CHAPTER X

CURRENT DEVELOPMENTS IN USING *MESO*-(TETRA)SUBSTITUTED PORPHYRINS FOR PDT

Yasser M. Shaker ^{a,b}; Ayman M. K. Sweed ^{a,b};
Claire Moylan ^a; Luke Rogers ^a; Aoife A. Ryan ^a;
Rosine Petitdemange ^a; Mathias O. Senge ^{a,c*}
E-mail: sengem@tcd.ie

^a *School of Chemistry, SFI Tetrapyrrole Laboratory, Trinity
Biomedical Science Institute, 152-160 Pearse Street, Trinity College
Dublin, The University of Dublin, Dublin 2, Ireland*

^b *Department of Chemistry of Natural and Microbial Products,
Division of Pharmaceutical and Drug Industries, National Research
Center, Dokki, 12622 Cairo, Egypt*

^c *Medicinal Chemistry, Institute of Molecular Medicine, Trinity
Centre for Health Sciences, Trinity College Dublin, St James's
Hospital, Dublin 8, Ireland*

meso-Substituted porphyrins present a readily accessible class of porphyrins which has found wide applications in biomedical sciences. Contemporary syntheses allow the generation of large libraries of compounds with variable *meso* substituents or the preparation of

bioconjugates. This chapter summarizes current synthetic strategies and medicinal chemistry applications of *meso*-substituted porphyrins in photodynamic therapy (PDT).

1. Introduction

The goal of this chapter is to describe recent advances in the development and use of *meso*-tetrasubstituted porphyrins in photodynamic therapy (PDT). The focus is on analogues of 5,10,15,20-tetrakis(3-hydroxyphenyl)chlorin (*m*-THPC) and related second and third generation photosensitizers (PSs) currently under development. There has been a rapid growth in the literature on this topic. The chapter summarizes the conjugation of *meso*-tetrasubstituted porphyrin photosensitizers with biologically active elements (amino acids, bile acids, peptides, folic acid, sugars, oligonucleotides, monoclonal antibody), where most of the contemporary work also focuses. Porphyrin isomers and core modified porphyrins will also be discussed, followed by a brief discussion of nanoparticle derived systems.

The story began in 1912, with the first ‘clinical trial’ of porphyrins in humans when Friedrich Meyer-Betz injected himself intravenously with 200 mg of the crude naturally occurring material isolated from haemoglobin, haematoporphyrin (Hp), and investigated its effect on his skin. Erythema (reddening), edema (swelling) and pain developed in the area exposed to light.¹ Over 40 years later Schwartz² treated crude Hp with acetic acid and sulfuric acid, followed by treatment with strong alkali base. The resulting compound was given the name haematoporphyrin derivative (HpD) and it demonstrated increased tumor localization and fluorescence properties, which could be implemented in diagnostic tools.³ HpD consists of a mixture of haematoporphyrin monomer, dimer and oligomers. Elimination of the Hp monomer (the less active porphyrin) by partial purification produced porfimer sodium which was clinically approved for use in PDT in 1993 under the trade name Photofrin.⁴ It is less heterogeneous than HpD but still contains a large number (approx. 60) of porphyrin compounds.⁵ Besides its poor water solubility, Photofrin has a low molar absorption coefficient (1,170

$\text{M}^{-1}\text{cm}^{-1}$ at 630 nm) and thus requires high drug concentrations and light doses to achieve tumor destruction. Moreover, Photofrin possesses a low tumor/normal tissue selectivity of less than one. Another limitation is the long lasting cutaneous photosensitivity due to its localization in the skin during which the patient should avoid sunlight for 4-6 weeks. Thus, while Photofrin was perceived as a breakthrough “compound”, in terms of opening a new treatment modality for cancer, it has significant drawbacks.

As a result, the search for improved photosensitizers with enhanced photophysical properties is of important requisite for future advances in PDT. The active phase of the synthesis of second generation photosensitizers focused on pure compounds with defined chemical composition and strong absorption at wavelengths between 650 nm and 800 nm for more effective PDT. Here, porphyrins, chlorins, and bacteriochlorins were the main targets as they have absorption maximum in the red region and have high singlet oxygen quantum yields.⁶ The classic example for a second generation porphyrin-based photosensitizer is 5,10,15,20-tetrakis(*m*-hydroxyphenyl)chlorin (*m*THPC, **2**, Fig. 1).^{6,7}

This compound received regulatory approval in Europe in 2001 from the EMA under the name Foscan (Temoporfin). Currently it is used for the palliative treatment of head and neck cancers. For example, in its early stage in a confirmation study carried out with 29 patients, it gave complete local reduction of squamous cell carcinoma of the head and neck as an alternative modality for surgery and radiotherapy.⁸ The parent porphyrin compound (5,10,15,20 tetrakis(*m*-hydroxyphenyl)porphyrin, *m*THPP, **1**) is 25-30 times as potent than HpD in PDT; however, smaller drug doses and less side effects are possible with *m*THPC and *m*THPBC.^{9,10}

Fig. 1. Chemical formulae of *m*THPC related compounds.

In a comparative study, *m*THPC showed superior uptake and tumor localization compared to *m*THPP.¹¹ The bacteriochlorin **3** was less photostable and thus **2** has received the most widespread use of these compounds.¹² It has a strong absorption band at 652 nm and a singlet oxygen quantum yield of 0.43, which is higher than *m*THPP. In addition, temocene (*m*-THPPo) **4** is an interesting example of a porphycene analogue of *m*THPC. It has excellent photophysical properties which make it a promising photosensitizer for PDT applications.¹³

One possible pitfall of porphyrin-based photosensitizers is their tendency to form aggregates, which affects their biodistribution.¹⁴ Here, introduction of a polar functional group can increase the solubility and enhance the pharmacokinetics of the drug. Thus, one aspect of contemporary research is a focus on amphiphilic porphyrins and related

bioconjugates, *e.g.*, glycoporphyrins.¹⁵ The hydrophilic part increases the solubility of the drug in the blood stream and prevents aggregation while the hydrophobic part aids uptake, membrane passage and localization. For example, with *m*THPC, the introduction of electron withdrawing groups or alterations of the hydroxy groups resulted in a decrease in the partition coefficient and a significant increase in solubility.¹⁶

The main focus of this review is on such FGI and bioconjugate strategies and how controlling the type of the substituent at the *meso* position and conjugation with biologically active elements can increase the efficiency of the porphyrin structure unit for PDT. Figure 2 is a representative summary of the various strategies possible on tetrapyrrole **5** in this context. Note, this chapter focuses exclusively on *meso* substituted porphyrins of the A_x- and ABCD-type.¹⁷

Fig. 2. Illustration of various bioconjugate strategies for porphyrin-based photosensitizers.

2. *m*THPC (Temoporfin)

*m*THPC (Temoporfin) can be synthesized in acceptable yields as

outlined in Figure 3. It's synthesis involves the condensation of pyrrole **7** with *m*-acetoxybenzaldehyde **6** in propionic acid under heating at reflux for 30 minutes followed by deprotection of the acetyl group to yield 5,10,15,20-tetrakis(*m*-hydroxyphenyl)porphyrin **1**. Reduction of **1** without protection of the phenolic groups can be achieved using *p*-toluenesulphonylhydrazide to form 5,10,15,20-tetra(*m*-hydroxyphenyl)chlorin (*m*THPC, **2**).⁹ A second reduction yields 5,10,15,20-tetra(*m*-hydroxyphenyl)bacteriochlorin (*m*THPBC, **3**).¹⁸

Fig. 3. Synthesis of *m*THPC.

The pharmacological properties of the basic photosensitizer can easily be modulated through simple transformations or formulation strategies. For example, encapsulating *m*THPC into nontoxic liposomes (Foslip[®]) or modified pegylated liposomes (Fospeg[®]) yields carriers of high capacity for the otherwise poorly water-soluble drugs.^{19,20} Additional benefits are different drug release times,²¹ different selectivities for tissue and cell types and more rapid tissue clearance,²²

improved pharmacokinetics and lower drug and light doses.^{23,24,25}

We have recently reviewed the state-of-the-art for *m*THPC and its development and the reader is referred to these articles for more details on chemical and biological studies of Temoporfin.^{7,10} This article focuses on recent advances with related compounds and aims to update these more comprehensive works.

3. New meso-Substituted Porphyrin Systems

3.1 *m*THPP-Related Systems

An ideal photosensitizer must have a long lived triplet state ($\tau_T > 5 \mu s$), as it is considered the most important precursor of the $O_2(^1\Delta_g)$ state. One such frequented strategy implemented to maximize the Φ_T of a given photosensitizer is through the heavy atom effect. Through the introduction of heavy atoms, *e.g.*, the halogenation of porphyrins as described below; one can enhance the rate of the transition of the PS from its singlet to triplet state (intersystem crossing), which is a spin-forbidden process.

Fig. 4. Brominated hydroxyphenylporphyrins.

As such, bromination of porphyrins favors reactive oxygen species (ROS) production by increasing the formation of triplet states and singlet oxygen generation. Laranjo *et al.* aimed to exploit this heavy atom effect by functionalizing hydroxylporphyrin derivatives with bromine *via* formation of 5,10,15,20-tetrakis(2-bromo-3-hydroxyphenyl)porphyrin **9** and 5,15-bis(2-bromo-3-hydroxyphenyl)porphyrin **10** as represented in

Figure 4.²⁶ *In vitro* studies with A375 and WiDr cells showed that for colorectal adenocarcinoma cells, IC₅₀ values of 464 nM for **9** and 180 nM for **10** could be determined. Melanoma cells, with an IC₅₀ of 100 nM for **10** and 50 nM for **9**, were more susceptible to both sensitizers. For *in vivo* studies Balb/c nu/nu mice were used and gave satisfactory results with **10**.

Despite the relative success of these brominated systems, the bulk of studies conducted tend to focus more on the creation of amphiphilic systems. A typical example is a study by Ben-Dror *et al.* using a series of *meso*-substituted porphyrins **11** with various degrees of hydrophobicity, closely related to *m*THPP **1** and *m*THPC **2** (Fig. 5).²⁷ The porphyrins used had alkyl chains with different lengths at the 5 position and 3-hydroxyphenyl substituents at the 10, 15 and 20 positions. These porphyrins were found to be very hydrophobic, with log P values in the range of 8.9 to 11.8. The correlation between log P and K_b (the binding constant of these porphyrins into liposomes) was found to be negatively linear. This indicates that an increase in hydrophobicity results in a decreased binding affinity to liposomes. However, all the synthesized porphyrins were taken up and penetrated into cells.

Fig. 5. *meso*-Substituted hydroxyphenylporphyrins with different degrees of hydrophobicity.

More recently, Rojkiewicz *et al.* [2013] developed a series of tetrakis(hydroxyphenyl)porphyrin derivatives containing long alkyl chains (*via* ether, ester and amide linkages) as potential photosensitizers in PDT. They determined the absorption, fluorescence spectra, triplet state lifetime and singlet oxygen generation capacity of the synthesized compounds.²⁸

More a step into the realm of compounds for electron transfer studies

are the porphyrin- C_{60} (P- C_{60}) **12** and its metal complex (ZnP- C_{60}) **13** dyads.^{29,30} These studies represented the first evaluation of dyads with a high capacity to form a photo-induced charge-separated state for PDT. The fluorescence quantum yield for both dyads were obtained in toluene and DMF and in both solvents these dyads show only very weak emission from the porphyrin moiety, indicating strong quenching of the porphyrin excited singlet state by the attached fullerene ($\sim 3.4 \times 10^{-3}$ for **12** and $\sim 1.5 \times 10^{-3}$ for **13**). Compound **12** in toluene exhibited a singlet oxygen quantum yield of 0.80, whereas in DMF the value changed to 0.18. A similar result was observed for **13** which displayed a singlet oxygen quantum yield of 0.62, whereas in DMF the value diminished to 0.04. Porphyrin **12** showed an inactivation of 80 % of Hep-2 human larynx-carcinoma cells with 54 J/cm² of irradiation; predominantly *via* apoptosis (Fig. 6).

Fig. 6. Porphyrin-fullerene dyads investigated in PDT.

3.2. Cationic Porphyrins

Cationic porphyrins have long captivated the attention of PDT researchers as they are endowed with a number of desirable traits, the most interesting of which being their increased water solubility. This family of tetrapyrroles is currently undergoing a renaissance of sorts in the field of anti-microbial PDT due to their rare ability to destroy Gram-negative bacteria. For example, four 5,10,15,20-tetraphenylporphyrin

(TPP) derivatives bearing either triphenylphosphonium (**14** and **15**) or triethylammonium (**16** and **17**) and terminated alkoxy groups at either the *para*- (**14** and **16**) or *meta*- (**15** and **17**) position of a *meso* phenyl residue were synthesized by Lei *et al.* (Fig. 7).³¹

Fig. 7. Cationic porphyrins used in PDT studies.

All compounds exhibited similar absorption and fluorescence spectra along with similar $^1\text{O}_2$ quantum yields. The more lipophilic nature of triphenylphosphonium ion over triethylammonium ions results in higher octanol/water partition coefficients for **14** and **15** compared to **16** and **17**. Confocal fluorescence microscopy proved that **14–17** accumulate in the mitochondria. MTT assays showed that these derivatives have significant phototoxicity and that their dark toxicity is negligible at concentrations of 6–10 μM towards the human breast cancer cell line MCF-7. The $^1\text{O}_2$ quantum yields of **14–17** were measured in acetonitrile and found to be 0.53, 0.50, 0.57, and 0.58, respectively. Porphyrin **14** exhibited the least dark cytotoxicity and **16** possessed the highest phototoxicity among the four porphyrins.

In a similar context Jensen *et al.* [2010] synthesized a series of cationic porphyrins **18–21** bearing one to four $-\text{N}(\text{CH}_3)_3^+$ groups linked to the *p*-phenyl positions.³² They investigated the effect of overall charge and its distribution on the cellular uptake, phototoxicity and intracellular localization using human carcinoma HEP2 cells. The dicationic porphyrin **19** accumulated the most within cells and preferentially localized within vesicular compartments and in mitochondria. The monocationic porphyrin **18** proved to be the most phototoxic of the series, localizing primarily in lipid membranes, including the plasma membrane, ER, mitochondria, and Golgi. Both the tricationic porphyrin **20** and the tetracationic porphyrin **21** localized subcellularly, primarily in the mitochondria, but of the two only **21** showed moderate phototoxicity ($\text{IC}_{50} = 8 \mu\text{M}$ at $1 \text{ J}\cdot\text{cm}^{-2}$).

Further studies looked at the role of amphiphilicity and zinc insertion on PDT efficacy. Pavani *et al.* evaluated the PSs **22** and **23** which exhibited similar photophysical properties ($\Phi_f \leq 0.02$; $\Phi_A \sim 0.8$).³³ Both an increase in lipophilicity and the presence of zinc in the porphyrin resulted in higher vesicle and HeLa cell uptake. Porphyrin **22** was more enriched in the mitochondria and the zinc derivative **23** had the highest global uptake. Zinc insertion decreased the interaction with isolated mitochondria and with the mitochondria of HeLa cells. Phototoxicity increased proportionally with membrane binding efficiency. Cytotoxicity tests showed that these porphyrins have no dark toxicity, whilst **23** exhibited good cytotoxicity with ~85% tumor eradication with a light

dose of 175 mJ.cm^{-2} .

Water-soluble fluoro-substituted porphyrins were designed for similar studies by Ko *et al.*³⁴ Of the porphyrins **24–27** the latter two exhibited significantly higher photocytotoxicity on HeLa cells. IC_{50} values were $0.87 \mu\text{M}$ for porphyrin **26** and $1.1 \mu\text{M}$ for porphyrin **27**, while IC_{50} values of $3 \mu\text{M}$ and $40 \mu\text{M}$ were obtained for **24** and **25** respectively. Regarding overall PDT efficacy, it was determined that compound **27** was the most effective PDT photosensitizer based on the studies conducted with HeLa cells.

Related studies focused on water-soluble tricationic porphyrins,³⁵ brominated cationic porphyrins,³⁶ and polymethine conjugated porphyrins as dual chromophores. These exhibited promising photodynamic activity either *in vitro* or *in vivo*.³⁷ This class of compounds still merits further investigations for both PDT and anti-microbial PDT.

3.3. Porphyrin-Polyamine Conjugates

Polyamines, *i.e.* molecules which contain two or more primary amino groups, are essential for normal cell growth. Rapidly proliferating cancer cells require more polyamines than their biosynthetic abilities allow, thus they tend to use a polyamine transport system (PTS) to fulfill their needs. The increased uptake of polyamines by cancer cells is an attractive chemotherapeutic approach. Previous studies have shown that the PTS can readily take up heterocyclic derivatives if conjugated to natural polyamines.³⁸

Hence, recent studies in PDT have focused on comparing the efficacy of porphyrin-polyamine conjugates in relation to Photofrin. These studies indicate a better efficiency of polyamine conjugates over the clinically approved PS. For example, Sarrazy *et al.*³⁹ synthesized a number of porphyrin-polyamine conjugates containing two or four spermidine or spermine units (Fig. 8).⁴⁰ Their phototoxicity was evaluated using human keratinocyte (HaCat) and human breast cancer (MCF7) cell lines and all conjugates were more efficient than Photofrin at inducing cell death *via* apoptosis. The *in vitro* cytotoxicity of the compounds was evaluated and their phototoxicity followed the order **31** ($65.4 \pm 22.5 \mu\text{M}$) > **28** > **29**, **32**

> **30** and **32** ($49.4 \pm 25.7 \mu\text{M}$) > **28** > **29** > **31** > **30** according to IC_{50} values for HaCat and MCF7, respectively.

Fig. 8. Spermidine and spermine appended porphyrins.

Fig. 9. Carboranyl- and polyamine-appended porphyrins.

Polyamine conjugates have also been employed in conjunction with Boron Neutron Capture Therapy (BNCT). Vicente *et al.* provided promising results on the synthesis of carboranyl-containing porphyrins and chlorins aimed at developing dual modality photosensitizers suitable for both PDT and BNCT cancer treatment.^{41,42} The results suggest that

carboranylchlorins are good candidates for this dual treatment modality and warrant further investigations. Recently, seven polyamine conjugates of a tri(*p*-carboranylmethylthio)-tetrafluorophenylporphyrin (**33-39**) were prepared in high yields by sequential substitution of the *p*-phenyl fluoride of tetrakis(pentafluorophenyl)porphyrin (TPPF) (Fig. 9).⁴³ The polyamines used were derivatives of the natural-occurring spermine with different lengths of the carbon chains, terminal primary amine groups and, in two of the conjugates, additional aminoethyl moieties. The tri(polyethylene glycol)conjugate **40** was also synthesized for comparison. The polyamine conjugates showed low dark cytotoxicity ($IC_{50} > 400 \mu M$) and low phototoxicity ($IC_{50} > 40 \mu M$ at $1.5 J \cdot cm^{-2}$). All polyamine conjugates, with one exception, showed higher uptake into human glioma T98G cells (up to 12-fold) than the PEG conjugate, and localized preferentially in the ER, Golgi and lysosomes.

3.4. *meso*-Tetrasubstituted Chlorins

Although *m*THPC is an effective PS for PDT it still suffers from drawbacks; amongst others is the ease of re-oxidation to the parent porphyrin **1**. Thus, attempts have been made to prepare related compounds with substituents at the reduced pyrrole ring. A classical approach is the use of dihydroxy derivatives which have been used by Rancan *et al.* to verify the *in vitro* effect of different substituents in dihydroxychlorins and the influence of amphiphilicity on intracellular uptake, subcellular localization, and photosensitizing activity (Fig. 10).⁴⁴ Variations in the substituents significantly influenced the intracellular uptake of these chlorins. For example, chlorin **42** which was the most polar sensitizer in this study, depending on incubation time, yielded an intracellular concentration in Jurkat cells 2–8 times higher than the unsubstituted chlorin **41**. These systems localize predominantly in lysosomes and increasing the amphiphilicity of the sensitizer molecules correlated with increased sensitizer uptake and increased number of necrotic cells after irradiation.

Recently, the synthesis of novel β -functionalized derivatives of *m*THPC was achieved *via* nucleophilic addition reactions to diketochlorin derivatives.⁴⁵ The intriguing β -substituted

dihydroxychlorin products **43–45** exhibited strong absorption in the red spectral region, high singlet oxygen quantum yields (0.97, 1.56 and 1.10 respectively), and were found to be highly effective in *in vitro* assays against HT-29 tumor cells. Chlorins **43** and **45** showed phototoxicity at concentrations of 2 and 10 μM respectively, and were similar in activity to *m*THPC **2**. Chlorin **44** displayed a lower activity and was only moderately effective at a concentration of 10 μM .

Fig. 10. Chlorins related to *m*THPC.

4. Bioconjugates of meso-Tetrasubstituted Porphyrins

4.1. Glycoconjugated Porphyrins

The synthesis of porphyrin-carbohydrate conjugates is based around the fact that on the surface of cancer cells an overexpression of specific extracellular proteins is observed. These proteins are known as lectins and they bind carbohydrates and therefore, porphyrins possessing carbohydrate groups could, in theory, enhance the uptake of the photosensitizer into, for example, retinoblastoma cells (Y79) bearing α -mannose and β -galactose receptors. Melanoma B16 cells and adenocarcinoma colon cells HT29 have been found to overexpress β -

galactose and β -glucose receptors respectively, and these cell lines could provide additional targets for such PS conjugates.⁴⁶ As a result, a significant body of information on sugar-appended porphyrins is available.⁴⁷

4.1.1. Carbohydrates Directly-attached to the *meso* Position

Two recent examples can serve to illustrate the synthetic strategies used for directly linked porphyrin-carbohydrate conjugates. Casiraghi *et al.* and coworkers constructed porphyrins bearing two and four glycosidic units directly linked at the *meso* positions.⁴⁸ Notably, the two palladium derivatives **46** (*C-meso*-linked aryl-uridinyI porphyrin) and **47** (tetra-*C*-glycosylated porphyrin) were proven to be effective for the selective cleavage of double stranded DNA into form II nicked circular DNA on exposure to visible light at RT in aqueous media (Fig. 11). Thus, some of the compounds illustrated here may have additional uses next to PDT.

Fig. 11. Directly *meso*-linked glycoporphyrins.

More directly related to PDT are photosensitizers prepared *via* fusion of the C6 of the pyranose sugar to the *meso* position of the porphyrin.⁴⁹

Examples are the C_2 -symmetric conjugates **48-55** and D_2 -symmetric derivative **56**. The tetraglyco-fused porphyrin **56** gave the highest cellular uptake and photocytotoxicity both in HeLa and HCT116 cells. Compounds **49**, **50** and **54** bearing electron donating groups showed high phototoxic effects in both human cancer cell lines compared to **51-53**.

4.1.2. Porphyrin-Linker-Carbohydrates Systems

(a) O-Glycosylated Porphyrins and Chlorins

From the outset of their pioneering studies in this area Maillard and coworkers identified the impact of porphyrin glycosylation on photosensitizers related to systems such as *m*THPC.⁵⁰ For example, they synthesized glucosyl conjugated tri- and tetra(*meta*-hydroxyphenyl)chlorins and investigated their PDT effect in HT29 human colorectal adenocarcinoma cells (Fig. 12).⁵¹ Typically the symmetrical tetraglycosylated chlorins displayed poor internalization and photoactivity compared to the asymmetric, more amphiphilic derivatives (**57**, **58** {1:1}). The latter showed better photoactivity compared to *m*THPC with light doses of 5-25 J.cm⁻². A comparison of the log P values of *m*THPC (>3), **57**, **58** (1.45) and the tetraglycosylated derivative (-1.31) again shows the importance of amphiphilicity in such structures. The fluorescence emission spectra of all compounds were similar; however, the glycosylated derivatives exhibited slightly better fluorescence quantum yields ($\phi_f = \sim 0.12$ vs. *m*THPC, $\phi_f = 0.095$).

Photosensitizers have been shown to bind to several plasma proteins depending on their amphiphilicity. The more hydrophobic photosensitizers have a tendency to bind to lipoproteins while hydrophilic photosensitizers tend to bind to albumin.^{52,53} Albumin is the most important drug carrier protein. During cellular uptake and phototoxicity tests, it was shown that **57** and **58** associated more to the albumin protein compared to *m*THPC. Both compounds were partly internalized by an active receptor-mediated endocytosis mechanism compared to *m*THPC which is mediated by an active uptake process *via* a low density lipoprotein (LDL).^{54,55} Passive diffusion must also play a role in internalization. Cellular uptake of **57** and **58** was 30 % lower

compared to *m*THPC and exhibited saturation. Maximum phototoxicity in HT29 cells was observed with concentrations as low as 2 μ M at light doses of 5-25 J.cm⁻². The roughly 4-fold better efficacy compared to *m*THPC was attributed to a higher accumulation of the former in the mitochondria.

Fig. 12. *O*-Linked glycoporphyrins related to *m*THPC.

The related porphyrin compound **59**, 5,10,15-tris(*meta-O*- β -D-glucosyloxyphenyl)-20-phenylporphyrin, was used for an *in vivo* study in healthy rats.⁵⁶ In order to establish the pharmacokinetics of this class of compounds, three different drug doses of **59** were administered (0.25, 0.5

and 1 mg.kg^{-1}), and compared to *m*THPC at 0.3 mg.kg^{-1} . The conjugate **59** was cleared more rapidly from the organism compared to *m*THPC, and its mean residence time was 5 h compared to *m*THPC, which had a residence time of 20 h with no metabolite of either PS identified. Looking at the biodistribution, a large amount of the glycoporphyrin was concentrated in organs such as the lungs, liver and spleen which are rich in reticulo-endothelial cells. The maximum PS concentration in the organs was observed at 14 h post-infection and within 48 h the PS was eliminated from all organs.

Fig. 13. Glycoporphyrins and -chlorins employed for uptake and PDT studies in HeLa cells.

Many related studies have focused on chlorins and the impact of different types and arrangements of the carbohydrate units. Initially progress was hampered by the lower stability of chlorins but significant improvements have been made through the preparation of glycochlorins.^{57,58,59,60} A typical *in vitro* study examined the cellular

uptake and photocytotoxicity of glycoconjugated chlorin derivatives with β -D-glucopyranosyl, β -D-galactopyranosyl, β -D-xylopyranosyl and β -D-arabinopyranosyl groups using HeLa cells and compared them to tetraphenylporphyrin tetrasulfonic acid (TPPS) (Fig. 13).⁶¹ The 5,10,15,20-tetrakis[3-(β -D-xylopyranosyloxy)phenyl]chlorin (**61mc**) showed a 50 times higher uptake than TPPS. All glycochlorins exhibited higher uptake than TPPS, and either equal or higher uptake than the porphyrin derivatives. The order of cellular uptake was as follows: **61mc**>**61pd**>**61md**>**61pa**>**61pb**>**61pc**>**61ma**>**61mb**>TPPS in line with the higher lipophilicity of the glycochlorins.

Studies on the photocytotoxicity of these compounds used 5,10,15,20-tetrakis[3-(β -D-xylopyranosyloxy)phenyl]chlorin **61mc** and 5,10,15,20-tetrakis[3-(β -D-glucopyranosyloxy)phenyl]chlorin **61ma** in HeLa cells. At a drug dose concentration of 2×10^{-7} M, their toxicity decreased in the order of **61ma** > **61mc** > TPPS, indicating that the photocytotoxicity was not related to cellular uptake. The LD₅₀ for **61mc** was $\sim 2 \times 10^{-7}$ M and 5×10^{-6} M for **60pa**.⁶²

Tetraphenylporphyrin-type systems have been adopted by several groups to investigate the effect of glycosyl groups at the periphery of the porphyrin macrocycle. Overall, the triglycosylated derivatives are more phototoxic than the parent tetrapyrrole or the symmetrical tetraglycosylated derivatives.⁵⁰ The photobiological properties of glycoporphyrins are dependent on the linker, the type of glycoside residue and the anomeric configuration.⁴⁷

Returning to the fundamental contributions by Maillard, his group synthesized diethylene glycol (Deg) linked glycoporphyrins and studied their PDT effect in human retinoblastoma cells (Y79).⁶⁰ Compounds **63-67** (Fig. 14) showed maximum fluorescence emission at 632 nm and a weak band at 720 nm with relatively low fluorescence quantum yields of ~ 0.03 . Compound **62** was internalized poorly by Y79 cell lines compared to the glycosylated derivatives. The most significant result was the high uptake of **65** and its good photoactivity (LD₅₀ = 0.35 μ M) compared to the parent compounds **62** and **67**. The singlet oxygen production was similar for all glycosylated compounds ~ 0.47 . The compounds were tested for uptake by murine melanocyte cells (B16) and human adenocarcinoma cells (HT29) and behaved similarly at 4 h incubation at

4 μ M concentration. Initial work carried out on the pre-incubation of cells in glycosylated albumin showed a 40-45% inhibition of uptake of TPP(*p*-Deg-*O*- β -GalOH)₃ and TPP(*p*-Deg-*O*- α -ManOH)₃ indicating a cell-sugar-receptor saturation.

Fig. 14. Diethylene glycol (Deg) linked glycoporphyrins.

Expanding on earlier work using **57**, **58** and **65** in HT29 and Y79 cell lines, a number of porphyrin and chlorin derivatives were explored for photocytotoxicity using weaker light doses of 1.8 J.cm^{-2} and excitation at $>540 \text{ nm}$ after 24 h incubation.⁶³ Even though the glycoconjugated derivatives had lower PDT activity compared to *m*THPC they displayed IC_{50} values of $2.4\text{--}0.05 \text{ }\mu\text{M}$ against Y79 cells. The *para*-*O*- β -glucose porphyrin derivative **68** was found to have the best phototoxicity compared to the parent porphyrin system with an IC_{50} of $0.9 \text{ }\mu\text{M}$ and no cytotoxicity in the absence of light with an $\text{IC}_{50} > 15 \text{ }\mu\text{M}$. However, this compound is less PDT active compared to the Deg-porphyrin conjugates described previously (**63–65**). The extension of the linker between the glycoconjugate and the chromophore appears to significantly increase the PDT activity. Also highlighted was the difference in the anomeric configuration with **64** ($\text{IC}_{50} = 0.05 \text{ }\mu\text{M}$) showing a 10-fold greater phototoxicity than **69** ($\text{IC}_{50} = 0.6 \text{ }\mu\text{M}$) in Y79 cells.

Hammerer *et al.* synthesized the carbohydrate-porphyrin-triphenylamine hybrid **70** containing a porphyrin linked at the *meso* position with triphenyl amine *via* an alkyne bond while the other three *meso* groups were conjugated with diethylene glycol mannose units (DEG-Man).⁶⁴ Interestingly, the hybrid **70** displayed high singlet oxygen quantum yields as well as good two photon absorption (2PA) cross section values as a result of the conjugating alkyne unit. However, compound **70** was inactive against HT-29 and retinoblastoma Y79 cells.

The number of available glycoporphyrins has increased significantly through the use of ‘click’-reactions. The 1,3-dipolar cycloaddition between an azide and an alkyne, that results in the formation of a 1,2,3-triazole moiety has recently been implemented by a number of research groups to obtain a wide range of *O*-glycoporphyrins. This synthetic strategy is considered an effective and highly efficient method for the conjugation of sugar units to the porphyrin periphery due to the high synthetic yields and the relatively simple and accessible experimental conditions.^{65,66,67,68,69}

Together with Scanlan we reported on mono-, di-, and trisubstituted glycoconjugated porphyrin compounds using ‘click chemistry’.^{70,71} This involved the Cu(I)-catalyzed reaction between various azido porphyrins and propargylic carbohydrates to form a library of glycoporphyrins using

microwave mediated reactions. Figure 15 gives a representative example for the synthesis of the difunctional glycoporphyrin **73** from azido porphyrins **71** and propargyl mannose **72** via a "double-click" process. The compounds were evaluated against esophageal squamous-cell carcinoma cell lines.

Fig. 15. Synthesis of a bisglycoporphyrin via 'click' reaction.

(b) *S*-Glycosylated Porphyrins and Chlorins

S-linked glycoconjugates are considered to be more stable than their *O*-linked counterparts and thus several porphyrin derivatives of the former have been reported. In several cases this has been combined with the use of fluorinated porphyrins which have high triplet quantum yields due to their enhanced intersystem crossing.⁷² Examples are the *S*-glycoside conjugated porphyrins **74** and **75** (Fig. 16).⁷³ The tetraglucose-porphyrin conjugate **74** showed better uptake compared to the galactose derivative **75** in the human breast cancer MDA-MB-231 cell line and also displayed better PDT activity.⁷⁴

Fig. 16. S-Linked glycoporphyrins.

A 'complete set' (mono-, *cis*-bis-, *trans*-bis-, tris- and tetrakis) of tetrakis(perfluorophenyl)porphyrins substituted with β -D-glucopyranosylthio groups were investigated by Hirohara *et al.*⁷⁵ The *S*-glucosylated porphyrin *trans*-**78** showed excellent photocytotoxicity ($EC_{50} < 5$ nM) in HeLa cells. The log P affecting the uptake of the porphyrin derivatives was determined and decreased from 5.1 to 0.8 in the order **76** > *cis*-**77** > *trans*-**78** > **79** > **74**. The photocytotoxicity was examined at a drug dose concentration of 100 nM in HeLa cells with photoirradiation at 16 J.cm⁻² and 24 h incubation post treatment. All drug molecules displayed no dark toxicity and the photocytotoxicities of **76**, *cis*-**77**, *trans*-**78**, **79**, and **74** were much higher than TPPS. EC_{50} values were <5 nM (*trans*-**78**), ca. 80 nM (**79**) and ca. 90 nM (**76**, *cis*-**77**, **74**). Porphyrin *trans*-**78** had a 20 times higher efficacy than the other *S*-glucosylated porphyrins.

In order to utilize the heavy atom effect to increase the ¹O₂ quantum yield, several metalloporphyrins were investigated. For example, 5,10,15,20-tetrakis(4-(2,3,4,6-tetra-*O*-acetyl- β -D-glucopyranosylthio)-2,3,5,6-tetrafluorophenyl)porphyrin **80a** and its Zn(II), Pd(II), and Pt(II) complexes **80b**, **80c**, and **80d** were synthesized and deprotected to give the *S*-glucosylated derivative **74** and its metal complexes **81b**, **81c**, and **81d**.⁷⁶ The singlet oxygen production increased in the order of free-base fluoroporphyrins (**80a**, 0.08 and **74**, 0.06) < Zn(II) complexes (**80b**, 0.055 and **81b**, 0.056) < Pd(II) complexes (**80c**, 0.003 and **81c**, 0.002). However, the ¹O₂-production of the Pt(II) complexes (**80d**, 0.001 and **81d**, 0.001) was very low. *In vitro* photocytotoxicity was measured with HeLa cells incubated for 24 h at a drug dose of 0.5 μ M (16 J.cm⁻²). The protected derivatives **80a**, **80b**, **80c**, and **80d** showed no photocytotoxicity while **74**, **81b**, and **81c** were active. Compounds **74**, **81b**, **81c**, and **81d** induced 90 % cell death (EC_{90}) at ~ 0.2 μ M in each case. This is about 25 times lower than observed for the *O*-glucosylated derivatives ($EC_{90} > 5$ μ M).⁷⁷

Hirohara *et al.* performed comparative analyses of a range of glycosylated TPPs and chlorins.⁷⁸ The *O*-glycosylated chlorins had promising photocytotoxicity in HeLa cells but were difficult to purify.^{79,80} Photosensitizers **80a**, **82**, **83**, **85**, **87** and **88** showed no significant photocytotoxicity at a concentration of 0.5 μ M (24 h, 16 J.cm⁻²

²), while the deprotected photosensitizers **74**, **75**, **84** and **86** were photocytotoxic (Fig. 17). The *S*-glucosylated photosensitizers **74** and **84** showed higher photoactivity than *S*-galactosylated **75** and **86** respectively. The drug concentrations inducing 50 % cell death (EC₅₀) of **74**, **75**, **84** and **86** were estimated to be ~0.15, 1.5, 0.07 and 0.3 μ M respectively.⁷⁸

Fig. 17. *S*-Linked glycoporphyrins and -chlorins.

Drain's group extended such studies to the bacteriochlorins and isobacteriochlorins.⁸¹ Compared to the porphyrin **74**, **84** exhibited a 6-fold increase in Φ_f , **89** a 12-fold increase, **90** had a Φ_f similar to **74**, however, it also possessed a considerably red-shifted absorbance to 730 nm with a nearly 50-fold greater absorbance. Previous studies with **74** indicated good uptake and photodynamic effects on several cancer cell lines, *e.g.*, K:Molv NIH 3T3 mouse fibroblasts and the MDA-MB-231 breast cancer cell line. Here, **84**, **89** and **90** were shown to be taken up at nanomolar concentrations.

(c) *Glycosylated Porphyrin Dimers and Oligomers*

Additionally, dimeric and oligomeric porphyrin glycoconjugates have been studied. Typical examples for neutral and cationic porphyrin dimers linked at the *meso* position *via* a flexible hydrocarbon chain are shown in Figures 18 and 19.⁸² The porphyrins **91-95** exhibited a $^1\text{O}_2$ production efficiency similar to that of haematoporphyrin. Photocytotoxicities of these compounds were evaluated with K562 human chronic leukaemia cell lines. The more hydrophilic bisporphyrin dimers **91**, **92** and **94** showed very low activity while compound **93** was the most active. It was however, only equivalent to Photofrin II after 120 minutes of irradiation and further incubation in the dark.

A different approach was taken by Maillard's group, who prepared a series of conjugated porphyrin dimers as new PDT agents (Fig. 20).⁸³ Apart from compound **96** all dimers produced twice as much $^1\text{O}_2$ than **65**. Determination of the cyto- and phototoxicity (HT-29 and Y79 cells) showed **96** and **98** to have only slight toxicity at high concentrations toward the Y79 cell line but also exhibited dark toxicity. Overall, the low solubility of these compounds in the culture medium impeded toxicity tests.

Fig. 18. Bisporphyrin glycoconjugates.

Fig. 19. Cationic bisporphyrin glycoconjugates.

Fig. 20. Conjugated bisporphyrins with carbohydrate units.

More promising are compounds **101-105** shown in Fig. 21.⁸⁴ They exhibit high two-photon absorption (TPA) properties at wavelengths suitable for biological tissues, which, combined with their high singlet oxygen quantum yields (from 0.43 up to 0.75), makes them promising PS.

Fig. 21. Conjugated bisporphyrins for TPA.

(d) Aminoglycosamide Porphyrins and Chlorins

In a further effort to improve the stability of glycoporphyrins to endogenous hydrolysis, aminoglycosamide derivatives were investigated (Fig. 22).⁸⁵ Similar to earlier results the HT29 cell line was more affected by the asymmetric and less hydrophobic glucosylated PSs **106** and **107**. The chlorin derivative **106** gave the better PDT effect ($\Phi^1\text{O}_2 = 0.73$, $\Phi_f = 0.16$) compared to the porphyrins **107** ($\Phi^1\text{O}_2 = 0.55$, $\Phi_f = 0.09$), **108** ($\Phi^1\text{O}_2 = 0.33$, $\Phi_f = 0.1$) and TPP ($\Phi^1\text{O}_2 = 0.55$, $\Phi_f = 0.11$). The cellular uptake of **107** (10 μM) after 24 h was ~ 11.5 -fold higher than TPP, whereas the accumulation of **106** and **108** was 6.4- and 5.0-fold respectively. *In vitro* studies indicated that **106** and **107** were more efficient than TPP and that these compounds accumulate in the endoplasmic reticulum.⁸⁶

Fig. 22. Selected aminoglucosamide porphyrins.

Related systems were described by Dallagnol *et al.* who synthesized porphyrin conjugates bearing thiourea, thiocarbamate and carbamate linkers (**109-111**).⁸⁷ They observed a strong correlation between the linker type and the photophysical properties of the porphyrin conjugates. The porphyrin derivatives with the carbamate linker gave the highest $^1\text{O}_2$ production ($\Phi_{\Delta} = 0.46$).

4.2. Porphyrin-Amino Acid Conjugates

Next to carbohydrates a wide range of amino acid conjugates have been

prepared in the past. A typical example are contemporary studies by Weimin *et al.* on the synthesis of porphyrin derivatives **112-116** (Fig. 23).⁸⁸ This involved double nitration of TPP followed by reduction to two amino groups and subsequent coupling with the amino acid. *In vitro* PDT studies with the Ec9706 cell line indicated a notable effect of the amino acid on the photocytotoxicity. The porphyrin complexes with Mn(II) showed higher photocytotoxicity than those with Co(II). Inhibition of the Ec9706 cell line increased with Co(II) insertion from 44 % to 74 %, while Mn(II) insertion resulted in an increase from 55 % to 100 %. It is interesting that insertion of metal ion enhanced the activity of mono-substituted porphyrins **112**, **115**, and **117** and, in contrast, decreased that of disubstituted porphyrins **113**, **114** and **116**.

Fig. 23. Amino acid appended porphyrins.

A valine-porphyrin conjugate **118** was prepared by Wang *et al.* [2008] using a modified Adler method.⁸⁹ The compound showed low cytotoxicity and good phototoxicity. In addition, porphyrin amino acid conjugates were also investigated in HeLa cells by Serra *et al.*⁹⁰ Such conjugates were stable in liposomes and penetrated efficiently into the cytoplasm of cultured cells and exhibited no dark cytotoxicity.

4.3. Porphyrin-Bile Acid Conjugates

In recent times, bile acids have become an attractive group for conjugation onto the porphyrin periphery due to their potential in substrate selectivity for saccharide cancer markers and also their affinity for apical sodium dependent bile acid transporters (ASBT), which are present in Barrett's esophagus. One of the first examples was the synthesis of the porphyrin-bile acid conjugates **119-122** by Koivukorpi *et al.* (Fig. 24).⁹¹ They showed good PDT activity in *in vitro* studies with A431NS, HeLaS3, 4T1 and FHC cell lines and ablated tumors in BALB/c mice which were subcutaneously transplanted with 4T1 mammary carcinoma cells.⁹² Binding studies showed that these compounds have the potential to recognize various oligosaccharides under physiological conditions and can be used for selective fluorescence detection of transformed cells expressing glycosylated markers.

We reported recently on a series of porphyrin and chlorin based bile acid conjugates (**123-126**) to impart greater selectivity and specificity on the photosensitizer for the treatment of esophageal cancer.⁹³ Chronic esophageal exposure to bile acids in patients with gastro-esophageal reflux disease is associated with the development of Barrett's metaplasia and associated molecular markers of inflammation which have been shown to support transformation, initiation and progression of tumor development. Another feature of Barrett's is the development of bile acid transporters such as the apical sodium dependent bile acid transporter (ASBT).⁹⁴ All four compounds were found to be highly soluble and successfully entered the cell, however, no phototoxicity was observed in SKGT-4 and OE22 cells and no $^1\text{O}_2$ was produced by the conjugates upon illumination. Intracellular aggregation or the presence of the triazole ring may have caused the lack of toxicity observed. A change in the linker group or site of conjugation may be necessary for further improvements.

Fig. 24. Bile acid appended porphyrins.

4.4. Porphyrin-Folic Acid Conjugates

Folic acid is an essential vitamin required for cell growth. It has a high affinity for specific binding to a folate receptor (FR). The combination of the photophysical properties of porphyrin with the targeting ability of folic acid successfully produced highly efficient conjugate systems for photodynamic therapy. Schneider *et al.* reported the design and synthesis of two folic acid conjugated TPPs **127** and **128** (Fig. 25).⁹⁵ The cellular uptake of both compounds by KB nasopharyngeal cells after 24 h incubation was 7-fold higher than the reference TPP, indicating an active

transport *via* receptor-mediated endocytosis.

Subsequently the same group reported the synthesis and photophysical properties of *m*THPC-like PS conjugated to folic acid *via* 2,2'-(ethylenedioxy)-bis-ethylamine units (**129**).⁹⁶ The conjugate **129** exhibited higher accumulation in KB tumors compared to tris(3-hydroxyphenyl)-4-carboxyphenylchlorin after 4 h post-injection.

A similar conjugate **131** showed 35 times higher cellular uptake in HeLa cells compared to the precursor porphyrin **130** after 24 h incubation.⁹⁷ Compound **131** also exhibited a very high photocytotoxicity (87 % cell growth inhibition) after irradiation and low dark cytotoxicity. Additionally, a lower photocytotoxicity for normal cells and folate-receptor negative cells was observed.

Fig. 25. Folic acid appended porphyrins.

4.5. Porphyrin-Peptide Conjugates

Each bioconjugate provides its own unique features to the porphyrin scaffold with peptides being no exception. Such derivatives have the potential as drug delivery agents, as they are relatively small in size and offer good tissue penetration. The excellent work carried out in proteomics makes them the most researched of all the bioconjugates and subsequently offers significant advanced targeting capabilities for medicinal applications including PDT.

One such example of porphyrin-peptide conjugates is the chlorin-ATWLPPR peptide conjugate **132**, which was prepared to specifically target tumor vasculature (Fig. 26).⁹⁸ The conjugate binds to recombinant NRP-1 protein and exhibits higher potency compared to the non-conjugated PS in human umbilical vein endothelial cells (HUVEC) expressing NRP-1. Further studies utilized MDA-MB-231 breast cancer cells, which strongly over-express the NRP-1 receptor.⁹⁹ A significant decrease of the conjugated PS after uptake was observed after RNA interference-mediated silencing of NRP-1. Use of a mouse model with U87 human malignant glioma cells demonstrated that only conjugated PS allowed a selective accumulation in endothelial cells lining the tumor vessels. The vascular effect induced by the conjugated PS was evident by a reduction in tumor blood flow of around 50 % during PDT. Nonetheless, affinity for NRP-1 remains low and further improvements are possible.

Cationic peptide-porphyrin derivatives have also been reported, including compounds **133-135**.¹⁰⁰ These compounds showed a moderate inhibitory activity toward aminopeptidase N, an enzyme responsible for tumor cell growth. Compound **135** showed the greatest potential for PDT with an $IC_{50} = 9.48 \mu M$.

Peptide PSs have also been developed for specific targeting in the newly emerging field of anti-microbial PDT. Doselli *et al.* conjugated neutral and cationic PSs to antimicrobial peptides (CAMPs), magainin and buforin (**136**, **137**) in order to increase the efficiency of bacterial inactivation (Fig. 27).¹⁰¹ The porphyrin-magainin conjugate increased the binding of the cationic and hydrophilic porphyrins with bacterial cells as indicated by the high photo-inactivation which was retained after

washing the bacterial suspension.

Fig. 26. Porphyrin-peptide conjugates.

Fig. 27. Anti-bacterial porphyrin-peptide conjugates.

Solid-phase synthesis and more complex reaction conditions have also found applications in this field. For example, the RGD tripeptide has been investigated as a bioconjugate group in PDT by Chaleix *et al.*¹⁰² Here, the cyclic peptide-porphyrin conjugate **138** (Fig. 28) contained a constrained RGD moiety formed by ring-closing metathesis and displayed similar singlet oxygen production to that of HP.

Fig. 28. A RGD-porphyrin conjugate.

4.6. Porphyrin-Oligonucleotide Conjugates

Porphyrin-oligonucleotide conjugates are of considerable interest in photomedical applications, specifically for the targeting of DNA and ultimately its photomodification.¹⁰³ A typical example is the synthesis of water-soluble porphyrin-oligonucleotide (ODN) conjugates **140a-c** by Mammana *et al.* (Fig. 29).¹⁰⁴ Similar to the situation with glycoporphyrins different linker units have been employed to modify the properties of such conjugates. For example, the zinc porphyrin-oligonucleotide conjugate **141** with a flexible linker was also prepared *via* solid support synthesis.¹⁰⁵

Fig. 29. Solid-phase synthesis of porphyrin-oligonucleotide conjugates.

4.7. Porphyrin-Antibody Conjugates (Photoimmuno-conjugates)

Some antigens are over-expressed in tumor tissues and thus targeting such antigens with their complimentary target-specific antibodies conjugated to porphyrins provides a logical targeting approach for PDT.¹⁰⁶ The utility of this approach is illustrated with the following examples.

Vrouenraets *et al.* conjugated typically unsuitable hydrophilic sensitizers with cMAb U36 and mMAb 425 by using a labile ester and a core PS (Fig. 30).¹⁰⁷ The conjugates showed no impairment of integrity on SDS-PAGE, were stable in serum and exhibited optimal immunoreactivity when the sensitizer:MAb ratio was <3. *In vitro* internalization experiments showed that **142** and ¹²⁵I-mMAb 425 conjugates were internalized by A431 cells, in contrast to ¹²⁵I-mMAbE48 conjugates. *In vitro* studies showed the internalized cMAb U36 and mMAb 425 conjugates were PDT active in A431 cells, unlike the non-internalized E48 conjugate and the unconjugated sensitizer. Biodistribution data of conjugates with sensitizer:¹²⁵I-cMAb U36 ratios varying from 1:1 to 3:1 in tumor-bearing nude mice revealed selective accumulation within the tumor.

Fig. 30. A Porphyrin-immuno conjugate.

Boyle's group investigated various porphyrin systems for their utility as immuno-conjugates. For example, of the two isothiocyanato porphyrins **143** and **144**, only the latter could be linked to single chain Fv

fragments (scFv) (Fig. 31).¹⁰⁸ Clearly, hydrophobic porphyrins are not useful in this context as they tend to form non-covalent complexes with the antibodies. However, the conjugate was found to be active against colorectal cancer Caco-2 cells.

Fig. 31. Porphyrin building blocks utilized by Boyle for immune-conjugate studies.

This work was continued by taking two cationic porphyrins **145** and **146** and conjugating them with three different MAb's; CD 326, CD 146 and CD 104.¹⁰⁹ Their PDT efficacy was tested against the human colon adenocarcinoma (LoVo) and CORL23 human large cell lung carcinoma (ECACC) cell lines. While the immune-conjugates had approximately 10-fold lower LD₅₀ values (ranging from 0.25-1.05 μ M), a non-specific PDT effect was observed in LoVo cells, which do not express the

CD146 antigen. The CD104 antigen is over-expressed in colorectal and bladder cancers,^{110,111} and thus, the porphyrin-anti-CD104 conjugate allows for targeting of these cancer tissues.

Very recently, Pereira *et al.* [2014] found that conjugation of a *meso* tetra-substituted porphyrin **148** with serum albumin and mAb anti-CD104 significantly increased the efficacy of the conjugate in targeted cancer destruction.¹¹² Compound **148** (derived from **147**) was conjugated with bovine and human serum albumin (BSA and HSA) and monoclonal antibodies (mAb anti-CD104 and mAb anti-Caf) to form the novel porphyrin-albumin and mAb immune-conjugates **149** for studies in the human bladder cancer cell line UM-UC-3 (Fig. 32).

Fig. 32. Synthesis of porphyrin albumin/immune conjugates.

5. Related Tetrapyrroles

5.1. Tetrabenzoporphyrins

Benzannulated porphyrins are of interest due to the increased stability of the macrocycle. Tetrabenzoporphyrin (TBP) is often considered an intermediate class between porphyrins and phthalocyanines.¹¹³ The unsubstituted tetrabenzoporphyrin can be obtained starting from phthalic

anhydride **150** which can be converted to **151**, followed by heating in aqueous ammonia to form the phthalimide derivative **152**. Condensation of **152** in the presence of zinc acetate forms the symmetric TBP **153**.¹¹⁴ However, condensation of tetrahydroisoidoles **154** with 4-substituted benzaldehydes **155** in the presence of $\text{BF}_3 \cdot \text{Et}_2\text{O}$ in methylene chloride followed by oxidation with DDQ yields the meso-tetraaryl-tetrabenzoporphyrins **157** (Fig. 33).¹¹⁵

Fig. 33. Synthesis of tetrabenzoporphyrins.

These synthetic advances have now been used for the preparation of bioconjugates for PDT studies. For example, Ménard *et al.* designed the

conjugated tetraglucosyl-tetrabenzoporphyrins **158** and **159** and the tetrapolyamine-tetrabenzoporphyrins **160** and **161**.¹¹⁶ All derivatives were tested against HaCaT and MCF-7 cells and proved less active than Photofrin II, possibly due to the altered photophysical properties of these highly nonplanar porphyrins.

Fig. 35. Tetrabenzoporphyrin bioconjugates.

5.2. Corroles

Corroles are aromatic tetrapyrroles containing one directly linked pyrrole-pyrrole unit which results in a smaller core cavity. By now their synthesis has advanced almost on par to that of porphyrins and Fig. 36

illustrates two approaches for the synthesis of *meso*-trisubstituted corroles (A_3 -corroles). Paolesse *et al.* modified the Adler-Longo method for the condensation of pyrrole and aromatic aldehydes (3:1 equivalents instead of 1:1 equivalents).¹¹⁷ Changing propionic acid to acetic acid yields corrole **162** as well as porphyrin **163** (Approach A). Alternatively, a condensation of excess pyrrole with aldehyde in a $H_2O/MeOH/HCl$ mixture yields the bilane **164** which can be oxidatively cyclized with DDQ to the corrole **162** (Approach B).¹¹⁸

Fig. 36. Synthesis of *meso* substituted corroles.

In terms of PDT applications, similar possibilities exist for corroles as has been shown with porphyrins. For example, a photophysical study of a series of halogenated mono-hydroxy corroles (**165-170**, Fig. 37) indicated the usual heavy atom effect on the intersystem crossing (ISC).¹¹⁹ Note, the long wavelength absorption of these corroles is much

stronger than that of TPP, which can aid PDT.

Fig. 37. Selected *meso*-hydroxyphenyl-corroles.

5.3. N-Confused Porphyrins

N-Confused porphyrin (NCP) is a porphyrin isomer in which one inverted pyrrole ring is connected to the two adjacent *meso* positions at the α - and β -positions (instead of two α -positions in case of porphyrin). Typically, these systems were identified first during variations of the condensation of pyrrole and aromatic aldehydes (Fig. 38).^{120,121}

Fig. 38. Synthesis of *N*-confused porphyrins and a water-soluble derivative.

Recently, Thomas *et al.* synthesized a water-soluble derivative of these N-confused porphyrins.¹²² Compound **173** has long-wavelength absorption bands >700 nm while its singlet oxygen quantum yield (0.70 in methanol and 0.55 in water) is comparable to structurally similar porphyrin derivatives. The compound exhibited no cytotoxicity in the absence of light but was highly phototoxic. *In vitro* studies with a series of human cancer cell lines [colon (HCT-116), breast (MCF7-ER, PR positive, and MDA-MB-231-ER, PR negative), pancreatic (MIA-PaCa-2), cervical (HeLa and SiHa), and oral (SCC-172 and SCC-131)] show promise for these kind of compounds. The best IC₅₀ value was found with breast adenocarcinoma cells (IC₅₀ = 6 μ M), which is comparable to other second generation PSs.

5.4. Thiaporphyrins

Thiaporphyrin macrocycles are core-modified porphyrins which can be obtained *via* replacement of one or more pyrrole rings with thiophene. Such a modification significantly alters the chemical properties compared to the N₄-porphyrins. Typical synthetic examples are shown in Fig. 39 where the functionalized *meso* tetrasubstituted 21-thiaporphyrins (N₃S porphyrins) and 21,23-dithiaporphyrins (N₂S₂ porphyrins) were prepared *via* condensation of the unsymmetrical thiophene diols **174** with either pyrrole and aryl aldehyde or 16-thiatripyrrane **176** under standard porphyrin synthesis conditions to yield **175** and **177** respectively.¹²³

A series of analogues of 5,20-diphenyl-10,15-bis(4-carboxylatomethoxy)phenyl-21,23-dithiaporphyrin **178-180** were investigated as potential PSs by Minnes *et al.*¹²⁴ Changing the chemical structure by varying the length of the side chain which links the carboxylic group with phenol residues at the *meso* position gave no significant alteration in the absorption and emission spectra. All singlet oxygen production values in methanol were close to unity. However, singlet oxygen quenching was observed with the elongated side chain linker in liposomes while the opposite was observed with the shorter side

chains in the cell lines, U936 and R3230Ac.

5.5. Porphycenes

Porphycene is a constitutional isomer of porphyrin which is synthesized easily *via* a McMurry reaction of 2,2'-bipyrrole-5,5'-dicarbaldehyde. Here, Temocene (*m*THPPo) is considered as the porphycene analogue of *m*THPC. Temocene **4** has excellent photophysical properties which makes it a promising PS for PDT applications. The synthesis of temocene **4** is illustrated in Fig. 40.^{13,125}

Fig. 40. Synthesis of Temocene.

García-Díaz *et al.* investigated the photodynamic activity of **4** and

compared to Temoporfin, Temocene **4** shows lower activity but also lower dark toxicity and superior photostability. Indeed, its singlet oxygen quantum yield ($\Phi_{\Delta} = 0.10$) is lower than that of Temoporfin ($\Phi_{\Delta} = 0.43$), but the kinetics of temocene and temoporfin of photobleaching under the same irradiation conditions showed that Temocene is substantially more photostable than Temoporfin. Temocene was less cytotoxic in the dark in HeLa cells and complete cell inactivation was achieved with 5 mM *m*THPPo at light doses of 3.5 J.cm^{-2} .¹³

The position and intensity of the Q-bands might give porphycenes an advantage for PDT applications relative to porphyrins as they require lower doses of drug and light to produce the same quantities of excited states. Other porphycene-type PS such as **187-189** with different substituents at position 9 of the tetrapyrrole macrocycle, were investigated *in vitro* by Scherer *et al.* using different human skin-derived cell lines (HaCaT, SCL I, SCL II) (Fig. 41).¹²⁶ HexoTMPn **187** was the most efficient in all three cell lines, especially in HaCaT cells with an EC_{50} of 14 nmol.L^{-1} , compared to 62 nmol.L^{-1} for **189** and 89 nmol.L^{-1} for **188**. Singlet quantum yields ranged from 0.27 to 0.36.

Fig. 41. Porphycenes used in PDT studies.

Similar photophysical data was reported by Arad *et al.* for 2,7,12,17-tetraphenylporphycene (TPPo) **190**, its Pd(II) **191** and Cu(II) **192** complexes.¹²⁷ The triplet ($\Phi_T = 0.33$) and singlet oxygen ($\Phi_\Delta = 0.23$) quantum yields of the former are comparable to that of other porphycenes, but much lower in comparison to the porphyrin isomer TPP ($\Phi_\Delta = 0.68$). In the case of **191**, fluorescence is inhibited by the internal heavy-atom effect and the triplet and singlet oxygen quantum yields are enhanced ($\Phi_T = \Phi_\Delta = 0.78$). Ragas *et al.* reported the synthesis of a tricationic water-soluble porphycene (Py₃MeO-TBPo) **193**.¹²⁸ While its photophysical parameters were similar to the other porphycenes, aggregation in aqueous media might limit its use in PDT. However, *in vitro* PDT cell killing *via* apoptosis was shown by Ruiz-González *et al.* in HeLa cells.¹²⁹

6. Nano-Formulation Strategies

Nanoparticles (NP) offer an attractive route for the administration of PSs and a relatively recent review details current strategies.²⁰ NPs allow for passive and active targeting of cancer tissues and choroidal neovascularization. Depending on the NP size, passage of the NP through endothelial barriers can be controlled, as has been shown by encapsulation of *m*THPP into biodegradable NP.¹³⁰ Even a very hydrophobic compound such as chloro(5,10,15,20-tetraphenylporphyrinato)indium(III), when encapsulated into NPs of diameters <200 nm, can be used as a PS to kill LNCaP prostate cancer tumor cells (conditions: 2 h, 1.8 $\mu\text{mol.L}^{-1}$, 45 J.cm⁻²) *via* apoptosis.¹³¹

Another recent example involves the use of an *m*THPP-type compound with four brucine quaternary ammonium salts bound to the *meta*-phenyl positions. This pigment was bound to gold nanoparticles and its PDT utility investigated both *in vitro* and *in vivo* using the basaloid squamous cell carcinoma PE/CA-PJ34.¹³² *In vitro* PDT experiments showed that the Au-NP-bound conjugates were less phototoxic than unbound conjugates, as they are more susceptible to aggregation in the culture media. In contrast, the *in vivo* experiments showed that the NP-bound PS were more effective than their free counterparts, possibly due to interaction of the conjugated-NPs with

plasma proteins to yield supramolecular complexes subject to the EPR-effect and complete regression of the PE/CA-PJ34 carcinoma after photodynamic treatment was observed.

NPs can also result in pH-controllable photosensitization, as shown through the interaction of the cationic 5,10,15,20-tetrakis(*N*-methyl-4-pyridyl)porphyrin (TMPyP) with bare SiO₂ nanoparticles.¹³³ At alkaline pH the functionalized SiO₂ nanoparticles act as a quencher of triplet and singlet oxygen with the PSs photoactivity “turned off”. In acidic solutions the PS is released from the SiO₂ surface and thus can generate ¹O₂ efficiently. This was indicated by singlet oxygen quantum yields of 0.45 in acidic solutions and 0.08 in basic solutions. The biological relevance of this approach was confirmed in initial studies with SK-BR-3 breast cancer cells.

Fig. 42. Král's cyclodextrin-based multimodality drug delivery system.

Another intriguing example is the free base porphyrin-cyclodextrin conjugate **194** developed by Král and coworkers.^{134,135} This nanosystem for multifunctional drug delivery and multimodal cancer therapy utilizes a combination of the binding of selected drugs in the cavity of the cyclodextrin with the photosensitizing properties of the porphyrin macrocycle (Fig. 42) and gave excellent accumulation of the conjugate in cancer tissue. The multimodality of this approach is illustrated by the

four parts of the system: drug (chemotherapy); porphyrin (targeted photodynamic therapy); metal (coordination with protein); protein (immunotherapy). This nanosystem can be used for efficiently targeting various therapeutic proteins.

7. Outlook

Looking forward and trying to predict the next paradigm shift in PDT is a difficult undertaking as most of the rational enhancements made to 2nd generation PS have not worked, as of yet. Nanoparticles designed for PDT have reached their zenith in terms of research and publications, with concerns still, over their bio-toxicity due to clearance rates. Bio-conjugates have become an ever increasing field with the belief that these biologically pertinent molecules attached to a PS will impart greater selectivity and improve the PS's targeting for the cancer cells. Sugars have received the biggest draw from researchers and, although some mild success has been achieved, none have managed to pose a threat to replace Photofrin[®], Foscan[®] or any of the other clinically approved PSs. Likewise, no matter the biological fragment; choice and location of the linker can be pivotal to the success of the PS. For example, while the ever popular "click" reaction is easy to perform, the biologically stable heterocycle formed has often resulted in no *in vitro* PDT activity due to ¹O₂ scavenging.

New emerging biological compounds of interest are folic acid, antibodies and bile acids. Since the discovery that folate receptors are over-expressed on the surface of numerous cancer cell lines, folic acid conjugates have been a logical route for improved targeting. Studies have shown folic acid conjugates with impressive ¹O₂ ΔΦ, whether these results can transfer into *in vitro* experiments, it is yet to be seen. In addition, issues regarding solubility and ease of purification arise as specialized equipment is generally needed to purify these conjugates due to the poor solubility of folic acid in organic solvent.

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