-800 nm) and are thus better than porphyrins in this respect (Ash et al., 2017).

The light sources used in topical PDT are traditional fluorescent, incandescent and vapour light as well as LEDs. Dye and diode lasers are applied for PDT of superficial tumours of internal hollow organs, as laser light can efficiently be coupled into an optic fibre to facilitate endoscopic application (Star WM, 1990; Brancalion and Moseley, 2002). Since light penetration into tissue is limited to only a few mm in depth, a so-called interstitial PDT is used by inserting one or more laser fibers via needles (or catheters) into thick or deeply-seated tumours to activate a photosensitiser (Shafirstein et al., 2017). As biological effect of PDT is related to the amount of light energy, a

proper use of light dosimetry is crucial for a successful PDT. The power of a light source is expressed in Watts (equal to Joules/sec) and the energy delivered is power $\times$ irradiation time (sec), expressed in Joules if the power is constant. As a surface is often irradiated in PDT, the power per unit of area, called irradiance, is expressed in W/m^2 . The light dose is equal to irradiance multiplied by exposure time ($J = \text{W/m}^2 \times \text{sec}$). The (energy) fluence rate (W/m^2) refers to the total radiant power incident from all directions onto a sphere divided by the cross-sectional area of that sphere. Multiplication of the fluence rate with the irradiation time generates the (energy) fluence (J/m^2). The fluence rate also describes the light intensity reaching a specific area of the target tissue. Although a suitable fluence or light dose to activate a photosensitiser is largely dependent upon the properties of the photosensitiser, the therapeutic PDT effect is also affected by other factors including photobleaching (that converts the photosensitiser to an inactive form) and oxygen consumption during light exposure. Generally, a high fluence leads to rapid photobleaching of a photosensitiser; while a high fluence rate increases a rate of oxygen consumption thus lowering the tissue oxygen tension. This is consistent with several studies reporting an increased PDT effect on tumour after light irradiation with a low fluence rate (Sitnik and Henderson, 1998; Middelburg et al., 2010).

1.1.4. Selective destruction of tumour by PDT

Effective PDT of cancer is based on preferential distribution of a photosensitiser in tumour tissue. This explains the early enthusiasm in the phenomenon of fluorescent photosensitisers in tumour (Policard and Leulier, 1924; Figge, et al., 1942) and current efforts on developing this modality. Dyes with various chemical properties localise in tumour with different degrees of selectivity. Generally, hydrophilic dyes are mainly distributed in the vascular stroma of tumour tissue; while hydrophobic drugs are preferentially localised in neoplastic cells (Kessel et al., 1987). Amphiphilic photosensitisers have biomembraneous binding of cells that is utilised for the principle of photochemical internalisation as described below (Berg et al., 1999). Moreover,

preincorporation of a photosensitiser into a 'delivery' molecule, such as antibodies, liposomes, etc., can enhance tumour selectivity of the photosensitiser and also affect the localisation patterns of the photosensitiser in the various compartments of tumour tissue (Allison et al., 1990; Ginevra et al., 1990; Kongshaug 1992; Castano et al., 2005; Abrahamse and Hamblin, 2016).

Since the diffusion of $^1\text{O}_2$ is only about 20 nm during its lifetime (Moan and Berg, 1991), intracellular and intratumoural localisation patterns of a photosensitiser are related to the targeting sites of its photodynamic action. PDT efficacy may therefore be enhanced by a photosensitiser with high and preferential distribution at PDT-sensitive sites of neoplastic tissue. Generally, a short drug-light interval (DLI) with a relative high dye concentration at the vascular structures predominantly leads to vascular damage in the tumours (Strauss et al., 1997). A longer DLI potentially destroys more tumour cells, as with time more photosensitiser may be distributed to the tumour cells.

1.1.5. Temoporfin-based PDT of Head and Neck cancer

The concept of PDT is attractive, as the combination of a tumour-localising photosensitiser with selective light delivery should provide a selective treatment for head and neck cancer with good cosmetic effect and low morbidity (Marchal et al., 2015). During the past >4 decades PDT with various photosensitisers has confirmed its usefulness in the treatments of patients with Head and Neck superficial tumours including those recurrent ones after previous surgery and/or radiation therapy (Chau et al., 2017; Civantos et al., 2018; Meulemans et al., 2019).

Effective PDT with HpD or porfimer sodium was shown in 1990s in the treatment of head and neck cancers (Feyh, 1996), but prolonged skin photosensitivity with limited treatment depth of tumour (Biel, 2010; Ikeda et al., 2013) led to look for second-generation photosensitisers with favourable properties of photochemistry, photophysics and photobiology (de Visscher et al., 2013). PDT with temoporfin has been reported to obtain complete response rates of 96% and 86% with good

functional and cosmetic outcome in the treatment of squamous cell carcinoma of the lip, oral and pharynx after a follow-up of 12 months and 37 months, respectively (Kubler et al., 2001; Copper et al., 2003). Temoporfin-PDT has also been used as an adjuvant purpose after salvage surgery with macroscopic and microscopic tumour residues in the margin areas in the patients with unacceptable risk for surgical resection or re-irradiation (Caesar et al., 2015; van Doeveren et al., 2018). Generally, the efficacy of temoporfin-based PDT is comparable to that of surgery for small tumours (up to 10 mm in thickness) (Karakullukcu et al., 2013; Cerrati et al., 2015; Meulemans et al., 2019). For larger lesions surgery is more effective, but with the potential side effects of severe morbidities. Interstitial irradiation of temoporfin with its strong absorption of far-red wavelengths can enhance treatment depth, so that it may make it possible to treat a larger tumour (Hopper, 2000; Tan et al., 2010; Civantos et al., 2018). However, the major challenge of temoporfin-PDT is the phototoxicity of normal tissues. mTHPC encapsulated into conventional liposomes as Foslip or into pegylated liposomes as Fospeg has been designed to improve the selective tumour distribution properties with possibly increased PDT efficacy (Reshetov, 2013; Gaio et al., 2016).

Although second-generation photosensitisers have significantly improved PDT efficacy with less systemic toxicity, the selectivity and specificity of PDT with the second-generation photosensitisers are still inadequate. New attempts have been made to further enhance the selectivity, specificity and efficacy (Selbo et al., 2000b; 2010) of PDT with targeted PDT and photochemical internalisation.

1.2. Targeted PDT

As described in the Section of 1.1.4, the conjugation of a photosensitiser with a targeting molecule that has an affinity for tumour can improve the selective tumour distribution of the photosensitiser. Photosensitisers used in targeted PDT are usually

covalently attached to molecular carriers including monoclonal antibodies (Sato et al., 2015), nanobodies (Hernández et al., 2020), peptides (You et al., 2015), carbohydrates (Park et al., 2011), somatostatin (Kascakova et al., 2014) and many others. The antibody-based targeted PDT, also called photoimmunotherapy (PIT) that conjugates a photosensitiser to a monoclonal antibody is within the scope of targeted PDT (Kobayashi and Choyke, 2016, 2019). Such photosensitiser-antibody conjugate is often referred to as a third-generation photosensitiser with improved selectivity and specificity of PDT. For example, epidermal growth factor receptor (EGFR) has been found to be over-expressed in several types of cancer including head/neck squamous cell carcinoma. Cetuximab is a chimeric (mouse/human) monoclonal antibody against EGFR. It was officially approved by EMA in 2004 and FDA in 2006 for the treatment of patients with squamous cell cancer of the head and neck in combination with radiation therapy for locally advanced disease (Concu and Cordeiro, 2018). However, cetuximab has considerable limitations with its toxicity and resistance development (Roe et al., 2006; Wheeler et al., 2008; Bardelli et al., 2010). Alternative treatment strategies to maximize the therapeutic effectiveness of EGFR as a molecular target are needed. Phthalocyanines, for example, photosens, a family of potent photosensitisers with their favourable properties of chemical stability, high fluorescence quantum yield and red-shifted light absorption for optimal tissue penetration, are already used for PDT of cancer patients in Russia (Trushina et al., 2008; Shiryayev et al., 2019). A phthalocyanine, IRDye-700DX (IR700DX) is commercially available and targeted PDT with the conjugation of EGFR antibodies and IR700DX, as shown in Figure 4, has attracted much attention during the past few years, as it can serve as both a diagnostic and a therapeutic agent.

Figure 4. Principle of Targeted PDT (Photoimmunotherapy)

(Adapted from <https://www.cancer.gov/news-events/cancer-currents-blog/2016/photoimmunotherapy-cancer>)

Based on a large number of preclinical studies with promising results (Mitsunaga et al., 2011; Moore et al., 2016), clinical trials were recently initiated by the Rakuten Aspyrian, Inc. (<https://rakutenaspyrian.com/our-pipeline/>) (Gillenwater et al., 2018) in patients with recurrent head and neck cancer.

1.3. Photochemical internalisation

Photochemical internalisation (PCI) is a new modality that uses the principle of PDT to induce direct and indirect tumour cell death with improved selectivity and specificity. In addition, this innovative modality is considered as a selective and efficient drug delivery system able to cytosolically release biologically active anticancer molecules that are entrapped in the endosomal and lysosomal vesicles of neoplastic cells (Berg et al., 1999; Høgset et al., 2004; Dietze et al., 2006; Selbo et al., 2010; Norum et al., 2017; Šošić et al., 2020; Jerjes et al., 2020).

In cell biology endocytosis is one of the major cellular uptake pathways that takes up substances unable to pass through cytoplasmic membrane. The endocytic process involves internalisation of extracellular substances through invagination of the cytoplasmic membrane, forming an intracellular bud, resulting in an endocytic vesicle containing the substances (Mukherjee et al., 1997; Berg and Moan, 1997). A membrane impermeable therapeutic molecule following the endocytic process has to escape from the endocytic vesicle before it reaches its intracellular targets, otherwise it is degraded in the vesicle.

Hydrophilic photosensitisers cannot easily pass through the hydrophobic cell membrane and are usually taken up by pinocytosis; while hydrophobic dyes may enter a cell by passive means. Amphiphilic photosensitisers with both hydrophilic and hydrophobic properties can bind to the cell membrane via the hydrophobic chemical groups, but not easily pass through the membrane due to the hydrophilic chemical groups. As a results, such dyes are localised in the membraneous structures of cytoplasm and a vesicle before and during an endocytosis process; respectively. In addition, a photosensitiser can be endocytosed into a cell via a receptor-mediated pathway when it is conjugated to a targeting moiety such as antibody. Upon light illumination these dyes produce ROS and $^1\text{O}_2$ to damage the membraneous structures of the endocytic vesicle.

Figure 5. Principle of Photochemical Internalisation

(Berg K, et al. Cancer Res 1999; 59:1180-1183)

Figure 5 schematically illustrates the endocytosis and PCI process (<http://pcibiotech.no/>). A cytoplasmic membrane-binding amphiphilic photosensitiser (S) and a membrane-trapped drug (D) (biologically active molecules) are endocytosed by a cell to be localised in an endocytically endosomal vesicle. The rupture of the vesicle membrane by activating the photosensitiser with light releases the drug into the cytosol and nucleus to exert its biological effects on its targets. Without such photodynamic action the trapped drug can be recycled and degraded in lysosomes with no therapeutic effects. It should be mentioned that hydrophilic dyes are also largely localised in endosomal and lysosomal vesicles via the pinocytotic process, but they are much less efficient or unable to induce a PCI effect due to their location in the matrix instead of membrane to photochemically inactivate the biologically active molecules (Prasmickaite et al., 2001).

Interestingly, an effective PCI effect can also be induced by a photochemical treatment prior to the delivery of biologically active molecules (Prasmickaite et al., 2002). The subcellular mechanism of this phenomenon is not known, but it has been assumed that the initially photochemically damaged vesicles may fuse and rupture newly formed vesicles to release active molecules (Prasmickaite et al., 2002). A PCI treatment with this protocol may have the advantage of abolishing the possible photochemical inactivation of the entrapped therapeutic molecules.

Biologically active molecules used for PCI in the thesis were bleomycin and gelonin. Bleomycin with the molecular weight of 1.4 kDa is a widely used chemotherapeutic drug for several types of cancer including head and neck cancer, Hodgkin's lymphoma, testicular tumours, etc. (Norum et al., 2009). However, its anti-cancer effect can be reduced due to the fact that it is taken up by tumour cells via endocytosis (Pron et al., 1999) with its possible degradation before reaching its targets (Selbo et al., 2000a). PCI has been proven to have the effect of bleomycin on both tumour-bearing animals *in vivo* (Norum et al., 2009; 2017; Sellevold et al., 2017) and clinical patients with head and neck cancer (Sultan et al., 2016). Gelonin with the molecular weight of 30 kDa is a protein toxin and belongs to the family of ribosome-inactivating protein toxins that

have widely been studied for the potential of cancer therapy (Thrush et al., 1996). Due to its lack of carbohydrate-binding domain gelonin is unable to pass through cytoplasm membrane. As a result, it is taken up by cells via endocytosis pathway and PCI is capable of releasing gelonin from endocytic vesicles to enhance its cytotoxic effect (Selbo et al., 2000a; 2001).

Cetuximab is the monoclonal antibody directed against the EGFR that is expressed on the cytoplasm membrane of many types of cancer cells. The photosensitiser IR700DX in the conjugate of cetuximab-IR700DX should be located in or close to the plasma membrane. Endocytosis of the conjugate and gelonin followed by light exposure may damage the membrane of endosomal and lysosomal vesicles to release the entrapped gelonin. Since cetuximab provides highly effective and specific EGFR targeting in the membraneous structures, the conjugate can thus facilitate a specific delivery of IR700DX to the membrane. This may lead to an improvement of efficacy and specificity of PCI effect. To avoid a direct destructive effect of targeted PDT on tumour cells a significant low targeted PDT dose with a low concentration of the conjugate and light dose is needed. We have hypothesized that a non-cytotoxic dose of targeted PDT may destroy the membrane of endo/lysosomal vesicles to release those endolysosomally entrapped chemotherapeutic drugs for their exerting anticancer effects. Such induction of PCI by the targeted PDT may eventually create a minimally invasive and highly efficient modality with a triple-model selectivity of a specific binding of IR700DX to tumour cells via cetuximab, release of biologically active molecules (eg. gelonin) and site-directed light activation.

1.4. Aims of the thesis

In order to improve the selectivity and specificity of PDT the aims of this thesis were

- (1). To investigate the effect of targeted PDT with cetuximab-IR700DX conjugate on human head and neck tumour cell lines *in vitro*.
- (2). To study the effect of targeted PDT with cetuximab-IR700DX conjugate on human head and neck tumour in a mouse skin-fold window-chamber model *in vivo*.
- (3). To optically monitor the effect of fluence rate on the efficacy of targeted PDT with cetuximab-IR700DX conjugate on a xenografted human head and neck tumour model in the flank of mice *in vivo*.
- (4). To investigate bleomycin-based PCI with mTHPC in human head and neck tumour cell lines *in vitro*.
- (5). To induce a gelonin-based PCI effect with targeted PDT using cetuximab-IR700DX conjugate on human squamous cell carcinoma cells *in vitro*.

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Chapter 2

Epidermal growth factor receptor (EGFR) density may not be the only determinant for the efficacy of EGFR-targeted photodynamic therapy in human head and neck cancer cell lines.

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ABSTRACT

Objective: The aim of this study was to investigate the effects of targeted PDT (ie. Photoimmunotherapy) *in vitro* on cell lines with various expression levels of epidermal growth factor receptor (EGFR) using an anti-EGFR targeted conjugate composed of cetuximab and IR700DX, phthalocyanine dye.

Materials and methods: Relative EGFR density and cell binding assay were conducted in three human head & neck cancer cell lines (scc-U2, scc-U8 and OSC-19) and one reference cell line A431. After incubation with the conjugate for 1 or 24 hours, cellular uptake and localisation were investigated by confocal laser scanning microscopy and quantified by image analysis. Cell survival was determined using the MTS assay and alamarBlue assay after the targeted PDT with a 690 nm laser to a dose of 7 J.cm^{-2} (at 5 mW.cm^{-2}). The mode of cell death was examined with flow cytometry using apoptosis/necrosis staining by Annexin V/propidium iodide, together with immunoblots of anti-apoptotic Bcl-2 family proteins Bcl-2 and Bcl-xL.

Results: A431 cells had the highest EGFR density followed by OSC-19, and then scc-U2 and scc-U8. The conjugates were localised both on the surface and in the cytosol of the cells after 1-h and 24-h incubation. After 24-h incubation the granular pattern was more pronounced and in a similar pattern of a lysosomal probe, suggesting that the uptake of conjugates by cells was via receptor-mediated endocytosis. The results obtained from the quantitative imaging analysis correlate with the level of EGFR expression. Targeted PDT killed scc-U8 and A431 cells efficiently; while scc-U2 and OSC-19 were less sensitive to this treatment, despite having similar EGFR density, uptake and localisation pattern. Scc-U2 cells showed less apoptotic cell death than in A431 after 24-h targeted PDT. Immunoblots showed significantly higher expression of anti-apoptotic Bcl-2 and Bcl-xL proteins in scc-U2 cell lines compared to scc-U8.

Conclusion: Our study suggests that the effectiveness of EGFR targeted PDT is not only dependent upon EGFR density. Intrinsic biological properties of tumours cell lines also play a role in determining the efficacy of targeted PDT. We have shown that in scc-U2 cells this difference may be caused by differences in the apoptotic pathway.

Introduction

Photodynamic therapy (PDT) of cancer is based on the local or systemic administration of an inactive photosensitiser followed by activation with visible light of a specific wavelength. The light-activated photosensitiser transfers the energy to molecular oxygen to produce highly cytotoxic reactive oxygen species (ROS), notably singlet oxygen, resulting in photodamage to tumour cells/tissues (1-3). A photosensitiser is, however, generally not selectively taken up by tumour cells (4), this can lead to significant damage to surrounding normal cells/tissues.

To overcome this limitation, targeted PDT first reported by Mew et al., in 1983 (5), involves the conjugation of a photosensitiser to an antibody that specifically targets a protein on the surface of tumour cells. Such conjugates have the potential for effective photosensitiser delivery to cause selective destruction of individual cancer cells after light irradiation. Cetuximab-IR700DX is a conjugate of a phthalocyanine dye and an antibody against human epidermal growth factor receptor (EGFR). IR700DX has a strong absorption in the red (690 nm) wavelength where the light penetration into tissue is close to optimal. However, as a single agent IR700DX is very little photodynamically active because it is highly water soluble and does not localise close to essential organelles (6). EGFR is highly expressed in many types of tumours including head and neck squamous cell carcinoma. This conjugate is currently under investigation for clinical use (7).

Targeted PDT efficacy using a different conjugate has previously been demonstrated in the human squamous cell carcinoma cell line A431 and MDAMB468-luc adenocarcinoma cell line *in vitro* (6). Both cell lines strongly over-express EGFR. Furthermore, destruction of tumour xenografts in mice has also been demonstrated after targeted PDT (7, 8). EGFR targeted PDT using nanobody conjugates has shown a positive relationship between EGFR expression and efficacy in EGFR over-expressing cell lines of A431 and UM-SCC-14C, whereas the Hela tumour cells with a low EGFR expression were spared (9).

The efficacy of cell death in PDT is mainly determined by the subcellular sites where a photosensitizer is located and therefore where singlet oxygen is generated. For targeted photosensitizers that are initially localised at the cell membrane this causes mainly necrosis by disruption of the cell membrane (9). In addition, intracellular located photosensitizer can induce various pathways of apoptosis (programmed cell death), such as upregulation of p53 or cytochrome C. Apoptosis is a form of cell death that is morphologically and biochemically distinct from necrosis (10, 11), and can be categorised into early and late stages. Early apoptosis is characterised by an intact plasma membrane with the exposure of phosphatidylserine on the cell surface; while late apoptosis is characterized by a permeable plasma membrane (12). Stress on various organelles of cells including the mitochondria, endoplasmic reticulum (ER), endo/lysosomes and nucleus can initiate specific apoptotic pathways (13-15). During apoptosis, Bcl-2 family proteins regulate apoptotic process including anti-apoptotic members such as Bcl-2 and Bcl-xL and pro-apoptotic members such as BAX and BAD (16, 17). Previous reports have shown that both Bcl-2 and Bcl-xL proteins induced the resistance of tumor cells to PDT (18).

It has previously been shown that the effectiveness of targeted EGFR therapies depends upon the cellular density of EGFR (9, 19). The aim of this study was to investigate targeted PDT responses using cetuximab-IR700DX in the human head and neck squamous cell carcinoma cell lines, scc-U8, scc-U2 and OSC-19. We studied the EGFR expression, microscopic localisation, cell survival and mode of cell death after targeted PDT using 1 or 24 hrs of incubation. The 1-hr short drug light interval was chosen to aim for membrane localised conjugate/EGFR receptor targeted responses; whereas the 24 hours incubation was to mimic a clinical scenario of using the conjugate.

Materials and methods

Cetuximab-IR700DX conjugate

IR700DX is a phthalocyanine-type photosensitiser; while cetuximab is a clinically used antibody against human cancers with the EGFR expression.

Cell lines

Three human head and neck (oral cavity) squamous cell carcinoma cell lines, scc-U8, scc-U2 (University of Michigan) and OSC-19 (University of Leiden); and one human cervical squamous cell carcinoma cell line, A431 (University of Oslo) as a reference, were used in this study. The A431, scc-U8 and scc-U2 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Invitrogen, UK) supplemented with 10% foetal calf serum (FCS), 100 U mL^{-1} penicillin, 100 $\mu\text{g mL}^{-1}$ streptomycin and 2 mM glutamine (PAA, Germany) at 37°C in a humidified 5% CO₂ atmosphere. The OSC-19 cell line was cultured in DMEM (Invitrogen, Carlsbad, CA) containing 4.5 g D-glucose L^{-1} , 110 mg L^{-1} sodium pyruvate L^{-1} , 580 mg L-glutamine L^{-1} supplemented with 10% FCS (Lonza, Basel, Switzerland), 400 IU mL^{-1} penicillin, 100 $\mu\text{g mL}^{-1}$ streptomycin (Invitrogen), 1×Minimal Essential Medium (MEM) non-essential amino acids solution and 1×MEM vitamin solution at 37°C in a humidified 5% CO₂ atmosphere. The passages of 10 to 40 of the cell lines were used in this study.

Relative EGFR expression and conjugate binding

Since IR700DX is a fluorescent dye, it is possible to directly detect IR700DX fluorescent signals of the conjugates to study cellular EGFR expression. Cells of each cell line were incubated with cetuximab-IR700DX conjugates at the concentration of 40 $\mu\text{g mL}^{-1}$ (approx. 263.8 nmol mL^{-1}) for 30 min at 4°C before being prepared for flow cytometry. This concentration was calculated to be sufficient to saturate all the EGFR receptors on the A431 cell line, based on the data from a previous study (20). The mean fluorescence intensity was used to measure the relative human EGFR

expression in various cell lines. Cells without the antibody were measured as background signal. In addition, a cell binding assay was conducted. For each cell line 8000 cells were seeded in a 96-well plate (Nunc, Roskilde, Denmark). After 24-h incubation for attachment, the plates were kept at 4 °C and cells were washed with cold DMEM binding medium containing 1% BSA and 25 mM HEPES without phenol red at pH 7.4. After incubation of cells with cetuximab-IR700DX conjugates (100 nM-0.39 nM, 1:2 serial dilution) for 2-h at 4 °C, unbound conjugate was washed away 3 times with binding buffer. The amount of bound conjugate was detected with Odyssey Infrared scanner using the 700 nm channel. Fluorescence intensities were plotted (in triplicate $\pm$ SD) versus the concentrations using the GraphPad Prism 7 software (GraphPad Software, San Diego, CA).

Cell survival

Cell proliferation was assessed with a standard MTS kit (CellTiter 96® AQueous One Solution Reagent, Promega Corp., Madison, WI) according to the manufacturer's recommendations using a 96-well plate reader (Molecular Devices, Sunnyvale, CA). It is a colorimetric method based on the cellular conversion of a tetrazolium compound into a formazan product, which can be detected by the 492 nm absorbance. Such absorbance measurements were not influenced by IR700DX. Cell viability was assessed with a standard alamarBlue Assay (alamarBlue™ Cell Viability Reagent) according to the manufacturer's protocol using a 96-well plate reader (Molecular Devices, Sunnyvale, CA). It is a fluorescent method based on a non-fluorescent molecule resazurin converting to a fluorescent molecule resorufin by reduction reactions in the cytoplasm of metabolically active cells. The resorufin fluorescence which can be measured at ex/em: 560/590 nm. Such fluorescence measurements were not influenced by IR700DX.

Dark cytotoxicity of cetuximab and cetuximab-IR700DX conjugates

A total of 15000 cells of each cell line were seeded per well in a 96-well plate. After attachment for 24-h, the cells were incubated with various concentrations (up to 2000 $\mu\text{g mL}^{-1}$) cetuximab and (from 10 $\mu\text{g mL}^{-1}$ to 2000 $\mu\text{g mL}^{-1}$) of cetuximab-IR700DX conjugates in the dark for 24-h. The cell proliferation was then evaluated by the MTS assay.

Subcellular localisation of cetuximab-IR700DX conjugates and quantitative image analysis

A total of 250000 cells of each cell line were seeded on the 0.25 mg mL^{-1} poly-lysine coated 24-mm cover slides and incubated in 10% FCS DMEM medium containing 40 $\mu\text{g mL}^{-1}$ of cetuximab-IR700DX conjugates for 1- or 24-h incubation. The cells were washed with medium once before being placed in a temperature-controlled 37°C mini incubator (Peecon, Germany). The subcellular localisation of fluorescent conjugates was imaged using the dual-channel Zeiss LSM 510 – Axiovert 200M (Carl Zeiss, Thornwood, NY) confocal microscopy (40 $\times$ /1.3NA Plan-neofluar oil objective) with an excitation wavelength of 633 nm and a 650 nm long-pass emission filter. The cells were optically sectioned and 10 groups of cells at two different optical sections (z-stack) were randomly chosen in each cell line with various incubation times to quantify the fluorescent intensities of conjugates at 3 various locations of the cytoplasm membrane, endo/lysosomes and cytosol of cells by the Fiji ImageJ software package.

Targeted PDT treatment protocols

Each cell line was seeded with 15000 cells in each well of a 96-well plate. After 24-h incubation for attachment, the cells were incubated in the dark with various concentrations of cetuximab-IR700DX conjugates from 10 to 100 $\mu\text{g mL}^{-1}$ for 1-h or 24-h at 37 °C. The cells were then washed with medium once and a volume of 100 μL medium was added in each well before being illuminated of a 690 nm laser (Modulight ML7700, Finland) using a shaker with a speed of 700 RPM to introduce a constant

amount of oxygen into the medium during irradiation. We measured the fluence rate to be constant across all of the illuminated wells. The light dose of 7 J.cm^{-2} at a fluence rate of 5 mW.cm^{-2} (22 minutes) were used in the experiments because our previous study showed that these treatment parameters were effective at killing A431 cells and did not induce any hyperthermal effects (21). Twenty-four hours after targeted PDT cell survival was determined with both cell proliferation MTS assay and cell viability AlamarBlue assay. Since previous reports from us and others have shown very little photodynamic effect on cells using IR700DX alone, we chosen not to include it as a control (6, 9).

Mode of cell death

The mode of cell death after targeted PDT was studied in the A431 and scc-U2 cell lines by flow cytometry using the commercial kit (VPS Diagnostics, Hoeven, the Netherlands) of Annexin V/propidium iodide to detect apoptosis and necrosis. The samples were prepared according to the manufacturer's instruction. Samples were analysed using a BD FACS Jazz (BD, Erembodigem, Belgium) and FlowJo V10.0.8 (FlowJo LLC). Cells positive for Annexin V only are considered in early apoptosis, while cells positive for PI only are considered necrotic. Cells that are positive for both Annexin V and PI are considered in late apoptosis or secondary necrosis.

Western blots

Protein extracts (40 $\mu\text{g/lane}$) of scc-U2 and scc-U8 from whole-cell samples were separated by electrophoresis on 10% SDS-PAGE and then electro-transferred to nitrocellulose membranes. After blocking in 5% milk in TBS buffer (0.1% Tween) for 2-h, the membranes were incubated overnight at 4°C with rabbit polyclonal IgG antibodies against Bcl-2, Bcl-xL or beta actin in TBS buffer (all from Santa Cruz, Heidelberg, Germany). After washing the membranes were incubated with HRP-conjugated anti-rabbit IgG. ECL detection reagent was used to visualize the reactive

bands. Finally, the western blot signals were quantified using the ChemiDoc XRS digital imaging system.

Statistical Analysis

The results were analysed (SigmaPlot® 12; Sysat software, Inc.) using the unpaired Student t-test for data with normal distribution; otherwise, the Wilcoxon signed rank test was used. The differences were considered statistically significant with P value <0.05.

Results

EGFR expression and conjugate binding in various cell lines

Figure 1a shows various amounts of EGFR expression determined using flow cytometry in scc-U8, scc-U2 and OSC-19 cell lines after incubation with the conjugates at 40 µgml⁻¹ for 30 min at 4°C. The EGFR expression in different head and neck cell lines is presented relative to the EGFR expression in the A431 cells (set to 100%): 36% in scc-U8, 38% in scc-U2 and 54% in OSC-19; respectively. The scc-U8 and scc-U2 had a similar level of EGFR expression; while OSC-19 expressed a slightly higher EGFR level. All three head and neck tumour cell lines had a much lower EGFR expression than the A431 cell line. Figure 1b shows the conjugate binding to A431, scc-U8, scc-U2 and OSC-19 cell lines. The fluorescence intensity detected at saturating levels (i.e. where a plateau is observed) is in agreement with the EGFR expression levels determined using flow cytometry. The binding affinity (K_d) was similar for the three head and neck cell lines (Table 1).

Figure 1. EGFR expression in the scc-U8, scc-U2, and OSC-19 cell lines, measured with flow cytometry (The data are presented as the percentages of EGFR expression of the A431 cells where the error bars are calculated from the full width at half maximum [FWHM] of the fluorescence signal) **(a)** and cell binding assay **(b)**.

Table 1. Binding affinity (Kd)

Cell line	Kd (nM) $\pm$ SD
A431	2.4 $\pm$ 0.09
OSC-19	0.4 $\pm$ 0.09
Scc-U2	0.2 $\pm$ 0.04
Scc-U8	0.3 $\pm$ 0.06

Intracellular localisation of cetuximab-IR700DX conjugates

After 1-h incubation the fluorescence of cetuximab-IR700DX was observed on the cell membranes as well as in a granular pattern within the cells, particularly in A431 cells. After 24-h incubation, the granular pattern of the conjugates became more pronounced in all cell lines (Fig. 2a). Co-incubation with an endo/lysosomal probe showed a similar pattern as seen in yellow overlap (Fig. 2b), suggesting that the conjugates were taken up through the endo/lysosomal pathway.

a

Cell lines

1-hour

24-hour

A431

Scc-U8

Scc-U2

OSC-19

b

Figure 2. Subcellular localization patterns of the conjugates in the A431, scc-U8, and scc-U2 and OSC-19 cell lines (a). The localization pattern of LysoTracker (green fluorescence), an endo/lysosomal probe, was also studied after its co-incubation with the conjugates (red fluorescence) in the cell lines. (b) is a representative of this study in a A431 cell. The fluorescence images were made by the confocal laser scanning microscopy.

Quantitative image analysis

After 1-h incubation conjugates were localised on the cell membrane and in the endo/lysosomes with significantly higher fluorescence signals in both locations in A431 cells than other cell lines ($p < 0.05$). Low fluorescence signals of the conjugates were seen in the cytosol in all cell lines (Fig. 3). After 24-h incubation, there were low fluorescence signals of the conjugates on the cell membrane and in the cytosol in all cell lines similar to those after 1-h incubation. However, the conjugates were taken up into endo/lysosomes significantly more in all the cell lines studied as compares to those after 1-h incubation ($p < 0.05$) (Fig. 3), although no statistical differences were found among the cell lines ($p > 0.05$). These results are in agreement with the EGFR expression and conjugate binding assay in the various cell lines.

Figure 3. Quantitative image analyses of various subcellular locations of the A431, scc-U8, scc-U2 and OSC-19 cell lines incubated with the conjugates (40 mgml^{-1}) for 1- or 24-hour.

Dark cytotoxicity of cetuximab and cetuximab-IR700DX conjugates

No cytotoxicity was found in the MTS assay after incubating cetuximab with A431, scc-U8, scc-U2 and OSC-19 cell lines at various concentrations up to $2000 \text{ }\mu\text{gml}^{-1}$ for 24 hours (data not shown). In addition, no dark cytotoxicity was found with the MTS assay after incubating the cetuximab-IR700DX conjugates with the same cell lines at various concentrations up to $2000 \text{ }\mu\text{gml}^{-1}$ for 24 hours (data not shown).

Targeted PDT treatment with the conjugates

Figure 4a shows the effect of targeted PDT using different concentrations of the conjugates up to $100 \text{ }\mu\text{gml}^{-1}$ for 1- or 24-h incubation determined by the MTS cell proliferation assay. A dramatic decrease in cell proliferation was seen at the concentration of only $10 \text{ }\mu\text{gml}^{-1}$ conjugates for 1-h incubation in the A431 and scc-U8 cell lines. No killing effects on the scc-U2 and OSC-19 cell lines were seen after the targeted PDT treatment even at the $100 \text{ }\mu\text{gml}^{-1}$ conjugates ($p < 0.05$ for all respective concentrations between A431 or scc-U8 and scc-U2 or OSC-19 cell lines). Scc-U8 showed a decrease in cell proliferation with increased concentrations of conjugates after 24-h incubation compared to 1-h incubation. A431, scc-U2 and OSC-19 had similar targeted PDT results after a 24-h incubation of the conjugates compared to the 1-h incubation. Considering the clinical importance of the 24-h drug light interval, the targeted PDT effect was evaluated using the alamarBlue assay for a limited subset of

treatment parameters. Figure 4b shows ca. 45% more targeted PDT effect in scc-U2 and OSC-19 cell lines determined with the alamarBlue compared to MTS assay. Both MTS and alamarBlue assays show significantly less response to targeted PDT in the scc-U2 and OSC-19 cell lines compared to the scc-U8 and A431 cell lines.

a

b

Figure 4. Cell survivals of A431, scc-U8, scc-U2, and OSC-19 cell lines at 24 hours after 1- or 24-hour incubation with the conjugates of cetuximab and IR700DX at various concentrations (as indicated) before being irradiated with a 690 nm laser (5 mW.cm^{-2} , 7 J.cm^{-2}). The cell proliferation and viability were measured by the MTS assay (a) and alamarBlue assay (b, 24-hour incubation with the conjugates). Each data point represents the mean of at least three separate experiments with at least five individual samples per experiment. The bars are SD.

Mode of cell death after targeted PDT with cetuximab-IR700DX

The A431 and scc-U2 cell lines were selected to study the mode of cell death because the A431 cell line was very sensitive to targeted PDT due to its high EGFR expression; whereas the scc-U2 cell line was less sensitive to the same treatment. Figure 5 shows that for the cells that could be interrogated using flow-cytometry, approximately 13% were in early apoptosis and 37% were in late apoptosis 4 hours after the targeted PDT with 1-hour incubation of the conjugates in the A431 cells. At 48 hours after the treatment 23% were in early apoptosis and 67% were in late apoptosis, indicating that targeted PDT led largely to late apoptosis of A431 cells. Similar results were found in the targeted PDT treatment with 24-hour incubation of the conjugates in the cells. However, in the case of scc-U2 cell line, only approximately 1% were in early apoptosis and 2% were in late apoptosis 4 hours and 48 hours after the targeted PDT with 1-hour incubation. Targeted PDT treatment with 24-hour incubation of the conjugates showed 1% of cells in early apoptosis and 4% of cells in late apoptosis at 4 hours and 5% in early apoptosis and 17% in late apoptosis at 48 hours post treatment, respectively; clearly showing the cells less sensitive to the targeted PDT treatment.

Figure 5. Apoptosis/necrosis of the A431 and scc-U2 at 4-, 24-, and 48-hour after the targeted PDT with 1- or 24-hour incubation of the conjugates. The apoptosis/necrosis was measured with flow cytometry using Annexin V/propidium iodide staining.

Immunoblots of anti-apoptotic proteins

The cell lines scc-U8 and scc-U2 were selected to study the difference in expression of anti-apoptotic proteins because they had a similar EGFR expression, but the scc-U2 was less sensitive to targeted PDT effect than scc-U8. Figure 6 (a) shows the expression of proteins Bcl-2 and Bcl-xL in the scc-U8 and scc-U2 cell lines; while (b) presents the Bcl-2/beta actin ratio and Bcl-xL/beta actin ratio in the 2 cell lines. Although there are different degrees of expression of Bcl-2 and Bcl-xL in the cell lines, scc-U2 cells have significantly higher ratios of both Bcl-2/beta actin and Bcl-xL/beta actin than scc-U8 cell lines ($p < 0.05$), suggesting that scc-U2 cells are more resistant to apoptotic death than scc-U8, since both Bcl-2 and Bcl-xL are anti-apoptotic proteins.

Figure 6. (a) Expression of Bcl-2 and Bcl-xL in the scc-U8 and scc-U2 cell lines examined by immunoblots and the data are representative of three separate experiments. (b) Bcl-2/beta actin ratio and Bcl-xL/beta actin ratio in the two cell lines.

Discussion

The efficacy of targeted cetuximab-IR700DX utilizing cell surface receptor, EGFR, is thought to be dependent on receptor density and to be mediated through a necrotic cell death pathway caused by the action of reactive oxygen species on the outer cell membrane (6, 10, 22). Cells that express high levels of EGFR should be more sensitive to targeted PDT than the cells with low levels of EGFR expression. The present study shows that this is an over-simplification. Targeted PDT efficacy is likely not only related to the receptor density, but also to a more complicated mechanism of cell death than that associated with necrosis at the cell membrane. Although there is no previous studies showing this in targeted PDT, there are numerous mechanisms involved in the

resistance to regular PDT treatments, ranging from the morphological alterations of the cells to different levels of various protein expressions in individual cell lines (18).

The cetuximab-IR700DX conjugate was localized on the cell membrane and in endo/lysosomes in A431 cells and targeted PDT resulted in effective cell kill. However, scc-U8 and scc-U2 have a similar level of EGFR expression, with subsequent localisation of cetuximab-IR700DX on the cell membrane, but responded significantly differently. Scc-U8 were easily killed, but the scc-U2 cells were less sensitive to the treatment with the light dose and fluence rate as applied in this study. In addition, OSC-19 cells expressed a slightly higher level of EGFR than scc-U8 and scc-U2 and showed also a limited response. It has been shown that different cell survival assays may show different responses, for example the MTT assay is known to be less sensitive than the alamar blue under certain treatment conditions (23). However, it is not immediately clear why we have found a 45% difference in cell survival between each assay. In each case the experimental conditions were identical using the same batch of conjugate and targeted PDT illumination protocol. Substantial decrease in cell survival of scc-U2 and OSC-19 only occurred at higher fluences and fluence rates (data not shown). However, the fluence rate was intentionally kept at the low 5 mW.cm^{-2} in this study to avoid exhaustion of oxygen or induction of a hyperthermal effect (which has been described to occur for other photosensitizers in a similar context) (24, 25).

Our results show that, in the case of OSC-19 and scc-U2 cell lines, there is no clear correlation between EGFR levels and targeted PDT-mediated cell death. This is not in agreement with the previous studies, for example, Heukers., et al. (9) who demonstrated that targeted PDT efficacy was correlated with the EGFR density of cells using EGFR targeted nanobodies. The reasons for this discrepancy are not known, but it might be explained by the use of different cell lines. It is also likely that nanobodies may interact differently with the cell membrane and cause a different balance of mechanisms of cell death compared to cetuximab.

It is generally understood that the main cell death mechanism is induced by primary necrosis caused by damage to the outer cell membrane. After light exposure, the scc-

U8 cells as well as A431 cells incubated with the conjugates were killed effectively. With flow cytometry using Annexin V/propidium iodide staining the mechanism of cell death was investigated, but it measured only fully intact cells and was therefore unable to detect small necrotic cell debris. Such exclusion of flow cytometric measurements may lead to underestimation of the fraction of cell death. Still, in the measurable fraction it is clear that cell death follows the apoptotic pathway (Fig. 5). The subcellular localisation plays an important role in the mechanism of cell death. Generally, hydrophobic sensitisers localise at the membranous structures including plasma membrane, endoplasmic reticulum, mitochondria, nuclear membrane; while hydrophilic dyes mainly at the endo/lysosomes. These organelles are the initial targets of a photodynamic process, since the cytotoxic singlet oxygen produced by light-activated photosensitiser can only diffuse 10 to 20 nm during its very short lifetime (21). Such initial PDT targets can be highly specific to trigger various signalling pathways of apoptotic induction. In this study the conjugate was found to be localised at the plasma membrane as well as endo/lysosomes after 1-hour and more in the endo/lysosomes after 24-hour incubation (Figs. 2a & 3) in all cell lines studied, particularly in A431. Several studies have shown that initial photodamage to plasma membrane or lysosomes can induce a marked delay or inhibition of apoptosis (26-28). These effects may play a role in our study, since the conjugates initially localise mainly at the plasma membrane and later in the endo/lysosomes, and destruction of these two locations after light exposure is expected. Furthermore, a relocation of the photosensitiser during light irradiation has been hypothesized to cause photoinactivation of those enzymes required for the apoptotic process including the initiator caspases of 8 and 9 and effector caspases of 3, 6 and 7 (26). However, the cell membrane/lysosomal damage may induce apoptosis, but not through mitochondria-mediated apoptosis (29), a finding consistent with ours in the A431 cells (Fig. 5).

The Bcl-2 family of apoptosis proteins acts as a central decision in the apoptosis pathway. Cell lines that over-express Bcl-2 and Bcl-xL have been shown to be more resistant to PDT (29-32). Chinese Hamster Ovary (CHO) cells transfected with an

antiapoptotic protein gene have been shown to be more resistant to PDT (33). Furthermore, overexpression of Bcl-2 can inhibit the activation-associated conformational change of the proapoptotic protein BAX (30). Such results indicate that the extent of Bcl-2 expression may determine the sensitivity of tumour cells to apoptosis and to overall PDT-mediated cell killing (34). In the present study, a significantly higher expression of Bcl-2 and Bcl-xL was found in the scc-U2 cells than scc-U8 cells (Fig. 6). This may explain the lower level of response of the scc-U2 cells to targeted PDT treatment.

It is important to stress that further studies are necessary to determine if these cellular effects are important *in vivo*, first in pre-clinical models and subsequently in clinical trials. The potential protective effect of anti-apoptotic proteins in some cells in head and neck tumours may result in variable targeted PDT treatment responses.

In conclusion, although cellular expression and localisation patterns of EGFR are similar in the three head and neck cell lines studied, Scc-U8 cell line is very sensitive to targeted PDT; while scc-U2 and OSC-19 are not. The EGFR density does not seem to be the only determining factor for the different responses to targeted PDT. We have shown that the resistance of scc-U2 cells to targeted PDT might be related to a lower degree of apoptotic induction due to a high expression of anti-apoptotic proteins of Bcl-2 and Bcl-xL.

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Targeted photodynamic therapy of human head and neck squamous cell carcinoma with anti-epidermal growth factor receptor antibody cetuximab and photosensitiser IR700DX in the mouse skin-fold window chamber model

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ABSTRACT

Targeted photodynamic therapy (PDT) in head/neck cancer patients with a conjugate of the anti-epidermal growth factor receptor (EGFR) antibody, cetuximab and a phthalocyanine photosensitizer IR700DX is under way, but the exact mechanisms of action are still not fully understood. In this study, the EGFR-overexpressing human head/neck OSC-19-luc2-cGFP tumor with transfected GFP gene was used in a skin-fold window chamber model in BALB/c nude mice. The uptake and localization of the conjugate in the tumor and its surrounding normal tissues were studied by an intravital confocal laser scanning microscopy with image analyses. The tumor was also irradiated with 690 nm laser light 24 h after conjugate administration. The vascular and tumor responses were examined by morphological evaluation and immunohistochemistry (IHC). The amount of conjugate in the tumor peaked at 24–48 h after injection. Image analyses of colocalization correlation parameters demonstrated a high fraction of the conjugate IR700DX colocalized in the GFP-expressing tumor cells. PDT-treated tumors showed extensive necrotic/apoptotic destruction with little vascular damage, while IHC showed no HIF-1 α expression and decreased EGFR and Ki67 expression with activated caspase-3 overexpression, indicating a direct killing of tumor cells through both necrotic and apoptotic cell death.

Introduction

The worldwide incidence of head and neck cancers is estimated to be more than 550 000 each year with the mortality rate of about 300 000 (1, 2). The tumors mainly arise from the squamous cell linings with more than 90% squamous cell carcinoma (3). Because of the complexity of the head and neck region with its critical structures, the treatment options do not only depend on the type and stage, but also the anatomic location of the tumor. The conventional treatment includes surgery or radiotherapy for early-stage I/II cancer (4-6), while combinations of surgery, radiotherapy and chemotherapy for advanced stage III/IV cancer (7-9). However, both surgery and radiotherapy often cause severe damage to surrounding normal tissues with a loss of their functions (10, 11). Such morbidities have encouraged the field to search for new treatment alternatives for this disease.

The concept of photodynamic therapy (PDT) is attractive for cancer treatment (12-14) because the combination of a tumor-localizing photosensitizer with selective light delivery has the potential to provide a selective treatment for cancer with low morbidity (15). Effective PDT with the first generation photosensitizer such as hematoporphyrin derivative or porfimer sodium was shown in 1990s in the treatment of head and neck cancers (16), but prolonged skin photosensitivity with limited treatment depth of tumor (17, 18) led investigators to look for second-generation photosensitizers with favorable properties of photochemistry, photophysics and photobiology (19, 20). The European Medicines Agency (EMA)-approved PDT for palliative treatment of head and neck cancer with meta-tetra(hydroxyphenyl)chlorin (mTHPC, temoporfin) as a photosensitizer has shown to obtain complete response rates comparable to surgical treatment as well as to maintain good functional and cosmetic outcome in the treatment of squamous cell carcinoma of the lip, oral cavity and pharynx (19, 21, 22). For larger lesions, surgery is more effective, but with the potential side effects of severe morbidities. Interstitial irradiation of temoporfin with its strong absorption of red wavelengths can enhance treatment depth, so that it may

make it possible to treat larger tumors (23-25). However, the collateral phototoxicity of normal tissues to mTHPC-based PDT requires strict light protection protocols to prevent unwanted PDT effects. This has led to a search for alternative approaches that spare normal tissues.

Targeted PDT based on a photosensitizer linked to a targeting moiety with an affinity for tumor cells can improve the selective tumor distribution of the photosensitizer. Such targeting moieties include monoclonal antibodies, peptides, carbohydrates, folic acid and others (26). Epidermal growth factor (EGF), a protein produced in the body, attaches to its receptor (EGFR) of cells to trigger cellular proliferation. EGFR has been found to be overexpressed on the cell surface of several types of tumors including head and neck squamous cell carcinoma. Cetuximab, a chimeric (mouse/human) monoclonal antibody, is able to block the effect of EGF by binding EGFR, and was approved by EMA in 2004 and FDA in 2006 as a therapy for the treatment of patients with locally advanced squamous cell carcinoma of the head and neck in combination with radiation therapy (27). Phthalocyanines, a family of potent photosensitizers with their favorable properties of chemical stability, high fluorescence quantum yield and red shifted light absorption for optimal tissue penetration, have already been used for PDT of cancer patients in Russia (28). In general, hydrophilic phthalocyanines, such as IR700DX, have little photodynamic efficacy due to poor localization and are therefore commonly used to form a conjugate with a targeting moiety (29-31). IR700DX conjugated with an EGFR antibody has been shown to serve as both a diagnostic and a PDT-therapeutic agent (30, 31). Based on a number of preclinical studies with promising results (31, 32), a clinical trial with cetuximab and IR700DX was recently initiated by Rakuten Aspyrian, Inc. (<https://clinicaltrials.gov/ct2/show/NCT02422979>) (33) in patients with recurrent head and neck cancer. Although a rapid direct necrotic killing effect on tumor cells *in vitro* was noticed after the targeted PDT with IR700DX (31, 32), the mode of cell death using this modality (including apoptosis) is still not fully understood. Further, the biological system of tumor cells cultured *in vitro* is significantly different from that of tumor tissue *in vivo*. Any antibody–dye conjugates

including cetuximab-IR700DX go through the vascular system (including endothelium and tissues beneath its basal layer) and interstitial tissue space before reaching tumor cells in tumor tissue after systemic administration. Tumor destruction by a targeted PDT may thus involve direct and indirect (via initial vascular damage) killing effects on tumor cells that depend on the intratumoral localization of the conjugate. To better understand the correlation of kinetic uptake and localization patterns of such a conjugate in tumor with vascular and tumor responses after PDT, we have utilized intravital microscopy with window chamber technologies, such as the dorsal skin-fold, to image real-time dynamic processes *in vivo* (34-36). With this approach and using a mouse window chamber human head and neck OSC-19-luc2-cGFP tumor model *in vivo* with EGFR expression and transfected GFP genes, the aims of this study were to investigate (1) the kinetic patterns of the uptake and localization of the conjugate, cetuximab-IR700DX, in the tumor, (2) the vascular and tumor responses after the targeted PDT and (3) the mechanisms of action of the targeted PDT on tumors with histopathology and immunohistochemistry.

Materials and methods

Human OSC-19-luc2-cGFP head and neck tumour cell line

The OSC-19-Luc2-cGFP (OSC-19) cell line was originally established in Japan from a patient with a well-differentiated squamous cell carcinoma of the tongue (37) and the OSC-19 cell line with transfected genes of luciferase 2 (luc2), and green fluorescent protein (GFP) was described previously (38). The cells were cultured in DMEM (Invitrogen, Carlsbad, CA) containing 4.5 g D-glucose L⁻¹, 110 mg L⁻¹ sodium pyruvate L⁻¹, 580 mg L-glutamine L⁻¹ supplemented with 10% FCS (Lonza, Basel, Switzerland), 100 IU mL⁻¹ penicillin, 100 mg mL⁻¹ streptomycin (Invitrogen), 1× minimal essential medium (MEM) nonessential amino acids solution and 1× MEM vitamin solution at 37°C in a humidified 5% CO₂ atmosphere. The passages of 10–40 of the cell line were used in this study.

Animals and skin-fold window chamber tumour model

An approval for the protocol of this study was obtained from the Erasmus University Medical Center and The Netherlands National Committee for the protection of animals used for scientific purposes. All experiments were conducted according to The National and European Ethical Committees' Guidelines on Animal Welfare. The skin-fold window chamber tumor model was made according to previously described procedures (36, 39). Briefly, female BALB/c athymic nude mice from Janvier Labs (Saint-Berthevin Cedex, France) were 12 weeks old, weighed between 17 and 25 g, fed with chlorophyll-free complete food for 2 weeks prior to experiments and kept under specific pathogen-free conditions. After being anesthetized with inhalation of isoflurane/O₂, a dorsal skin flap of a mouse was made by dissecting the dorsal skin under aseptic conditions and a window chamber was then implanted on the dorsal skin flap. The OSC-19 cells had subcutaneously been inoculated into the dorsal skin flap (5×10^4 cells suspended in 10 μ L serum-free medium) before a cover glass was placed to close the chamber. The window chamber was made of a synthetic material, polyether ether ketone (PEEK) that is inert and does not provoke immune reactions. The animals with the chamber were individually kept in a cage in a climate-controlled room with 32°C and 50%–60% humidity. The individual housing of mice prevents potential damage to the window chamber by other mice, while the high temperature and humidity avoid the cooling down and dehydration of the skin flap. Mice fitted with this chamber showed normal behaviors with full capacity of motion and climbing. The general conditions of the window chamber together with tumor growth and its blood circulation were regularly checked by a confocal microscope at a low magnification to determine a best time to start the experiment with an average of 7 days after implantation of tumor cells (Fig. 1).

Figure 1. Microscopic green fluorescent protein (GFP)-expressing human head and neck OSC-19 squamous cell carcinoma in a mouse skin-fold window chamber model. The image was made with the intravital LSM (bar: 500 μm). For the details, see the Section of Materials and Methods

Intravital imaging

Experiments were started when the tumor showed microvascularization and growth (area: 0.5–2.5 mm^2). The animals ($n = 8$) were sedated with the inhalation of isoflurane/ O_2 and placed on a 37°C temperature-controlled stage of a confocal microscope. The fluorescent tumor GFP (Ex/Em: 488/BP505–530 nm) and conjugate IR700DX (Ex/Em: 633/LP650 nm) in tumor and its surrounding normal tissues were optically sectioned to a 9 μm thickness and imaged by the Zeiss laser scanning microscope (LSM) 510–Axiovert 200M (Carl Zeiss, Thornwood, NY) with a 10 $\times$ objective at 0, 4, 24 and 48 h after i.v. administration of the conjugate cetuximab–IR700DX (100 μL of 1 mg mL^{-1} from Rakuten Aspyrian Inc. San Diego, USA) via the tail vein. A mode of the system was set to enable the PC to control the microscope, xy-table, z-positioning and image capture with three detection channels of transmission, GFP and IR700DX. During the study period of 48 h, the animals were kept under reduced light conditions to avoid possible phototoxic effects of the conjugate, after which all animals were sacrificed by cervical dislocation.

Since one image made by the LSM system with a 10 $\times$ objective cannot cover the field of a whole tumor and its surrounding normal tissues, nine images were acquired in each of the three channels to visualize an area large enough to include a tumor and

its surrounding normal tissues. Based on the transmission and tumor GFP images, the areas of tumor, vessels and connective tissue were identified. At least three regions of interest were randomly chosen in the areas of each type of tissues for image analyses with *ImageJ* to study the kinetic uptake of the conjugate by tumor and its surrounding normal tissues.

For the colocalization study, a total of eight live mice with 1–3 tumors per mouse were used. Eight optically sectioned serial confocal fluorescent images (9 μm thickness) of conjugate IR700DX and eight corresponding tumor GFP images were acquired using the Zeiss LSM 510 with a 20 $\times$ objective in each tumor at 0, 4, 24 and 48 h after i.v. administration of conjugate via the tail vein. One of the eight conjugate IR700DX images, randomly chosen with its respective tumor GFP image, was used to carry out the colocalization image analyses with the *Fiji* software (<http://fiji.sc>) of the image processing program *ImageJ*. The background (including dark current of the microscopy) of the images of the tissue samples taken from the animals prior to the conjugate injection was subtracted. The coloc2 plugin in the *Fiji* version was used to calculate pixel-intensity correlation-based colocalization parameters of Pearson's R value (a measure of the strength of the linear relationship between two different types of fluorescent signal images) and Mander's tM1 parameter (a measure of the colocalization coefficient between two images).

Targeted PDT in four tumor-bearing animals was performed on the tumors under 690 nm laser irradiation (ML7700, Modulight, Inc., Finland) through a frontal light distributor (Medlight SA, Ecublens, Switzerland) to a dose of 100 J cm⁻² (at 50 mW cm⁻²) 24 h after conjugate administration. Normal and tumor vascular responses prior to and 5-min, 2, 24 and 48 h after light exposure were determined by transmission microscopy with a 10 $\times$ objective (Leica SP5 AOBS Multiphoton lasers, Wetzlar, Germany). In addition, the fluorescent rhodamine dextran 2 MDa was immediately (<10 min) imaged after its i.v. administration (100 μL of 1 mg mL⁻¹) with the same microscopic system (Ex/Em: 555/580 nm) to study vascular leakage in the tumor and surrounding normal tissues at 2 h after targeted PDT.

Histopathology and immunohistochemistry (IHC)

The destructive effects of the targeted PDT on two of the four tumors were examined by histopathological evaluation and IHC. The other two tumors were too small to be used for such study. Three untreated tumors were also included as controls. Normal and tumor tissues of the skin-fold window chamber were harvested at 48 h after the targeted PDT, immediately frozen in liquid nitrogen and stored in -80°C before use. The tissues were fixed in a 10% buffered formalin solution and paraffin-embedded. Three μm tissue sections were then made and stained with hematoxylin and eosin (H&E). In addition, the sections were immunostained using the Dako EnVision + system (K8012, Dako Cooperation, CA, USA) and Dako Autostainer. Deparaffinization, rehydration and target retrieval were performed in a Dako proteinase K (for EGFR) or a Dako PT-link (for hypoxia-inducible factor 1- α (HIF-1 α), Ki67, cleaved caspase-3 and LC3) and EnVision Flex target retrieval solution. Endogenous peroxidase was blocked using a Dako blocking reagent for 5 min followed by incubation at 4°C overnight with primary antibody against EGFR (mouse monoclonal antibody, Clone H11, 1:200 dilution, Dako Corporation, CA, USA) and at room temperature for 30 min with primary antibodies against HIF-1 α (rabbit polyclonal antibody, 1:300 dilution, Novus Europe, UK), Ki67 (mouse monoclonal antibody, Clone MIB-1, 1:150 dilution, DakoCytomation, Denmark A/S), cleaved caspase-3 (rabbit polyclonal antibody, 1:100 dilution, Novus Europe, UK) and LC3 (#2775, 1:1000 dilution, Cell Signaling, The Netherlands). Thereafter, the sections were incubated with a Dako EnVision FLEX + mouse or rabbit linker for 15 min followed by incubation with Dako EnVision FLEX/horseradish peroxidase for an additional 30 min. For visualization of staining, the sections were treated with 3'3-diaminobenzidine tetra-hydrochloride (DAB) for 10 min, counterstained with hematoxylin and mounted in a toluene-free mounting medium (Dako, Denmark A/S).

Statistical Analysis

Student's *t*-test was used to analyze differences in the fluorescence intensities of the conjugate in tumor and normal tissues. The same test was also employed to analyze the colocalization data obtained from *ImageJ*. A $p < 0.05$ value was considered to be statistically significant.

Results

Uptake, localisation and colocalisation of the conjugate in tumour

Figure 2 shows an example of transmission, GFP and IR700DX images that were used to quantify the uptake of conjugate by tumor and its surrounding normal tissues. The red fluorescent IR700DX of the conjugate was already seen in the tumor and its surrounding vessels (arrow in Fig. 2) with some vascular branches in the tumor areas at 4 h after its administration. At 24 and 48 h, more conjugate was distributed in the tumor with little in the vessels (arrows in Fig. 2). Quantitative measurements with image analyses demonstrate that the conjugate in the tumor tissue increased with times after i.v. injection with a peak at 24–48 h and was significantly higher than that in the surrounding blood vessels and normal connective tissue ($P < 0.05$; Fig. 3).

Figure 2. Kinetic localization patterns of the cetuximab-IR700DX conjugate in the OSC-19 tumor and its surrounding normal tissues in the skin-fold window chamber at 0, 4, 24 and 48 h after i.v. injection. The images were obtained from one of the tumors examined including transmission, green tumor GFP signals and red cetuximab-IR700DX conjugate together with arrows for the blood vessels in the surrounding normal tissue. The images were acquired with the intravital LSM (bars: 500 μ m). For the details, see the Section of Materials and Methods.

Figure 3. Kinetic patterns of uptake of the cetuximab-IR700DX conjugate by the OSC-19 tumor and its surrounding normal blood vessels and connective tissue in the skin-fold window chamber at various times after i.v. injection. The amounts of the conjugate in the tissues were measured by quantifying the fluorescent signals of the conjugate IR700DX with the intravital LSM as described in the Section of Materials and Methods.

Figure 4 shows a negative value of Pearson's R value in the samples taken before conjugate injection. At 4 h after conjugate injection, very low values of Pearson's R value were seen between the conjugate IR700DX and tumor GFP. The analyses of the images taken at 24 and 48 h after conjugate injection show higher values of the correlation parameter (Fig. 4), demonstrating a higher degree of colocalization of the two different fluorescent signals. Furthermore, high values of Mander's tM1 at all time points illustrate that the fraction of conjugate IR700DX was largely colocalized with the tumor GFP areas (Fig. 4). Statistically, Pearson's R value shows a significant difference between 4 and 24 h ($P = 0.002$) with no significant difference between 24 and 48 h ($P = 0.153$). These statistical data may indicate a significant difference between 4 and 24–48 h in terms of the degree of pixel-intensity correlation of conjugate IR700DX and

tumor GFP. However, the statistical analyses of Mander's tM1 show no significant difference between 4 and 24–48, suggesting a high degree of colocalization correlation of the two different signals between the different time points.

Figure 4. Image analyses of the colocalization correlation parameters of (A) Pearson's R value and (B) Mander's tM1 for the conjugate IR700DX and tumor GFP using the coloc2 plugin of ImageJ. Zero hour in the plots is prior to the conjugate administration. The background of the 0 h images is subtracted in Mander's tM1 plot.

Vascular responses to targeted PDT

Four animals with the skin-fold window chamber tumor model were used to study normal and tumor vascular responses in real-time during targeted PDT (Fig. 5). The extent of the vascular responses was also semiquantified and presented in the Table 1. No changes in the large blood vessels in normal tissue around tumors were seen at 5 min after targeted PDT in two out of four animals (T-1 & T-2), but the vessels were slightly constricted at 2 h and severely contracted at 24 h. At 48 h after targeted PDT, the function of all vessels had recovered with visible blood flow in the two animals (Fig. 5, Table 1). In the third animal, a slight constriction of the large blood vessels was observed at 5 min after targeted PDT followed by apparent dilatation of the vessels at 2 h before the recovery of their function at 24 h (Fig. 5, Table 1). Similarly, the large vessels were dilated at 2 h after PDT in the fourth animal with a recovery at 24 h (Fig. 5, Table 1). No permanent damage to the large blood vessels was observed at 48 h after PDT in all the four animals (Fig. 5). Furthermore, the vascular responses in the

four animals were determined by imaging rhodamine dextran 2 MDa extravasation at 2 h after targeted PDT. Figure 6 shows no leakage of rhodamine dextran 2 MDa in all the four tumors, indicating that the targeted PDT does not cause a severe damage to tumor vascular structures. However, there was some Rhodamine leakage in the tumor-surrounding normal tissues (red regions highlighted in Fig. 6).

Figure 5. Kinetic changes of vascular responses of four treated tumor-bearing animals in the window chamber were determined by the intravital transmission LSM at prior to and 5 min, 2, 24 and 48 h after the targeted PDT (bars: 2000 μm). For the details, see the Section of Materials and Methods.

Table 1. Vascular responses to targeted PDT

Tumors	Prior to	5 min	2 h	24 h	48 h
Tumour-1	-	-	+	++	-
Tumour-2	-	-	+	++	-
Tumour-3	-	+	+	-	-
Tumour-4	-	-	+	-	-

EGFR-overexpressing human head and neck OSC-19-luc2-cGFP tumour in the mouse skin-fold window chamber was irradiated with a 690 nm laser at a dose of 100 J.cm⁻² (50 mW.cm⁻²) at 24 hours after the i.v. injection of cetuximab-IR700DX conjugate. The vascular responses were semi-quantified by (-) no changes in the vascular lumen with normal blood flow; (+) slightly changes in narrowing or dilating the vascular lumen, but still with visible blood flow and (++) severely changes in narrowing the vascular lumen and/or shutting down the vessels with no detectable blood flow.

Figure 6. The fluorescent rhodamine dextran 2 MDa was imaged immediately after its i.v. injection as a marker for vascular leakage in four tumor-bearing animals at 2 h after the targeted PDT. The images were acquired with the intravital LSM including transmission, green tumor GFP, yellow rhodamine dextran 2MDa and merge of GFP and Rhodamine (bars: 1000 μ m). For the details, see the Section of Materials and Methods. The red regions in the T-2 and T-4 indicate some leakage of rhodamine dextran 2 MDa from the normal vessels around the tumors.

Tumor destruction by targeted PDT

The tumor tissue response to the targeted PDT was examined by histopathology and IHC. Untreated tumors in the skin-fold window chamber demonstrate viable tumor cells with no damaged alterations by histopathology with H.E. staining (Control in Fig. 7). IHC of the control tumors showed homogenous cell surface expression of EGFR and nuclear staining of the proliferative factor Ki67 in individual tumor cells (Fig. 7). In addition, some positive cleaved caspase-3 cells were seen (Fig. 7). The tumors treated with the targeted PDT have shown an extensive damage with necrotic and apoptotic (nuclear condensation) cell death (H.E. in Fig. 7). Some slight damage in the overlying normal skin tissue was also seen (Fig. 7). In addition, inhomogenous, weak staining of EGFR and of the proliferative factor, Ki67 suggests a decreased expression of EGFR and Ki67 in tumor cells. Moreover, the cleaved caspase-3, a pivotal factor for the execution of cell apoptosis, was apparently overexpressed in the tumor cells, indicating apoptotic cell death after the targeted PDT (Fig. 7). LC3, a central protein in the autophagy pathway and a marker of autophagosomes, was not expressed (data not shown), suggesting no involvement of autophagy in the tumor cell death. Interestingly, no congestion and hemorrhage of blood vessels were seen in the tumors and surrounding normal tissues (Fig. 8). IHC showed typical brownish nuclei of positive staining of HIF-1 α , a well-known transcription factor for regulating cellular response to hypoxia, in the epidermal cells, but not in the beneath tumor cells (Fig. 8). This was consistent with no severe vascular damage demonstrating that the tumor destruction by the targeted PDT was largely due to a direct killing effect rather than an indirect effect via vascular damage.

Figure 7. Hematoxylin–eosin (H.E.) staining and immunohistochemistry of EGFR, Ki67 and cleaved caspase-3 of tumors at 48 h after the targeted PDT (the bars in H.E. images: 100 μm for the left column of images and 50 μm for the middle column of images). EPI, epidermis; T, tumor. A control tumor is also included (the bar in the H.E. image is 50 μm for the right column of images).

Figure 8. Immunohistochemistry of HIF-1 α in a tumor at 48 h after the targeted PDT. EPI, epidermis; T, tumor. Epidermal cells show HIF-1 α staining of brownish nuclei served as “internal” positive controls, while no HIF-1 α is seen in the subcutaneous tumor cells. The bars: 100 μ m for the left image and 50 μ m for the right images.

Discussion

A dorsal skin-fold window chamber tumor model in combination with confocal imaging technologies can offer a unique opportunity to quantitatively analyze in real-time the fluorescence-based pharmacokinetics and biodistribution of the cetuximab-IR700DX conjugate in the OSC-19 tumor and its surrounding normal tissues in living athymic mice. In addition, this technique can visualize the dynamic patterns of normal and tumor vascular responses to the targeted PDT. Such studies would help to optimize parameters affecting the targeted PDT efficacy. Cetuximab-IR700DX was taken up by the tumor cells already at 4 h and peaked at 24 and 48 h after administration. It was still detectable in the blood at 4 h after administration and less at later time points. A 24 h drug–light interval was therefore chosen for the subsequent

targeted PDT of the tumor in this study. The same interval is also being used in the ongoing clinical trial of the targeted PDT in head and neck cancer patients. While cetuximab is a chimeric (mouse/human) antibody against human EGFR, it does not bind to mouse EGFR. The increase in fluorescence in normal mouse tissues observed in this model is likely to be the result of content in the blood circulation and unspecific uptake of the conjugate. It should be mentioned that IR700DX in a free form was not included in this study because this water-soluble dye has been shown in several reports to be little taken up by tumor tissues with little subsequent photodynamic effects (29-31, 40).

The microscopy-based methods to study colocalization of markers in biological systems are often qualitatively descriptive rather than quantitative. For example, to overlap a green and a red fluorescent image for two various markers with a resultant yellow color image is still commonly used to assess colocalization. However, such a visual estimation does not allow quantification of the degree of colocalization. Based on the initial work by Pearson in 1896 (41), several statistical methods of pixel-intensity correlation have been developed to quantify the degree of colocalization of two fluorescence channels (42, 43). The significant lower level of Pearson's R value at 4 h than 24 h (Fig. 4) is in agreement with the pixel-intensity-based "uptake" data with a lower amount of IR700DX in the tumors at 4 h than 24–48 h (Fig. 3). Further, the relative high values of Manders' tM1 as a colocalization quantifier demonstrate a high fraction of positive IP700DX areas that colocalizes with the tumor GFP. No significant difference of Mander's tM1 in the images taken at 4, 24 and 48 h suggests that IR700DX was localized in the tumor cells with a relative high specificity during the study period.

Microvascular structures of a tumor are usually vulnerable to PDT. This starts with recoverable functional disturbance, such as vasoconstriction and vasodilatation, in the arterioles, capillaries and postcapillary venules. This often results in aggregation of blood cells and thereby reduced blood flow and possibly finally complete stasis (44-46). The vasoconstriction may be caused by the release of thromboxane, a potent vasoconstrictor formed from damaged cell membrane lipids such as from platelets

(47). Conversely, histamine, a powerful vasodilator released from the degranulation of mast cells, may induce the vasodilatation (48). Furthermore, if a photosensitizer is localized in the collagen fibers and connective tissue elements of the subendothelial zone of the capillary wall, this may lead to permanent vascular damage after light activation. No permanent vascular damage in this study suggests that the targeted PDT may not damage the subendothelial zone of the vasculature. Normally, the effects of hypoxia/anoxia on tumor due to functional alterations of vessels are limited if they do not last for a long time, as an effective PDT usually requires a permanent vascular damage (49). The initial mild to severe constriction followed by dilation of the vascular responses was observed up to 24 h after the targeted PDT in the present study. This might be due to a nonspecific photodynamic effect of circulating conjugates on the vascular walls, since some conjugate in the blood vessels was still seen at 24 h after injection (Fig. 3). Such microvascular effects were not permanent, as the vascular functions were recovered at 48 h after the targeted PDT. Moreover, the rhodamine dextran 2 MDa extravasation confirmed no leakage of the tumor vessels, suggesting no structural damage of the tumor vessels. Consistently, no expression of HIF-1 α in the tumor cells (Fig. 8) indicates that the targeted PDT-induced transient vascular alterations did not result in a severe hypoxic condition in the tumors.

Histopathology and IHC demonstrate an extensive necrotic destruction of the two tumors 48 h after the targeted PDT with no signs of hemorrhage. Thus, such tumor damage may not involve the blood vessels. Only slight damage of the overlying epidermis was observed, most likely the result of unspecific uptake of the conjugate. The binding of the conjugate to the EGFR of the OSC-19 tumor cells most likely played a crucial role in the direct killing of tumor cells by the targeted PDT.

EGF, a small mitogenic protein, binds to EGFR on the cell surface to induce autophosphorylation of several tyrosine residues in the C-terminal domain of EGFR. This leads to a stimulation of its intrinsic tyrosine kinase activity. It is well known that overexpression of EGFR promotes cell cycle progression from the G1 to S phase to increase cell proliferation. In this study, the down-expression of EGFR with

downregulation of the proliferative factor Ki67 of the tumor cells as seen by IHC supports the tumor damage via targeting EGFR of the targeted PDT.

Since the first report by Agarwal et al. in 1991 on the apoptotic induction by PDT with chloroaluminium phthalocyanine (50), a large number of studies have concluded that PDT can induce cell death through apoptosis. Apoptosis, or programmed cell death, is a form of cell death distinct from necrosis with respect to the characteristics of morphology and biochemistry. Cytosolic aspartate-specific (cysteine) proteases, called caspases, are responsible for the induction of apoptosis. All apoptotic caspases exist in cells as an inactive form. When cells undergo apoptosis, these caspases become activated through one or more sequential proteolytic events that cleave the single peptide precursor into large and small fragments. Among them, the caspase-8, caspase-9 and caspase-3 appear to play a crucial role in the degradative events in apoptosis. Currently, there are two well-characterized pathways of caspase-activating cascades that regulate apoptosis: the cell surface death receptor pathway via cleavage of caspase-8 and the mitochondrial-initiated pathway via cleavage of caspase-9. The cleaved caspase-8 or caspase-9 then activates the downstream executioner, caspase-3. The nuclear condensation of some tumor cells with the upregulation of cleaved caspase-3 in this study suggests an apoptotic induction probably through a receptor-mediated pathway after directly targeting EGFR by the targeted PDT. No positive detection of the LC-3 as a marker of autophagy may indicate no involvement of the autophagic process in this study.

Conclusions

The fluorescent kinetic uptake and localization patterns of the cetuximab-IR700DX conjugate in the EGFR-overexpressing human head and neck OSC-19 tumors in the mouse skin-fold window chamber were studied in real-time. The amounts of the conjugate peaked at 24–48 h in the tumor tissue. The light exposure then caused initial contraction and subsequent dilatation of the normal blood vessels followed by

functional recovery at 48 h. No leakage of the rhodamine dextran 2 MDa within the tumor tissue, together with negative staining of HIF-1 α in the tumor cells by IHC, suggests no substantial damage to the tumor vascular structure, neither a severe hypoxic condition in the tumors after the targeted PDT. Furthermore, the extensive tumor necrosis shown by histopathology with H.E. staining and the cleavage of caspase-3 by IHC in the tumor cells demonstrates both necrotic and apoptotic cell deaths are involved in the tumor destruction. These results suggest that such an effective modality was largely due to a direct effect on the EGFR-overexpressing OSC-19 tumor cells via the specific light-activated cetuximab–IR700DX conjugate rather than an indirect effect from vascular damage. This study demonstrates that targeted PDT with the cetuximab–IR700DX conjugate may have a potential for treating EGFR expressing tumors.

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Chapter 4

***In vivo* optical monitoring of the efficacy of epidermal growth factor receptor targeted photodynamic therapy: The effect of fluence rate**

4

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ABSTRACT

Targeted photodynamic therapy (PDT) has the potential to improve the therapeutic effect of PDT due to significantly better tumor responses and less normal tissue damage. Here we investigated if the efficacy of epidermal growth factor receptor (EGFR) targeted PDT using cetuximab-IR700DX is fluence rate dependent. Cell survivals after treatment with different fluence rates were investigated in three cell lines. Singlet oxygen formation was investigated using the singlet oxygen quencher sodium azide and singlet oxygen sensor green (SOSG). The long-term response (to 90 days) of solid OSC-19-luc2-cGFP tumors in mice was determined after illumination with 20, 50, or 150 mW·cm⁻². Reflectance and fluorescence spectroscopy were used to monitor therapy. Singlet oxygen was formed during illumination as shown by the increase in SOSG fluorescence and the decreased response in the presence of sodium azide. Significantly more cell death and more cures were observed after reducing the fluence rate from 150 mW·cm⁻² to 20 mW·cm⁻² both *in vitro* and *in vivo*. Photobleaching of IR700DX increased with lower fluence rates and correlated with efficacy. The response in EGFR targeted PDT is strongly dependent on fluence rate used. The effectiveness of targeted PDT is, like PDT, dependent on the generation of singlet oxygen and thus on the availability of intracellular oxygen.

Introduction

Photodynamic therapy (PDT) involves the administration of a photosensitizer (PS) that after exposure to light, and in the presence of oxygen, leads to the formation of reactive oxygen species, predominantly singlet oxygen [1]. The efficacy of the treatment is dependent on many factors that dynamically interact during treatment. Ground state oxygen is needed for formation of singlet oxygen, which means that tissue oxygenation strongly influences treatment efficacy [2]. Illumination with high fluence rates can deplete oxygen levels in both tumor and normal tissue, limiting efficacy [3,4]. Vascular responses that can occur during PDT, such as constriction and thrombus formation, can additionally decrease tumor oxygenation and negatively affect the PDT response [5-8]. Conversely, vascular responses may potentially enhance tumor necrosis due to vascular occlusion [5,9]. The dynamic interaction between light, photosensitizer, cells and vasculature can potentially change optical properties influencing light delivery to tissue. Therefore, a full understanding of light delivery, tissue optical properties, oxygenation levels and photosensitizer localization are important if PDT is to be optimized. We and others have shown that rate of fluorescence photobleaching of some photosensitizers during PDT is related to formation of singlet oxygen and can be used for implicit dosimetry [3,10-12]. Most fluorescence measurements techniques, however, do not account for the influence of tissue optical properties on fluorescence signals that have been shown to be different for different tissues and change during PDT. Single fiber reflectance (SFR) spectroscopy combined with fluorescence (SFF) spectroscopy can be used to determine the absorption and scattering properties of light through tissue and to calculate the intrinsic fluorescence intensity [13]. These types of in-situ measurements during treatment have potential for monitoring the efficacy of PDT.

Targeted strategies to deliver PSs specifically to tumor cells have the potential to improve the therapeutic effect of PDT due to significantly better tumor responses, less normal tissue damage and decreased skin photosensitization [1,14,15]. In this

approach different, and in many cases increased, expression levels of receptors on tumor cells can be used as a target. In head and neck cancer it has been shown that 83% of tumors show overexpression of epidermal growth factor receptor (EGFR) which is commonly utilized in targeted therapies [16]. One way to target a PS to the EGFR receptor is to conjugate with an antibody, first reported by Mew et al. [17]. The phthalocyanine dye IR700DX, is a hydrophilic PS that by itself has little photodynamic efficacy due to a poor cellular localization and is commonly used to form a conjugate with a targeting molecule. Cetuximab (ErbixTM) is an antibody against human EGFR that is approved by the FDA and EMA to treat colorectal and late stage head and neck cancer. A conjugate of cetuximab and IR700DX (cetuximab-IR700DX) has the potential for effective photosensitizer delivery to tumor cells and has been shown to cause selective tumor destruction after light irradiation [18-24]. *In vitro* studies investigating cetuximab-IR700DX have shown cell membrane localization and rapid internalization, and cell death after light exposure [20,21,23]. Sato et al. [20] and Nagaya et al. [21] both demonstrated a delay in growth of a preclinical tumor after cetuximab-IR700DX PDT using either one light fluence or repetitive treatments. Recently we have shown in an intravital imaging study that tumor localization, minimal vascular responses and PDT induced tumor necrosis occur using an illumination scheme with a single fluence of $100 \text{ J}\cdot\text{cm}^{-2}$ delivered at a fluence rate of $50 \text{ mW}\cdot\text{cm}^{-2}$ [24]. To our knowledge the effect of different fluence rates on the efficacy of targeted PDT has not been investigated *in vivo*.

Cetuximab-IR700DX is currently under investigation for the treatment of recurrent head and neck squamous cell carcinoma in a phase III trial [25]. In a phase I/IIb clinical trial of 30 patients, Gillenwater et al. [26] show promising responses using a drug-light-interval (DLI) of 24 h between drug administration and light exposure for 4 to 6 min. Although explicit details on the treatment parameters (fluence and fluence rate) were not reported, the short illumination times described means it is most likely that fluence rates were high, because this reduces illumination times, in the range of $150 \text{ mW}\cdot\text{cm}^{-2}$.

Only mild and moderate treatment related side effects such as tumor hemorrhage, swelling and pain were observed.

These promising preclinical and clinical results encouraged us to investigate the effects of fluence rate on cetuximab-IR700DX mediated PDT. The use of a photosensitizer with a moderate singlet oxygen quantum yield suggests that photodynamic action is the source of damage in the antibody targeted IR700DX mediated PDT. The literature on this subject is however contradictory, some studies performed experiments in the presence of reactive oxygen species or singlet oxygen scavengers and could not completely remove the effect leading authors to invoke other mechanisms of cell death [18,19]; whereas other studies have shown a singlet oxygen only mechanism [22]. In designing strategies for treatment optimization it is important to know to what extent singlet oxygen mediates PDT response and if oxygen availability is a limiting factor.

Therefore in the present study we investigated the involvement of singlet oxygen and the effect of different fluence rates on head and neck cancer derived OSC-19-luc2-cGFP, scc-U2 and scc-U8 cells *in vitro*. We continued this study *in vivo* investigating long-term effects of different fluence rates on the growth of a subcutaneous OSC-19-luc2-cGFP solid tumor in the flank of a mouse. We hypothesized that treatment with a low fluence rate would be more effective due to efficient use of the tissue oxygenation. We utilized single fiber reflectance spectroscopy and fluorescence spectroscopy to monitor PDT response during illumination and investigated whether there was a correlation between this and treatment efficacy [27]. Our results show that the response to EGFR targeted PDT is the result of singlet oxygen formation, that this response is dependent on the fluence rate used, and that in-vivo optical monitoring of IR700DX fluorescence photobleaching has the potential for predicting therapeutic efficacy.

Materials and methods

Cell lines and culture

Three human head and neck (oral cavity) squamous cell carcinoma cell lines, scc-U2, scc-U8 (University of Michigan), and OSC-19-luc2-cGFP [41] (University of Leiden) were used in this study. The scc-U2, and scc-U8 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, ThermoFisher scientific, Waltham, MA, USA) supplemented with 10% fetal calf serum (FCS, Lonza, Basel, Switzerland), 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM glutamine (Gibco, ThermoFisher scientific, Waltham, MA, USA) at 37 °C in a humidified 5% CO₂ atmosphere. The OSC-19-luc2-cGFP cell line was cultured in advanced DMEM (Gibco, ThermoFisher scientific, Waltham, MA, USA) containing 4.5 g/L D-glucose, 110 mg/L sodium pyruvate, 580 mg/L glutamine supplemented with 10% FCS (Lonza, Basel, Switzerland), 1400 IU/mL penicillin, 100 mg/mL streptomycin, 1× Minimal Essential Medium (MEM) non-essential amino acids solution, and 1× MEM vitamin solution (Gibco, ThermoFisher scientific, Waltham, MA, USA) at 37 °C in a humidified 5% CO₂ atmosphere. The passages of 10–40 of the cell lines were used in this study.

Targeted photosensitizer

Cetuximab-IR700DX was provided by Aspyrian Therapeutics, Inc., San Diego, CA, USA.

PDT illumination *in vitro*

Each cell line was seeded with 15,000 cells in each well of a 96-well plate. After 24 h incubation for attachment, the cells were incubated in the dark with 40 µg/mL cetuximab-IR700DX for 24 h at 37 °C. Cells were washed and a volume of 100 µL medium was added to the cells. Illumination was performed with a 690 nm laser (Modulight ML7700, Tampere, Finland) using a shaker with a speed of 700 RPM to facilitate oxygen diffusion as much as possible. The fluence rate was determined at the

surface of the 96 well plate to account of scattering and reflection of the incident irradiance using an isotropic detector (Medlight SA, Ecublens, Switzerland) connected to an optical power meter (National Instruments NI PXI-1045 and PX 2000-306, Woerden, The Netherlands). Cells were illuminated with a fluence rate of 150, 125, 100, 72, or 25 mW·cm⁻². The total fluence delivered was different for the different cell lines to compensate for the difference in sensitivity to PDT treatment. Scc-U8 cells were illuminated to a fluence of 7 J·cm⁻² and scc-U2 and OSC-19-luc2-cGFP cells to a fluence of 15 J·cm⁻² [23]. Cell survival was determined 24 h after PDT using the cell proliferation MTS assay. Since previous reports from us and others have shown very little photodynamic effect on cells using IR700DX alone, we chose not to include it as a control [18,42].

Cell survival

Cell proliferation was assessed with a standard MTS kit (CellTiter 961 AQueous One Solution Reagent (Promega Corp., Madison, WI, USA) according to the manufacturer's recommendations using a 96-well plate reader (Molecular Devices, Sunnyvale, CA, USA). The formazan product detected by the 492 nm absorbance was not influenced by the IR700DX.

Formation of reactive oxygen species

The formation of singlet oxygen formation was investigated using two different experiments. In the first experiment PDT was performed in the presence of the singlet oxygen quencher sodium azide. In the second experiment the formation of singlet oxygen was visualized using confocal imaging during PDT in the presence of singlet oxygen sensor green (SOSG, Molecular Probes, Oregon, OR, USA).

- Quenching singlet oxygen

Each cell line was seeded with 15,000 cells in each well of a 96-well plate. After 24 h incubation for attachment, the cells were incubated in the dark with 40 µg/mL cetuximab-IR700DX for a total of 24 h at 37 °C. After 20 h incubation with cetuximab-

IR700DX, sodium azide (NaN_3) at 50 mM (Sigma-Aldrich, Zwijndrecht, NL, USA) was added for the remaining 4 h. Cells were then washed at a volume of 100 μL medium before illumination. PDT and cell survival measurements were performed in the same manner as described previously (*in vitro* PDT illumination).

- **Detection of reactive oxygen species**

Scc-U2 and scc-U8 cell lines were used to investigate the formation of singlet oxygen using SOSG during confocal imaging. This assay could not be performed on OSC-19-luc2-cGFP due to the GFP expression of the cell line which conflicts with the fluorescence detection of SOSG endoperoxide (SOSG-EP), the product of the reaction between SOSG and singlet oxygen.

The scc-U2 and scc-U8 cell lines were seeded with 8000 cells in a well of a 96-well plate (one well per plate to avoid cross illumination). After 24 h incubation for attachment, the cells were incubated in the dark with 40 $\mu\text{g}/\text{mL}$ cetuximab-IR700DX at 37 °C. After 24 h the cells were washed 3 times with saline and incubated with singlet oxygen sensor green (10 μM in saline) for 2 h at 37 °C. After washing 3 times with saline the cells were supplied with 50 μL saline and placed on the heated stage of a Leica SP5 Microscope equipped with a 10 $\times$ Plan-Neofluar objective. It is well known that SOSG and SOSG-EP can themselves both act as photosensitizers [37,43], therefore the imaging parameters (488 nm excitation light intensity), pixel dwell time and number of images collected were carefully defined in control experiments on the cells incubated with only SOSG for 2 h.

The conjugate fluorescence was recorded before and after illumination using 633 nm excitation and 650–800 nm HyD detection. The SOSG-EP fluorescence images were recorded before, during and after illumination using 690 nm light delivered at either 150 $\text{mW}\cdot\text{cm}^{-2}$ or 20 $\text{mW}\cdot\text{cm}^{-2}$ to a fluence of 15 $\text{J}\cdot\text{cm}^{-2}$. Cells illuminated with 150 $\text{mW}\cdot\text{cm}^{-2}$ were imaged 3 times; immediately before, halfway (50 s) and immediately after illumination. Cells illuminated with 20 $\text{mW}\cdot\text{cm}^{-2}$ were imaged 9 times; before, every 100 s during illumination and after illumination. ImageJ 1.48v was used to analyze the images recorded. The pre-PDT fluorescence image of cetuximab-IR700DX

was used to create regions of interest (ROI) around the individual or small clustered cells by the thresholding and analyze particles feature. This was used to determine the fluorescence intensity in both the red and the green channel in the images recorded. The intensities of SOSG-EP fluorescence and cetuximab-IR700DX fluorescence determined per cell or groups of cells and averaged (weighted mean) per image. Per cell line and fluence rate 3 to 5 sets of images were recorded and averaged (weighted mean). The rate of photobleaching was determined as the slope of the reciprocal of the normalized fluorescence using linear regression fitting. The rate of SOSG-EP fluorescence increase was determined as the slope of the fluorescence increase using linear regression fitting. Data of ROI's with a max of 255 in the red channel, or a R2 lower than 0.90 in linear regression fitting were excluded from data analysis.

Solid tumor model

Female BALB/c nude mice (Charles River) aged 8–11 weeks were injected with 1×10^6 OSC-19-luc2-cGFP cells subcutaneously in the flank. Volumes of the ellipsoid-shaped tumors were calculated from the 3 diameters that were measured using a caliper every 2 or 3 days after they became palpable. The formula used was $V = (D1 \times D2 \times D3) \times \pi/6$. Only animals with tumors showing exponential growth were included in the study (80%). Experiments were started when the tumors reached a volume of approximately 40–70 mm³ and the treatment volume was defined 100%. After illumination tumors were again measured with a caliper every 2 or 3 days until 500% of the treatment volume or up to 90 days after treatment by HB and/or WP blinded to the treatment parameters. The points in time at which the regrown tumor reached a volume of respectively 100%, 200%, 300%, 400%, and 500% were linearly interpolated and averaged for each treatment group (n=4–5 per group). The effectiveness of each treatment scheme was determined by comparison of the mean tumor volume doubling time, defined as the number of days the regrown tumor required to double its pre-treatment volume. A tumor was considered cured when it reduced its size until it was not palpable and remained so for 90 days after treatment. Tumor tissue that had

reduced in size but remained palpable and did not grow was excised on 90 days post treatment for pan-Keratin and CD45 staining. The Netherlands National Committee for the protection of animals used for scientific purposes approved the protocol (AVD1010020151636). All applicable institutional and/or national guidelines for the care and use of animals were followed.

PDT illumination *in vivo*

Illumination was performed under gas anesthesia 24 h after i.v. administration of cetuximab-IR700DX. Mice injected with physiological saline served as a light only control ($n = 5$). Just before illumination the skin overlying the tumor was loosely stretched using two sutures and the quadfurcated fiber, for collecting the reflectance and fluorescence spectra, was placed underneath the skin in touch with (but not pressing against) the tumor (Figure 1). Reflectance and fluorescence spectra were recorded before, during and until max 5 min after illumination without repositioning the fiber. A 690 nm laser (ML7700, Modulight Inc., Tampere, Finland) and a frontal light distributor (Medlight SA, Ecublens, Switzerland) were used to deliver a dose of $100 \text{ J}\cdot\text{cm}^{-2}$ at a of 20, 50, or $150 \text{ mW}\cdot\text{cm}^{-2}$ ($n = 4$ in each group).

Figure 1. Schematic drawing of the illumination and single fiber spectroscopy setup.

Single fiber reflectance and fluorescence spectroscopy

The experimental setup used to collect reflectance and fluorescence spectra is based on what has been described in detail previously [44] and is shown in Figure 1. In short, the setup utilizes a single optical fiber connected to a quadfurcated optical fiber polished under an angle of 15° to minimize internal specular reflections from the fiber tip. White light reflectance spectra were obtained after excitation of white light from a halogen light source HL-2000-FHSA (Ocean Optics, Duiven, The Netherlands) via one arm and collection of the reflected light via a second arm connected to a spectrophotometer SD-2000 (Ocean Optics, Duiven, The Netherlands). A calibration procedure was performed to account for internal reflections, variability in lamp-specific output and in fiber-specific transmission properties [45]. Fluorescence spectra were obtained immediately after the reflection measurement using excitation light split from the 690 nm therapeutic laser (at a fluence rate that approximately matched the treatment fluence rate in each group) connected to the third arm of the quadfurcated fiber. The emitted fluorescence was collected through the fourth arm of the quadfurcated fiber, filtered through the 695 LP filter and connected to a second spectrograph (QE-6500, Ocean Optics, Duiven, The Netherlands).

Mathematical analysis of spectra

Single fiber reflectance spectra were analyzed using an analytical model to describe the wavelength-dependent optical properties to extract physiological and morphological information from the sampled tissue as previously described [13]. In short, attenuation due to absorption within the tissue is modeled using a modified Beer–Lambert law and is a function of both the tissue absorption coefficient (μ_a) and the single fiber photon path length. The reflectance amplitude, as well as the single fiber photon path length, depends on the scattering properties of the tissue, with a dependence on the reduced scattering coefficient (μ'_s) and on the angular distribution of scattering (phase function). The dependence of the single fiber reflectance signals on the phase function parameter γ results from the overlapping source–detector areas utilized in single fiber measurements, for which the diffusion approximation does not hold [46]. To fit the data, the fiber core diameter, numerical aperture of the fiber and the refractive index of the solid tumor under investigation were taken into account. The reduced scattering coefficient was fitted as a power-law function [45], and γ was assumed to be constant over the fitted wavelength range. Furthermore, instead of fitting γ as a free parameter, we kept γ fixed at 1.4, 1.6, and 1.8, which are an expected range for γ in biological tissues [47]. We have verified that this did not result in differences in the estimated absorption coefficient of more than 6% compared to other fitting approaches.

Since we have not previously performed measurements on the OSC-19-luc2-cGFP solid tumor, our initial model assumed that absorption was attributable to oxygenated (HbO_2) and deoxygenated hemoglobin (Hb) confined within the local microvasculature and bilirubin. A Levenberg–Marquardt algorithm was used to estimate the parameter values for the microvascular hemoglobin oxygen saturation StO_2 , the blood volume fraction (BVF) and average blood vessel diameter (VD) by minimizing the chi-squared metric between measured reflectance data and model predictions. Confidence intervals on parameter estimates were calculated from the square root of the diagonal of the covariance matrix [48]. Parameter values were averaged over repeated

measurements, weighted by the confidence interval of individual spectral fits, and reported with the associated weighted standard deviation.

The fluorescence spectra from all individual measurements were corrected for the absorption and scattering as described before [27]. Spectral deconvolution was used to divide the corrected fluorescence spectrum into autofluorescence and photosensitizer fluorescence. Subtraction of the autofluorescence spectrum then yielded the intrinsic fluorescence spectrum. The resulted intrinsic photosensitizer fluorescence spectrum was related to the product of photosensitizer absorption coefficient μ_a^f (mm^{-1}) and quantum yield Q_f (–) by integration over the wavelengths. A basis spectrum of IR700DX was constructed from 3 measurements on 3 different mice and used to fit the intrinsic fluorescence spectrum. From the fitted curve we were able to determine the intrinsic fluorescence intensity and the 95% confidence interval. The rate of photobleaching over the first $5 \text{ J}\cdot\text{cm}^{-2}$ was determined as the slope of the reciprocal of the normalized fluorescence using linear regression fitting.

Statistics

All results are presented as mean $\pm$ SD, except for data collected using single fiber spectroscopy. The fitted values for StO₂, BVF, VD and $Q\cdot\mu_a^f$ were weighted by its confidence interval to result in a weighted mean $\pm$ SD. The significant difference was determined using the Student's *t*-test/ANOVA/SNK and $p < 0.05$ was considered significant. For the tumor growth delay data multivariate analysis and Chi quadrant analysis were used.

Results

Effect of different fluence rates *in vitro*

The effect of fluence rate on the cell survival was investigated on the three head and neck cell lines of OSC-19-luc2-cGFP, scc-U2 and scc-U8 with an EGFR expression relative to the expression in the OSC-19-luc2-cGFP (set to 100%) of 66% in scc-U8 and 70% in scc-U2 as previously determined [23]. Illumination with $150 \text{ mW}\cdot\text{cm}^{-2}$ after 24 h incubation with cetuximab-IR700DX resulted in $53 \pm 6\%$, $69 \pm 11\%$, and $83 \pm 10\%$ cell survivals in the OSC-19-luc2-cGFP, scc-U2 and scc-U8 cell lines respectively (Figure 2). The total fluence delivered was $15 \text{ J}\cdot\text{cm}^{-2}$, $15 \text{ J}\cdot\text{cm}^{-2}$, and $7 \text{ J}\cdot\text{cm}^{-2}$ respectively to obtain a similar cell survival for the different cell lines [23]. Reducing fluence rate from 150 to $100 \text{ mW}\cdot\text{cm}^{-2}$, without changing the total fluence, did not decrease cell survival statistically significant for all three cell lines. Only when fluence rate was reduced to $75 \text{ mW}\cdot\text{cm}^{-2}$ the cell survival was statistically significantly decreased with $p < 0.05$ compared to other fluence rates used for scc-U2 and OSC-19-luc2-cGFP. Further reduction of fluence rate to $20 \text{ mW}\cdot\text{cm}^{-2}$ resulted in a significantly decreased survival of $10 \pm 2\%$ for OSC-19-luc2-cGFP ($p < 0.05$), $12 \pm 5\%$ for scc-U2 ($p < 0.01$), and $22 \pm 1\%$ for scc-U8 ($p < 0.01$) compared to all other fluence rates used, suggesting that oxygen availability can be an issue.

Figure 2. Cell survivals of OSC-19-luc2-cGFP (black bar), scc-U2 (striped bar) and scc-U8 (white bar) treated with cetuximab-IR700DX using a 24 h DLI and illuminated with different fluence rates to a fluence of 15 J.cm⁻² for OSC-19-luc2-cGFP and scc-U2 and 7 J.cm⁻² for scc-U8. † statistically significantly different from all other fluence rates investigated with the same cell line with $p < 0.05$, ‡ statistically significantly different from all other fluence rate investigated with the same cell line with $p < 0.01$.

Formation of singlet oxygen

- Quenching singlet oxygen

PDT treatment in the presence of the singlet oxygen quencher NaN₃ significantly diminished the effect of PDT with $p < 0.001$ in all cases (Figure 3). No significant difference in cell survival could be observed between NaN₃ only and NaN₃ + PDT treated cells for all three cell lines investigated, suggesting that cell death after PDT is singlet oxygen dependent.

Figure 3. Cell survivals of OSC-19-luc2-cGFP (black bar), scc-U2 (dashed bar) and scc-U8 (white bar) cells treated with either NaN₃ only, cetuximab-IR700DX mediated PDT or the combination using a 24 h DLI and illuminated with 20 mW.cm⁻² to a fluence of 15 J.cm⁻² for OSC-19-luc2-cGFP and scc-U2 and of 7 J.cm⁻² for scc-U8. # statistically significantly different from PDT only with p<0.001.

- Detection of singlet oxygen

To investigate the formation of singlet oxygen during *in vitro* PDT, SOSG-EP fluorescence was imaged using confocal fluorescence microscopy. Control experiments, imaging scc-U8 cells incubated with SOSG for 2 h, confirmed that imaging with 488 nm could result in self-sensitized singlet oxygen as shown by the increase of SOSG-EP fluorescence in absence of cetuximab-IR700DX (**Figure S1**). Imaging with very low power 488 nm light did not result in an increase in SOSG-EP fluorescence. Additionally, imaging with 633 nm light, to excite cetuximab-IR700DX or 690 nm light to perform PDT, did not result in any increase of SOSG-EP. Similar results were obtained for scc-U2 cells (data not shown). Based on these experiments we chose to image SOSG-EP with very low intensity 488 nm light and collected a maximum of 10 images during illumination.

Figure S1. Effects of imaging parameters and PDT illumination on the SOSG photosensitization of scc- U8 cells incubated with SOSG for 2 h. (a–e) Set of images, collected using different illumination parameters, comprising (a) transmission image of cells, (b) first fluorescence image collected using 1% 488 nm light, (c) 10th fluorescence image collected using 1% 488 nm light, (d) first fluorescence image collected using 50% 488 nm light, (e) 10th fluorescence image collected using 50% 488 nm light. (f) SOSG-EP fluorescence intensity determined in the cells in consecutive images collected using 1% 488 nm light (open squares, left y-axis) and 50% 488 nm light (closed squares, right y-axis). (g–i) Different set of images, collected during PDT illumination with 690 nm light, using 1% 488 nm light and 633 nm light to collect cetuximab-IR700DX in future experiments, comprising (g) transmission image, (h) pre illumination and (i) post illumination. (j) SOSG-EP fluorescence intensity determined in the cells in consecutive images collected using 1% 488 nm light (open squares) during illumination with 690 nm light. Bar is 100 μm .

Incubation of cetuximab-IR700DX for 24 h resulted in fluorescence at the membrane and in the endo/lysosomes in both cell lines as shown in Figure 4 and in agreement with what was shown previously [23]. Confocal imaging after 2 h of SOSG incubation using the established illumination parameters showed auto fluorescence and low levels of SOSG-EP fluorescence. After illumination, cetuximab-IR700DX fluorescence decreased and an increase in SOSG-EP fluorescence was observed. This increase in SOSG-EP fluorescence was not limited to the location of cetuximab-IR700DX, but observed throughout the cytosol of the cells (Figure 4).

Figure 4. Examples of 2D images and 3D surface plots from images collected pre and post PDT of scc-U2 and scc-U8 cells incubated with cetuximab-IR700DX for 24 h and singlet oxygen sensor green (SOSG) for 2 h. White arrows point to some of the endo/lysosomes showing cetuximab-IR700DX fluorescence. Blue arrow points to blebs formed in response to the PDT treatment. Bar is 20 μm. Height of the peaks correspond to the fluorescence intensities of SOSG (green).

Low magnification imaging during illumination gave the opportunity to investigate multiple cells. Figure 5 shows a gradual increase in SOSG-EP fluorescence in both scc cell lines. At the start of illumination almost no SOSG fluorescence was detectable (Figure 5b). During PDT SOSG fluorescence increased which varied from cell-to-cell (Figure 5c,d). No statistically significant difference was observed in mean SOSG-EP

fluorescence increase and rate of increase after illumination with 20 or 150 $\text{mW}\cdot\text{cm}^{-2}$ (Figure 5e). We also observed no significant difference in extent and rate of cetuximab-IR700DX fluorescence photobleaching after illumination with 20 or 150 $\text{mW}\cdot\text{cm}^{-2}$ (Figure 5f). This is in agreement with the SOSG results and suggests that, given the illumination conditions of this part of the study, the rate of singlet oxygen formation is similar for the different fluence rates investigated.

Figure 5. Example of a set of fluorescence images recorded of scc-U8 cells incubated with cetuximab-IR700DX for 24 h and SOSG for 2 h, illuminated with 20 $\text{mW}\cdot\text{cm}^{-2}$ to a fluence of 7 $\text{J}\cdot\text{cm}^{-2}$. (a) transmission image of cells pre illumination. (b) Cetuximab-IR700DX fluorescence (red) and SOSG fluorescence (green) at the start of illumination. (c) Cetuximab-IR700DX fluorescence (red) and SOSG fluorescence (green) at the end of illumination. (d) Zoomed in image of region shown in image c. White bar is 200 μm and black bar is 50 μm . Blue arrows point to examples of blebs formed in response to the PDT treatment. (e) The rate of SOSG-EP fluorescence increase in counts per $\text{J}\cdot\text{cm}^{-2}$ during illumination with either 20 or 150 $\text{mW}\cdot\text{cm}^{-2}$ in scc-U2 (orange) and scc-U8 (violet) cells. Open circles express the weighted mean for a set of images as shown in a-c and the solid bar expresses the weighted mean over 3–5 sets of images. (f) The rate of cetuximab-IR700DX photobleaching in counts per $\text{J}\cdot\text{cm}^{-2}$ during illumination with either 20 or 150 $\text{mW}\cdot\text{cm}^{-2}$ in scc-U2 (orange) and scc-U8 (violet) cells. Open circles express the weighted mean for a set of images as shown in a-c and the solid bar expresses the weighted mean over 3–5 sets of images.

Single fiber reflectance and fluorescence spectroscopy on tumor surface *in vivo*

- Reflectance spectroscopy

Single fiber reflectance spectroscopy was used to monitor the response to PDT in the surface of OSC-19-luc2-cGFP tumors located subcutaneously on the flank of BALB/c nu/nu mice. An example of a collected reflectance spectrum, the fitted spectrum and the scattering background model in absence of absorption is shown in Figure 6a. Blood oxygen saturation (StO_2) in this example was $14.9 \pm 25.6\%$, blood volume fraction (BVF) was $4.2 \pm 0.6\%$ and mean vessel diameter (VD) was $5.6 \pm 0.7 \mu m$.

Figure 6. Reflectance measurements during PDT using different fluence rates. **(a)** Example of a collected reflectance spectrum recorded pre illumination (cyan), fitted spectrum (black solid line), and scattering background (dashed line) to determine StO_2 , BVF, and VD. **(b)** Weighted mean StO_2 determined during PDT at 20 $mW \cdot cm^{-2}$ (blue diamonds), 50 $mW \cdot cm^{-2}$ (red squares), and 150 $mW \cdot cm^{-2}$ (green circles). **(c)** Weighted mean blood volume fraction (BVF) determined during PDT at 20 $mW \cdot cm^{-2}$ (blue diamonds), 50 $mW \cdot cm^{-2}$ (red squares), and 150 $mW \cdot cm^{-2}$ (green circles). **(d)** Weighted mean vessel diameter (VD) determined during PDT at 20 $mW \cdot cm^{-2}$ (blue diamonds), 50 $mW \cdot cm^{-2}$ (red squares), and 150 $mW \cdot cm^{-2}$ (green circles). No significant differences in vessel density and blood volume fraction were observed among tumors in each group.

Figure 6b–d show the weighted mean of four animals for blood oxygen saturation, blood volume fraction and mean vessel diameter during illumination with either 20, 50, or 150 $\text{mW}\cdot\text{cm}^{-2}$. No significant difference between the groups could be determined. During PDT no systematically significant changes occurred, except that blood oxygen saturation gradually decreased. We found no correlation between initial individual values for these vascular parameters and corresponding subsequent tumor growth delay.

- **Fluorescence spectroscopy**

Single fiber fluorescence spectroscopy was used in combination with reflectance spectroscopy to determine the intrinsic IR700DX fluorescence intensity in tumor. Figure 7a shows an example of a fluorescence spectrum recorded during illumination. Fluorescence kinetics during illumination is shown in Figure 7b,c. The total amount of photobleaching, reached at the end of illumination, and the initial rate of photobleaching in the first 10 $\text{J}\cdot\text{cm}^{-2}$ was significantly different between the three fluence rates used. Illumination with 20 $\text{mW}\cdot\text{cm}^{-2}$ to 100 $\text{J}\cdot\text{cm}^{-2}$ resulted in photobleaching to 1.5 ± 0.8 % of the initial fluorescence intensity, however, already after approximately 30 $\text{J}\cdot\text{cm}^{-2}$ the weighted mean fluorescence intensity was not significantly different from zero. Illumination with 50 $\text{mW}\cdot\text{cm}^{-2}$ to 100 $\text{J}\cdot\text{cm}^{-2}$ resulted in photobleaching to $17.7 \pm 11.2\%$ which was significantly more compared to 20 $\text{mW}\cdot\text{cm}^{-2}$ ($p < 0.05$). Illumination with 150 $\text{mW}\cdot\text{cm}^{-2}$ to 100 $\text{J}\cdot\text{cm}^{-2}$ resulted in photobleaching to $51.6 \pm 4.7\%$ of the initial fluorescence which was significantly more than the two other treatments with $p < 0.01$. The presence of fluorescence at the end of illumination with 50 and 150 $\text{mW}\cdot\text{cm}^{-2}$ suggests that these treatments were less effective.

Figure 7. Fluorescence measurements during PDT using different fluence rates. **(a)** Example of a collected fluorescence spectrum recorded during illumination (azure blue) and the IR700DX basis spectrum (black). **(b)** Weighted mean intrinsic fluorescence intensity determined during PDT at 20 $mW \cdot cm^{-2}$ (blue diamonds), 50 $mW \cdot cm^{-2}$ (red squares) and 150 $mW \cdot cm^{-2}$ (green circles). **(c)** Weighted mean of reciprocal of normalized fluorescence during the first 5 $J \cdot cm^{-2}$ delivered at 20 $mW \cdot cm^{-2}$ (blue diamonds), 50 $mW \cdot cm^{-2}$ (red squares) and 150 $mW \cdot cm^{-2}$ (green circles) and their corresponding linear regression line.

The initial photobleaching of cetuximab-IR700DX followed a second order decay as illustrated by the linearity of the reciprocal of the normalized fluorescence, as a function of light fluence (Figure 7c). Illumination with 20 $mW \cdot cm^{-2}$ led to a significantly faster photobleaching rate with respect to the fluence delivered in comparison to 50 and 150 $mW \cdot cm^{-2}$ with $p < 0.05$. Illumination with 50 $mW \cdot cm^{-2}$ led to a faster photobleaching rate with respect to the fluence delivered compared to 150 $mW \cdot cm^{-2}$, but this was not significant for the first 5 $J \cdot cm^{-2}$.

Tumor response to PDT

The mean tumor treatment volume for all treated animals before light exposure was $58.9 \pm 11.4 \text{ mm}^3$ ($n = 17$, range 30.4–77.2 mm^3) and not significantly different among the four groups (ANOVA, $p = 0.07$). Control animals, receiving no drug and no light, showed a mean tumor volume doubling time of $3.4 \pm 0.3\%$ days (Figure 8a).

Figure 8. Effects of PDT using different fluence rates on the growth of OSC-19-luc2-cGFP tumor. **(a)** Relative tumor volume in time after treatment with control (black), 20 mW·cm⁻² (blue diamonds), 50 mW·cm⁻² (red squares), and 150 mW·cm⁻² (green circles). **(b)** Kaplan–Meier plot of the percentage of tumors that didn’t grow to more than 200% of the initial treatment volume after treatment with control (black), 20 mW·cm⁻² (blue), 50 mW·cm⁻² (red), and 150 mW·cm⁻² (green).

In the first few days after PDT treatment it was difficult to measure volumes of treated tumors due to edema in the illuminated area. Illumination with a fluence rate of 20 mW·cm⁻² resulted in crust formation of skin and hemorrhage underneath skin. This was not observed in animals treated with 150 mW·cm⁻².

In the 20 mW·cm⁻² group, two out of four animals showed no tumor after the edema resolved up to 90 days post treatment and were considered a cure. One animal showed a small residual mass up to 50 days post treatment after which this was not palpable for the rest of the observation period, also considered cured. And one animal showed a residual tumor that decreased in volume up to the day 50 post treatment and remained stable around 26% of the initial volume up to the day 90 post treatment. Immuno-histological and H&E staining of the formalin fixed paraffin-embedded (FFPE) tissue showed negative staining for pan-Keratin and CD45, suggesting non-vital tissue (Figure S2). Note that the lack of regrowth of the tumor results in a relative low mean tumor volume in the growth curve of Figure 8a. This data is better visualized using a Kaplan Meier plot of the percentage of tumors that didn’t grow larger than 200% of the initial treatment volume (Figure 8b).

Figure S2. Microscopic images of sections stained with H&E and anti-pan Keratin or anti CD45 immunohistologically. Top panel depicts sections of control, untreated tumor. Middle panel shows sections of residual tissue still palpable 90 days post treatment with $50 \text{ mW}\cdot\text{cm}^{-2}$. And lower panel depicts the sections of residual tissue still palpable 90 days post treatment with $20 \text{ mW}\cdot\text{cm}^{-2}$. Bars represent $100 \mu\text{m}$.

In the $50 \text{ mW}\cdot\text{cm}^{-2}$ group 1 out of 4 animal showed residual tumor that remained stable between 100% and 150% of the initial tumor volume up to 90 days post treatment. H&E staining of the FFPE tissue showed cell rich fibrotic tissue with areas of necrosis and thickened blood vessel walls. Immuno-histological analysis showed negative staining for pan-Keratin and CD45, suggesting this is not vital tissue (Figure S2). The other three animals showed tumor regrowth to more than 200% of their initial treatment volumes within 16.2 ± 2.2 days.

In the $150 \text{ mW}\cdot\text{cm}^{-2}$ group 1 out of 4 animals showed no regrowth of tumor from day 13 to 90 days post treatment and was considered cured. The other three tumors showed regrowth of tumor to more than 200% of their initial treatment volumes within 19.9 ± 7.4 days, not significantly different from $50 \text{ mW}\cdot\text{cm}^{-2}$.

Discussion

We have demonstrated that tumor growth delay and *in vitro* cell survival show a clear dependence between fluence rate and efficacy during EGFR targeted PDT. Significantly less cell survival is observed using a fluence rate of $20 \text{ mW}\cdot\text{cm}^{-2}$ compared to $150 \text{ mW}\cdot\text{cm}^{-2}$ *in vitro* and significant less tumors were cured after illumination with $150 \text{ mW}\cdot\text{cm}^{-2}$ or $50 \text{ mW}\cdot\text{cm}^{-2}$ compared to $20 \text{ mW}\cdot\text{cm}^{-2}$ *in vivo*. It is well known that fluence rate is an important factor for therapeutic outcome of non-targeted PDT [3, 4, 10, 12]. Illumination with a high fluence rate has shown to result in less tissue damage during 5-aminolevulinic acid based protoporphyrin IX PDT [3]. Similar results were reported on tumor growth after Photofrin [28] and 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a mediated PDT [29]. Foster et al. already described in 1991 that oxygen consumption for singlet oxygen formation during a high fluence rate illumination exceeds the rate at which oxygen can be resupplied from the vasculature [30]. Schouwink et al. showed for mTHPC that the median $p\text{O}_2$ significantly dropped in RIF1 tumors during illumination with $100 \text{ mW}\cdot\text{cm}^{-2}$ [31]. Coutier et al. reported for mTHPC that illumination with a fluence rate of 30 and 5 $\text{mW}\cdot\text{cm}^{-2}$ did not change the intratumor $p\text{O}_2$ and resulted in a significant longer tumor growth delay compared to higher fluence rate illuminations [32]. The rate and extent of photobleaching can be used for implicit dosimetry [3, 10-12]. In the present study we observed a slower rate of photobleaching for high fluence rate illuminations of 50 and $150 \text{ mW}\cdot\text{cm}^{-2}$ compared illumination with $20 \text{ mW}\cdot\text{cm}^{-2}$. We also observed less photobleaching for high fluence rate illuminations. Here the effective PDT dose delivered during a high fluence rate illumination is lower than during a low fluence rate. The differences in cell survival, tumor response and cures in the present study are clearly the result of a difference in effective PDT dose delivered, due to the choice of fluence rate. Targeted photosensitizers are potentially sensitive to oxygen depletion since they are designed to deliver the photosensitizer to tumor cells at high concentrations using a targeting molecule that is aimed at overexpressed receptors and usually conjugated with more

than 1 photosensitizer molecule. A recent study reported that the degree of conjugation of IR700DX to cetuximab was approximately 3 [20]. We expect that the clinical formulation of cetuximab-IR700DX will have a similar degree of conjugation. During illumination this results in a high demand for oxygen in those targeted tumor cells that are far from the oxygen supply. High fluence rate illumination might therefore be particularly disadvantageous for targeted PDT using cetuximab-IR700DX.

Although illumination with a low fluence rate of $20 \text{ mW}\cdot\text{cm}^{-2}$ showed significant better responses, the duration of the treatment might be a problem clinically. Fluorescence data showed complete photobleaching long before the end of illumination with $20 \text{ mW}\cdot\text{cm}^{-2}$. From studies investigating 5-aminolevulinic acid mediated PDT it is known that continuing illumination after complete PpIX photobleaching does not lead to an increase in PDT efficacy [33]. We have shown previously, in ALA mediated PDT, that the normalized extent of photobleaching can be used as a predictor of PDT response [3, 33, 34]. It is likely that this also applies for IR700DX and it would be interesting to investigate this, as for clinical implementation shortening the low fluence rate illumination without loss of efficacy would be beneficial.

Cetuximab-IR700DX localizes at the membrane and in the endo-lysosomes of the cell supporting our previous results [23]. In general, photosensitizers accumulated in the endo-lysosomes become aggregated. Upon illumination the endo-lysosomes disrupt, content is released into the cytosol and aggregated photosensitizers become de-aggregated. While the aggregated state of a photosensitizer, in general, has a low quantum yield; de-aggregation of a photosensitizer would lead to an increase in fluorescence and photoactivity [35,36]. Hypothetically, fluorescence intensity would increase shortly after the start of illumination and the higher efficacy of low fluence rate illuminations would be due to a longer illumination time. Our current results, however, show a continuous decrease in fluorescence intensity during illumination, suggesting that this effect is not occurring in EGFR-targeted PDT.

We found no correlation between treatment outcome and vascular parameters monitored during PDT. Blood oxygen saturation, blood volume fraction, and mean vessel diameter did not show any clear changes during illumination at different fluence rates. Cetuximab is a chimeric (mouse/human) monoclonal antibody and therefore not expected to bind to mouse EGFR. Vascular responses due to direct damage of endothelial cells were therefore not expected and the current results show that also indirect vascular responses are not occurring during PDT. We observed hemorrhage at 1 or 2 days post treatment, but this was not related to an increase in blood volume fraction during PDT. This suggests that the majority of vascular responses occur after PDT. This observation is supported by a previous study in which we showed vascular leakage and vasoconstriction after PDT using cetuximab-IR700DX [24]. Our photobleaching results suggest oxygen depletion, but vascular monitoring shows no vasoconstriction. This confirms that the rate of oxygen consumption for the singlet oxygen generation indeed exceeds the rate at which oxygen can be resupplied from the vasculature during high fluence rate illuminations. The limiting factor most likely is diffusion of oxygen from vessels to cells.

Reports in literature on the role of oxygen availability and the formation of singlet oxygen in the response to antibody targeted photosensitizers *in vitro* are inconclusive [18, 19, 22]. Both Mitsunaga et al. [18] and Shirasu et al. [19] showed only partial reduction in effectiveness of antibody-PS mediated PDT in the presence of sodium-azide, a known singlet oxygen quencher. Whereas Kishimoto et al. [22] found complete loss of effectiveness in similar experiments investigating a different antibody-PS conjugate. While we previously reported that scc-U2 and ssc-U8 have different sensitivities to targeted PDT, in the present study we demonstrate that both cell lines show increasing cell death for lower fluence rates. Furthermore, we were able to diminish the effect of treatment completely when illuminated in the presence of NaN_3 , supporting our hypothesis that singlet oxygen is the source of damage in the targeted PDT. Mitsunaga et al. [18] already showed that the capacity of NaN_3 to quench singlet

oxygen was dose dependent, suggesting that the NaN_3 dose used, and probably also the NaN_3 incubation time applied, might play a role in the reported inconsistencies.

Involvement of singlet oxygen in the response to targeted PDT is further confirmed, in the present study, by the observed increase in fluorescence of singlet oxygen sensor green during illumination. Gollmer et al. demonstrated the drawbacks utilizing SOSG as an accurate and reproducible probe for intracellular singlet oxygen [37]. For example, binding of SOSG to a protein in medium could adversely influence a number of processes by which SOSG could enter the cell. For this reason, in the present study, cells were incubated with SOSG in saline. Intracellularly SOSG will bind to proteins but this will not prevent SOSG to react with singlet oxygen to form SOSG-EP and fluoresce, as shown by Gollmer et al. [37]. We performed controls to define the imaging conditions to make sure that SOSG-EP fluorescence increase was due to singlet oxygen formed by illuminated cetuximab-IR700DX. Our high magnification imaging of single cells, or small clusters of cells, showed SOSG-EP fluorescence increase throughout the whole cell and not just localized to the location of cetuximab-IR700DX. Gollmer et al. found that it was difficult to obtain systematic and reproducible results upon irradiation of SOSG-containing cells, some cells showed clearly increases in fluorescence intensity of SOSG-EP, whereas other cells did not [37]. We observed this also in the current study. For this reason, we continued our study imaging a large proportion of a 96 well, containing numerous cells to determine the SOSG-EP fluorescence increase. The increase in SOSG-EP fluorescence during PDT using different fluence rates was, however, not significantly different, suggesting that oxygen availability was not limited during high fluence rate illumination. This is in disagreement with the results on the cell survival where we did see differences between illumination with high or low fluence rates. This discrepancy between results is probably related to differences in experimental circumstances. The cell survival results were obtained with 100 μL medium in each well and the 96-wells plate placed on a shaker to facilitate oxygen diffusion as much as possible. The SOSG-EP fluorescence measurements were performed on static microscopic stage and with only

50 μL of saline in each well. The diffusion of oxygen from air through fluid to cells is highly dependent on the level of fluid on top of the cells [38]. Furthermore, the oxygen tension in water is higher than in medium [39]. While we have no data on cell death after SOSG-EP fluorescence measurements, it might be that the difference in experimental conditions account for the difference in fluence rate effects observed.

Two of the treated tumors, one treated with $20 \text{ mW}\cdot\text{cm}^{-2}$ and another treated with $50 \text{ mW}\cdot\text{cm}^{-2}$ were still palpable after 90 days, but did not regrow. H&E and immunohistological staining of these tissues showed cell rich fibrotic tissue and no sign of inflammation or vital tumor tissue. These two tumors were not considered as unsuccessful treated since it did not grow back to more than 200%, but also not considered as cured. It is unclear why the treated tissue remained present in the mouse and was not cleared. Tissue remaining present at the treated tumor or lymph node sites is occasionally observed in patients treated for head and neck cancer. Establishing that the treatment killed all viable tumor cells is known to be difficult even on excised tissues and possess a clinical issue.

The data presented here show that a low fluence rate used for EGFR-targeted PDT improves the treatment outcome in an immune compromised nude mouse model. It is known that the immune response contributes to the PDT response and that this is higher after low fluence rate compared to high fluence rate illumination [4,29,40]. Although this needs to be confirmed in a model with a functional immune system, it is likely that this is also true for targeted PDT. From a clinical perspective the use of low fluence rate illumination may therefore even be more beneficial.

In summary, we show that the source of damage in EGFR antibody targeted PDT is the generation of singlet oxygen. The effectiveness of targeted PDT is dependent on the fluence rate used. Clinical PDT may benefit from a low fluence rate illumination and might be dosed on the photobleaching of the photosensitizer.

Conclusions

Our results show that the damage in targeted PDT is highly dependent on the fluence rate used. The effectiveness of targeted PDT is, like PDT, dependent on the generation of singlet oxygen and thus on the availability of intracellular oxygen.

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Chapter 5

Photochemical internalisation (PCI)-mediated enhancement of bleomycin cytotoxicity by liposomal mTHPC formulations in human head and neck cancer cells

5

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ABSTRACT

Background and objective: Photodynamic therapy (PDT) with photosensitizers that locate in endocytic vesicles of cancer cells can be exploited to promote the intracellular release of anticancer drugs entrapped in endolysosomal vesicles. This new approach is commonly referred to as Photochemical Internalization (PCI). Here we report on the PCI effects of three different formulations (Foscan, Foslip, and Fospeg) of the clinically approved photosensitizer, meta-tetrahydroxyphenyl chlorin (mTHPC) on the anticancer drug bleomycin (BLM) in the head and neck cancer cell lines.

Materials and methods: Uptake and localization of Foscan, Foslip, and Fospeg in head and neck cancer cells were evaluated by fluorescence spectrophotometry and fluorescence microscopy. Photodynamic efficacy of Foscan, Foslip, and Fospeg were compared with cell proliferation assay. Moreover, PCI effects of Foscan, Foslip, and Fospeg on BLM were compared using protocols in which PDT was applied after or before BLM treatment.

Results: Cellular uptake of Foscan, Foslip, and Fospeg increased in a dose-dependent fashion with consistent higher uptakes of Foslip and Fospeg than Foscan. Fluorescence microscopy showed diffuse intracellular localization pattern for Foscan, Foslip, and Fospeg similar to that of a specific ER probe. However, the subcellular localization pattern of the Rhodamine-labelled same type of pegylated liposomes as Fospeg was similar to that of a specific endolysosomal probe, suggesting that Fospeg uptake appeared to initially proceed via endolysosomal trafficking. Foscan, Foslip, and Fospeg showed no apparent PCI-mediated cytotoxicity when PDT was performed after BLM treatment. However, significantly increased cytotoxicity of BLM ($P < 0.05$) was observed for both Foslip and Fospeg when PDT was performed before BLM treatment. The observed differences of PCI-mediated cytotoxicity between these two timing protocols appear to be related to the differential intracellular trafficking and localization of Foscan, Foslip, and Fospeg.

Conclusion: Liposomal formulations of mTHPC may be candidates for developing mTHPC-based PCI protocols to enhance the efficacy of anticancer drugs entrapped in endolysosomal vesicles.

Introduction

Photodynamic therapy (PDT) of cancer is based on the application of an essentially inactive photosensitizer (PS) followed by its light-mediated activation, resulting in the local production of highly cytotoxic reactive oxygen species [1-4]. Currently, meta-TetraHydroxyPhenyl Chlorin (mTHPC), trademark Foscan[®], is the only clinically approved PS in the European Union, Norway & Iceland for the palliative treatment of patients with advanced head and neck cancer who have failed prior therapies and are unsuitable for radiotherapy, surgery or systemic chemotherapy [5, 6].

Photochemical internalisation (PCI) is a novel approach that exploits PDT for light-enhanced cytosolic release of anticancer drugs that are entrapped in the endo/lysosomal vesicles of cancer cells [7, 8]. The mode of action of PCI is based on the fact that a PS and an anticancer drug may colocalize in the same or proximal endolysosomal vesicles. Light activation of the PS induces damage to the membranes of these vesicles resulting in the release of the entrapped drug into the cytosol, which may then better reach its specific site of action e.g. the cell nucleus. PCI has been shown to potentiate the biological activity of a large variety of therapeutic agents, including the chemotherapeutic agent bleomycin (BLM). Previously, it was reported that the chlorin-based PS "Amphinex" shows potent PCI-mediated enhancement of the antitumor activity of BLM in head and neck cancer patients (University College Hospital London, personal communication).

Foscan is one of the most potent second generation photosensitizers and is activated with non-thermal light at 652 nm four days after intravenous administration. However, its hydrophobic nature may result in an aggregation which reduces its bioavailability and lowers its accumulation in cancer cells. Recently, Foscan has been formulated into both conventional liposomes (hereafter designated by Foslip) and PEGylated stealth liposomes (hereafter designated as Fospeg) in order to improve tumour-selective accumulation and antitumor PDT efficacy [5, 6, 9-11].

It is well-established that drug-loaded liposomes are usually taken up by tumour cells via endo/lysosomal intracellular trafficking pathways. Therefore, we hypothesized that liposomal formulations of Foscan may be exploited for the PCI-mediated enhancement of the antitumor activity of BLM. Here, we present for the first time an *in vitro* study comparing the PCI effects of Foscan, Foslip and Fospeg on the BLM antitumor activity in human head and neck cancer cell lines.

Materials and methods

Chemicals

mTHPC was formulated in a sterile aqueous mixture solution of 20% ethanol, 30% propylene glycol and 50% water as Foscan, in conventional liposomes as Foslip and in PEGylated liposomes as Fospeg. All the 3 formulations of mTHPC were provided by Biolitec Pharma Ltd. (Dublin, Ireland). In control experiments we used Rhodamine-labelled PEGylated liposomes (DPPE) (Avanti Polar Lipids, Alabaster, USA) with the same phospholipid composition as Fospeg. ER-Tracker Green™ and LysoTracker™ Blue DND-167 were from Molecular Probe (Eugene, OR). BLM was purchased from Pharmachemie BV Haarlem, The Netherlands. Stocks of BLM (15 IU in 1.5 ml saline) were freshly prepared for each experiment. The chemical structure of mTHPC and absorption spectrum of Foscan are shown in the Figure 1A.

Cell lines

Head and neck cancer (oral cavity) cell lines scc-U2 and scc-U8 were used [12]. Both cell lines were cultured in RPMI-1640 cell culture medium supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine at 37 °C in a humidified 5% CO₂ atmosphere.

Cell proliferation assay

Cell proliferation was assessed using a commercially available standard MTS kit (CellTiter 96[®] AQueous One Solution Reagent (Promega Corp., Madison, WI) according to the manufacturer's recommendations using a 96-well plate reader (Perkin Elmer Precisely 1420 multilabel counter VICTOR3[™]). It is a colorimetric method based on the cellular conversion of a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium, inner salt, MTS) into a formazan product, which can be detected by 492 nm absorbance. Absorbance measurements were not influenced by mTHPC.

Dark toxicity of mTHPC

Tumour cells were seeded in a 96-well plate at a density of 5,000 cells per well. After a 24-hour of attachment the cells were incubated with mTHPC in one of the formulations at various concentrations up to 1.0 µg/ml for 24 hours in the dark. The survival of the cells was then evaluated by the MTS assay.

Cellular uptake of mTHPC

Tumour cells were seeded in a 24-well plate at a density of 50,000 cells per well. After a 24-hour of attachment the cells were incubated with Foscan, Foslip or Fospeg at various concentrations in the dark at 37°C for 4 hours. In another experiment, the cells were incubated with one of the drugs at the concentration of 0.1 µg/ml at 4°C for 4 hours to see the effect of temperature on the uptake of drugs by cells. The cells were then washed 3 times with phosphate buffered saline (PBS) and cell scrapers were used to make single-cell suspensions of samples. The intracellular amounts of mTHPC in the 3 formulations were measured by recording fluorescence emission wavelength of 652 nm using the Perkin-Elmer LS-5 Luminescence Spectrometer (Norwalk, CT). The excitation wavelength was 420 nm. A 10-nm slit width and a 1.0×0.4 cm² quartz cuvette were used for the measurements.

Subcellular localization of mTHPC

The attached cells in 6-well plates were incubated in 10% FCS RPMI-1640 medium containing 1 µg/ml Foscan, Foslip or Fospeg for 3.5 hours followed by adding 1 µM of an ER probe, ER-Tracker Green™ for 30 min as well as 4 µg/ml of a nuclear probe, Hoechst 33342 for 5 min. After being washed twice with PBS containing 10% FCS, a cover glass was gently put on the cells. The subcellular localization patterns of mTHPC in the 3 formulations were examined by fluorescence microscopy (Nikon Eclipse E800, Nikon, Tokyo) with a 100 W halogen lamp. In addition, the cells were incubated with Rhodamine-DPPE (1 µg/ml) for 3.5 hours followed by a fluorescent lysosomal probe, LysoTracker™ Blue DND-167 (1 µM) for 30 min. Fluorescence images were made with a highly light sensitive thermo-electrically cooled charge coupled device (CCD) camera (ORCAII-ER, Hamamatsu, Japan). The filter combinations consisted of a 380-420 nm excitation filter, a 430 nm beam splitter and a 590 nm long-pass emission filter for mTHPC in various formulations; a 465-495 excitation filter, a 505 nm beam splitter and a 515-555 nm band-pass emission filter for ER-Tracker Green™; a 330-380 nm excitation filter, a 400 nm beam splitter and a 420 nm long-pass emission filter for Hoechst 33342; a 530-550 nm excitation filter, a 565 nm beam splitter and a 575-630 nm band-pass emission filter for Rhodamine-DPPE; and a 330-380 nm excitation filter, a 400 nm beam splitter and a 420 nm long-pass emission filter for LysoTracker™ Blue DND-167.

PDT treatment protocols

After incubation of cells in the 96-well plates with mTHPC in one of the formulations at various concentrations in the darkness for 1, 4 or 24 hours the cells were washed with medium once before being illuminated using a diode laser (CeramOptec GmbH, Bonn, Germany) emitting at 652 nm, the second major absorption peak of mTHPC as shown in the Figure 1A. The light was delivered at a fluence rate of 1 mW/cm² and fluence of 1 J/cm². Five days after PDT treatment the cell survival was measured with the MTS assay.

Cytotoxicity of BLM

The cells were seeded in the 96-well plates at a density of 5,000 cells per well. After a 24-hour attachment the cells were incubated with different concentrations of BLM for 1 or 4 hours in the darkness followed by washing once with medium and incubating the cells for 1, 3 or 5 days. The cell survival was then measured by the MTS assay.

PCI treatment protocols

Two protocols were used in the PCI experiments. In the conventional 'light after BLM' protocol, the cells were incubated with Foscan, Foslip or Fospeg in the darkness for 3 hours followed by adding BLM (0.002 IU) for another hour. The cells were then washed with medium once before being exposed to the laser. In the 'light before BLM' protocol, the cells were incubated with Foscan, Foslip or Fospeg in the darkness for 4 hours and then washed once with the medium before being irradiated. Immediately after light exposure the cells were incubated with BLM for 1 hour and then washed once with medium. The same fluence rate (1 mW/cm²) and fluence (1 J/cm²) were used for both protocols. Five days after PCI treatments the cell survival was measured with the MTS assay. Furthermore, the degree of interaction between PDT and BLM was examined by the method (13) and is given by

$$\alpha = SF^a \times SF^b / SF^{ab}$$

where SF^a and SF^b represent surviving fractions with PDT and BLM respectively; SF^{ab} is the surviving fraction after combined treatments. In this calculation, $\alpha = 1$ indicates an additive effect, $\alpha > 1$ is a synergistic effect; while $\alpha < 1$ is an antagonistic effect.

Statistical analysis

The results were analysed (SigmaPlot® 11; Systat software, Inc) using the unpaired Student *t*-test for data with normal distribution; otherwise, the Wilcoxon signed-rank test was used. The differences were considered statistically significant when *p* value < 0.05.

Results

Dark toxicity of Foscan, Foslip and Fospeg

Figure 1B shows no dark toxicity of Foscan on the cells after incubation for 24 hours at the concentrations up to 1 $\mu\text{g/ml}$.

A

B

Figure 1. A, Absorption spectrum of Foscan with 2 major peaks at 420 and 652 nm. The chemical structure of mTHPC is also included. **B**, Dark toxicities of Foscan, Foslip and Fospeg on the scc-U8 cells *in vitro*. Each data point represents the mean of three separate experiments with at least 5 samples per experiment. The bars are SD.

Cellular uptake of Foscan, Foslip and Fospeg

The cellular uptake of mTHPC in the 3 formulations was studied by fluorescence spectrophotometry after incubation for 4 hours at various concentrations. The Figure 2A shows the kinetic uptake patterns of the 3 formulations of mTHPC in the scc-U2 and scc-U8 cells. Generally, the cellular uptake was increased with increasing concentrations of a drug, but both Foslip and Fospeg were taken up significantly ($p < 0.05$) more than Foscan at the concentration 1.0 $\mu\text{g/ml}$. Interestingly, when the cells were incubated with these drugs at 4°C for 4 hours, the cellular uptake of Foscan was not much affected, but the uptake of Foslip and Fospeg was significantly inhibited (Figure 2B). This finding suggests that Foscan may largely be taken up by a passive

diffuse mechanism; while the uptake of Foslip and Fospeg is at least partly via energy-dependent endocytosis pathway.

B

Figure 2. A, Uptake patterns of Foscan, Foslip and Fospeg in the scc-U2 and scc-U8 cells after incubation with various concentrations for 4 hours. **B**, Uptake of Foscan, Foslip and Fospeg by the scc-U8 cells after incubation for 4 hours at 4°C or 37°C (normalized to 1 for the cellular uptake at 4°C). Each data point represents the mean of 3 separate experiments with at least 3 samples per experiment. The bars are SD.

Intracellular localization of Foscan, Foslip and Fospeg

The subcellular localization patterns of mTHPC in the 3 formulations were studied by fluorescence microscopy after a 4-hour incubation in the scc-U2 and scc-U8 cells. A diffuse pattern of all the 3 drugs was seen in the cells, a similar pattern to that of ER-Tracker (Figures 3A & 3B), indicating that all drugs were localized in the ER of cells. However, a granular pattern of the fluorescently labelled Rhodamine-DPPE with the same type of pegylated liposomes of Fospeg was found in the cells with a similar pattern of a LysoTracker probe (Figure 3C), suggesting that Fospeg, was initially taken up through the endo/lysosomal pathway, followed by a diffuse process from the endo/lysosomes to ER membrane structures. Similar intracellular localization patterns of Foscan, Foslip and Fospeg were seen in both scc-U2 and scc-U8 cells.

Figure 3. Subcellular localization patterns of Foscan, Fospeg, ER-Tracker, Rhodamine-DPPE, LysoTracker in the scc-U8 cells. The detailed method is described in the section of Materials and Methods. **A**, Fluorescence images of Foscan (a), ER Tracker (b), merged a/b images (c) together with Hoechst 33342 fluorescent image; and phase-contrast image (d). **B**, Fluorescence images of Fospeg (a), ER Tracker (b), merged a/b images (c) together with Hoechst 33342 fluorescent image; and phase-contrast image (d). **C**, Fluorescence images of Rhodamine DPPE (a), LysoTracker (b), merged a/b image (c) and phase-contrast image (d).

PDT treatment protocols

The cells in the 96-well plates had been incubated with one of the 3 drugs for 1 (Figure 4A), 4 (Figure 4B), or 24 (Figure 4C) hours before being exposed to light irradiation. The concentrations used for PDT were decreased with increasing incubation times, as a longer incubation time was expected to cause a higher uptake

of a drug by cells. PDT with Foslip and Fospeg at the concentrations of 0.5 and 1.0 $\mu\text{g/ml}$ for a 1-hour incubation shows a significantly higher cell-killing effect than Foscan (Figure 4A) in both scc-U2 and scc-U8 cell lines ($p < 0.05$). Similar results were also obtained after PDT with a 4-hour or 24-hour drug incubation (Figures 4B & 4C), indicating that Foslip and Fospeg are more powerful photosensitizers at killing cancer cells after light irradiation.

A

B

C

Figure 4. PDT of scc-U2 and scc-U8 cells with Foscan, Foslip or Fospeg at various concentrations for a 1-hour (A), 4-hour (B) or 24-hour (C) incubation. The cell survival was measured by MTS assay. Each data point represents the mean of 2 to 4 separate experiments with at least 5 samples per experiment. The bars are SD.

Cytotoxicity of BLM

Figure 5A shows the toxicities of BLM on the cells. Interestingly, there was a huge difference in such cytotoxicities, depending on the length of time after BLM treatment. When the cell survival was measured on day-1 and day-3 after a 1-hour BLM incubation at the concentrations of 0.1 and 0.3 IU, there was not much difference in BLM cytotoxicities. However, on day-5 some cells were killed even at a 100- or 150-fold lower concentrations of 0.001 and 0.002 IU, indicating that BLM needs at least 5 days to exert its cytotoxic effect. However, no difference was found in the cytotoxicity when the cells were treated with BLM at the concentrations of 0.0005 to 0.002 IU for 1 and 4 hours (data not shown). Figure 5B demonstrates the cell survival on day-5 after a 1-hour treatment of BLM at low concentrations. Increasing BLM doses from 0.0005 to 0.002 IU does not significantly enhance the cytotoxic effect on the cells. It appears that scc-U2 cells are more sensitive than scc-U8 cells to BLM

A

B

Figure 5. Cytotoxicities of BLM. **A**, The scc-U8 cells were treated with BLM for 1 hour at various concentrations as indicated. The cell survival was measured on day-1, day-3 and day-5 after treatment. **B**, The cells were treated with BLM for 1 hour at various concentrations as indicated and the cell survival was measured on day-5 after treatment. Each data point represents the mean of 3 separate experiments with at least 5 samples per experiment. The bars are SD.

5

PCI-mediated enhancement of BLM cytotoxicity

PCI effects of BLM mediated with Foscan, Foslip or Fospeg were studied. Proper parameters affecting the efficacy of PDT (e.g. sensitizer and light doses) as well as of BLM (e.g. concentration) were chosen to obtain a suboptimal effect of cell killing after individual treatments, so that it made it easier to observe a PCI effect after a combined treatment. Figure 6A shows no significant enhancement of the BLM effect on cell killing by PDT with one of the 3 drugs ($p > 0.05$) when the conventional 'light after BLM' protocol was used. However, when the cells were treated with the 'light before BLM' protocol, PDT with Foslip or Fospeg did significantly increase the effect of BLM on the cell killing (Figure 6B). Figure 6C clearly demonstrates that the 'light before BLM' protocol with both liposomal formulations of mTHPC at the concentration of 0.05 $\mu\text{g/ml}$ induced a PCI effect of BLM on the cells. Furthermore, figure 7 exhibits the degree of interactions between PDT and BLM treatment. All alpha values of Foslip and

Fospeg with the 'light before' protocol are higher than 1; while none of all 3 drugs produced the alpha values larger than 1 after the treatments with the 'light after' protocol. These results indicate that the 'light before' protocol can generate a synergistic effect on cell killing. No interaction between BLM and laser light was seen.

A

B

C

Figure 6. PCI of BLM on the cells with the 'light after BLM' protocol (**A**) or 'light before BLM' protocol (**B**) and comparison the 'light after BLM' protocol and 'light before BLM' protocol (**C**). The concentrations of photosensitizers were indicated in **A** and **B** and 0.05 $\mu\text{g/ml}$ in **C**. The concentration of BLM was 0.002 IU; while the light dose was 1 J/cm^2 . The cell survival was measured on day-5 after treatment. Each data point represents the mean of 2 to 4 separate experiments with at least 5 samples per experiment. The bars are SD.

Discussion

Foscan, Foslip and Fospeg demonstrate no dark toxic effect on the cell survival at the concentrations up to 1 $\mu\text{g/ml}$ (Figure 1B). The uptake study shows that the amounts of intracellular mTHPC in the 3 formulations increase with increasing concentrations (Figure 2A). However, Foslip and Fospeg were significantly taken up more than Foscan. A similar finding has recently been reported with a higher cellular uptake of Fospeg than Foscan in a human prostate cancer cell line [14]. At 4°C the uptake of Foscan by the cells was not apparently affected; while that of Foslip and Fospeg was significantly inhibited (Figure 2B). These results may indicate that Foscan is taken up through a passive diffuse mechanism; whereas Foslip and Fospeg via endocytosis pathway, a well-known phenomenon that liposomes are generally taken up by cells via endocytosis. The subcellular localization of Fospeg (Figures 3B, 3C) also supports this finding with a granular pattern of the fluorescently labelled Rhodamine-DPPE similar to that of the LysoTracker, a lysosomal probe, in the cells (Figure 3C); as DPPE has the same phospholipid compositions as Fospeg.

Efficacy of PDT of cancer cells depends largely on the amount and localisation of a photosensitizer in the cells. Such intracellular amount is related to the concentration of the photosensitizer used and the length of its incubation with cells. In this study 3 different incubation times with various photosensitizer concentrations from 0.1 to 1 $\mu\text{g/ml}$ for a 1-hour incubation, 0.01 to 0.1 $\mu\text{g/ml}$ for a 4-hour incubation and 0.0025 to 0.01 for a 24-hour incubation were used. After light irradiation via a laser at the fluence of 1 J/cm^2 , both cell lines were killed with different extents. Foslip and Fospeg killed significantly more cells than Foscan did ($p < 0.05$) (Figures 4A, 4B, 4C), suggesting that Foslip and Fospeg may be more effective candidates for further development of mTHPC-based PCI treatment.

BLM with a molecular weight of 1.5 kDa, initially discovered by Umezawa et al [15], is a glycopeptic antibiotic. It is toxic to eukaryotic cells because of its ability to bind and degrade DNA [16]. The present study has shown that BLM needs at least 5 days

to effectively exert its cytotoxic effect (Figures 5A & 5B). For a period of shorter than 5 days BLM still has a toxic effect, but requires at least a 100-fold higher concentration. Furthermore, the toxicities of BLM on the cells do not increase with increasing concentrations from 0.0005 to 0.002 IU (Figure 5B), suggesting that BLM might be entrapped in the endosomes and lysosomes of the cells and degraded by bleomycin hydrolases before reaching its targets in the nucleus. This is in good agreement with a previous finding that BLM was taken up by endocytosis leading to an inefficient cytotoxic effect [17].

The conventional 'light after BLM' PCI protocol did not increase the cytotoxicity of BLM by PDT with Foscan (Figure 6A). This may be due to the fact that Foscan localizes in the membraneous structures rather than endo/lysosomes. However, surprisingly, such 'light after BLM' protocol with Foslip or Fospeg (Figure 6A) did not induce a PCI effect either, even though both drugs are supposed to be at least partly taken up by the cells via endocytosis. When the 'light before BLM' protocol was used, PDT with Foslip or Fospeg did significantly enhance the effect of BLM on the cell killing in both cell lines (Figure 6B), indicating a clear PCI effect of BLM (Figure 6C). Analyses of the degree of interactions between PDT with Foslip or Fospeg and BLM show a synergistic effect of the combined two modalities in the case of 'light before' protocol, but not in the case of the 'light after' protocol (Figure 7). Although the reason for this finding is not known, similar results were also reported by others [18], who hypothesized that the endo/lysosomes damaged by initial PDT further fused and ruptured the BLM-containing vesicles of endo/lysosomes to release BLM into cytosol and nucleus, resulting in cell death. This is well consistent with our results of an initial endo/lysosomal localization of Fospeg (Figures 3B & 3C) where these endo/lysosomes are supposed to be targeted after light exposure. Such explanation is also supported by a recent report demonstrating that mTHPC in the Fospeg was moved away from the PEGylated liposomes to the membrane structures of ER and Golgi apparatus after an initial uptake of Fospeg by the cells via endocytosis [19]. Since photochemical treatment can potentially damage light-sensitive anticancer drugs, the major

advantage of using the 'light before BLM' protocol over the conventional 'light after BLM' protocol may avoid such destruction of those light-sensitive and biologically active molecules during PCI treatment.

Figure 7. The degree of interactions (α) between PDT and BLM treatment. The conditions of treatments are the same as described in the figure 6C.

Conclusion

The PCI effects of BLM were compared among 3 formulations of mTHPC in two human Head and Neck cancer cell lines scc-U2 and scc-U8 *in vitro*. These included mTHPC in an aqueous formulation as Foscan, in conventional liposomes as Foslip and in PEGylated liposomes as Fospeg. Foscan was taken up by the cells largely via a passive diffuse manner; while Foslip and Fospeg might initially go through endocytosis pathway followed by a diffuse from endo/lysosomal vesicles to ER. PDT with the 3 formulations did not enhance the cytotoxicity of BLM in both cell lines when it was performed after BLM treatment. However, PDT with Foslip or Fospeg did significantly enhance the toxicity of BLM on these cell lines when it was performed before BLM administration. The results of this study suggest that the liposomal mTHPC would be good candidates for further development of mTHPC-mediated PCI of human head and neck cancer.

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Chapter 6

Photochemical internalisation (PCI) of gelonin by EGFR-targeted photosensitiser delivery for treatment of human squamous cell carcinoma cells

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ABSTRACT

Background and objective: Photochemical internalisation (PCI) is a PDT-based therapeutic modality that has shown promise for enhancing intracellular delivery of biologically active molecules including proteins, genes and chemo-therapeutics. However, the specificity of current PCI protocols appears suboptimal. The aim of this study was to explore the possibility of using a targeted PDT with the cetuximab-IR700DX conjugate to induce a PCI effect of the biologically active molecule, gelonin, on the human A431 squamous cell carcinoma cells *in vitro*.

Materials and methods: Subcellular localisation of the cetuximab-IR700DX conjugate was studied by the DeltaVision OMX 3D-SIM super-resolution imaging system in the A431 human squamous cell carcinoma cells incubated with the conjugate for 1- or 24-h in the darkness. The lysosomal-associated membrane protein 1 (LAMP-1) was also imaged with its antibody to see if it matches the localisation pattern of conjugates. For the targeted PDT with the conjugate and the targeted PDT-mediated PCI with gelonin, the cell viability was measured by the MTT assay. In the PCI case the targeted PDT was used before or after the gelonin application. The PCI effects with the two protocols on cell viability were compared.

Results: The cetuximab-IR700DX conjugate was localised primarily on the plasma membrane of the cells after 1-h incubation. After 24-h of incubation the conjugate was found intracellularly in a granular pattern in addition to the localization close to the plasma membrane. The granularly located conjugate was partly located close to LAMP-1. The targeted PDT with the conjugate killed the cells, but with the help of gelonin the targeted PDT killed significantly more cells ($p < 0.05$). Such a PCI effect occurred in both cases of light exposure given before and after addition of gelonin, but the treatment protocol with the light irradiation before gelonin administration was found significantly more cytotoxic than that with the light given after gelonin application ($p < 0.05$) at both conjugate concentrations. The results suggest that photoactivation of IR700DX of the conjugate has led to rupture endosomal and lysosomal vesicles to release endo/lysosomal-localising gelonin into the cytosol of cells, thus producing a PCI effect.

Discussion: The results of this study indicate for the first time that the targeted PDT with cetuximab-IR700DX conjugate is able to induce a PCI effect with gelonin on the tumour cells *in vitro*. Such approach may have the advantage of enhancing a specificity of a PCI effect with antibody-mediated more specific binding of IR700DX to EGFR on the plasma membrane and further to endo/lysosomal membranes (via phagocytosis) of tumour cells. This finding is promising, but preliminary and needs to warrant further studies in *in vitro* and *in vivo* biological models.

Introduction

Photochemical internalisation (PCI) is a modality that uses the principle of PDT for destruction of endo/lysosomal membrane to induce cytosolic release of biologically active molecules (or drugs) that are entrapped in the endolysosomal vesicles of diseased (e.g. cancer) cells (1-6). This treatment is currently undergoing evaluation in clinical trials (7). The induction of a PCI effect is largely based on a photosensitizer (e.g. TPCS2a or ALPcS2a) with an amphiphilic chemical property that favors incorporation into the lipid bilayer of cell membranes, but unable to penetrate through the membranes due to the sulfonate groups. Such photosensitizers will enter the cells through adsorptive endocytosis and localize in the inner leaflet of the endocytic compartments.

Cetuximab is a monoclonal antibody with specificity for the epidermal growth factor receptor (EGFR) that is highly expressed on the cytoplasm membrane of many types of cancer cells including head and neck cancer. When cetuximab is used to conjugate the photosensitizer IR700DX, the conjugate of cetuximab-IR700DX initially binds to the EGFR on the plasma membrane, then moves to the membrane of or inside endosomal and lysosomal vesicles during endocytosis. Finally, photochemical reaction of the conjugated IR700DX with light exposure damages the membranes of endosomal and lysosomal vesicles to release those entrapped biologically active molecules. Since cetuximab provides highly specific EGFR targeting in the membraneous structures, the conjugate can thus facilitate a specific delivery of IR700DX to the membrane of endolysosomes. This may lead to an improvement of the specificity of the PCI effect.

The aim of this study was to explore the possibility of using a targeted PDT with the cetuximab-IR700DX conjugate to induce a PCI effect on a biologically active molecule, gelonin, on the human A431 squamous cell carcinoma cells *in vitro*.

Materials and methods

Cetuximab-IR700DX conjugate

IR700DX is a phthalocyanine-type photosensitiser; while cetuximab is a clinically used antibody against human cancer with the expression of EGFR.

Cell line

Human squamous cell carcinoma cell line of the skin, A431 was used in this study. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Invitrogen, UK) supplemented with 10% foetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine (PAA, Germany) at 37°C in a humidified 5% CO₂ atmosphere.

Subcellular localization of the cetuximab-IR700DX conjugate

A total of 2.5×10^5 A431 cells were seeded on a 0.25 mg/ml poly-lysine coated 24-mm cover slide in each well of 6-well plates. After 24-h incubation for attachment, the cells were incubated in 10% FCS DMEM medium containing 40 µg/ml of cetuximab-IR700DX conjugate in the darkness for 1- or 24-h. The cells were washed once with the medium before being fixed with 4% formaldehyde (Ultra Pure EM Grade, Polysciences Inc) in PBS (10 minutes at room temperature). The fixed cells were permeabilized with 0.05% saponin in PBS and stained with a mouse anti-LAMP1 antibody (mouse monoclonal H4A3, Developmental Studies Hybridoma Bank), a donkey anti-mouse antibody labelled with Alexa Fluor 488 (Jackson ImmunoResearch Laboratories, Cambridgeshire, UK), and Hoechst33342. The cells were then mounted on object slides with SlowFade Diamond Antifade Mountant (ThermoFisher). The cells were imaged on a Deltavision OMX V4 microscope (Cytiva) using an Olympus ×60 NA 1.42 Plan Apochromat objective, an InSightSSI™ widefield illumination module and three cooled sCMOS cameras. Three-channel images including z-stacks (0.25 µm section spacing) covering the whole cells of interest were recorded. The fluorescence was detected

through the band-pass filters for Hoechst (Ex. 396/29 nm, Em. 436/31 nm), AF488 (Ex. 477/32 nm, Em. 528/48 nm) and IR700DX (Ex. 646/15 nm, Em. 683/40 nm). Raw images were deconvolved and aligned using the Softworx software (GE/Cytiva). Illustrations were maximum intensity projections of z-stacks and prepared using the Fiji/ImageJ software.

PDT treatment of tumour cells

The A431 cells (8×10^3) were seeded in each well of a 96-well plate. After 24-h incubation for attachment, the cells were incubated with 1 or 2 $\mu\text{g/ml}$ of the cetuximab-IR700DX conjugate in the darkness for 24-h. The cells were then washed with medium twice before being illuminated using an in-house built 690 nm LED lamp. The lamp has a fluence rate of 17 mW.cm^{-2} and the light was delivered at a fluence of 7 J.cm^{-2} . Twenty-four hours after PDT treatment the cell viability was measured with the MTT assay as described below.

PCI treatment of tumour cells

Two protocols were used in the PCI experiments. In the conventional 'light after' protocol, the cells were incubated with 1 or 2 $\mu\text{g/ml}$ of the cetuximab-IR700DX conjugate in the darkness for 20-h followed by adding gelonin ($0.5 \mu\text{M}$) for another 4-h. The cells were then washed with medium twice before being exposed to a 690 nm LED lamp. In the 'light before' protocol, the cells were incubated with 1 or 2 $\mu\text{g/ml}$ of the cetuximab-IR700DX conjugate in the darkness for 24-h and then washed with the medium twice before being irradiated. Immediately after light exposure the cells were incubated with gelonin for 4 hours and then washed with medium. The same light fluence rate (17 mW.cm^{-2}) and fluence (7 J.cm^{-2}) were used for both protocols. Twenty-four hours after PCI treatments the cell viability was measured with the MTT assay.

Cell viability assay

The cell viability was measured by means of the MTT assay. At 24-h after treatment the culture medium was removed and 100 μ l of 0.25 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma) dissolved in fresh medium was added. After 2-h incubation the medium was removed and the cells and formazan crystals were solubilized in 100 μ l dimethyl sulfoxide. The plates had been shaken for 15-min before absorbance was measured at 570 nm using a plate reader (Original Multiscan EX Labsystems, Global Medical Instrumentation, Ramsey, MN, USA). The cell viability is presented relative to untreated control cells.

Statistical analysis

The results were analysed (SigmaPlot-11; Systat software, Inc) using the unpaired Student t-test. The differences were considered statistically significant when p value < 0.05.

Results and discussion

The subcellular localisation patterns of the cetuximab-IR700DX conjugate in the A431 human squamous cell carcinoma cells after incubation for 1- or 24-h (40 μ g/ml) were, for the first time, investigated by the DeltaVision OMX super-resolution imaging system with a triple staining of cetuximab-IR700DX (Red), LAMP-1 (Green) for specific endo/lysosomal membrane and Hoechst (Blue) for nucleus. Figure 1 demonstrates the cellular localisation of cetuximab-IR700DX conjugate and its colocalisation with LAMP-1 at 1- and 24-h after cetuximab-IR700DX incubation. The conjugate appeared mainly located on or close to the plasma membrane of cells at 1-h after administration with little, if any, colocalisation with LAMP-1. After 24-h incubation the cetuximab-IR700DX conjugate was still located to a large extent on the plasma membrane, but many red spots from the conjugate were also found in the cytosol of cells, partly colocalizing with the LAMP-1. These results clearly indicate that the cetuximab-IR700DX conjugate

is located in endo/lysosomal vesicles besides plasma membrane after 24-h incubation probably via endocytosis. This endolysosomal localisation of the conjugate provides a biological basis for inducing a PCI effect when a biologically active endocytosed molecule is combined with PDT treatment. LAMP-1 is found primarily in lysosomes and not in the early endosomes, which may indicate that a fraction of the conjugate is localized in early endocytic vesicles.

Figure 1. Subcellular localisation of the cetuximab-IR700DX conjugate in the A431 tumour cells. Triple staining was done with cetuximab-IR700DX conjugate (1-h or 24-h incubation at 40 $\mu\text{g/ml}$ (Red), LAMP-1 (Green) for endo/lysosomal membrane and Hoechst (Blue) for nucleus. The images were made by the DeltaVision OMX super-resolution imaging system. They show the cetuximab-IR700DX conjugates localised on the surface of tumour cells after 1-h incubation (left) and some red granules of the conjugates inside of green endo/lysosomal vesicles after 24-h incubation (middle and right). Bars: 10 μm for the left and middle images and 2 μm for the right image.

Figure 2 shows the A431 cell viability after the treatment with the cetuximab-IR700DX-based targeted PDT or with the targeted PDT-mediated PCI using a biologically active molecule, gelonin, *in vitro* as a function of the conjugate concentrations. The targeted PDT killed about 50% and 90% of the cells when 1 $\mu\text{g/ml}$ and 2 $\mu\text{g/ml}$ of the conjugate were applied, respectively. Interestingly, when in combination with gelonin the targeted PDT killed significantly more cells than the targeted PDT alone ($p < 0.05$), indicating a clear induction of a gelonin-based PCI effect. Such a PCI effect occurred in both cases of light exposure given before and after addition of gelonin, but the treatment protocol with the light irradiation before gelonin administration (Figure 2, Left) was found significantly more cytotoxic than that with

the light given after gelonin application ($p < 0.05$) at both conjugate concentrations (Figure 2, Right). A similar finding was also reported by others (8) as well as by us (Chapter 5 of this Thesis). The results suggest that photoactivation of IR700DX of the conjugate has led to rupture endosomal and lysosomal vesicles to release endo/lysosomal-localising gelonin into the cytosol of cells, thus producing a PCI cytotoxic effect.

Figure 2. Cell viability after targeted PDT-induced gelonin-based PCI. 8×10^3 A431 cells were seeded in each well of a 96-well plate. After attachment for 24-h the cells were incubated with the conjugate at various concentrations as indicated for 24 h before the light exposure (7 J.cm^{-2} ; 17 mW.cm^{-2}). Gelonin at a concentration of $0.5 \text{ }\mu\text{M}$ was given for 4 h before or after light.

In conclusion, the targeted PDT may have an advantage of enhancing the specificity of the PCI effect due to the specific binding of IR700DX to EGFR of tumour cells via the cetuximab antibody. The targeted PDT-mediated PCI may eventually create a minimally invasive and highly efficient modality with a triple-model selectivity of a specific binding of a sensitizer to tumour cells via a targeting molecule, release of a biologically active molecule and site-directed light activation. Although this study demonstrates, for the first time, the induction of a PCI effect by the targeted PDT, the current *in vitro* results are preliminary and warrant further studies in *in vitro* and *in vivo* biological models.

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Chapter 7

General discussion and future perspectives

A high incidence of head and neck cancer with inadequate selectivity and specificity of standard treatments leads to high morbidity and mortality of patients (Ferlay et al., 2015). Alternative modalities are therefore required to improve the selectivity and specificity. The concept of conventional PDT is generally attractive, as the combination of a tumour-localising photosensitizer with selective light delivery should provide a good therapeutic contrast to target tumour areas with surrounding normal tissue less damaged (Dolmans et al., 2003; Agostinis et al., 2011; van Straten et al., 2017). Such a minimally invasive approach with good cosmetic effects is ideally suitable for the treatment of lesions in the head and neck region with anatomic complexity. The second-generation photosensitizer, temoporfin, approved by EMA for the PDT treatment of head and neck cancer (de Visscher et al., 2013), lacks the selectivity and causes toxicity in normal tissues.

Targeted PDT conjugates a photosensitizer with a monoclonal antibody for specifically targeting molecules that are highly expressed in tumour cells. Such dye/antibody/light-based targeted PDT has attracted much attention during the past years. Since EGFR has been found to be over-expressed in several types of cancer including head/neck squamous cell carcinoma, a number of EGFR-targeting drugs have been developed. Although EGFR monoclonal antibodies have been used in preclinical and clinical studies, these drugs have considerable limitations with their toxicity and resistant development (Roe et al., 2006; Wheeler et al., 2008; Bardelli et al., 2010). Alternative treatment strategies to maximize the therapeutic effectiveness of EGFR as a molecular target are needed. Cetuximab, a chimeric (mouse/human) monoclonal antibody against EGFR, is the only FDA fully approved drug for the human cancer treatment, although trastuzumab has recently been approved by FDA for the treatment of HER2-positive breast cancer. Phthalocyanines, a family of potent photosensitizers with their favourable properties of chemical stability, high fluorescence quantum yield and red-shifted light absorption (optimal tissue penetration), are already used in PDT of cancer patients in Russia (Sokolov et al., 1996).

It is tempting to investigate the targeted PDT of human head and neck tumour with cetuximab conjugated to the commercially available phthalocyanine, IR700DX.

This thesis studied the effects of targeted PDT on human head and neck cancer cell lines *in vitro* with the cetuximab-IR700DX conjugate. In **Chapter 2** the killing effect of targeted PDT is not correlated with the amount of EGFR expression in tumour cells. For example, scc-U8, scc-U2 and OSC-19 cell lines had a similar level of EGFR expression on the cell membrane, but they responded significantly different to the targeted PDT. Scc-U2 and OSC-19 cell lines were significantly less sensitive to the targeted PDT than scc-U8 cells with a light dose of 7 J.cm^{-2} (5 mW.cm^{-2}). However, with a higher light dose of 15 J.cm^{-2} (20 to 100 mW.cm^{-2}) the killing effects of these two less sensitive cell lines were increased (Figure 1 in **Chapter 4**). Such different cellular sensitivities to the targeted PDT cannot simply be explained by cellular necrosis due to targeted PDT-mediated destruction of cytoplasm membrane. Generally, cellular sensitivity to PDT is mainly related to the features of cells and PDT protocols (Casas et al., 2011). Among the features, besides drug efflux transporters, the susceptibility to apoptosis plays an important role. Inactivation of pro-apoptotic factors or over-expression of anti-apoptotic factors are the main pathways involved (Aniogo et al., 2019). For example, the Bcl-2 family of apoptosis proteins often acts as a central decision in the apoptosis pathway. Cell lines that over-express Bcl-2 and Bcl-xL have been shown to be more resistant to PDT (Granville et al., 1999; Ichinose et al., 2006; Usuda et al., 2008), indicating that the levels of those factors may determine the sensitivity of tumour cells to apoptosis and also to overall PDT-mediated cell killing (Usuda et al., 2003). In the present thesis a significantly higher expression of Bcl-2 and Bcl-xL was found in the scc-U2 cells than scc-U8 cells. This may explain the lower level of response of the scc-U2 cells to the targeted PDT treatment. These results may suggest that various approaches to overcome this targeted PDT resistance may include the use of agents to minimize the efflux pumps of cells, to block anti-apoptotic factors and to activate pro-apoptotic proteins.

In **Chapters 3** and **4** a dorsal skin-fold window chamber tumour model in combination with confocal imaging technologies was used to quantitatively study in real-time the fluorescence-based pharmacokinetics and bio-distribution of the cetuximab-IR700DX conjugate in the human OSC-19 head and neck tumour and its surrounding normal tissues in living athymic nude mice. With this technique it was possible to visualise the dynamic patterns of normal and tumour vascular responses to the targeted PDT. Such studies would help to optimize parameters affecting the targeted PDT efficacy. The results of **Chapter 3** with a more uptake of the conjugate by tumour tissue than surrounding normal tissues at 24-48 h after intravenous administration are consistent with the drug-light interval of 24 h in the recently ongoing clinical trial of the targeted PDT in head and neck cancer patients (<https://clinicaltrials.gov/ct2/show/NCT03769506>).

Generally, a conventional PDT with a raw photosensitizer causes structural destruction of vessels in a tumour due to the fact that the free sensitizer is non-specifically localized in the vascular structure after its administration. The directly visual evidence with the microscopic imaging system in **Chapter 3** has confirmed no damage to the vascular structures by the targeted PDT. This finding is further supported by no expression of HIF-1 α in the tumour cells. The little effect of the targeted PDT on the vessels may be due to no binding of the conjugate to the vascular wall. As a result, no hypoxic condition in the tumour is generated and the efficacy of the targeted PDT may thus be improved with sufficient oxygen molecules.

Biological effects of PDT result from interactions between a photosensitizer and light in the presence of oxygen. The cytotoxic agents produced are ROS, $^1\text{O}_2$ in particular. Since $^1\text{O}_2$ has a short lifetime in cells and tissues with the radius of its action only about 10-20 nm, PDT acts only on targets with high concentrations of a photosensitizer (Moan and Berg, 1991). The selective PDT of tumour relies thus on the selectivity of tumour localization of the photosensitizer. With the microscopic imaging system **Chapter 3** was the first report to quantitatively confirm the colocalization of the conjugate and GFP expressing OSC-19 tumour *in vivo* with the Pearson's method

(Pearson 1896; Manders et al., 1992; Manders et al., 1993). Such selective tumour localization of the conjugate leads to selective destruction of the tumour after light exposure.

H.E. and IHC in **Chapter 3** show the nuclear condensation of tumour cells and the upregulation of cleaved caspase-3, the apoptosis executioner, after the targeted PDT. This is consistent with the results of apoptotic induction by the targeted PDT in the tumour cell lines *in vitro*, indicating the involvement of apoptotic cell death. The mechanism of action involved in killing tumour cells by targeted PDT is still far from understood. Better understanding of such mechanism would help to improve the treatment protocols to enhance the effectiveness of this modality. For example, apoptotic induction of tumour cells in both *in vitro* and *in vivo* by targeted PDT with the cetuximab-IR700DX conjugate in this thesis is likely immunogenic cell death (ICD), a consistent finding by others (Ogawa et al., 2017). Host dendritic cells can present the ICD antigens to cytotoxic T cells to initiate a specific host anticancer immune response (Zhou et al., 2019). Such systemic antitumour immunity may also lead to responses to distant metastatic tumour cells to inhibit tumour recurrence.

Light is one of the three determinants of PDT. Manipulation of light irradiation can affect the PDT effect. In **Chapter 4** various fluence rates of light irradiation during the targeted PDT of human head and neck tumour cells were compared in both *in vitro* and *in vivo* models. Significant more tumour cell death *in vitro* and more tumour cures *in vivo* were obtained after reducing the fluence rate from 150 mW.cm⁻² to 20 mW.cm⁻². Photobleaching of IR700DX was increased with lower fluence rates and correlated with the enhanced targeted PDT efficacy. These results indicate the effectiveness of targeted PDT is dependent on the fluence rate and on the ¹O₂ generation. Thus, it would be very interesting to study this finding in clinical settings in order to confirm such benefits of targeted PDT with low fluence rates.

This thesis also investigated PCI effects on tumour cells *in vitro*. PCI is a light-mediated therapeutic modality that has been shown to induce a cytosolic release of a number of macromolecules including chemotherapeutics, targeted proteins, genes,

etc. in tumour cells (Høgset et al., 2004; Berg et al., 2010). Disulfonated tetraphenyl chlorin (TPCS_{2a})-induced PCI of bleomycin has recently been introduced to treat head and neck cancer patients with promising results (Sultan et al., 2016).

Although temoporfin based PDT is very efficient in terms of the drug dose used as compared to the first-generation photosensitizer porfimer sodium, the selectivity and specificity are still low, so that it induces considerable local and systemic phototoxicity of normal tissues (de Visscher et al., 2013). **Chapter 5** explored the possibility of a PCI effect of bleomycin with temoporfin. Since the principle of PCI is based on a photosensitizer that localizes on the membrane of endo/lysosomal vesicles, temoporfin is not ideal for PCI because its chemical hydrophobicity makes it to diffusely localize in all biomembraneous structures of a cell. The thesis examined mTHPC prepared in conventional liposomes as Foslip and in PEGylated liposomes as Fospeg. Such liposomal formulations of mTHPC localise endo/lysosomal vesicles via an endocytic process. As a result, a PCI effect of bleomycin was induced with both Foslip and Fospeg when PDT was applied before bleomycin treatment; but not in the case when PDT was performed after the use of bleomycin. The reason for this finding is not known, but similar results were also reported by others (Prasmickaite et al., 2002). They hypothesized that the endo/lysosomes damaged by initial PDT would further fuse and rupture the bleomycin-containing vesicles of endo/lysosomes to release bleomycin into cytosol and nucleus, resulting in cell death. The results of **Chapter 5** indicate that liposomal formulations of mTHPC may be candidates for developing mTHPC-based PCI protocols.

Although PCI has been shown to enhance cytosolic release of anticancer drugs that are entrapped in the endo/lysosomal vesicles of cancer cells, the targeting of photosensitisers to the membrane of endocytic vesicles is not specific enough, so that in practice a PCI effect is always a combination of PCI with PDT where the relative strengths of each is determined by the photosensitiser localisation and the amount of ¹O₂ produced. Theoretically, if a selective targeting of a photosensitizer to the endocytic membrane can be assured and the amount of ¹O₂ that is required to release

anticancer drugs is lower than that needed to destroy cells by PDT, there is an optimal 'PDT dose' for PCI. Targeted PDT may enable to deliver a photosensitizer to endocytic membrane structures in a more specific manner through a specific antibody-based receptor-mediated endocytic pathway. This may improve the specificity of a PCI effect.

Chapter 6 used the targeted PDT with the cetuximab-IR700DX conjugate to generate a PCI effect of the biologically active molecule, gelonin, on killing the A431 human squamous cell carcinoma cells *in vitro*. The results demonstrate that the targeted PDT with gelonin killed significantly more A431 tumour cells than the targeted PDT alone, clearly indicating an induction of a PCI effect. Targeted PDT may have potential for enhancing specificity of PCI effect.

As clinical conventional PDT with temoporfin continues, a number of new photosensitisers are being tested in preclinical biological models including 5,10,15,20-tetrakis[(5-diethylamino)-pentyl]porphyrin (Li et al., 2015) and oligosaccharide-conjugated chlorin (Nishie et al., 2016). In addition, clinical trials of PDT using new dyes such as 3-(10-hexyloxyethyl) pyropheophorbide (Rigual et al., 2013; Shafirstein et al., 2016) or talaporfin sodium (Ikeda et al., 2018) in head and neck cancer patients have shown promising results. Such preclinical and clinical studies with new promising sensitisers would lead to the improvement of efficacy of PDT in head and neck cancer.

Due to limited light penetration into tissue conventional PDT is usually used for the treatment of superficial lesions with a few mm in thickness (D'Cruz et al., 2004; Shafirstein et al., 2016). For larger or deeply seated tumours interstitial PDT with intra-tumour light delivery via laser fibers through needles may be applied to achieve therapeutic effects. With the help of various imaging technologies for guiding light delivery and monitoring real-time dosimetry the effectiveness of interstitial PDT would be further improved (Lou et al., 2004; Jager et al., 2004; Shafirstein et al., 2017). EMA has approved interstitial PDT with temoporfin for patients with refractory locally-advanced head and neck cancer (Shafirstein et al., 2017; Civantos, et al., 2018).

Targeted PDT has huge potential for enhancing PDT efficacy of tumour with improving selectivity and specificity, but antibodies are relatively large molecules with

limited tissue penetration and long half-lives. The use of small nanobodies (antigen-binding fragments of variable domains of heavy chains of an antibody) such as EGFR-targeted nanobody-photosensitiser conjugate has shown selective and effective antitumour effects after light exposure in a preclinical model of head and neck cancer (van Driel et al., 2016). Such nanobodies may have advantages over antibodies for targeted PDT. Targeted PDT may be useful as an adjuvant treatment in treating macroscopic and microscopic recurrent head and neck tumours after surgery (Meulemans et al., 2019). Effectiveness of targeted PDT may also be enhanced in combination with existing standard treatment modalities of cancer including radiotherapy, chemotherapy, immunotherapy, etc.

Although there is an ongoing Phase-3 clinical trial with the targeted PDT using cetuximab-IR700DX conjugate in head and neck cancer patients (<https://clinicaltrials.gov/ct2/show/NCT03769506>), there remain the basic questions of what determines its efficacy *in vivo*, how various parameters influence the *in vivo* treatment dosimetry and how the targeted PDT dose can be measured. These problems are obviously critical for the implementation of effective targeted PDT. As used in the **Chapter 4** of this thesis it is possible to use reflectance and fluorescence spectroscopic techniques, such as multidiameter single-fiber reflectance (MDSFR) system (Wilson et al., 1997; Robinson et al., 1998; van Leeuwen-van Zaane et al., 2013, 2014), to measure in real-time the pharmacokinetic pattern of IR700DX of the conjugate, local tissue oxygenation and $^1\text{O}_2$ production before, during and after cetuximab-IR700DX-based targeted PDT. Such measurements should be correlated with blood perfusion and tumour responses. These studies would help to find an optimal timing for subsequent light irradiation of tumour after conjugate administration. It is also to use these data to optimize parameters (including light dose, fluence rate, etc.) affecting the targeted PDT efficacy and to predict the treatment responses.

PCI may be considered as a 'fortified PDT'. The combination of PCI with targeted PDT may eventually create a minimally invasive and highly efficient treatment. This modality

has a triple-model selectivity including (1) a specific binding of IR700DX to tumour cells through targeting EGFR by cetuximab antibody, (2) cytosolic release of biologically active molecules from endo/lysosomal vesicles and (3) site-directed light activation. Although the thesis for the first time induced a gelonin-based PCI effect with the targeted PDT in the A431 human tumour cells *in vitro*, more studies are needed to confirm this finding in both *in vitro* and *in vivo* tumour models before developing a new strategy of PCI with targeted PDT to improve selectivity and specificity.

It is hoped that targeted PDT and targeted PDT-mediated PCI would further improve the selectivity, specificity and efficacy of the clinical treatment for squamous cell carcinoma (or field cancerization) of head and neck (the lip, oral and pharynx) with reduced phototoxicity in normal tissues. In addition, they may be used as adjuvant treatments after surgery with macroscopic and microscopic tumour residues in the margin areas. Their effectiveness may also be enhanced in combination with existing standard cancer treatment modalities.

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Chapter 8

Summary
Samenvatting
Oppsummering

Summary

Photodynamic therapy (PDT) is an established minimally invasive modality of cancer treatment. The technique is based on a combination of light-activated photosensitizer with local light illumination to generate ROS, $^1\text{O}_2$ in particular; that cause the oxidation of lipids, amino-acids and proteins to induce necrosis and apoptosis of tumour cells.

PDT with the first-generation photosensitizer, porfimer sodium (a mixture of several porphyrin compounds), is used to treat various types of cancer including head and neck cancer with promising results. The second-generation dye, temoporfin, improves the efficacy of PDT, but the inadequate selectivity and specificity lead to phototoxicity of normal tissues, that limits considerable clinical application.

Targeted PDT uses the principle of PDT with a conjugate of a second-generation photosensitizer and an antibody directly against a molecule that is over-expressed in tumour cells. The photosensitizers conjugated with antibodies to enhance selectivity and specificity of PDT are often regarded as third-generation dyes.

Photochemical internalization (PCI) is a technique that uses the principle of PDT for light-enhanced cytosolic release of anticancer drugs that are entrapped in the endo/lysosomal vesicles of cancer cells. This modality can induce tumour cell death with improved selectivity and specificity.

In order to improve the selectivity and specificity of PDT this thesis started with investigating killing effects of targeted PDT using cetuximab-IR700DX conjugate on 3 human head and neck tumour cell lines (OSC-19, scc-U2 and scc-U8) *in vitro*. Such therapeutic effect was then studied in a xenografted human OSC-19 head and neck tumour in a mouse skin-fold window-chamber model *in vivo*. In addition, the effect of fluence rate of light irradiation on the efficacy of the targeted PDT was evaluated in a xenografted human OSC-19 head and neck tumour model in the flank of mice *in vivo*. The thesis was ended with exploring the possibilities of bleomycin-based PCI with mTHPC in 2 human head and neck tumour cell lines (scc-U2 and scc-U8) *in vitro* and gelonin-based PCI with targeted PDT using cetuximab-IR700DX conjugate in the A431 human squamous cell carcinoma cells *in vitro*.

In **Chapter 2** targeted PDT with cetuximab-IR700DX conjugate killed scc-U8 and A431 cells efficiently; while scc-U2 and OSC-19 were less sensitive to this treatment, despite having similar EGFR density, uptake and localization pattern. Immunoblots showed significantly higher expression of anti-apoptotic Bcl-2 and Bcl-xL proteins in the scc-U2 cell line as compared to scc-U8 cell line. These findings suggest that the effectiveness of EGFR targeted PDT is not only dependent upon EGFR density. Intrinsic biological properties of tumor cells also play a role in determining the targeted PDT efficacy.

In **Chapter 3** the targeted PDT effect with the conjugate on the same type of human OSC-19 tumour cells was evaluated using a dorsal skin-fold chamber model in Balb/c nude mice *in vivo*. The amount of conjugate in the tumour peaked at 24-48 hours after intravenous injection, significantly higher than that in the surrounding normal tissues. Image analyses of colocalization correlation parameters demonstrated a high fraction of the conjugate IR700DX colocalised in the GFP-expressing tumour cells. PDT-treated tumours showed extensive necrotic/apoptotic destruction with little vascular damage; while IHC showed a lack of HIF-1 α expression, and decreased EGFR and Ki67 expression with activated caspase-3 over-expression, indicating a direct killing of tumour cells through both necrotic and apoptotic cell death.

In **Chapter 4** the effects of fluence rates of light irradiation on targeted PDT efficacy were studied in human tumour cells *in vitro* and tumours *in vivo*. Significant more tumour cell death *in vitro* and more tumour cures *in vivo* were observed after reducing the fluence rate from 150 mW.cm⁻² to 20 mW.cm⁻². Photobleaching of IR700DX was increased with lower fluence rates and correlated with the enhanced treatment efficacy. These results indicate that effectiveness of the targeted PDT is strongly dependent on a fluence rate.

Chapter 5 describes PCI effects of bleomycin on 2 human head and neck cancer cell lines (scc-U2 and scc-U8) with mTHPC formulated in two different types of liposomes. The PCI effects were induced only when a PDT was applied before bleomycin treatment. This finding may provide a new strategy to develop a more effective PCI

protocol with possible advantage of avoiding destruction of light-sensitive anticancer drugs and biologically active molecules during PCI treatment. Such modality may also have potential of reducing or avoiding the side-effect of phototoxicity of normal tissues with temoporfin in clinical practice.

Since the targeting of a photosensitizer to the endocytic membrane is random in a 'standard' PCI, **Chapter 6** explored the possibility of using the cetuximab-IR700DX conjugate to deliver the dye IR700DX more specific to the endocytic membrane via the cetuximab endocytosis. This may trigger a more selective release of an anti-cancer agent by a more specific photochemical rupture of the endocytic membrane. The results obtained so far demonstrate a clear induction of a gelonin-based PCI effect by the targeted PDT with the cetuximab-IR700DX conjugate on the A431 human squamous cell carcinoma cells.

Samenvatting

Fotodynamische therapie (PDT van 'Photodynamic therapy') is een gevestigde, minimaal invasieve, kankerbehandeling. De behandeltechniek combineert een licht gevoelige stof, ofwel geactiveerde photosensitizer, met gerichte belichting om reactieve zuurstofcomponenten te genereren. Deze reactieve zuurstofcomponenten, met name $^1\text{O}_2$, veroorzaken oxidatie van vetten, aminozuren, en eiwitten waarmee necrose en apoptose van tumorcellen wordt geïnduceerd.

PDT met de eerste generatie photosensitizer porfimer sodium (een mengsel van verscheidene porfyrine verbindingen) wordt al in klinische settings gebruikt. Deze behandeltechniek wordt ingezet bij verschillende vormen van kanker, onder andere die van het hoofd-halsgebied, en toont veelbelovende resultaten. PDT met een tweede generatie photosensitizer temoporfine verbetert de effectiviteit van PDT, maar veroorzaakt door een lagere selectiviteit fototoxiciteit in gezonde weefsels waardoor deze combinatie klinisch minder goed inzetbaar is.

Gerichte PDT maakt gebruik van het werkingsmechanisme van PDT maar combineert dit met een conjugaat van een tweede generatie photosensitizer en een antilichaam dat gericht is op moleculen welke in hoge concentratie aanwezig zijn op de kankercel door overexpressie. Een conjugaat van een photosensitizer met een kankerselectief antilichaam wordt vaak beschouwd als een derde generatie photosensitizer.

Fotochemische internalisatie (PCI van 'Photochemical internalization') maakt gebruik van PDT om antikankermedicatie, welke door de tumorcellen in de endosomen en lysosomen is opgenomen, versterkt te laten vrij komen in het cytosol en zo de tumorcel te doden. Deze modaliteit verhoogt de selectiviteit en specificiteit van antikankermedicatie.

Een belangrijk doel van deze these was om de selectiviteit en specificiteit van PDT te verbeteren. Het onderzoek begon met het bestuderen van het *in vitro* dodend vermogen van gerichte PDT met cetuximab-IR700DX conjugaat op humane tumorcellen van het hoofd-halsgebied van drie verschillende tumor cellijnen (OSC-19, scc-U2 and scc-U8). Vervolgens werd het *in vivo* therapeutische effect van deze

behandelmethode onderzocht op humane xenotransplantaten van OSC-19 tumorcellen in muizen, door gebruik van huidplooï venster implantatie.

Daarna werd de invloed van de belichtingsintensiteit, ook wel fluence rate, van de gerichte belichting op de *in vivo* effectiviteit van PDT onderzocht in humane xenotransplantaten van OSC-19 tumorcellen op de flank van muizen. Vervolgens onderzochten wij het *in vitro* effect van PCI met bleomycine in combinatie met mTHPC, een tweede generatie photosensitizer, op twee humane hoofd-hals tumor cellijnen (scc-U2 and scc-U8). Deze these eindigde met *in vitro* onderzoek naar de behandelbaarheid van PCI met gelonine in combinatie met gerichte PDT met cetuximab-IR700DX conjugaat op A431 humane plaveiselcelcarcinoom cellen.

Hoofdstuk 2 beschrijft de effecten van *in vitro* gerichte PDT met cetuximab-IR700DX conjugaat: deze therapie doodde scc-U8 en A431 tumorcellen effectief, maar bleek minder doeltreffend in het doden van scc-U2 en OSC-19 tumorcellen, ondanks het gegeven dat de vier cellijnen een vergelijkbare densiteit, opname en lokalisatie van EGFR eiwitten hadden. Immunoblots lieten een significant hogere expressie van de anti-apoptotische eiwitten Bcl-2 en Bcl-xL zien in de scc-U2 tumorcellen vergeleken met de scc-U8 tumorcellen. Deze bevindingen suggereren dat de effectiviteit van op EGFR gerichte PDT niet alleen afhangt van de EGFR densiteit in de tumorcel. Ook intrinsieke biologische eigenschappen van de tumorcellen beïnvloeden de effectiviteit van gerichte PDT.

Hoofdstuk 3 bespreekt het effect van *in vivo* gerichte PDT met cetuximab-IR700DX conjugaat op hetzelfde type OSC-19 tumorcellen met gebruik van dorsale huidplooï venster implantatie in Balb/c naakte muizen. De concentratie van het conjugaat was in de tumor het hoogst tussen 24 en 48 uur na intraveneuze injectie. De concentratie was in de tumor significant hoger dan in het omliggende gezonde weefsel. Microscopische beeld analyses van tumorcellen met GFP expressie toonden hoge concentraties van IR700DX conjugaat in de tumorcellen. Met PDT behandelde tumoren lieten uitgebreide necrotische en apoptotische schade zien, terwijl er maar weinig vasculaire schade was. De immunohistochemie toonde een verminderde expressie van HIF-1 α ,

EGFR en Ki67, maar overexpressie van geactiveerd caspase-3. Dit indiceert een werkingsmechanisme met directe destructie van de tumorcellen door necrotische en apoptotische celdood.

Hoofdstuk 4 beschrijft de effecten van de fluence rate van lichtbestraling bij de *in vivo* en *in vitro* behandeling van humane tumorcellen met gerichte PDT. Vermindering van de fluence rate van 150 mW.cm^{-2} naar 20 mW.cm^{-2} ging gepaard met significant meer tumorceldood *in vitro* en meer tumor genezing *in vivo*. Bij een lagere fluence rate trad er meer vervaging, ofwel *photobleaching*, op van de toegevoegde photosensitizer IR700DX, wat correleerde met verhoogde behandel-effectiviteit. Dit indiceert dat de effectiviteit van gerichte PDT sterk afhangt van de gehanteerde fluence rate.

Hoofdstuk 5 bespreekt de effecten van PCI met bleomycine en mTHPC, geformuleerd in twee typen liposomen, bij scc-U2 en scc-U8 tumorcellen. De PCI behandeling had alleen effect als gestart werd met PDT alvorens bleomycine toe te dienen. Deze bevinding kan richting geven in de ontwikkeling van effectievere behandelstrategieën en behandelprotocollen voor PCI. Een potentieel voordeel van de sequentiële behandeling is dat licht-sensitieve antikankermedicatie en biologisch actieve moleculen niet beschadigd worden door de gerichte lichtbestraling. Deze modaliteit heeft de mogelijkheid om fototoxische schade van gezond weefsel te verminderen, of zelfs te vermijden, bij de behandeling met temorfine in klinische settings.

Hoofdstuk 6 beschrijft de mogelijkheid om het cetuximab-IR700DX conjugaat te gebruiken om IR700DX specifiek aan het endosoom membraan te laten hechten door cetuximab endocytose. In een 'standaard' PCI is de binding van photosensitizers aan het endosoom membraan namelijk willekeurig. Een specifiekere opname kan een meer selectief vrijkomen van de antikankermedicatie teweegbrengen doordat deze via een fotochemische scheur van het endosoom membraan in de tumorcel vrij komt. De huidige resultaten demonstreren een duidelijk versterkt behandel-effect van PCI met

gelonine door toevoeging van gerichte PDT met cetuximab-IR700DX conjugaat bij A431 tumorcellen.

Oppsummering

Fotodynamisk terapi (PDT fra "Photodynamic therapy") er en etablert minimal invasiv form for kreftbehandling. Teknikken er basert på en kombinasjon av et lysaktivert lyssensibiliserende stoff med lokal belysning for å generere reaktive oksygenkomponenter. Disse reaktive oksygenkomponentene, spesielt $^1\text{O}_2$, oksiderer lipider, aminosyrer og proteiner som induserer nekrose og apoptose i kreftceller. Førstegenerasjons lyssensibiliserende stoffet, porfimer sodium, er en blanding av flere porfyrinforbindelser og brukes allerede i klinikker. Denne behandlingsteknikken brukes til å behandle ulike former kreft, inkludert i hode- og hals området, og har vist lovende resultater. Andregenerasjons lyssensibiliserende stoffet temoporfin forbedrer effekten av PDT, men har lavere selektivitet og spesifisitet som fører til fototoksisitet av normalt vev, noe som betydelig begrenser muligheten for klinisk bruk.

Målrettet PDT er basert på PDT med et konjugat bestående av et andregenerasjons lyssensibiliserende stoff og et antistoff rettet direkte mot et molekyl som overuttrykkes i tumorceller. Lyssensibiliserende stoff konjugert med antistoffer for å forbedre selektivitet og spesifisitet av PDT blir ofte sett på som tredjegerasjons fargestoffer. Fotokjemisk internalisering (PCI fra "Photochemical internalization") bruker PDT til å forbedre frigjøringen av kreftmedisiner som finnes i endosomene og lysosomene i cytosolen, noe som dreper tumorceller. Denne modaliteten øker selektiviteten og spesifisiteten til legemidler mot kreft.

Et viktig mål for forskningen i denne doktorgraden er å forbedre selektiviteten og spesifisiteten til PDT. Studiet startet med å undersøke effektiviteten av målrettet PDT med cetuximab-IR700DX-konjugat mot tre forskjellig humane hode- og hals kreftceller (OSC-19, scc-U2 og scc-U8) *in vitro*. Den terapeutiske effekten ble deretter studert i xenotransplantert humane OSC-19 kreftceller i en mus ved bruk av '*skin-fold window-chamber model*' *in vivo*. I tillegg ble påvirkningen av fluensrate av lysbestråling på effektiviteten av målrettet PDT evaluert i en xenografted human OSC-19 hode- og hals tumormodell i flanken av mus *in vivo*. Oppgaven konkluderte med å forske på muligheten for bleomycin basert PCI med mTHPC i 2 humane hode- og hals

kreftcellelinjer (scc-U2 og scc-U8) *in vitro* og gelonin basert PCI med målrettet PDT ved bruk av cetuximab-IR700DX-konjugat i A431 humane plateepitelkarsinomceller *in vitro*.

I **kapittel 2** viser målrettet PDT at bruk av cetuximab-IR700DX-konjugat effektivt drepte scc-U8- og A431-celler; mens scc-U2 og OSC-19 var mindre følsomme for denne behandlingen, til tross for at de hadde lignende EGFR- uttrykk, opptak og lokaliseringsmønster. Immunoblots viste en signifikant høyere ekspresjon av anti-apoptotiske Bcl-2 og Bcl-xL proteiner i scc-U2 cellelinjen sammenlignet med scc-U8 cellelinjen. Disse funnene antyder at effektiviteten av EGFR-målrettet PDT ikke bare er avhengig av EGFR- uttrykk. De intrinsiske biologiske egenskapene til kreftcellene spiller også en rolle i å bestemme den målrettede PDT-effekten.

I **kapittel 3** ble den målrettede PDT effekten med cetuximab-IR700DX-konjugat vurdert *in vivo* med den samme type humane OSC-19 kreftceller ved hjelp av en Balb/c nakenmus '*skin-fold window-chamber model*'. Konsentrasjonen av konjugatet i svulsten var på sitt høyeste etter 24 til 48 timer etter intravenøs injeksjon. Konsentrasjonen var betydelig høyere enn i det omkringliggende normale vevet. Mikroskopisk bildeanalyse viste at en høy prosentandel av konjugatet IR700DX var kolokalisert i GFP-uttrykkende tumorcellene. PDT-behandlede tumor viste omfattende nekrotisk/apoptotisk effekt med liten vaskulær skade; mens immunhistokjemi viste mangel på HIF-1 α -ekspresjon, og reduserte EGFR- og Ki67-ekspresjon med aktivert caspase-3-overuttrykk, noe som indikerer en direkte avlivning av kreftceller gjennom både nekrotisk og apoptotisk celledød.

I **kapittel 4** ble effekten av fluensrate av lysbestråling på målrettet PDT-effekt studert i humane kreftceller *in vitro* og tumorer *in vivo*. Det ble observert signifikant mer tumorcelledød *in vitro* og flere tumorcurer *in vivo* ved å redusere fluensrate fra 150 mW.cm⁻² til 20 mW.cm⁻². Fotobleking av IR700DX ble økt med lavere fluensrate og korrelert med den forbedrede behandlingseffekten. Disse resultatene indikerer at effektiviteten av målrettet PDT er sterkt avhengig av fluensrate.

Kapittel 5 beskriver PCI-effekten av bleomycin på 2 humane hode-hals krefcellelinjer (scc-U2 og scc-U8) med mTHPC formulert i to forskjellige typer liposomer. PCI-effekten ble kun indusert når PDT ble påført før bleomycin-behandling. Dette funnet kan gi en ny strategi for å utvikle en mer effektiv PCI-protokoll med mulig gevinst å unngå ødeleggelse av lysfølsomme kreftmedisiner og biologiske aktive molekyler under PCI-behandling. Slik modalitet kan også ha potensiale for å redusere eller unngå bivirkningen fototoksisitet av normalt vev med temoporfin i klinisk praksis.

Siden målretting av lyssensibiliserende stoffer mot den endocytiske membranen er tilfeldig i en 'standard' PCI, utforsker **kapittel 6** muligheten for å bruke cetuximab-IR700DX-konjugatet for å levere IR700DX mer spesifikt for endocytisk membran via cetuximab-endocytosen. Dette kan utløse en mer selektiv frigjøring av et kreftmiddel ved et mer spesifikt fotokjemisk brudd i den endocytiske membranen. Resultatene som er oppnådd så langt viser en klar induksjon av en gelonin basert PCI-effekt av målrettet PDT med cetuximab-IR700DX-konjugat på A431 kreftceller.

Chapter 9

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Chapter 10

About the author

Wei Peng was born on July 2nd, 1991 in Oslo, Norway. He grew up there with his parents and had attended Oslo International School until he graduated the International Baccalaureate programme at the age of 17. In August 2009 he started the International Bachelor of Medicine, Global Health Profile, at the University of Groningen, The Netherlands. During his bachelor programme, Wei was invited to the Bachelor's Honours College, an honours programme with an emphasis on broadening and deepening of knowledge and development of his academic skills and mind-set. As part of the bachelor thesis, Wei got involved in a research project with the Department of Oral and Maxillofacial Surgery under the supervision of Dr. M.J.H Witjes and Prof. Dr. J.L.N. Roodenburg. The project was awarded by the Association of Universities in The Netherlands for the best poster presentation for undergraduate research in The Netherlands and Belgium in 2011 and set the building bricks for his entering the MD/PhD programme in 2015. The PhD project was performed at three university hospitals (University Medical Center Groningen, Erasmus Medical Center and Oslo University Hospital) under the supervision of Dr. M.J.H Witjes, Prof. J.L.N. Roodenburg, Dr. D. Robinson, Dr. H.S. de Bruijn and Prof. K. Berg. Wei was also a member of the Postgraduate Student Committee at Molecular Medicine, Erasmus Medical Center. The findings of his MD/PhD project are presented in this thesis. Wei understood the importance of combined his scientific career with his clinical one. In 2016 Wei graduated his Master of Medicine after completing clinical rotations at the University Medical Center Groningen, Isala Hospital in Zwolle and his final pre-registration clerkship at the Department of Otorhinolaryngology and Head and Neck Surgery, Oslo University Hospital. Alongside his regular master programme, Wei managed to complete the Master's Honours College, an honours programme which focuses on different aspects of leadership and development of his leadership style. In 2018 Wei had started his residency in general surgery at the Akershus University Hospital before he moved over to otorhinolaryngology and head and neck surgery. He was offered soon afterwards a residency position in general internal medicine at the Stavanger University Hospital, which he accepted to gain a broader knowledge within

medicine before going back to the Department of Otorhinolaryngology and Head and Neck Surgery. In 2020 Wei started working as a otorhinolaryngological, head and neck surgical resident at the Vestfold Hospital Trust, Norway.