New strategies to enhance photodynamic therapy for solid tumors
Broekgaarden, M.

Citation for published version (APA):
Broekgaarden, M. (2016). New strategies to enhance photodynamic therapy for solid tumors

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 12
Discussion, outlook, and conclusion

1. Current status of photodynamic therapy

In order for photodynamic therapy (PDT) to be considered a relevant last line therapy in the treatment of terminal cancer patients, it is essential that it achieves a high efficacy while maintaining low morbidity. Theoretically, PDT has the potential to realize this by utilizing a non-toxic photosensitizer and non-toxic laser light irradiation. With the ability of the photosensitizer to accumulate at high concentrations in the tumor tissue and the selective irradiation of the tumor tissue, PDT should theoretically be able to exert cytotoxic effects specifically towards malignant tissue. In practice however, the potential of PDT has not come to full fruition. Although PDT has achieved promising results in the treatment of non-resectable hilar cholangiocarcinoma with a clear survival benefit (16 months post-diagnosis for stenting + PDT versus 6 months for stenting only) for patients has been described, the first-generation photosensitization strategy utilized in those studies has led to prolonged skin photosensitivity in patients. As such, further treatment of these patients with the current standard of PDT was deemed unethical. Currently, palliative care for patients with non-resectable perihilar cholangiocarcinoma consists of biliary stent placement and a subsequent chemotherapy regimen of gemcitabine plus cisplatin, which only achieves a median survival of 11.7 months post diagnosis versus 8 months when patients received gemcitabine only. Thus, if PDT could be enhanced in terms of efficacy and morbidity, it could replace the current standard of palliative care to substantially increase patient survival and quality of life. In order to achieve this, photochemical, pharmacokinetic, and biological hurdles must be overcome. Therefore, this thesis aimed to present novel strategies to overcome these hurdles to ultimately improve PDT for solid cancers.

A pitfall in many current clinical investigations is the use of first- and/or second-generation photosensitizers that do not possess the most optimal physicochemical characteristics with respect to wavelength absorption and molar extinction coefficient. There are a plethora of photosensitizers with a wide range of physicochemical properties available. Of these, the metallated phthalocyanines possess high molar extinction coefficients, i.e., are highly efficient at absorbing excitation light, and absorb at clinically relevant wavelengths in the therapeutic optical window for PDT, typically around 674 nm. In comparison to HpD, porfimer sodium, and protoporphyrin IX, metallated phthalocyanines do not absorb light below 600 nm, and thus are less prone to undergo excitation upon exposure to broad spectrum (day)light. With respect to the importance of light absorption at longer wavelengths, it was shown that in comparison to porfimer sodium-PDT (630 nm), meso-tetrahydroxyphenylchloride (mTHPC) PDT (650 nm) for perihilar cholangiocarcinoma resulted in a substantial increase in the necrotic zone following PDT, which was essential for a more complete removal of the tumor. The significantly enhanced PDT-efficacy was attributed to mTHPC’s ability to absorb light of a longer wavelength (650 nm) with higher efficiency due to a high molar extinction coefficient. However, in comparison to mTHPC, zinc phthalocyanine (ZnPC) absorbs at an even longer wavelength (674 nm), and has an almost 10-fold higher molar extinction coefficient ($3 \times 10^5$ M/cm$^{-1}$) in comparison to mTHPC. Thus, selecting a more suitable photosensitizer in the form of a metallated phthalocyanine is a logical first step in the improvement of PDT.
2. Targeted liposomes for the tumor specific delivery of ZnPC

In addition to the contemptible physicochemical properties of currently used first- and second-generation photosensitizers, photosensitizers that are systemically administered in free form give rise to non-specific biodistribution, leading to prolonged accumulation in the skin. Thus, the future of PDT for terminal cancers lies in the development of third-generation photosensitizers, i.e., second-generation photosensitizers that are encapsulated in a drug delivery system that targets the photosensitizing agents with high specificity towards the tumor tissue. A theoretical background for multiple targeted liposomes for applications in PDT is given in Chapter 2. Subsequently, Chapters 3, 4, and 5 further explore the feasibility of encapsulating the second-generation photosensitizer zinc ZnPC into interstitially targeted liposomes (ITLs), tumor cell targeted liposomes (TTLs), and tumor vascular endothelium targeted liposomes (ETLs), respectively.

2.1 Interstitially targeted liposomes

In Chapter 3, we first set out to determine whether the encapsulation of ZnPC in liposomes yielded photodynamically active vesicles, and to optimize and test the liposomal formulation to obtain in vitro proof-of-concept. It was shown that liposomal encapsulation of ZnPC is photodynamically feasible as the ZnPC-containing liposomes were photodynamically active and capable of oxidizing lipids, proteins, and small molecules. The liposomes were minimally taken up by tumor cells, but exerted cytotoxic effects towards tumor cells when used for PDT. The liposomal formulation that was explored in this chapter was based on previously established stealth liposomes (Table 1), i.e., liposomes that escape absorption by the liver and do not readily interact with immune cells or cells that comprise the reticuloendothelial system. As such, these liposomes exert relatively long circulation times and have been shown to accumulate in the tumor tissue as a result of the enhanced permeability and retention (EPR) effect. The EPR effect is the result of a fenestrated vascular endothelium, distorted blood flow, and impaired lymphatic drainage, which are characteristic for the tumor vasculature. Thus, the results of this chapter imply that these stealth liposomes can be used to deliver ZnPC specifically towards the tumor interstitial spaces and subsequent PDT. Hence, these liposomes were termed interstitially targeted liposomes (ITLs). By accumulating in the interstitial spaces, PDT with ZnPC-ITLs may target both cancer cells and non-cancer cells and may even damage extracellular matrix constituents, as these are sensitive to oxidation and may denature as a result. Thus, these liposomes provide a means to disrupt tumor structures that are not normally targeted with other forms of therapy.

2.2 Tumor cell targeted liposomes

Another method to obtain specific accumulation of liposomes in tumor tissue is by functionalizing the vesicles with a tumor-recognizing antibody, yielding immunoliposomes or tumor cell targeted liposomes (TTLs). Although the extent of liposomal accumulation in the tumor tissue is not always higher with immunoliposomes, vesicles that bind tumor cells are subsequently internalized. As such, the liposomally encapsulated drug is able to exert its effects intracellularly. Many studies have utilized monoclonal antibodies or Fab’ fragments thereof that were randomly labeled of the antibody to the liposomes and have reported low labeling yields using these methods. Chapter 4 reports a biochemical approach to label the VHH fragment of an anti-EGFR single domain antibody (nanobody) in a site-specific manner to ZnPC-containing liposomes. In comparison to conventional antibodies, nanobodies have superior characteristics with respect to stability, manufacturing costs, and ease of modification. Thus, their exploitation in the production of TTLs represents a potential improvement over TTLs prepared with conventional antibodies. The site-specific conjugation of the nanobody to the maleimide groups of polyethylene glycol (PEG)-functionalized phospholipids was achieved with high labeling efficiency. Similar to other studies, it was reported that the immunoliposomes were highly and specifically taken up by EGFR-overexpressing cells. Taken together,
this study has yielded similar results regarding uptake selectivity and efficacy, but utilized a method that allows for more efficient, site specific labeling of a nanobody that is more stable and easier to modify than conventional antibodies.

### 2.3 Tumor endothelium targeted liposomes

An interesting discovery has been the preferential accumulation of cationic liposomes at the tumor vasculature. Although the exact mechanism remains elusive, it is speculated that a distorted blood flow in angiogenic tumor vessels and the consequential shedding of the barrier-forming glycocalyx of endothelial cells is believed to be the underlying mechanism for an increased interaction of the cationic liposomes and the anionic endothelial cell membranes. It should be noted that the use of non-PEGylated cationic liposomes has been associated with notable toxicological events in vivo, which can be diminished by PEGylation of the vesicles. As such, the liposomal formulations listed in Table 1 were well tolerated in mice. Since the liposomal formulation developed, optimized, and tested in Chapter 5 share similar lipid bilayer compositions as those presented in Table 1, it was hypothesized that these tumor vascular endothelium targeted liposomes (ETLs) would also be well tolerated in vivo. Indeed, the in vivo toxicological assessment did not show any notable toxicity in mice. Moreover, in comparison to ITLs and TTLs, the ETLs were most effectively taken up by cells in vitro. The ETLs also demonstrated enhanced accumulation in tumor tissue in mice although the exact intratumoral localization needs to be further investigated. Given the convincing literature

<table>
<thead>
<tr>
<th>Type</th>
<th>Formulation</th>
<th>Drugs</th>
<th>Application</th>
<th>Outcome</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITL</td>
<td>DPPC:DOPC:Chol:PGlC/UA (1:1:0.5)</td>
<td>BPD-MA</td>
<td>PDT, mice bearing Meth A sarcoma</td>
<td>- Increased PDT efficacy #</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>HSPC-Chol:DSPE-PEG (10.33:0.33)</td>
<td>Doxorubicin</td>
<td>CTX, human multiple myeloma, phase III trial</td>
<td>- Well tolerated</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>DPPC-Chol:DSPE-PEG (10.45:0.06)</td>
<td>ZnPC</td>
<td>PDT in vitro</td>
<td>- Low levels of uptake</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Toxicity evaluation in mice</td>
<td>- Therapeutically feasible</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Absence of long term toxicity</td>
<td>13</td>
</tr>
</tbody>
</table>

| ETL  | HSPC:Chol:DC-6-14:DSPE-PEG (2.1:0:2.0:2) | Oxaliplatin | CTX, mice bearing B16B16 melanoma | - Effective uptake by tumor vascular endothelial cells | 14 |
|      | HSPC:Chol:DC-6-14:DSPE-PEG (2.1:0:2.9:2) | Oxaliplatin | CTX, mice bearing Lewis lung carcinoma | - Effective uptake by tumor vascular endothelial cells | 15 |
|      | DOTAP:DOPC (1:0.94) | Paclitaxel | CTX, human breast cancer, phase I trial | - Increased suppression of tumor growth # | 15 |
|      | DPPC:DC-Chol:Chol:DSPE-PEG (1.0.38:0.076:0.08) | ZnPC | PDT in vitro | -High uptake efficiency in vitro | 17 |
|      | | | Toxicity evaluation in mice | - High therapeutic efficacy * | |
|      | | | | - Absence of long term toxicity | |

| TTL  | DPPC:Chol (3:2) + 0.5% DSPE-PG/Sm + Cetuximab Fab | Doxorubicin/Epirubicin | CTX, mice bearing MDA-MB-368 xenografts | - No increase in drug delivery efficiency * | 18 |
|      | DPPC:Chol (3:2) + 0.5% DSPE-PG/Sm + Cetuximab Fab | Doxorubicin/Epirubicin | Various EGFR positive human malignancies, phase I trial | - Increased therapeutic efficacy | 19 |
|      | DPPC-Chol:DSPE-PEG/Galn (1:0.45:0.06) | ZnPC | PDT in vitro | - Reduced intracellular drug levels | 20 |
|      | | | | - Increased therapeutic efficacy * | |

Table 1: Liposomal formulation used throughout the literature that have formed a basis for the composition of the lipid bilayer of the liposomes developed in this dissertation. It should be noted that this table is not meant as a complete overview of the literature. Asterisks indicate a comparison between targeted and non-targeted liposomes, pound signs indicate a comparison between liposomal drugs and non-liposomal drugs. Abbreviations: DPPC, dipalmitoyl phosphatidylcholine; DOPC, dioleoyls phosphatidylcholine; Chol, cholesterol; PGlC/UA, palmitoyl-glucuronic acid; BPD-MA, benzoporphyrin derivative monoacid ring A; HSPC, hydro soy phosphatidylcholine; CTX, chemotherapy; DC-6-14, O,O'-ditetradecanoyl-N-(alpha-trimethyl ammonio acetyl) diethanolamine chloride; DOTAP, dioleoyl trimethylammonium-propane. 6, 11-20
in which *in vivo* proof-of-concept has been obtained \(^{14-16}\), it is expected that the ETLs developed in *Chapter 5* display a similar affinity for accumulation at the tumor vascular endothelium. Therefore, the use of ZnPC-ETLs may serve as a functional addition to the use of ZnPC-ITLs and ZnPC-TTLs, inasmuch as a different intratumoral location is photosensitized.

### 2.4 Outlook

The *in vitro* proof-of-concept studies described in this dissertation warrant further exploration *in vivo*. Preliminary data indicate that the ZnPC-ITLs, and ZnPC-ETLs were well tolerated in mice. Experiments to obtain *in vivo* proof-of-concept regarding drug delivery and PDT efficacy are currently underway. In this respect, it should be emphasized that the lipid compositions of the ITLs, ETLs, and TTLs were based on liposomal formulations that have been clinically approved or for which extensive *in vivo* proof-of-concept of drug delivery efficacy has been demonstrated (Table 1). Therefore, it is hypothesized that these liposomes act in accordance with the prevailing literature and will individually show robust therapeutic potential *in vivo*. Ultimately, a comprehensive drug delivery modality could be achieved upon combining all the liposomal formulations into a single entity (*Chapter 2*). It is hypothesized that this drug delivery modality will result in substantially higher photosensitizer doses in the tumor tissue, which upon light irradiation will result in excessive oxidative damage at multiple intratumoral locations. As a consequence, PDT could achieve improved therapeutic efficacy, better disease management, increased sensitivity to adjuvant agents (*e.g.*, chemotherapy), reduced morbidity, and increased patient survival upon successful translation to the clinic.

### 3. Identification of molecular targets for adjuvant therapeutics

In recent years, it has become apparent that tumors possess the ability to adapt to therapies such as chemo- and radiotherapy, which results in the generation of therapy-resistant cells, tumor regrowth, and dismal therapeutic outcomes. Since therapy recalcitrance has also been reported for nasopharyngeal and bladder carcinomas, a literature study was performed to identify the strategies tumor cells engage to adapt to the PDT-induced oxidative stress and post-therapeutic hypoxic and microenvironment. The ultimate goal of this literature review was to find molecular signaling cascades that contribute to PDT recalcitrance and that represent feasible druggable targets for adjuvant therapy that sensitize tumors to PDT. An elaborate survey of the literature yielded five pathways of which many individual participating proteins have been implicated in the resistance of tumors to PDT. These were classified as the antioxidant response, the inflammatory response, the hypoxic stress response, the immediate early stress response, and the proteotoxic stress response. These pathways have been elaborately reviewed in *Chapter 6*, and experimental evidence for the activation of these pathways by suboptimal PDT in human perihilar cholangiocarcinoma cells has been described in *Chapter 7*.

#### 3.1 HIF-1

It was found that hypoxia, and thus the activation of the hypoxic stress response mediated by hypoxia-inducible factor 1 (HIF-1), is a preexisting physiologic condition in most – if not all – tumors \(^{28}\). Therefore, the initial focus of *Chapter 8* was to investigate the whether the HIF-1 pathway was feasible a target for adjuvant therapy to sensitize tumors to PDT. In order to validate HIF-1 as a target protein for inhibition strategies, we first set out to investigate whether HIF-1 was activated by PDT. However, when cells were kept at standard normoxic culture conditions, no increases in HIF-1 stabilization could be observed, which was in contrast to various previous studies \(^{29,30}\). The most convincing activation of HIF-1 was found when *in vivo* cancer models were used \(^{31,32}\), and thus it was hypothesized that HIF-1 activation was a result the vascular shutdown that ensues PDT, given that other post-therapeutic circumstances could be adequately reproduced *in vitro*. Indeed, when vascu-
lar shutdown was simulated by placing cells in a hypoxic incubator directly after PDT, stabilization of HIF-1 could be detected in human epidermoid carcinoma cells. Although HIF-1 stabilization also occurred in untreated cells placed in hypoxic conditions, PDT exacerbated the extent of HIF-1 protein levels and HIF-1 induced gene expression. As such, these results provided a rationale for adjuvant HIF-1 inhibition during PDT, and demonstrate an important flaw in conventional 2D culture experiments with PDT.

It was initially hypothesized that HIF-1 inhibition would reduce the ability of tumor cells to maintain energy homeostasis via anerobic glycolysis. Although PDT on A431 human epidermoid carcinoma cells pretreated with the HIF-1 inhibitor acriflavine indeed resulted in a higher PDT efficacy, there were only minor alteration in glycolytic activity and no notable induction of glycolysis-associated genes. Instead, PDT induced the expression of PTGS2 and VEGF, which were reduced upon the pretreatment of cells with acriflavine. Given the abundant evidence that the products of these genes, COX-2 and VEGF, are potent survival stimulators that reduce the susceptibility of tumor cells to PDT, the increased susceptibility of ACF pretreated A431 cells was attributed to the reduced expression levels of these genes. Thus, the results of this study indicate that HIF-1 inhibition with acriflavine in combination with PDT is feasible, and that tumor cells that activate HIF-1 under hypoxic conditions are sensitized through reduced transcription and translation of PTGS2 and VEGF.

However, initial studies regarding HIF-1 stabilization also included the Sk-Cha1 cell line, the results of which are described in Chapter 9. Strikingly, these cells neither activated HIF-1 to a similar extent as A431 cells, nor expressed PTGS2 and VEGF at higher levels after PDT + hypoxia in comparison to hypoxia alone. Although ACF pretreatment exerted a beneficial effect on these cells, it did not appear to be related to reduced expression of neither PTGS2 nor VEGF, nor a range of other survival-related genes including those related to glycolysis. Moreover, ACF exerted its adjuvant effect in Sk-Cha1 cells under both under normoxic and hypoxic conditions, which was in sheer contrast to the effect observed in A431 cells. Thus, the results discussed in Chapter 9 pointed towards an alternative causality of ACF’s adjuvant effect in this cell line. With the notion that Sk-Cha1 cells retain an intact connection between DNA-damage and apoptosis through a functional p53 protein, and that ACF has been shown to inhibit topoisomerases, it was proposed that ACF exerts its adjuvant activity through topoisomerase inhibition and subsequent accumulation of DNA damage. Indeed, Sk-Cha1 cells exerted DNA damage, cell cycle stalling, and apoptosis upon prolonged exposure to ACF. In doing so, it was successfully combined with PDT albeit in a different manner in comparison to A431 cells.

Thus, although the selection of ACF as an adjuvant agent for PDT is therapeutically feasible in vitro, its effects may not be solely attributable to inhibition of HIF-1. Nonetheless, tumor cells that activate HIF-1 under the hypoxia that manifests itself after PDT appear susceptible to HIF-1 inhibition. These results underscore the potential of survival pathway inhibition in conjunction with PDT.

3.2 NF-κB

It was hypothesized that inhibition of nuclear factor κB (NF-κB), in a similar fashion as HIF-1, would result in higher PDT efficacy given its prominent survival-stimulating effects. However, it was expected that NF-κB inhibition would also affect the immunogenicity of the dying cells since the NF-κB transcription factor induces many genes that stimulate inflammation. As such, the beneficial effect of NF-κB inhibition might affect the antitumor immune response. In order to prove this hypothesis, a test system was devised in which the medium from PDT-treated EMT-6 murine mammary carcinoma cells was used to stimulate murine RAW 264.7 macrophages. Subsequently, the immunogenicity of NF-κB inhibition in the tumor cells was determined by measuring macrophage activation and cytokine excretion, as described in Chapter 10.

Initial observations confirmed that the medium from PDT treated EMT-6 cells was immunogenic and resulted in macrophage activation. However, in contrast to our hypothesis, the inhibition of NF-κB using siRNA yielded cells that were less susceptible to PDT. Even more confusing was the observation that tumor cells in which NF-κB was inhibited exerted higher immunogenicity than
those undergoing sham siRNA transfection, hinting towards a immunosuppressive role for NF-κB in tumor cells. Analysis of the excreted cytokines demonstrated substantially increased interleukin 6, tumor necrosis factor α, and chemokine C-C motif ligand 2 levels. These results again contradicted our hypothesis, in which it was predicted that NF-κB inhibition would result in reduced immunogenicity, whereas increased macrophage activation and cytokine excretion were observed. Comparisons with similar studies in the literature show greatly deviating results regarding cytokine production and function following PDT, making it difficult to draw firm conclusions on the effects of our observations.

As such, the results of this study underscore the importance of investigating the effects of pathway inhibition. As a tumor is not simply composed of tumor cells, but also contains (myo)fibroblasts, endothelial cells, and infiltrating immune cells that all communicate with one another, it is important to realize that thwarting with the effects of a transcription factor in tumor cells might disturb the function of cancer-associated cells within the tumor microenvironment.

3.3 Outlook

Although the signaling cascades that tumor cells initiate following PDT appear to be largely focused on survival, and many of them have been implicated as such, the inhibition of these pathways may not necessarily yield the expected results. Therefore, a deeper understanding of the described pathways is required in order to identify valid targets for adjuvant therapy in conjunction with PDT. Given the complexity of the signaling cascades, the interplay between tumor cells and tumor associated cells, and the molecular diversity of varying tumor types, it is advisable to follow up on the in vitro studies using in vivo models of therapeutically relevant cancer types. It should be noted that ACF has been used in humans as an antiviral agent and as a contrast agent, and was found to be well tolerated. In addition, a siRNA against transforming growth factor β (Trabedersen, Antisense Pharma, Regensburg, Germany) is currently in clinical trials for pancreatic cancer and malignant melanoma. Those developments emphasize the clinical translability of these types of pathway inhibitors. Further exploration on druggable targets within the identified survival pathways may therefore yield a plethora of compounds that may be utilized to sensitize tumors to PDT.

4. Development of multi-agent containing liposomes for photodynamic and chemotherapeutic combination treatment

4.1 Potential of a combination treatment of chemotherapy and PDT

Given the effective exploitation of the hypoxic tumor microenvironment that manifests itself after PDT through the inhibition of HIF-1, it became an intriguing notion to utilize adjuvant agents that only exert cytotoxic effects under low oxygen tensions. As such, the drug tirapazamine (TPZ) caught our attention. Under normoxic conditions, TPZ undergoes a futile oxidation/back oxidation cycle fueled by nuclear reductases, during which it is converted into a TPZ-radical and back to TPZ. However, since the back-oxidation consumes oxygen, the TPZ radicals accumulate in the nucleus under hypoxic conditions. Subsequently, these radicals have been shown to oxidize DNA, resulting in the formation of base damage, single strand breaks, and double strand breaks. This led us to hypothesize that TPZ could be activated post-PDT to exert an adjuvant cytotoxic effect on tumor cells. In order to confirm this hypothesis, the effects of TPZ were studied in p53-deficient A431 cells and p53 proficient Sk-Cha1 cells.

In Chapter 11, TPZ was shown to be well tolerated under normoxic conditions by both A431 and Sk-Cha1 cells. Under hypoxic conditions, TPZ induced oxidative stress and was substantially more toxic to both cell lines. The slow proliferating Sk-Cha1 cell line was more capable in coping with TPZ in comparison to A431 cells, in which the compound was highly toxic under normoxic conditions. Using roughly the IC50 concentrations of both TPZ and ZnPc-ETL PDT, it was shown that a
combination therapy of TPZ pretreatment and subsequent PDT with vascular shutdown simulation resulted in higher levels of cell death in comparison to either treatment alone. These adjuvant effects were more prominent in the Sk-Cha1 cells, and these effects were also observed under normoxic conditions. Analysis of the amount of DNA damage induced by TPZ showed that DNA damage was a preexisting condition in A431 cells, but that TPZ induced cell death in both cell lines under normoxic and hypoxic conditions. So, although TPZ was generally more toxic fast-proliferating (and p53-deficient) cells, adjuvant effects regarding PDT-efficacy were more prominent in p53-proficient cells. Thus, it was concluded that TPZ is a feasible adjuvant chemotherapeutic for PDT-combination therapies.

4.2 A combination therapy liposomal formulation containing both chemotherapeutics and photosensitizers

In the assessment of TPZ as a suitable adjuvant agent for combination therapy in conjunction with PDT, it was challenging to quantify the actual uptake of TPZ. Regarding the uptake of TPZ, it has been shown that only 1% of TPZ is taken up when administered to cells in free form. In addition, the clinical trials in which TPZ has studied for the treatment of cervical cancer have failed to yield convincing results and TPZ has also been associated with severe adverse events. Poor pharmacokinetic properties are most likely the cause of both the disappointing clinical results and the related morbidity. Thus, we subsequently sought to find means to improve the uptake and biodistribution of TPZ.

Research towards potentiating the selectivity of chemotherapeutics for tumor tissue has fueled the clinical approval of liposomal doxorubicin (Doxil), the first liposomal formulation approved for patient treatment. Doxorubicin represents a highly effective chemotherapeutic agent for the treatment of a wide range of tumor types, but its use is associated with severe adverse events. Congestive heart failure is the most lethal, owing to the affinity of doxorubicin for mitochondria and the high density of mitochondria within cardiac tissue. Thus, a liposomal formulation that delivers and releases doxorubicin specifically towards the tumor site would be a substantial improvement over conventional free administration of the drug. As such, Doxil, a liposomal formulation containing doxorubicin that yielded significantly higher intratumoral drug doses, intratumoral drug retention, and treatment efficacies, was developed and has received clinical approval in 1995. The liposomal formulation of Doxil is comparable to ITLs in that a neutrally charged lipid bilayer and steric stabilization with DSPE-PEG was employed. However in contrast to doxorubicin, TPZ's logP (http://www.drugbank.ca/drugs/DB04858) most likely does not support slow release into the tumor microenvironment. As an alternative to ITLs, ETLs were shown to be effectively taken up in vitro and to release the intraliposomal drugs within the tumor cells (Chapter 5). Thus, we hypothesized that the inclusion of TPZ into liposomes would facilitate higher intracellular drug doses, and a higher therapeutic efficacy at lower doses. By including ZnPC in the lipid bilayer of these liposomes, a single liposomal formulation could be forged for the utilization of both PDT and adjuvant hypoxia-activated chemotherapy.

By using a straightforward drug-loading method during the preparation of the liposomes in which the lipid film was hydrated with a solution containing TPZ, we successfully produced ETLs that contained both ZnPC and ETLs. These TPZ-ETLs were able to significantly increase intracellular drug levels and improve the PDT-efficacy to a higher extent as compared to the free compound. Moreover, substantially lower amounts of TPZ were used to obtain these results. Although TPZ exerted some ROS-scavenging capability in cell-free systems, it is anticipated that these were of minimal effect on the PDT-efficacy due to the mainly nuclear mechanism of action of TPZ, which is an intracellular site where ZnPC does not localize.
**4.3 Outlook**

PDT with adjuvant DNA-damaging chemotherapy has demonstrated in the treatment of perihilar cholangiocarcinoma, where it was shown that PDT was more effective when patients received adjuvant fluoropyrimidine chemotherapy. The enhanced efficacy of this combination therapy may lie in the induction of an auxiliary cell death pathway via DNA damage, which is not typically induced with PDT. With respect to the combination of TPZ and PDT, Baas et al. initially found only a minor increase in tumor response in mice bearing RIF-1 tumors that were treated with TPZ + PDT in comparison to PDT alone. Those results are in agreement with the minor beneficial effects of free TPZ found in clinical studies, which illustrates the necessity for a drug delivery modality for TPZ. Though the potential of liposomes for the selective delivery of chemotherapeutics such as doxorubicin and oxaliplatin to the tumor tissue has been well established, we are the first to encapsulate TPZ in a liposomal formulation. As such, this study may pave the way for further studies towards the efficacy of TPZ-ETLs, but may also stimulate investigations towards the feasibility of encapsulation other chemotherapeutics or adjuvant agents (such as ACF) with the aim of enhancing their selectivity and efficacy. In conclusion, the current study shows that chemophotodynamic therapy is possible with a single liposomal formulation that carries both photosensitizers and chemotherapeutics.

**5. Conclusions**

This thesis describes the development and *in vitro* proof of concept of the individual components of a comprehensive liposomal drug carrier system that delivers the photosensitizer ZnPC specifically towards the tumor cells, tumor vasculature, and the tumor interstitial spaces. Substantial increases in therapeutic efficacy may be achieved by combining PDT with adjuvant agents that block specific survival promoting signaling pathways. Additional combination therapies are potentiated by the inclusion of chemotherapeutics into photosensitizer-containing liposomes, yielding multifunctional liposomes with which both chemotherapy and PDT can be executed using a single liposomal formulation.

Thus, after obtaining thorough *in vivo* proof-of-concept and by understanding the underlying mechanisms of action, the strategies presented here may be further developed to ultimately provide a beneficial treatment for patients with therapy-resistant cancer. Improved destruction of larger tumor masses using the comprehensive photosensitization strategy, improved susceptibility of the tumors using targeted inhibitors of survival mechanisms, and reduced recurrence due to adjuvant and selective chemotherapy may be realized to achieve patient survival.

In conclusion, the strategies to improve the efficacy of PDT that are presented presented in this thesis may culminate in substantial improvements in PDT for the treatment and management of cancer.

**References**