Chapter 33

Bacteriochlorophyll Sensitizers in Photodynamic Therapy

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Summary

Recent progress in the bioproduction and chemical manipulation of bacteriochlorophyll (BChl) *a* has opened the way for utilization of highly potent sensitizers in photodynamic therapy. Although less stable than their chlorin analogues in their native form, BChl derivatives provide a superior optical and biophysical profile for the generation of reactive oxygen species (ROS). In fact this pigment family probably represents the most efficient light collectors and radical generators in nature. Here we describe a recent development of this exciting family of drugs, with particular emphasis on vascular targeted therapy (VTP).

I. Introduction

The spectra, photophysics and photochemistry of native bacteriochlorophylls (BChls) have made them efficient sensitizers with optimal light-harvesting properties: they have clear advantages as sensitizers in photodynamic therapy (PDT) even when compared to their chlorophyll (Chl) counterparts. In particular, these molecules have very high extinction coefficients at long wavelengths (λ_{max}= 760–780 nm, $\varepsilon = (4–10) \times 10^4$ $M^{-1}cm^{-1}$), where light penetration into tissues is maximal (Pandey and Zheng, 2000). Moreover, they generate reactive oxygen species (ROS) at a higher quantum yield, depending on the central metal and, to some extent, on peripheral substituents (Musewald et al., 1998; Vakrat-Haglili, 2002). Unfortunately, BChls are much less stable than Chl analogues. In fact, the most common route of BChl degradation is by chemical or photochemical oxidation to their corresponding Chl form (Lindsay-Smith and Calvin, 1966; Henderson et al., 1991). Additionally, BChl biosynthesis usually requires light and anaerobic conditions: these are two major obstacles to large-scale production.

During the last decade, genetic manipulations (Ghosh, 2003), the development of illuminated flow-fermenters (Tsygankov et al., 1994, 1997) and especially the isolation of bacteria that grow vigorously under aerobic conditions in the dark while producing large concentrations of BChl *a* (Doi et al., 1991) have made BChl *a* a viable chemical precursor for the drug industry. In parallel, Smith et al. (1992) developed a chemical procedure that generates a BChl analogue from Chl *a*, thereby relieving, to a certain extent, the need for bacterial production of BChl *a*. Concurrently, several groups found ways to chemically modify BChls and purify the newly-formed chemical derivatives to the high degree required for pharmaceutical and chemical studies (Struck et al., 1992; Mironov et al., 1993; Scherz et al., 1994; Kozyrev et al., 1996). In view of this progress, BChl derivatives are expected to become principal participants in PDT during the coming decade. Among the more interesting properties of BChl-based photosensitizers is their relatively low redox potential (Watanabe and Kobayashi, 1991; Geskes et al., 1995; Noy et al., 1998) compared with other porphyrinoids. Thus, photoactivation of BChl and BChl derivatives under aerobic conditions is expected to enhance the production of superoxide and hydroxyl radicals that are significantly more potent sensitizers than singlet oxygen.

Tumor destruction generally results from direct cytotoxic effects, from hypoxia and starvation caused by vascular shutdown, and from the inflammatory response (Macdonald and Dougherty, 2001). Traditionally, PDT is thought to destroy tumor cells due to preferentially accumulated photosensitizers (Macdonald and Dougherty, 2001). However, early studies of BChl-based PDT (Henderson et al., 1991; Henderson and Dougherty, 1992) excluded the possibility of a direct BChl-PDT effect on the tumor cells because such BChl-based drugs were found ineffective when administered long before irradiation. Despite the superior optical properties and significant yield of ROS generation in vitro, no significant tumor regression was observed when irradiated for more than a few hours after administering the sensitizer. Soon, it was found that BChl derivatives act as antivascular drugs (van Leengoed et al., 1993; Zilberstein et al., 1997) which led to extensive developments towards a vascular targeted-PDT (VTP) approach (Scherz

Abbreviations: BChl – bacteriochlorophyll; BChl-Ser – bacteriochlorophyllide *a* L-serine ester; BChlide – bacteriochlorophyllide; BChn – bacteriochlorin; BOLD MRI – blood oxygen level-dependent magnetic resonance imaging; BPhe – bacteriopheophytin; BPheide – bacteriopheophorbide; BPP – bacteriopurpurin; Chl – chlorophyll; Chn – chlorin; HDL – high-density lipoprotein; i.v. – intravenously; IgG – immunoglobulin G; ISC – intersystem crossing; LD_{50} – median lethal dose; LDL – low-density lipoprotein; PDT – photodynamic therapy; PP – purpurin; ROS – reactive oxygen species; s.c. – subcutaneously; TOOKAD® – [Pd]-bacteriochlorophyllide *a*; VTP –vascular-targeted PDT; ϕ_Δ – quantum yield of singlet oxygen

et al., 1994, 2000, 2004). The research efforts and development programs of many groups, however, are still aimed at specifically attacking the tumor cells by ROS generation during illumination of cell-targeted BChl and BChl derivatives. In this chapter, we will present and discuss each group of the currently developed BChl-based sensitizers.

II. Photosensitizers Derived from Bacteriochlorophyll *a*

A. Bacteriochlorophyll a

1. General Description and Chemistry

BChl *a* (see formula in Chapter 1, Scheer) is a 7,8,17,18-tetrahydrophytoporphyrin structurally similar to the 17,18-dihydrophytoporphyrin, Chl *a*, in which the 3-vinyl group is replaced by an acetyl substituent. BChl *a* can be easily recovered from cultivated purple photosynthetic bacteria. As in Chl *a*, the optical spectrum, redox potentials, and overall reactivity of BChl *a* are mainly determined by the π electrons of the macrocycle and the incorporated metal. As a result, BChl *a* can be a potent photosensitizer with a strong absorption at 770–780 nm and high yield for ROS generation (quantum yield of singlet oxygen, ϕ_Δ, may reach 0.6, depending on the molecular environment). However, it easily undergoes isomerization, demetalation and oxidative degradation, including restoration of the 7,8-double bond (Lindsay-Smith and Calvin, 1966; Henderson et al., 1991). These disadvantages and the insolubility of BChl *a* in aqueous solutions limit its practical use. Metal substitutions in BChl modify the macrocycle's redox potentials, electronic transition energies (Chapter 2, Senge et al. and Chapter 34, Yerushalmi et al.), solubility and coordination properties; therefore, such modifications may dramatically affect the application of BChl as a photodynamic reagent. Furthermore, some of these modifications increase stability to harsher chemistry that could otherwise result in oxidation of the bacteriochlorin to the chlorin macrocycle with a consequent blue shift of the Q_y maximum absorption wavelength. Synthetic modifications of the BChl *a* periphery and metal replacement, aimed at higher stability and solubility, should facilitate its application as a PDT agent.

Targeting. A catalytic amount of photodegradable BChl-intruded liposomes containing encapsulated Ca^{2+} enabled release of a larger population of drug-loaded conventional liposomes using a Ca^{2+}-dependent lytic process (Wymer et al., 1998; Gerasimov et al., 1999).

2. Pre-Clinical Studies and Efficacy

a. In Vitro Studies

Photocytotoxicity of BChl *a* was tested against radiation-induced fibrosarcome (RIF) tumor cell lines and found to have only moderate and limited effectiveness due to rapid photobleaching of the sensitizer (Henderson et al., 1991).

b. In Vivo Studies

PDT with BChl *a* induced 80%-cure of spontaneous mouse mammary tumor (SMT-F) and RIF tumors on day 90 in mice, when irradiation (270 J cm^{-2}, 780 nm) was carried out for 2 h, but not 24 h, after intravenous (i.v.)-administration (5 mg kg^{-1}), which indicated rapid clearance and vascular shutdown (rapid or delayed) as the main factor in tumor necrosis. Normal skin tissue photosensitivity totally disappeared after 5 days, probably due to chemical instability of BChl in the biological milieu (Henderson et al., 1991).

B. Bacteriochlorophyllide a and Derivatives

1. General Description and Chemistry

Enzymatic hydrolysis or transesterification of BChl with amino acids as well as chemical amidation of the propionic side chain significantly increased its water-solubility (Scherz et al., 1994; Fiedor et al., 1996). Serine-conjugated bacteriochlorophyllide (BChlide) *a* seems to retain the photophysical properties of BChl (Eichwurzel et al., 2000). Significant generation of hydroxyl radicals in aqueous solutions has been reported (Katz et al., 1998). Unfortunately, like BChl *a*, BChlide *a* and its derivatives undergo fast photo-oxidation, demetalation in slightly acidic solutions, and biodegradation (Rosenbach-Belkin et al., 1996), preventing their wide application.

The replacement of the central Mg atom by Pd in BChlide *a* improved its stability and photodynamic reactivity (Scherz et al., 2000). The absorption band shifted to 763 nm, while maintaining its intensity. [Pd]-BChlide *a* was modified by esterification or amidation at the C-17 propionate residue (Scherz et

al., 2001) and by transesterification at the C-13[3] carboxylate residue (Scheer et al., 2001). These derivatives differed in their amphiphilicity. [Pd]-BChlide *a* underwent intersystem crossing (ISC) with quantum yield ~1. ROS generation with [Pd]-BChlide *a* was dependent on the molecular environment. The ϕ_Δ dropped from ~1 in hydrophobic solutions to ~0.5 in aqueous and micellar solutions, while the yields of hydroxyl and superoxide radicals increased to ~0.2%. Superoxide and hydroxyl radical generation by [Pd]-BChlide probably involves oxidation of the macrocycle that serves as both an electron and proton donor (Vakrat-Haglili, 2002).

Targeting. The conjugation of BChlide *a* with peptides, hormones and proteins as cell-specific ligands such as melanocyte stimulating hormones for site-specific PDT of melanoma, can be carried out via chemically-activated amidation of the BChlide *a* propionic acid residue at C-17 (Scherz et al., 1994).

[Pd]-BChlide *a* ethyl ester was modified to form inclusion complexes with dimeric cyclodextrins (Roehrs et al., 1995; Moser, 1998).

2. Pre-Clinical Studies and Efficacy

a. In Vitro Studies

BChlide *a* and BChl-Ser showed high antitumor activity in vitro in M2R mouse melanoma cells (median lethal dose (LD_{50}) of 0.2–0.5 μM) (Rosenbach-Belkin et al., 1996). Effective phototoxicity of the BChl derivatives, even under hypoxic conditions (L. Chen et al., 1998), probably occurs by the unusual mechanism of photosensitized oxidation by means of hydroxyl radicals (Katz et al., 1998). Substituting Pd for the Mg atom increased the photodynamic efficacy against M2R melanoma cells and other cell lines, decreasing the LD_{50} value to ~0.01–0.03 μM, with no dark toxicity (Scherz et al., 2001).

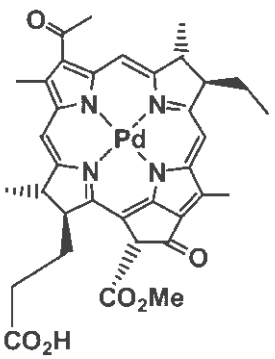
Targeting. BChlide *a*, conjugated to rabbit IgG, was 30 times more phototoxic in the inactivation of protein A-presenting *Staphylococcus aureus* compared to free BChl-Ser, indicating that site-specific generation of ROS can substantially increase the biological effect over that obtained by free sensitizer (Gross et al., 1997).

b. In Vivo Studies

Studies in mice, subcutaneously (s.c.) implanted with M2R mouse melanoma xenografts, displayed rapid (16 h) clearance of BChl-Ser from all tissues tested with only transient accumulation in the liver, which implied potential minimization of prolonged skin photosensitivity (Rosenbach-Belkin et al., 1996). Under the selected treatment protocol, when irradiated without delay after drug administration, the photodynamic effect (108 J cm^{-2}, 580–750 nm, shortly after 20 mg kg^{-1} i.v.) was predominantly conferred to the tumor vascular bed with vessel occlusion and complete blood stasis (Zilberstein et al., 1995, 2001). PDT with BChl-Ser resulted in a high cure rate (>80%) of pigmented melanoma in mice (Zilberstein et al., 2001) where sensitizing light penetration in the pigmented tumor was as deep as 0.9 cm (Zilberstein et al., 1997). BChl-Ser-PDT was also shown to be efficient against massive SD sarcoma xenografts in rats (Kelleher et al., 1999, 2003): here, PDT and hyperthermia were found to be synergistic when using BChl-Ser (Kelleher et al., 1999, 2003). When monitored with a tissue-inserted oxygen microsensor, the local photodynamic response of the tumor to BChl-Ser-PDT was associated with rapid light-dependent depletion of tumor oxygen levels, presumably due to ROS formation (Zilberstein et al., 1997).

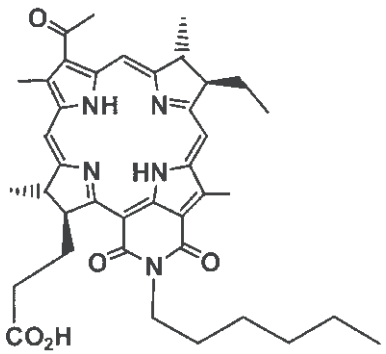
[Pd]-BChlide *a* (trade name TOOKAD®, see Fig. 1) was found to have an exceptionally short clearance time with no skin phototoxicity in mice and rats (Scherz et al., 2000) and also in dogs (Hetzel F, private communication). High cure rates were demonstrated following TOOKAD®-PDT (90 J cm^{-2}, 770 nm immediately after 4 mg kg^{-1} i.v.) of massive s.c. human prostate cancer xenografts and bone metastases in mice (Koudinova et al., 2003).

Hemorrhagic necrosis of the entire prostate gland, accompanied by preservation of the urethra and urinal functions, was shown in dogs (200 J cm^{-2}, 763 nm after only 5 mg kg^{-1} i.v.): this demonstrated the expected benefit of the strong near infra red (NIR) excitation (763 nm) by inducing effective PDT to the depth of 2 cm from the optical fiber (Q. Chen et al., 2002a,b). PDT with TOOKAD®, aimed primarily at the tumor vasculature, efficiently eradicated solid C6 glioma tumors in mice, reducing the rate of groin and lung metastasis (Schreiber et al., 2002). Antivascular TOOKAD®-PDT also indiscriminately destroyed both multidrug resistant and wild type HT29 colon carcinoma tumors (Preise et al., 2003). Selective tumor eradication, with minimal damage to the surrounding tissue, probably resulted from the different responses of tumor- and normal-blood vessels



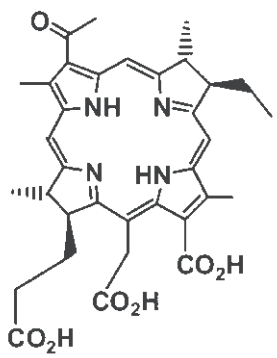
Palladium-Bacteriopheophorbide *a*

TOOKAD® (WST09)



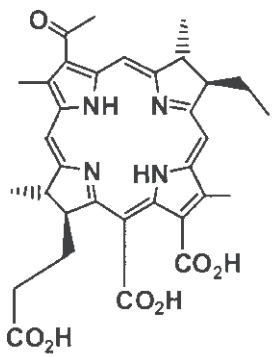
3^1-oxo-7,8-*trans*-dihydro-rhodochlorin-15-carboxy-13^1,15^1-N-hexylimide

BPP N-hexyl imide



3^1-oxo-7,8-*trans*-dihydro-rhodochlorin-15-acetic acid

BChn *a*



3^1-oxo-7,8-*trans*-dihydro-rhodochlorin-15-carboxylic acid

BChn p_6

Fig. 1. Structures of some BChl derivatives used as active photosensitizers (IUPAC-approved names are followed by the trade names or abbreviations).

(Mazor et al., 2002; Borle et al. 2003; Gross et al., 2003a). Massive photosensitization of TOOKAD® in the circulation was demonstrated with blood oxygen level-dependent magnetic resonance imaging (BOLD MRI), creating the possibility of online imaging of the photodynamic process and enabling accurate light guidance (Gross et al., 2003b).

C. Bacteriopheophorbide a and Derivatives

1. General Description and Chemistry

BPheide *a*, obtained from BChl *a* by acidic elimination of both Mg and the esterifying alcohol, either phytol or geranylgeraniol, has a lower extinction coefficient than BChl(ide) *a* near 760 nm, higher

dark and light stability and a ϕ_Δ value of about 0.6 (Krasnovsky Jr et al., 1990).

2. Pre-clinical Studies and Efficacy

a. In Vitro Studies

BPheide *a* and 13^2-hydroxy-BPheide *a* alkyl esters were efficient in treating several melanotic cell lines (Moser, 1993). These sensitizers were characterized by negligible threshold doses in comparison with Photofrin® and Pheide *a* (Moser et al., 1994b, 1995), with subcellular localization in the cytoplasm and Golgi apparatus (Moser et al., 1992, 1994a).

The photocytotoxicity observed with EMT-6 cells, using BPheide 4-hydroxybutylamide, was 2400 × higher than that of Photofrin®. Similar to Pheide analogs (see above), a significant efflux of the drug from the cells is promoted by high and low density lipoproteins (HDL and LDL, respectively), suggesting rapid clearance from normal tissue with low phototoxicity (Dagan et al., 1995; Gatt et al., 1996).

b. In Vivo Studies

Biodistribution and pharmacokinetics analysis of 13^2-hydroxy-BPheide *a* methyl ester in mice bearing Lewis lung carcinoma, revealed fairly rapid (4 h) clearance of the drug from the blood, but with a longer (up to 168 h) retention time in the internal organs and tumor, coupled with metabolic processes (Röder et al., 1994).

D. Bacteriochlorin a and Derivatives

1. General Description and Chemistry

Bacteriochlorin *a* (BChn *a*, Fig. 1), a structural relative of chlorin e_6 (Chn e_6), absorbs at 760 nm and generates not only singlet oxygen at relatively low ϕ_Δ value of 0.05 and 0.33 in phosphate buffer and liposomes, respectively (Damoiseau et al., 2001; Hoebeke and Damoiseau, 2002), but also produces superoxide and hydroxyl radicals, equal to the yield of singlet oxygen in buffer solution (Hoebeke et al., 1997). However, the preparation of BChn *a* as yet gives variable results (Post et al., 1996), and, being insoluble, its formulation is a suspension (Schuitmaker et al., 1991; van Leengoed et al., 1993; van Tenten et al., 2002), which complicates dose-response estimation (Post et al., 1996). BChn *a* amides and their metallocomplexes, however, are water-soluble: they were obtained recently from the corresponding BPheide *a* precursors by regioselective aminolysis and have proven to be efficient sensitizers for vascular targeted PDT (Scherz et al., 2004).

2. Pre-Clinical Studies and Efficacy

a. In Vitro Studies

BChn *a* in a serum-deficient medium was found to be an effective photosensitizer when tested on Chinese hamster ovary and T24 (human bladder) carcinoma cells, but ineffective in a (lipo)protein-rich cell culture medium. Association with lipoproteins (20% with LDL and 60% with HDL) is probably responsible for BChn *a* uptake by malignant neoplasms (Schuitmaker et al., 1995).

b. In Vivo Studies

BChn *a* induced complete necrosis of an isogeneic mammary tumor (100 J cm^{-2}, 760 nm, 0.25–1 h after 20 mg kg^{-1} i.v.) (van Leengoed et al., 1993) and a re-growth delay of both RIF tumors in mice (van Geel et al., 1995) and of model liver metastases in rats (using 10 mg kg^{-1}) (Rovers et al., 1998). Both vascular and direct tissue cell (mitochondrial) damage contributed to necrosis (Schuitmaker et al., 1991; van Leengoed et al., 1993).

PDT with BChn *a* for prevention of posterior capsule opacification after cataract extraction seemed promising (van Tenten et al., 2001; van Tenten et al., 2002).

A water-soluble derivative of [Pd]-BChn *a* showed rapid clearance from murine blood and all tissues within 30 min, with the hepatic clearance route having ~20 min half-life period: this suggests negligible diffusion of the pigment from the vasculature into organs other than liver, lungs and spleen. PDT (30 J cm^{-2}, 755 nm, immediately after 9 mg kg^{-1}) provided selective necrosis of solid M2R melanoma tumors in mice with a 70% cure on day 90 (Scherz et al., 2004). Even higher cure rates were found with HT29 colon carcinoma xenografts (Scherz et al, unpublished data).

E. Bacteriopurpurin 18, Bacteriochlorin p_6 and Derivatives

1. General Description and Chemistry

Bacteriopurpurin (BPP) *18*, obtained from a crude

extract of BChl *a* by a procedure similar to that used for preparation of purpurin (PP *18*), proved to be a stable compound with a strong absorption maximum at 818 nm (Mironov et al., 1993). Alkaline cleavage of the anhydride ring produced water-soluble BChn p_6 (Fig. 1), that absorbed at 766 nm (Mironov et al., 1993). Partial reduction transformed BPP *18* into a δ-lactone derivative with a 3-(1-hydroxyethyl) substituent, that was more stable towards acidic and basic cleavage, but the major absorption maximum shifted to 724 nm (Mironov et al., 1998). By opening the cyclic anhydride ring in BPP *18* with 1-hexylamine, the corresponding cyclic isoimide (λ_{max} 804 nm) and imide (λ_{max} 822 nm) derivatives (see Fig. 1) were obtained (Kozyrev et al., 1996). The more nucleophilic hydroxylamine provided [3^1-oxime]-BPP *18* N-hydroxyimide (λ_{max} 812 nm) in one reaction (Mironov et al., 2002).

2. Pre-Clinical Studies and Efficacy

a. In Vitro Studies

Targeting. Conjugates for doubly targeted delivery (cell and nuclear specific) were constructed using BChn p_6 and chimeric modular recombinant transporters, expressed in *Escherichia coli*. The transporters included an internalizeable ligand (α-melanocyte-stimulating hormone), a nuclear localization factor (viral T-antigen), a hemoglobin-like carrier protein and an endosomolytic factor (diphtheria toxin domain). The conjugates were 250-fold more photodynamically active in mouse melanoma cells than free BChn p_6 (Rosenkranz et al., 2003). Interestingly, the melanocyte-stimulating hormone was previously proposed to be used for in-situ PDT targeting of different BChl derivatives into melanoma cells (Scherz et al., 1994).

b. In Vivo Studies

Converted into a series of alkyl ether analogues, BPP *18* N-hexylimide derivatives showed similar in vivo efficacy in mice, but with notable differences in their activity in RIF tumors (135 J cm^{-2}, 785 nm, 24 h after 0.2 μmol kg^{-1} i.v.). The heptyl ether derivative was the most effective photosensitizer (80% tumor cure), with selective localization in mitochondria, but not via the peripheral benzodiazepine receptor (Y. Chen et al., 2002c).

III. Clinical trials

Steba Biotech, France, has recently accomplished Phase I/II clinical trials of TOOKAD®-PDT for prostate cancer therapy in 25 patients, with localized prostate cancer after radiation failure, using escalation of drug doses and irradiation fluence. Experiments conducted in two centers in Canada showed no severe adverse effects at the highest doses examined. The sensitizers were rapidly cleared from the circulation with no skin toxicity 3 h or less after administration to the patients. Local necrosis up to 3-cm diameter, induced by an interstitial diffuser-optic-fiber, was observed by MRI seven days after treatment (Elhilali, 2003; Trachtenberg J, personal communications). Early Phase II trials have already begun.

IV. Conclusions and Perspectives

PDT had already been proven to be a viable and interesting procedure for palliative and, in a limited number of cases, as a curative alternative to current, less selective treatment procedures for cancer. Yet, the application of PDT for first-line treatment is rare, and the authors of this review suggest that this delay in curative application of PDT reflects the search for and discovery of new sensitizers that may enable better targeting to the tumor cells. Vascular targeting, however, seems to us a more promising avenue for the future of PDT. Here, BChl derivatives with long wavelength absorption, high yield of ROS generation and fast clearance rates, together with simple diode lasers and sophisticated blood imaging procedures, may advance PDT into a 'key player' role in first-line tumor treatment. We believe that the concept of 'differential tissue response' rather than 'differential tissue uptake' may prove to be the major principle underlying selective PDT treatment and should become a key guideline in the future design of BChl- and Chl-based photosensitizers.

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