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Review

Photodynamic Therapy of Oral Cancer and Novel Liposomal Photosensitizers †

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† This paper is dedicated to the memory of Krystyna Konopka, formerly Professor of Microbiology and of Biomedical Sciences at the Arthur A. Dugoni School of Dentistry.

Abstract: Photodynamic therapy facilitates the selective destruction of cancer tissue by utilizing a photosensitizer drug, the light near the absorbance wavelength of the drug, and oxygen. Methylene Blue, 5-aminolevulinic acid (the precursor of the photosensitizer, protoporphyrin IX), porphyrin, Foscan, Chlorin e6, and HPPH have been used successfully as photosensitizers in the treatment of oral verrucous hyperplasia, oral leukoplakia, oral lichen planus, and head and neck squamous cell carcinoma. "Theranostic" liposomes can deliver a contrast agent for magnetic resonance imaging and a photosensitizer for the image-guided photodynamic therapy of head and neck cancer. Liposomes incorporating photosensitizers can be targeted to cell surface markers overexpressed on cancer cells. Novel porphyrinoids have been developed in our laboratories that are highly effective as photosensitizers. Tribenzoporphyrazines encapsulated in cationic liposomes have produced IC_{50} values up to 50 times lower compared to the free photosensitizers. It is anticipated that targeting these drugs to cancer stem cells, using upconversion nanoparticles for the near-infrared irradiation of tumors to activate the photosensitizers, and overcoming tumor hypoxia will enhance the efficacy of photodynamic therapy of tumors accessible to light sources.

Keywords: oral squamous cell carcinoma; pharyngeal cancer; nanoparticles; photodynamic therapy; photosensitizer; targeted liposomes; singlet excited state; theranostics; tribenzoporphyrazines

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1. Introduction

The World Cancer Research Fund International reports that, in 2020, the incidence of mouth and oral cancer was 744,994 throughout the world. The number of deaths during this period was 364,339 [1]. The Oral Cancer Foundation estimates that the worldwide burden of cancers of the oral cavity and oropharynx is 657,000 new cases per year, and more than 330,000 deaths.

Of the newly diagnosed persons, only slightly more than half will be alive within 5 years. This outcome has not changed significantly over several decades, except in the case of HPV16-positive oral cancers that respond better to current treatments. Surgery generally leads to disfiguration. Chemotherapy and radiation therapy cause difficulty in swallowing, chewing and talking, jaw pain, mouth sores, and dysfunctional salivary glands.

Photodynamic therapy makes possible the selective destruction of cancer tissue, and may complement surgery, radiotherapy and chemotherapy. Photodynamic therapy utilizes

a photosensitizer drug, the light at or near the absorbance wavelength of the drug, and oxygen. The incidence of light on the photosensitizer, which is initially in the low energy ground "singlet state" (S_0) and has two electrons with opposite spins (corresponding to a total spin angular momentum of *zero*), generates the "singlet-excited state" (S_1 or S_2) with the electrons in an orbital with a higher energy (Figure 1) [2]. The singlet excited-state is not stable and loses its energy by alternate mechanisms: (i) emitting light, observed as fluorescence at a higher wavelength (i.e., lower energy; the Stokes shift); (ii) heat production via a process termed "internal conversion"; or (iii) "intersystem crossing", forming the excited "triplet state" with a total spin angular momentum of *one*, arising from parallel spinning electrons. The triplet simply means that, in this state, the molecule exhibits three spectral lines of light absorption.

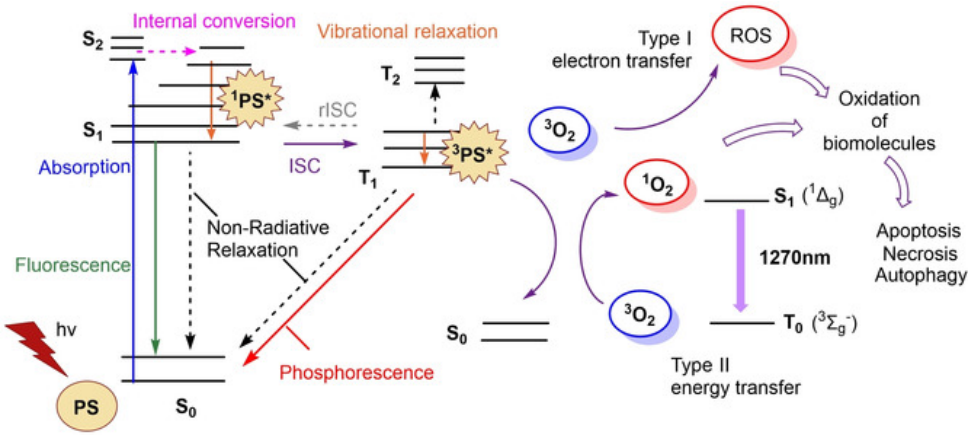


Figure 1. Simplified Jablonski diagram, showing the pathways after photoactivation of a photosensitizer (PS). When the photosensitizer absorbs light (with an energy given by $h\nu$, where h is Planck's constant, and ν is the frequency of light) an electron in the singlet ground state is energized into a high-energy singlet state. This state can lose energy by emitting a photon at a higher wavelength (fluorescence) or by internal conversion (non-radiative relaxation). The spin of the high-energy electron may flip in intersystem crossing (ISC), forming the excited triplet state, which has a relatively long half-life. Superoxide and hydroxyl radicals are formed in the presence of molecular oxygen in Type I reactions. Singlet oxygen is formed in Type II reactions. These reactive oxygen species can damage amino acids, lipids and nucleic acids (Reproduced with permission from Melissari et al. [3]).

In the ensuing "Type I" reaction, the photosensitizer in the triplet state can interact with a neighboring molecule, which may be an electron donor or electron acceptor, forming a radical anion or a radical cation, which can then react with oxygen to form the superoxide radical ($O_2^{-}\bullet$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (HO•) (Figure 1). In the "Type II" reaction, the energy of the triplet state photosensitizer is transferred to ground state triplet molecular oxygen (O_2), producing the highly reactive excited singlet oxygen ($^1O_2$) (Figure 1). These reactive oxygen species (ROS) can then oxidize proteins, nucleic acids and lipids, resulting in cytotoxicity to a cancer cell or a microorganism. Photosensitizers employed in cancer treatment mostly utilize the Type II mechanism [2].

An oxygen-independent mechanism of photodynamic therapy, termed Type III, has been described whereby excited photosensitizers directly degrade nucleic acids and proteins [4,5].

Photodynamic therapy can cause an increase in the intracellular Ca^{2+} concentration and the activation of phospholipase A_2 [6]. It can down-regulate the epidermal growth factor receptor at the cell surface. It can cause the accumulation of ceramide, leading to an increase in mitochondrial membrane permeability and the release of cytochrome c. Photodynamic therapy can result in the expression of interleukin-6 and integrin damage.

The anti-cancer effect by photodynamic therapy may be caused by (i) direct cytotoxicity to cancer cells, (ii) occlusion of the tumor vasculature, thus causing hypoxia and cell death,

or (iii) induction of a systemic immune response directed at the tumor [6]. Cytotoxicity may be mediated by apoptosis, necrosis, and possibly autophagy. For example, photodynamic therapy with 5-aminolevulinic acid induces apoptosis in the oral cancer cell line, CA9-22, via the NF-kB/JNK pathway, and involves both caspase 8-and caspase-9 [7]. Pheophorbide a is a photosensitizer that was synthesized by the removal of a magnesium ion and a phytyl group from chlorophyll-a [8]. Photodynamic therapy in human oral squamous cell carcinoma cells, YD-10B, utilizing pheophorbide a, inhibits the proliferation of the cells, increases the number of apoptotic cells via the inactivation of the ERK pathway, and also induces autophagy, shown by the increased expression of microtubule-associated protein 1 light chain 3B and the accumulation of acidic vesicular organelles [8]. Another important mechanism that contributes to photodynamic therapy therapeutic performance involves the activation of the immunological system. Thus, at some point, photodynamic therapy can be considered as a cancer immunotherapy. Treatment with various classes of photosensitizers is known to trigger immunogenic cell death (ICD) where the T-cell adaptive immune response is activated, leading to the formation of long-term immunological memory [5,9].

A major advantage of photodynamic therapy over conventional treatments is its minimal invasiveness, as well as relatively selective tumor destruction and the preservation of healthy tissues [10]. Some photosensitizers can concentrate in tumors relative to the surrounding healthy tissue [11–13]. Liposomes targeted to the transferrin receptor can mediate an 18-fold higher accumulation of the photosensitizer, aluminum phthalocyanine tetrasulfonate, in bladder tumors compared to normal urothelium [14]. These properties are significant in the treatment of head and neck squamous cell carcinoma, where loss of normal tissue may cause functional problems and disfiguration. Photodynamic therapy may also be used in combination with conventional treatments [15].

Below, we discuss the use of various photosensitizers in the treatment of head and neck squamous cell carcinoma in patients, in animal models, and in vitro, as well as describe the novel photosensitizers developed in our laboratories.

2. 5-Aminolevulinic Acid

The compound 5-aminolevulinic acid is a precursor of the photosensitizer, protoporphyrin IX, in the heme biosynthetic pathway [10,16]. Exogenous 5-aminolevulinic acid (Figure 2) inhibits the first step of porphyrin synthesis, which thus results in the accumulation of protoporphyrin IX in the tissue.

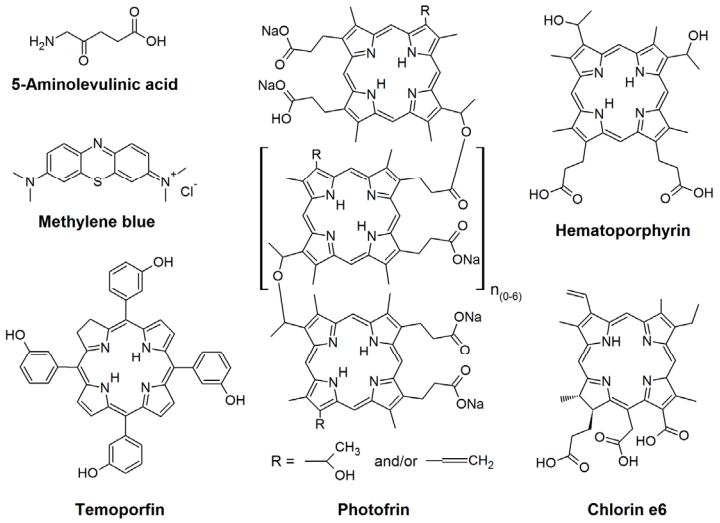


Figure 2. Molecular structures of photosensitizers.

Photodynamic therapy with 5-aminolevulinic acid has been used fairly widely in clinical studies against oral leukoplakia, which has the potential to become malignant [17]. Correspondingly, oral leukoplakia presents a convenient clinical model for cancer preventive

approaches [18]. One of the earlier attempts to treat oral leukoplakia with 5-aminolevulinic acid was that of Kübler et al. [19], which involved the application of a 20% 5-aminolevulinic acid cream, locally for 2 h, to 12 patients who had had oral leukoplakia for many years. After removing the cream, monochromatic red light at 630 nm from an argon-dye laser (at a radiant exposure of 100 J/cm^2) was applied to the leukoplakia lesions of the patients for 1 h. After 3 months of therapy, five patients were cured completely.

Four patients showed a partial response, while three patients did not show any response [19]. Similar to this study, Sieron et al. [20] reported that a complete response was achieved in 10 out of 12 patients suffering from oral leukoplakia when they were treated with a 10% 5-aminolevulinic acid emulsion (O/W) topically with 6–8 irradiation sessions utilizing an Argon-pumped dye laser at 635 nm (delivering a total dose of 100 J/cm^2 per session). To examine treatment efficacy in different diseases and treatment protocols, Chen et al. [21] topically applied a 20% 5-aminolevulinic acid gel to 32 patients, including 8 patients with oral verrucous hyperplasia and 24 patients with oral leukoplakia. Oral verrucous hyperplasia lesions showed a better response than oral leukoplakia lesions to this photodynamic therapy. Oral leukoplakia lesions required the application of the protocol twice a week to show even a partial response, while oral verrucous hyperplasia lesions showed a complete response in fewer than six treatments once a week [21].

Siddiqui et al. [22] reported the effects of photoactivated aminolevulinic acid as an oral cancer therapy. The regimen of the photosensitizing agent is normally 60 mg/kg divided into three doses, which leads to accumulation of the photoactive product protoporphyrin IX (PpIX), followed, after 0.5–1 h, by illumination of the tumor with 100 J/cm^2 LED light at 635 nm. Complete tumor response was achieved in 76% of patients.

Recently, Yao et al. [23] applied an ablative fractional laser to oral leukoplakia lesions in the oral cavity of 48 patients to improve the clinical success of 5-aminolevulinic acid-mediated photodynamic therapy with the aim of enhancing the tissue penetration of the photosensitizer. After this procedure, a 20% gel of 5-aminolevulinic acid was applied topically to the lesions for 3 h, and the areas were illuminated subsequently by red light with a Yage LED-IB at a wavelength of 630 nm (180 J/cm^2). After one month, 30 patients had complete recovery and 12 patients had partial recovery. The recurrence and malignant transformation rates were 37.5% and 8.3%, respectively, after 3 years of follow-up of the patients [23].

3. Methylene Blue

Oral lichen planus is a chronic inflammatory disease that has the risk of transformation into malignant squamous cell carcinoma [24]. The standard treatment for this condition is topically applied corticosteroids that have local side effects, including secondary candidiasis, hypopigmentation, and delayed wound healing, in long-term use [25]. Methylene blue (Figure 2), which has been used as a photosensitizer in the treatment of basal cell carcinoma, Kaposi's sarcoma and melanoma [26], has also been studied for its efficacy and safety in photodynamic therapy for oral lichen planus. Aghahosseini et al. [27] used methylene blue for photodynamic therapy on 13 patients with 26 oral lichen planus lesions. After gargling with a 5% methylene blue solution, laser light was applied to the lesions for 2 min (diode laser, 632 nm, 120 J/cm^2). At the end of 12 weeks of follow-up, 16 of the lesions showed significant reduction in size, with an average reduction of around 44%. In addition, no serious side effects were observed in the patients [27]. In a similar study, 20 patients with oral lichen planus underwent methylene blue-mediated photodynamic therapy. Four weeks after the treatment, 17 of 20 patients responded to treatment. Three patients did not respond to treatment because of longer-term lesions and because the implied duration of the lesion may be a determinant in the response to treatment [28].

Bakhtiari et al. [29] compared the efficacy of methylene-blue-mediated photodynamic therapy with conventional topical corticosteroid treatment on 30 patients with oral lichen planus. After 60 days of follow up, photodynamic therapy was found to be as effective as corticosteroid therapy and had no side effects [29]. Mostafa et al. [30], however, reported

that methylene-blue-mediated photodynamic therapy (diode laser, wavelength 660 nm, intensity 100–130 mW/cm^2) gave better results in terms of size reduction in the lesions and of pain compared to topical corticosteroid treatment. These clinical trials indicate that photodynamic therapy with methylene blue has the potential to be used as an alternative to conventional corticosteroid therapy [30].

4. Porphyrin Photosensitizers

Photofrin (dihematoporphyrin ether) and hematoporphyrin derivatives are referred to as first-generation sensitizers [10] (Figure 2). Biel has studied the treatment of a large group of head and neck squamous cell carcinoma patients with Photofrin. Early on, 'true' cancer of the larynx was treated successfully. A complete response was observed in about 90% of patients, even in those who failed an initial therapy (usually radiation) [15,31–33]. Biel [33] reported the use of Photofrin photodynamic therapy on 110 patients with recurrent or primary laryngeal tumors. The therapy involved the intravenous injection of 2 mg/kg photosensitizer, and treatment 48 h later with light from an Nd:Yag pumped-dye laser (Laserscope) at 630 nm, using a 400-mm fused silica optical fiber (Laserguide) and a microlens. The light dose rate was 80 J/cm^2 and 150 mW/cm^2 in the larynx. A 5-year cure rate of 90% was achieved, and all the recurrences could be treated with photodynamic therapy, surgery or radiation.

Biel [33] has suggested that photodynamic therapy should be considered for the treatment of primary and recurrent Tis (in situ carcinoma in the superficial lining of the oral cavity), T1 (tumor $\leq$ 2 cm across), and T2 (tumor > 2 cm and <4 cm across) squamous cell carcinoma of the larynx.

Porfimer sodium (Photofrin)-mediated photodynamic therapy was employed to treat 18 patients with squamous cell carcinoma and 7 with epithelial dysplasia with hyperkeratosis in the oral cavity [34]. The patients received intravenous Photofrin at a dose of 2 mg/kg 48 h before laser irradiation. The lesions were irradiated with an excimer dye laser (PDT-EDL1 from Hamamatsu Photonics) at 630 nm, with an irradiation output of 4 mJ/pulse/cm^2, and a repetition rate of 40 Hz. Light was directed to the tumor by means of a 400 µm flat-tipped quartz fiber. Ninety six percent of the patients were cured.

5. Foscan (Temoporfin; mTHPC)

Foscan [5,10,15,20-meta-tetra(hydroxyphenyl)chlorin, Temoporfin, mTHPC] (Figure 2) has been used in the treatment of early oral squamous cell carcinoma in 114 patients who had floor-of-the-mouth, lip, and anterior tongue lesions [35]. Foscan (0.15 mg/kg) was given intravenously and the lesions were exposed to laser light at 652 nm with a total dose of 20 J/cm^2, at a fluence rate of 100 mW/cm^2. The light was delivered to the tumor over 200 s through an optical fiber and microlens diffuser. The response rate was 93% for T1 lesions and 58% for T2 lesions. All patients sustained an excellent functional status after therapy, and none of them required airway management.

D'Cruz et al. [36] reported data on 128 patients with incurable or recurrent disease. Fifteen patients had multiple lesions. Four days after the administration of mTHPC, the tumor surface was illuminated with a nonthermal diode laser using a microlens fiber at a light dose of 20 J/cm^2, and an intensity of 100 mW/cm^2 at 652 nm. Incident light was perpendicular to the tumor surface and illuminated an area 0.5 cm beyond the visible tumor. About 16% of patients achieved a complete response. Thus, it appears that this group of patients, who had already had extensive surgery and radiation, could still benefit from 'salvage' photodynamic therapy [36].

Foscan may also be used in the treatment of lip cancer with better functional outcomes than those achievable by surgery and radiation [37], early oral squamous cell carcinoma [35], as well as advanced head and neck cancer [38].

One hundred and seventy patients, with early-stage (Tis, T1, T2) oral cavity and oropharynx squamous cell cancers or carcinoma in situ, were treated with intravenous Temoporfin at 0.15 mg/kg body weight, followed, after 96 h, by exposure to a diode laser

at 652 nm and a dose of 20 J/cm^2. The overall response rate in this study was 91%, with a complete response rate of 71% [39].

Copper et al. performed photodynamic therapy on 25 patients with T1–T2 N0 tumors (tumors that have not spread to the local lymph nodes) of the oral cavity and/or oropharynx. Patients received meta-tetra(hydroxyphenyl) chlorin (mTHPC) (Foscan) as a photosensitizer. The trial resulted in complete remission for 25 patients [40].

In another study, 21 patients with stage IV advanced and/or recurrent tongue base carcinoma were treated with photodynamic therapy using mTHPC (0.15 mg/kg) as a photosensitizer. Fifteen patients did not report problems after the treatment. Photodynamic therapy, in this case, significantly reduced tumor-associated symptoms such as breathing, swallowing, and speech (voice) problems [41].

6. Chlorin e6 and HPPH

Sobaniec et al. studied photodynamic therapy in 23 patients with oral leukoplakia. Patients were treated with chlorin e6 (Photolon®) (Figure 2), containing 20% chlorin e6 and 10% dimethylsulfoxide as a photosensitizer. The appointments were scheduled for PDT treatment biweekly and this treatment reduced the size of the oral leukoplakia lesion [42] by 55% (average).

In a clinical trial involving patients with oral dysplasia, carcinoma in situ, or early-stage head and neck squamous cell carcinoma, patients were given 3-(1′-hexyloxyethyl) pyropheophorbide (HPPH) (Figure 3) at a dose of 4 mg/m^2 systemically, 22–26 h before light delivery. The tumor was illuminated with 50 to 140 J/cm^2. At day 28, there was 58% complete response, 11% partial response, and 11% stable disease [43].

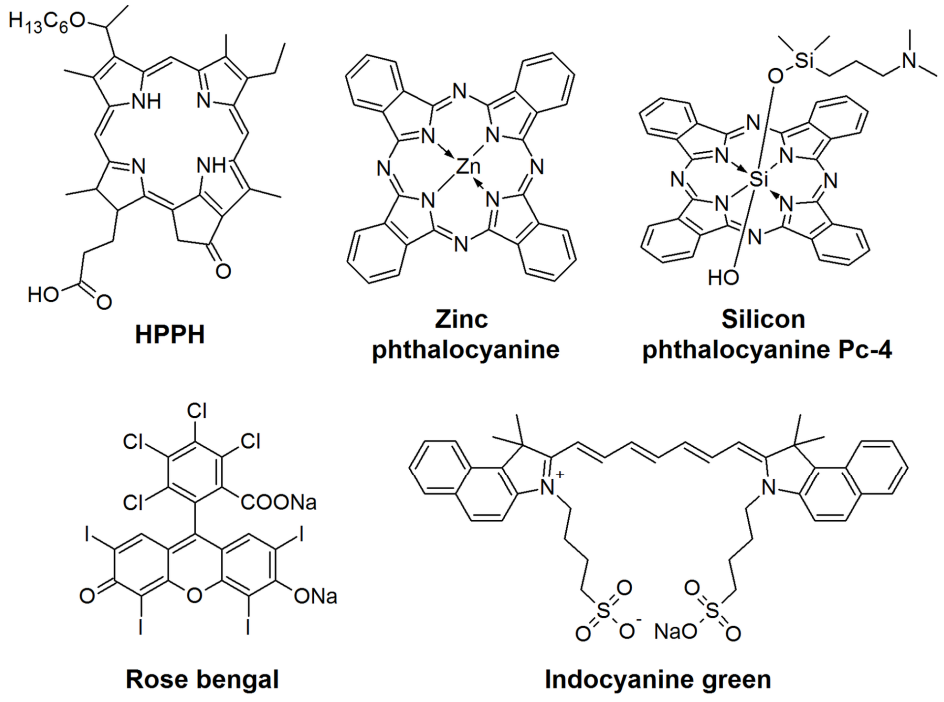


Figure 3. Molecular structure of photosensitizers.

7. Theranostics and Photodynamic Therapy

The theranostic approach relies on combining several modalities, such as imaging, delivery of therapeutics, as well as stimuli-responsive delivery within one system. This strategy is applied to achieve more precise drug delivery, monitor therapy outcomes, enhance tumor penetration, and provide better biocompatibility and more controllable drug release. Sophisticated multifunctional platforms are designed to include all the required modalities.

Theranostic agents have been investigated in the field of photodynamic therapy in various cancer models, with some examples in oral cancer. Recently, we reported on theranostic liposomes delivering a contrast agent (a hybrid of a phospholipid and gadopentetic acid) for magnetic resonance imaging (MRI) and the therapeutic agent, a second-generation photosensitizer, zinc(II) phthalocyanine (ZnPc) (Figure 3). The system has been developed for image-guided photodynamic therapy of head and neck cancer. We observed that, in comparison to liposomes containing only a contrast agent (without ZnPc), the theranostic liposomes (with both Gd(III) chelate and ZnPc) had higher relaxivity. The improved relaxation of theranostic liposomes (resulting from the presence of ZnPc), may possibly enhance MRI contrast, and thus potentially allow a reduction in the Gd(III) chelate dose. The positive influence of ZnPc on relaxivities of theranostic liposomes was attributed to the changes that occur inside the liposomal bilayer that affect water permeability across the liposomal membrane, enabling the interaction of water molecules with paramagnetic centers. Regarding photodynamic efficacy, ZnPc, loaded into theranostic liposomes, exhibit IC_{50} of 0.22–0.61 μM in two oral cancer cellular models (SCC-25 and FaDu) [44].

To reduce the toxicity and improve the performance of photodynamic therapy, targeted platforms for theranostic applications have been developed. Wang et al. [45] studied targeted iron-oxide nanoparticles for photodynamic therapy and MRI of head and neck cancer. The system involved a second-generation photodynamic therapy drug, Pc 4, a cancer-targeting ligand, fibronectin-mimetic peptide (Fmp), and iron oxide nanoparticles. Non-targeted and targeted nanoparticles accumulated in xenograft tumors with higher concentrations than non-formulated Pc 4 and reduced the size of head and neck squamous cell carcinoma xenograft tumors.

Another strategy that allows controllable drug release is the stimuli-responsive drug delivery system [46]. These systems respond to the unique properties of the tumor microenvironment involving acidic pH, the overexpression of specific enzymes, and high levels of ROS. In studies of light-responsive drug delivery systems, a combination of photodynamic therapy and photothermal therapy has been reported, of which the synergistic therapeutic effect was verified [46]. For example, Song et al. [47] designed a chlorin e6-linked drug delivery system co-loaded with cisplatin and metformin for the treatment of head and neck squamous cell carcinoma. The photosensitizer chlorin e6 showed laser-triggered photothermal therapy and photodynamic therapy effects, while cisplatin and metformin served as the chemotherapeutic core [47].

A novel near-infrared-triggered drug release system for combined photothermal therapy, photodynamic therapy, and chemotherapy was investigated by Wang et al. [48]. Nanoparticles were formed using human serum albumin, indocyanine green (Figure 3) and cisplatin. The nanoparticles ensured site-specific drug delivery/release and reduced chemotherapy's systemic toxicity, demonstrating the synergistic effects of photodynamic therapy, photothermal therapy, and chemotherapy with in vitro and in vivo experiments.

Therapeutic protocols for head and neck cancer usually involve chemotherapy, radiotherapy, and/or immunotherapy. Combination therapy is a common trend in oncology. By using various carriers, researchers have proposed a multimodal treatment approach combining chemotherapy and optical therapy, which seem to be beneficial in treating oral cancer [48].

Wang et al. [49] developed novel nanoplatforms for the photothermal therapy of oral cancer with Rose Bengal (Figure 3) as a photodynamic agent and gold nanorods as a photothermal agent. Green laser light was used to activate Rose Bengal, and red laser light for gold nanorods, during an in vitro study with Cal-27 cells. In a study with hamster cheek pouches, an animal model, they found that Rose Bengal–gold nanorods, for combined photodynamic therapy and photothermal therapy, can provide enhanced anti-cancer efficacy against oral cancer. In addition, nanoparticles combined with a laser can be effectively used in photothermal therapy, attacking tumor cells without significant damage to other cells [49].

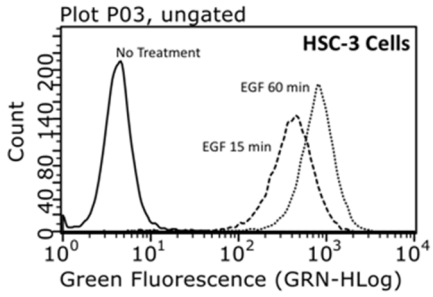
Ren et al. [50] synthesized hybrid nanoparticles composed of poly(ethylene glycol)-polycaprolactone, with incorporated organic compound (C3) and indocyanine green as photothermal therapy and photodynamic therapy agents, respectively. This nanoplatform was able to simultaneously produce hyperthermia through C3 and produce reactive oxygen species and a fluorescence-guided effect through indocyanine green to kill oral squamous cell carcinoma cells [50].

Another example of a theranostic system developed to treat head and neck squamous cell carcinoma was a combination of photodynamic therapy with gene therapy. The efficacy of photodynamic therapy can be improved by inhibiting the Wnt/β-catenin signaling pathway, which is involved in the activation of the epithelial-to-mesenchymal transition. This transition can lead to tumor recurrence and progression. Ma et al. [51] efficiently delivered siRNA targeting Wnt-1 into the cytoplasm of photodynamic-therapy-treated oral cancer cells using poly(ethylene glycol)-polyethyleneimine-chlorin e6 nanoparticles. This treatment significantly inhibited oral squamous carcinoma cell growth and enhanced the killing effect on cancer cells [51].

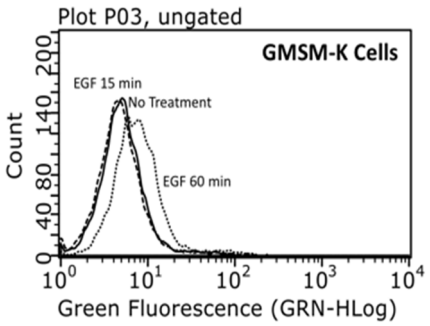
8. Targeting Cancer Cells

Although the intravenous administration of photosensitizers has been employed in most clinical trials, the potential side effects should be alleviated. Among the problems of photodynamic therapy are tissue selectivity for the photosensitizers, tissue hypoxia, and tissue penetration of light [52]. The main side effect of photodynamic therapy is systemic, off-target photosensitization resulting from the intravenous administration of the photosensitizer [53,54]. Thus, the intratumoral delivery of the photosensitizer in liposomes, and the potential retention of the liposomes locally via the liposome design, will be highly advantageous. We envision that photosensitizer-encapsulating liposomes can be injected directly, targeting the liposomes to markers overexpressed in cancer cells and will minimize delivery to normal cells around the malignant lesion. Another disadvantage of photodynamic therapy is the necessity to direct the light source to the tumor. Thus, the primary indication for this therapy is in the treatment of superficial and easily accessible cancers [54]. The intravenous injection of the Foscan formulation of mTHPC is painful, results in severe weight loss and acute liver toxicity in CAL-33 tumor-bearing nude mice, whereas the Lipidot nano-emulsion does not cause these side-effects [55], indicating that an association with a lipidic carrier can alleviate some of the toxic effects of photosensitizers. However, this method still does not overcome the systemic photosensitivity of the treated patient.

Although the direct delivery of photosensitizers into oral squamous cell carcinoma lesions may be possible, photocytotoxicity to normal cells surrounding the cancer cells is a side-effect that should ideally be avoided. Using flow cytometry, we examined the binding of fluorescent epidermal growth factor (EGF) to EGF receptors on the surface of HSC-3 human oral squamous cell carcinoma cells, and control, non-tumor-derived GMSM-K cells (Figure 4). We observed extensive binding of EGF to HSC-3 cells and minimal binding to GMSM-K cells, even at 60 min. Confocal fluorescence microscopy showed the extensive internalization of fluorescein-labeled EGF by HSC-3 oral squamous cell carcinoma cells (Figure 5). These observations can be extended to the use of photosensitizer-incorporating liposomes with EGF or anti-EGF receptor antibodies attached covalently to their surface.



(**a**)



(**b**)

Figure 4. Flow cytometry of EGF binding to HSC-3 (**a**) and control GMSM-K cells (**b**).

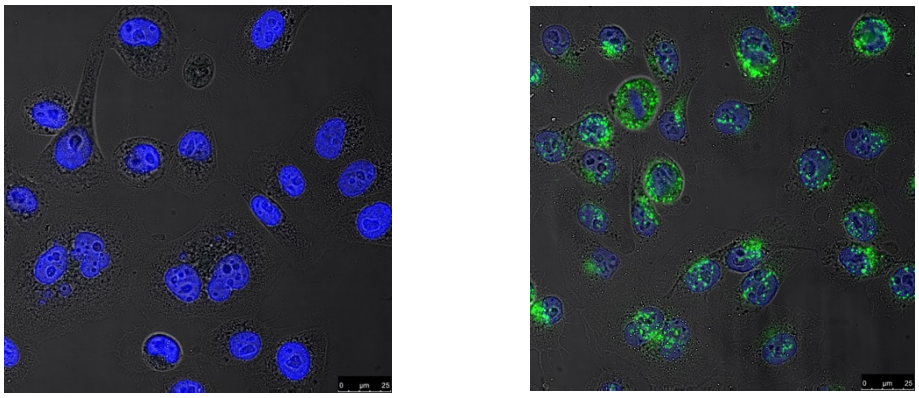


Figure 5. Confocal fluorescence microscopy of fluorescein-labeled EGF internalized by HSC-3 cells following a 15 min incubation with 1 μg/mL EGF. **Left** panel: untreated cells with Hoechst dye; **Right** panel: treated cells.

9. Novel Porphyrinoids with High In Vitro Cytotoxic Activity against Oral Cancer Cells

9.1. Sulfanyl Porphyrazines with Fluoroalkyl and Diether Chains

Our laboratories have been working on the development of novel photosensitizers and their photocytotoxicity on oral cancer cells. Two sulfanyl porphyrazines, possessing 4-fluorobutyl (**1a**) and 2-(2-ethoxyethoxy)ethyl (**1b**) substituents (Figure 6), were studied using human oral squamous cell carcinoma cell lines derived from the tongue (HSC-3) and

the buccal mucosa (H413) [56]. Porphyrazines **1a**,**b** in the concentration range 1–50 μM showed no dark toxicity, i.e., without light exposure. At 50 μM, however, both **1a** and **1b** aggregated, as noted under the microscope.

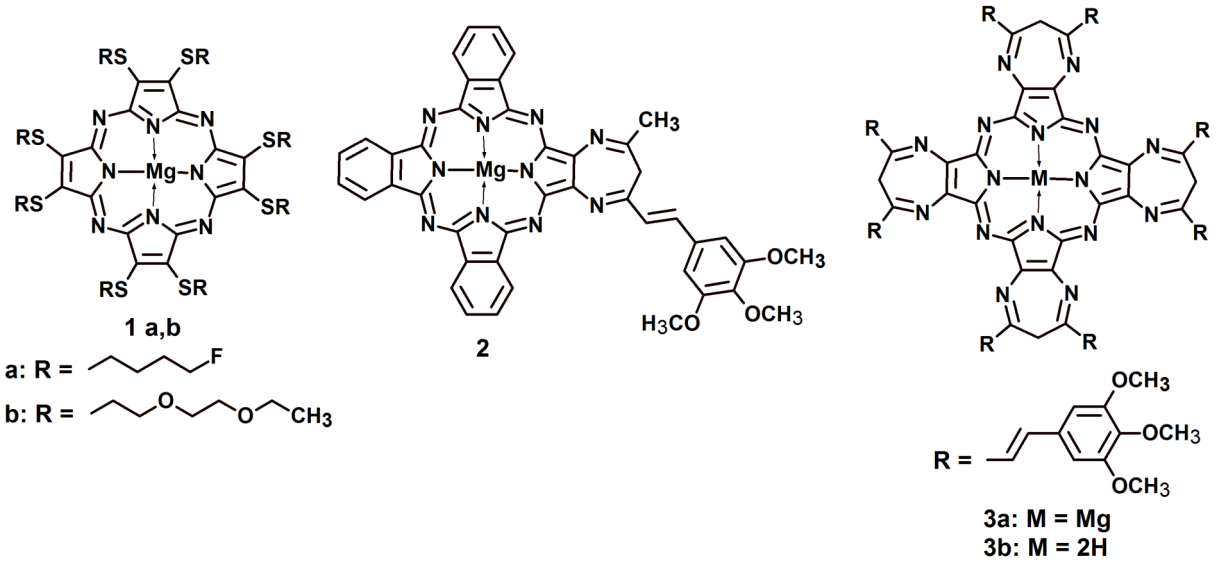


Figure 6. The chemical structure of porphyrazines **1**–**3**.

The light-induced toxicity was tested at 1 and 5 μM after exposure to light of 600–850 nm. No light-induced toxicity was observed for porphyrazine **1b** on both cell lines, and for **1a** for HSC-3 cells. By contrast, **1a** reduced the H413 cell viability by about 30–35% at both concentrations. Thus, the phototoxicity of the sulfanyl porphyrazine with fluoroalkyl substituents was found to be cell-dependent, although these cells are derived from the same type of tumor.

9.2. Porphyrazines and Tribenzoporphyrazine with Annulated Diazepine Rings

Further studies using HSC-3 and H413 cells were performed with porphyrazines and tribenzoporphyrazine possessing annulated diazepine rings (**2**, **3**, Figure 6) [57]. Dark toxicity experiments in the concentration range 0.1–10 μM showed that only tribenzoporphyrazine **2** on H413 cells showed some dark toxicity at concentrations higher than 1 μM. Photocytotoxicity studies after LED light irradiation at 690 nm revealed that the viability of the H413 cells was reduced by 90% at 1.0 μM of compound **2**, and by **3a** at 10 μM by about 25%, whereas **3b** did not show any significant viability reduction. In the case of HSC-3 cells, a photocytotoxic effect of about 95% was found for compound **2** at 1 μM and 87% for 10 μM of **3b**. There was no significant reduction in HSC-3 cell viability by porphyrazine **3b**.

The photodynamic efficiency of compounds **2** and **3b** toward HSC-3 cells was also examined after incorporation into four different liposome formulations: (i) L-α-phosphatidyl-D,L-glycerol (PG, from chicken eggs):1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), (ii) PG:POPC:cholesterol (Chol), (iii) N-[1-(2,3-dioleoyloxy)-propyl]-N,N,N-trimeth -ylammonium chloride (DOTAP):POPC, and (iv) DOTAP:POPC:Chol. All four liposomes containing tribenzoporphyrazine **2** showed high light-induced photodynamic activity [57]. On the contrary, there was no significant photocytotoxic effect of liposomal compound **3b**. The IC_{50} calculations of the four types of liposomes with **2** revealed that the negatively charged liposomes composed of **2**:PG:POPC were the most active. Moreover, their activity was about three times higher than the free form of photosensitizer **2**, indicating that these liposomes are the most potent delivery systems for this photosensitizer.

9.3. Potential Methods of Liposome Administration

We envisage liposomal photosensitizers to be administered in several ways. Analogous to our studies on the injection of suicide genes complexed to transferrin-associated cationic liposomes into orthotopic oral squamous cell carcinoma tumors in mice that result in the arrest of tumor growth following the delivery of the prodrug, ganciclovir [58], they can be injected directly into the tumors. This method may be enhanced by the simultaneous delivery of agents that degrade the tumor microenvironment, enabling the liposomes easier access to the entire tumor. Liu et al. [59] employed collagenase, encapsulated in pH-responsive nanoscale coordination polymers that released their enzyme in the mildly acidic medium of the tumor microenvironment, resulting in a loosened extracellular matrix structure, enhanced tumor perfusion, and relieved hypoxia. They showed that liposomal chlorin-e6 mediated more effective photodynamic therapy following collagenase treatment. Kohli et al. [60] reported that the distribution of liposomal doxorubicin in the tumor matrix was improved after the depletion of tumor hyaluronan. Concerns about the induction of metastases following enzyme treatment have not been borne out [61]. Liposomal photosensitizers may be administered transmucosally by enabling their stable adhesion to the mucosa [62], for example, by the use of cationic liposomes (vide infra). Liposomes may also be potentially embedded in oral films to facilitate their transmucosal transport [63].

9.4. Sulfanyl Porphyrazines with 4-Bromobenzyl and 4-Biphenylylmethyl Substituents

The photocytotoxic effects of free and liposome-encapsulated sulfanyl porphyrazines containing 4-bromobenzyl (**4a**) and 4-biphenylylmethyl substituents (**4b**, Figure 7), on oral cancer cells derived from the tongue (CAL 27, HSC-3), and HeLa human cervical epithelial adenocarcinoma cells, was investigated [64].The photosensitizers in free form did not have any significant photocytotoxicity on any of the cells. The liposomal formulations of these porphyrazines were prepared using two different types of nanoparticles, composed of PG:POPC or DOTAP:POPC. Porphyrazine **4a**, incorporated into the positively charged DOTAP:POPC liposomes, showed high photocytotoxicity with the reduction in the cell viability by about 90% at 10 μM. The viability of cells at 1 μm was decreased by 47% for HSC-3 and 34% for CAL 27 cells. There was no significant reduction in the HeLa cell viability. The phototoxicity of liposomal formulations containing the biphenylyl analog **4b** was much lower. Cationic DOTAP:POPC liposomes showed light-induced toxicity against HSC-3 cells, reducing cell viability by about 30% at 10 μM, but not against other cells. The negatively charged **4b**:PG:POPC liposomes did not have any photocytotoxicity. The results indicated that DOTAP:POPC liposomes could be a promising drug delivery system for sulfanyl porphyrazines, but their effectiveness also depends on the photosensitizer structure.

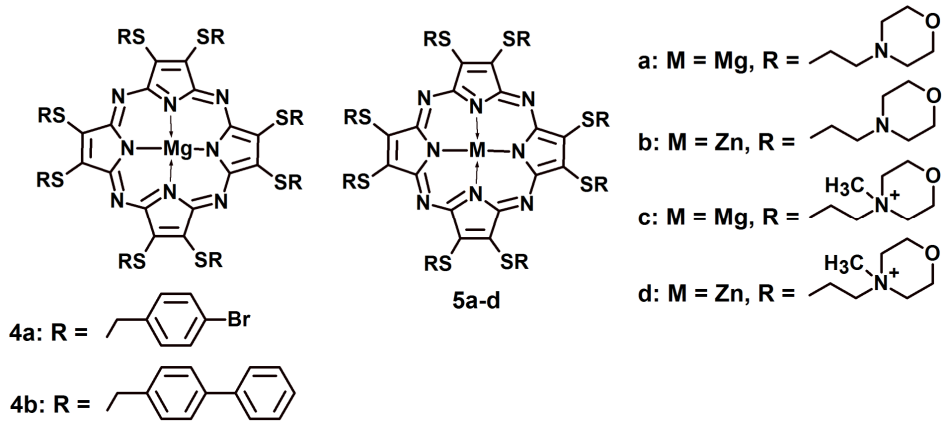


Figure 7. The chemical structure of porphyrazines **4**,**5**.

9.5. Sulfanyl Porphyrazines with Morpholinoethyl and N-Methylmorpholinoethyl Substituents

Magnesium(II) and zinc(II) porphyrazines, bearing morpholinoethyl (**5a,b**) and cationic N-methylmorpholinoethyl substituents (**5c,d**, Figure 7), were tested on squamous cell carcinoma cell lines SCC-25 and CAL-27 derived from the tongue [65]. They were tested in free forms administered to the cell culture medium in the range 0.01–5.0 μM, and in POPC:POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-1′-rac-glycerol sodium salt) liposomes at 0.01–2.0 μM. Despite their similar origin, CAL 27 cells were found to be less susceptible to photodynamic treatment than SCC-25 cells. Magnesium(II) porphyrazine, with morpholinoethyl groups (**5a**), showed high photocytotoxicity on both cell lines, with IC_{50} values of 0.75 and 1.20 μM for SCC-25 and CAL 27 cells, respectively. In addition, the liposomal formulation of **5a** was even more active, with IC_{50} = 0.64 μM for SCC-25 and 0.69 μM for CAL 27 cells. The zinc analog **5b** revealed lower effectiveness, with the phototoxic effect observed only against SCC-cells (IC_{50} = 6.46 μM), which increased almost twice after liposomal incorporation (IC_{50} = 3.61 μM). However, porphyrazines containing cationic N-methylmorpholinoethyl substituents (**5c,d**) did not show any significant cytotoxicity on tested cancer cells.

9.6. Sulfanyl Tribenzoporphyrazines with Dendrimeric Moieties

Sulfanyl tribenzoporphyrazines were another important subgroup of photosensitizers subjected to studies against oral cancer cells. Three such macrocycles with dendrimeric moieties (**6a**–**c**, Figure 8) were tested on CAL 27 and HSC-3 cells [66]. After being exposed to LED light of 690 nm, all three photosensitizers showed a photocytotoxic effect against tested cells. Tribenzoporphyrazine **6a** with branched G_1-dendrimeric substituents showed moderate activity toward CAL 27 cells with an IC_{50} value of 3.13 μM, but much higher activity against HSC-3 cells (IC_{50} = 0.64 μM). The decrease in the dendrimeric substituent generation to G_0 in the case of tribenzoporphyrazine **6b** resulted in reduced photocytotoxicity, as the IC_{50} values reached 6.66 and 10.6 μM for CAL 27 and HSC-3 cells, respectively. However, the lowest nanomolar IC_{50} values of 10 nM for CAL 27 and 42 nM for HSC-3 were reached by **6c**, in whose structure butoxycarbonyl substituents were reduced to hydroxymethyl groups. In further studies, porphyrazine **6b** was subjected to an oxidation reaction, resulting in the oxidative breaking of the one pyrrole ring and the formation of the S-seco-tribenzoporphyrazine analogue **7** (Figure 8) [66]. The photodynamic activity of the seco-derivative **7** on CAL 27 and HSC-3 cells was very potent, with IC_{50} values of 0.61 and 0.18 μM, respectively. This activity was much higher than those for the precursor **6b**.

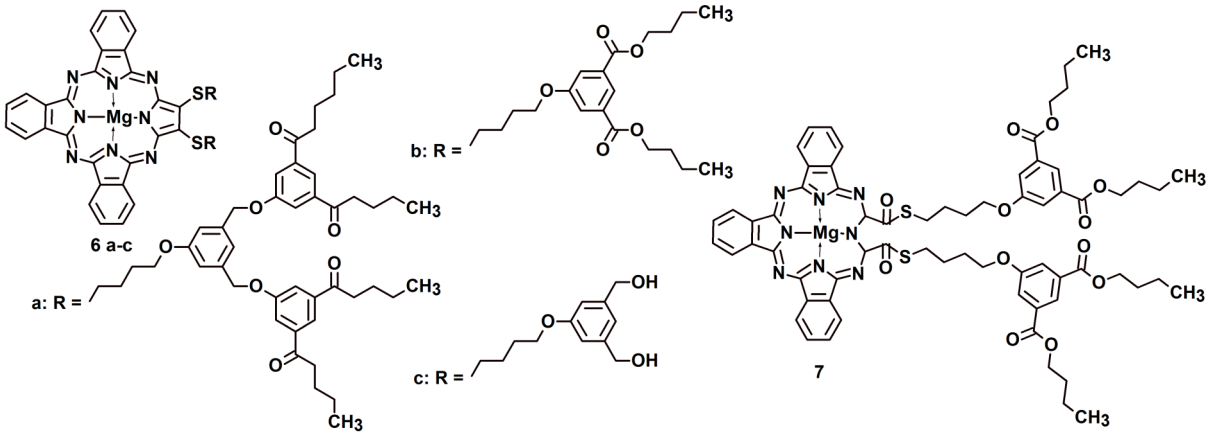


Figure 8. The chemical structure of porphyrazines **6**,**7**.

Photosensitizers **6a**–**c** and **7** were also tested after incorporating them into four liposomal formulations [67,68]. Zwitterionic lipids POPC or 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) constituted the main components of the liposomes, whereas PG or DOTAP were added to provide a negative or positive charge, respectively. For

all tested tribenzoporphyrazines, cationic DOTAP:POPC liposomes had the highest photodynamic activity. Notably, the IC_{50} values for this formulation were up to 50 times lower compared to the free forms of tribenzoporphyrazines, and the oral cancer cells, CAL 27 and HSC-3, were more sensitive to photodynamic treatment than the HeLa cervical adenocarcinoma cells.

9.7. Phthalocyanines

Phthalocyanines constitute another class of porphyrinoids that we tested on oral cancer cells. Two commercially available compounds, zinc phthalocyanine (ZnPc) and aluminum phthalocyanine chloride (AlPc, Figure 9), were examined on HSC-3 and HeLa cells [69]. The photosensitizers were prepared in free forms dissolved in the culture medium, as well as incorporated into negatively charged PG:POPC liposomes. Both phthalocyanines revealed a phototoxic effect, which was dependent on cell type. Zinc phthalocyanine was more effective against HSC-3 cells, whose viability was reduced to 22% at 1.0 μM photosensitizer, whereas the viability of HeLa cells decreased to 53% at the same concentration of ZnPc. By contrast, HeLa cells were more sensitive to the treatment with aluminum phthalocyanine chloride. The viability of cells after photodynamic treatment with 1.0 μM of AlPc decreased to 15 and 57% for HeLa and HSC-3 cells, respectively. Moreover, liposomal incorporation enhanced the cytotoxic effect of both phthalocyanines. A lethal photodynamic effect on both cell types was observed for liposomes containing 1.0 μM of ZnPc, while AlPc-liposomes at the same concentration caused a lethal effect on HeLa cells, but the viability of HSC-3 cells was reduced to only 21%.

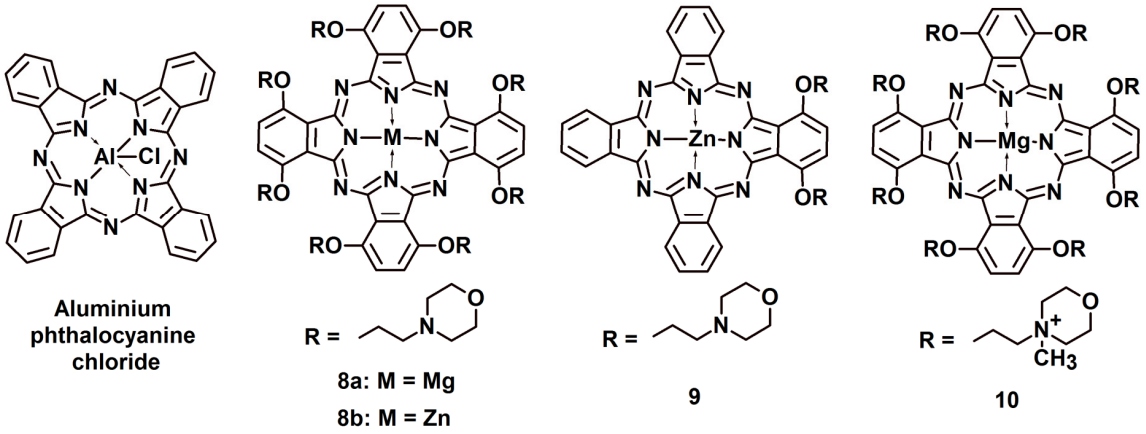


Figure 9. The chemical structure of AlPc and phthalocyanines **8**–**10**.

In another study, zinc phthalocyanine was incorporated in two types of liposomes composed of POPG:POPC or POPG:DOPE [70]. The photodynamic activity of obtained liposomes, both extruded and non-extruded, was tested on CAL27 cells and FaDu pharyngeal carcinoma cells. Surprisingly, POPG:DOPE liposomes did not have photocytotoxicity against tested cell lines. On the other hand, zinc phthalocyanine in free form, and incorporated into extruded POPG:POPC liposomes, showed a significant photodynamic effect, which was higher on CAL 27 cells, compared to FaDu cells. The ZnPc, dissolved in culture medium at 0.1 and 0.5 μM concentrations, decreased the viability of CAL 27 cells by about 20% and 70%, respectively. The viability of FaDu cells was not affected at 0.1 μM and reduced by about 60% at 0.5 μM. The incorporation of ZnPc in extruded POPG:POPC liposomes resulted in highly increased photocytotoxicity; at 0.1 and 0.5 μM concentrations, a lethal effect was observed on CAL 27 cells. In the case of HeLa cells, a 0.5 μM concentration was also lethal, but, at 0.1 μM, cell viability was decreased to 6%.

Novel phthalocyanines synthesized by our team were also examined on oral cancer cells. Magnesium and zinc phthalocyanines (**8a**, **b**), as well as zinc tribenzoporphyrazine (**9**) containing 2-(morpholin-4-yl)ethoxy substituents (Figure 9), were examined on HSC-3 and

H413 cancer cells derived from the tongue and buccal mucosa, respectively [71]. Tribenzoporphyrazine **9** definitely showed the highest photocytotoxic effect. However, significant dark toxicity was observed with H413 cells, reaching 21 and 74% at 1.0 and 10 μM concentrations. HSC-3 cells were unaffected by **9** in this concentration range in the dark, while, after irradiation, the cell viability was reduced by >90% and >80% at 0.1 and 1.0 μM. Photosensitizers **8a**, **b**, and **9** were also incorporated into PG:POPC and DOTAP:POPC liposomes, but, surprisingly, they did not strongly affect biological activity against HSC-3 and H413 cells. Another study on HSC3 and H413 cells involved phthalocyanine containing cationic N-methylmorpholiniumethoxy substituents (**10**, Figure 9) in free form and incorporated into PG:POPC liposomes [72]. Surprisingly, besides a high photodynamic activity against both Gram-positive and Gram-negative bacteria, no significant photocytotoxicity toward oral cancer cells was observed.

9.8. Other Porphyrinoid Photosensitizers

The photodynamic activity of other porphyrinoid photosensitizers on various cancer cells has been examined recently, both in free forms and in drug delivery systems, including liposomal formulations [5,73,74]. However, the reports concerning oral cancer cells are very limited. Thomas et al. [75] investigated the photosensitizing activity of a water-soluble N-confused porphyrin with 4-sulfonatophenyl substituents on oral squamous cell carcinoma cell lines SCC-131 (IC_{50} = 13 μM) and SCC-172 (IC_{50} = 11 μM) [75]. Chin and coworkers studied the photodynamic activity of three glycerol substituted phthalocyanines on MCF-7 breast carcinoma, HCT-116 colon carcinoma, and HSC-2 oral squamous cell carcinoma cells. Non-peripherally tetra-glycerol-substituted and mono-iodo tri-glycerol-substituted phthalocyanines showed promising activity with IC_{50} values in the range of 2.8–3.2 μM and 0.04–0.06 μM for tetra-glycerol and mono-iodo tri-glycerol analogs, respectively [76]. A chlorin-based photosensitizer, pheophorbide a, was used on YD10B and YD38 oral squamous cell carcinoma cell lines [77]. The viability of YD10B cells decreased by 70% after treatment, and that of YD38 cells by 60%. A greater increase in ROS generation, and in the number of apoptotic cells, were also observed for YD10B cells compared to the YD38 cells. Moreover, the RUNX3 gene related to apoptosis was selected as a potential marker for determining sensitivity to photodynamic therapy with pheophorbide a. It was found that the expression level of RUNX3 was proportional to the percentage of PDT-induced cell death [77]. Chu and coworkers [78] studied the effect of vandetanib, a blocker of epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor-2 (VEGFR-2), on the efficiency of photodynamic therapy. The studies performed with Chlorin e6 on CAL 27 oral cancer cells revealed that adding vandetanib enhanced photocytotoxicity. This result was explained by both the direct and indirect effects of vandetanib on the cellular DNA repair machinery and tumor microenvironment [78].

10. Future Directions in the Photodynamic Therapy of Oral Cancer

10.1. Targeting Photosensitizers to Cancer Stem Cells

Cancer stem cells are a small subpopulation of existing tumors or cancer cells that can regenerate the original tumor or cancer, first recognized in leukemia [79,80]. Cancer stem cells have also been identified in head and neck squamous cell carcinoma [81–83]. Although CD44 is a surface marker for cancer stem cells, it is not entirely specific. Cells that express both CD44 and CD271 upregulate cancer stem-cell related genes and show higher tumorigenicity than cells expressing only CD44 [84]. These observations raise the possibility of targeting photosensitizers embedded in or encapsulated inside liposomes via the use of antibodies against CD44 and against CD271, similar to the example given above with epidermal growth factor.

10.2. Upconversion Nanoparticles for Near-Infrared Irradiation of Tumors

The absorption wavelengths of the photosensitizers used in photodynamic therapy are too short to enable sufficient tissue penetration to reach cells located deeper in tumors.

Upconversion involves the generation of high-energy (shorter wavelength) light from near-infrared, low-energy radiation that can reach deeper tissues [85,86]. As a case in point, Sawamura et al. [87] used nanoparticles composed of a lanthanide, which, upon excitation with near-infrared light (980 nm), emits light of wavelengths in the Soret band (405 nm), and one of the a Q bands (540 nm), of the photosensitizer, protoporphyrin IX PPIX. The lanthanide nanoparticles were derivatized with amino groups to bind to the human gastric cancer cell line, MKN45, and irradiation of the system with near-infrared light caused extensive cytotoxicity. Chlorin e6 complexed with upconversion lanthanide nanoparticles that emit in the ultraviolet, blue, and red regions when excited at 980 nm had a significant cytotoxic effect on MCF-7 human breast cancer cells cultured as tumor spheroids [88]. This effect was stronger than that induced by irradiation at 660 nm.

10.3. Overcoming Tumor Hypoxia

The tumor microenvironment exhibits acidic pH and hypoxia resulting from the high metabolic activity of dividing cancer cells. The oxygen tension (pO_2) in tumors is usually less than 5 mmHg, whereas it is in the range 10 to 80 mmHg in normal tissue [89]. The therapeutic effect of type-II photodynamic therapy is such that the photosensitizers in the triplet state transfer their energy directly to $^3O_2$ to produce singlet oxygen ($^1O_2$). This process obviously requires the presence of O_2, and thus, increasing the O_2 concentration of the tumor is likely to enhance the efficacy of photodynamic therapy. Some of the methods to counter tumor hypoxia are to introduce exogenous O_2 to the tumor, generate de novo O_2 in the tumor, degrade the tumor microenvironment, and inhibit the signaling pathway for hypoxia-inducible factor 1 (HIF-1) [89].

11. Conclusions

Current standard treatments for squamous cell carcinoma of the head and neck are inadequate. Photodynamic therapy may be advantageous over conventional treatments because of its minimal invasiveness, its selective tumor destruction and, thus, the preservation of healthy tissues. Some photosensitizers may localize preferentially in tumors, and liposomes carrying photosensitizers may be targeted to receptors overexpressed on certain cancer cells to mediate a much higher accumulation of the photosensitizer compared to normal cells. The use of Foscan in the treatment of early oral squamous cell carcinoma resulted in a response rate of 93% for T1 lesions and 58% for T2 lesions. In a group of 170 patients with early-stage oral cavity and oropharynx squamous cell cancers or carcinoma in situ, treated with intravenous Foscan followed by light exposure, an overall response rate of 91%, and a complete response rate of 71%, were obtained. The theranostic approach to the delivery of photosensitizers integrates imaging and stimuli-responsive therapeutic delivery within one system. One example of this approach is poly(ethylene glycol)-polycaprolactone nanoparticles containing the organic compound, C3, for photothermal therapy, and indocyanine green for photodynamic therapy to kill oral squamous cell carcinoma cells. We have synthesized and tested the anti-cancer activities of derivatives of sulfany porphyrazines, sulfanyl tribenzoporphyrazine, porphyrazines, and tribenzoporphyrazines, both as free drugs and incorporated in the liposome membrane. Tribenzoporphyrazines encapsulated in positively charged liposomes exhibited the highest photodynamic activity, with IC_{50} values up to 50 times lower compared to the free forms of the drugs. Thus, the avid binding of these photosensitizers to oral cancer cells, and their internalization, enables a much higher photocytotoxicity. Current and future studies on targeting liposomal photosensitizers to oral cancer cells, on the generation of liposomes or other nanoparticles enabling deeper penetration of near-infrared irradiation of tumors, and on the generation of local oxygen in tumors, are highly likely to enhance our ability to treat oral premalignant or cancerous lesions.

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