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Chapter 11

Porphyrin and Phthalocyanine Photosensitizers as PDT Agents: A New Modality for the Treatment of Melanoma

Shawn Swavey and Matthew Tran

Additional information is available at the end of the chapter

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1. Introduction

1.1. Melanoma — Background

In the United States, skin cancer is the most common type of cancer, which is further divided into three types of cancer: basal cell, squamous cell and melanoma. Basal cell and squamous cell cancers are the most common types of skin cancer, but they are less deadly and more readily treated than melanoma. While it is the least common of the skin cancers, more than 68,000 Americans are diagnosed with melanoma each year, and an additional 48,000 are diagnosed with early forms of cancer that involve the top layer of the skin. [1] It is the leading cause of death from skin cancer, due to its metastatic behavior. The frequency of melanoma has more than doubled in the past thirty years, especially in Western industrialized societies. [2]

Melanoma is a malignant tumor that originates in the melanocytes. Melanocytes are found randomly throughout the basal cells and are found in the deepest portions of the epidermis. They are responsible for making the pigments (melanin) found in the skin. The level of pigment released by melanocytes is directly related to skin color. It has been found that exposure to ultraviolet radiation triggers the melanocytes to create more pigments, which leads to what we observe as tanned skin. Studies show that melanoma has different, common locations of occurrence in men and women. [1] Men have been found to commonly develop melanoma on the skin of the head, neck, between the shoulders and the hips, whereas women often develop melanoma on the skin of the lower legs or between the shoulders or hips. Melanoma can occur on any skin surface, but it is more prone to develop on preexisting moles. There are five stages of melanoma [1]; Stage 0; Melanoma is only located in the top layer of the skin and the basil lamina is intact. This is referred to as *melanoma in situ*, Stage I; the melanoma lies in the

epidermis. The tumor is no more than 1 mm thick and has penetrated the basil lamina and extended into the papillary dermis, Stage II; The tumor is between 1 and 2 mm thick and extends into the papillary-reticular dermis interface, Stage III; The tumor extends into the reticular dermis and the melanoma cells have spread into at least one lymph node or to nearby tissues, and Stage IV; The cancer cells have metastasized to the lungs or other organs, skin areas, or lymph nodes far from the original growth. The tumor has also extended into the subcutaneous fat. Detecting melanoma early is crucial to the survival rate of the patient.

2. Risk factors for melanoma

There are several risk factors attributed to the development of melanoma in individuals. Exposure to ultraviolet radiation has been shown to be a major risk factor in the development of melanoma. Severe sunburns increase the likelihood of developing melanoma later in one's life. Although tanning decreases the likelihood of sunburns, it is not a method of counteracting cancer, for it increases the duration of sun exposure. In addition, individuals with fair skin or who are prone to severe, blistering sunburns are at a high risk of developing melanoma. Melanoma is far less common in dark skinned individuals. In addition, the duration of sun exposure over one's life is a factor in the risk of radiation induced melanoma. A person who has lived most of his/her life in an area of high sun exposure is more likely to develop melanoma. Similarly, people who live in higher altitudes, where the sun is stronger, are at a greater risk for developing melanoma than those who live at lower altitudes. Another risk factor is personal history of melanoma. An individual who has been diagnosed with melanoma before is likely to contract another form of melanoma. In addition, someone who has been diagnosed with basal cell or squamous cell skin cancer is at a high risk of developing another form of skin cancer, including melanoma. Family history of the disease is also a contributing factor. Individuals who have ancestral cases of melanoma are at a high risk of developing the disease themselves. The risk of developing melanoma is increased when the family incidences are in one's immediate family. Approximately 8-12% of cases of cutaneous melanoma are inherited. [3]

3. Current treatment regimens for melanoma

There are several existing treatments for melanoma, including surgery, chemotherapy [4], radiation therapy [5], biological therapy [6], and to a lesser extent, photodynamic therapy. These treatments can be used in any combination to varying degrees of success. Since melanoma is typically a more invasive form of skin cancer, many of these treatments act as deterrents rather than cures. However, if the melanoma is diagnosed and treated early enough, it can be cured with a high degree of success.

The primary treatment for melanoma is surgery. In the surgical process, the tumor is excised along with some of the surrounding healthy tissue so that there is a minimal chance of leaving

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cancer cells in the area. Chemotherapy is another fairly common procedure for treating melanoma. Chemotherapy is a type of cancer treatment that relies on drugs in order to stop or slow the growth of cancer cells. However, chemotherapy indiscriminately harms healthy cells that also grow rapidly, such as those located in the mouth, intestines, and hair. The effectiveness of chemotherapy relies on the type and severity of the cancer in question, and depending on the aforementioned factors, it can cure, control, or ease cancer. Chemotherapy can be administered alone, but it is typically used in conjunction with other cancer treatments, such as biological therapy, radiation therapy, or surgery. The treatment can be administered in a variety of ways: injection, intra-arterial, intraperitoneal, intravenous, topical, or oral. The therapy sessions are spread out so that there is a recovery period in between treatments.

Radiation therapy is another type of therapy used in the treatment of melanoma. Like chemotherapy, radiation therapy can cure, stop, or slow cancer growth depending on the type and severity of the disease. Also similar to chemotherapy, radiation therapy is indiscriminate in its treatment, affecting nearby healthy cells in addition to the afflicted cells. Radiation therapy employs radiation to kill cancer cells, and this radiation can be administered either internally or externally. Because internal radiation therapy places the radiation source inside the body, it allows for a more precise treatment of the cancer in question than external beam treatment. Radiation therapy also possesses serious side effects, many of which can be more severe if chemotherapy is received in addition to radiation therapy. The most common side effects include fatigue and skin changes such as dryness, itching, peeling, or blistering. Biological therapy is also used as a cancer treatment. Biological therapy, like chemotherapy, relies on drugs in order to combat cancer. However, biological therapy differs from chemotherapy because it aids the immune system in fighting cancer. Like many of the other treatments, biological therapy can be used to stop or slow growth, and it makes it easier for the immune system to destroy cancer cells.

Although it has been around for over three decades and has been successfully used to treat other forms of skin cancer, photodynamic therapy (PDT) has not received as much attention especially in the United States as a treatment option for melanoma. The major reason for this lack of attention stems from the fact that PDT requires visible light of specific wavelengths to be effective; however, the pigments found particularly in melanoma block this light making PDT less effective. As will be described in this chapter, researchers are looking at innovative ways to develop PDT into a useful treatment option for melanoma.

4. Photodynamic Therapy (PDT)

4.1. Discovery and applications

Photodynamic therapy (PDT), the therapeutic use of light, was first discovered by Raab [7] when he reported that the combination of acridine orange and light could destroy living organisms. In the 1920's it was noted by Policard [8] that the effects of fluorescence were inherently more for tumor tissue than the healthy tissue. A great deal of research has been done on PDT, which represents an interesting treatment modality for numerous

health related conditions, especially but not limited to cancer treatment. It is now believed that PDT also treats immunological effects (new antibiotics) [9], inflammation [10] and bacterial infections. [11]

Traditional cancer treatment includes radiation, surgery and/or chemotherapy, all of which have deleterious side effects. As an alternative to these treatments PDT offers a more targeted and less invasive treatment regimen. Until now, photodynamic therapy has been used for treatment of bladder cancers, brain cancers, breast metastases, skin cancers, gynecological malignancies, colorectal cancers, thoracic malignancies, and oral, head and neck cancers. [12]

Although it is not completely understood, researchers have reported that PDT activates and suppresses the immune system, [13] by a combination of effects that begins after the light treatment, the curative properties arise from the death of the irradiated cancer cells. The damage to the plasma membrane and membrane of the cellular organelles by singlet oxygen can trigger other events with far reaching consequences. The mechanism by which PDT induces specific immune responses has been suggested by Korbelik. [14] Summarizing, PDT-treated tissue release large quantities of cell debris, and inflammatory signals, cytokines and chemo-tactic agents which trigger the tissue to secrete immunosuppressive factors. The work of Musser [15] showed that PDT-induced immune suppression was also a function of the photosensitizer used.

Another PDT-induced effect is inflammation. It is reported that vascular destruction, observed after PDT is similar to the inflammatory response after tissue injury or bacterial infection. [16] This process is characterized by the release of a wide range of potent mediators including vasoactive substances, components of clotting cascades, proteinases, peroxidases, radicals, leucocytes, chemoattractants, cytokines, growth factors, and other immunoregulators.

5. Mechanisms of action for PDT

PDT typically utilizes a photosensitizer, molecular oxygen and light to destroy cancer cells. Two mechanisms [17] of the actions for PDT are recognized, Fig 1. In anoxic environments, the light induced excitation of the photosensitizer can promote an electron to a higher energy state. At this point a variety of reactions can take place. For example, this excited photosensitizer can react directly with organic substrates by electron exchange, filling the hole vacated by the excited electron, producing an oxidized substrate and reduced photosensitizer. Guanine, the most susceptible base to oxidation, is the presumed target leading to the formation of various oxo-guanine complexes and ultimately the decomposition of cellular DNA. [18] The reduced photosensitizer can react with oxygen to produce superoxide anions (O_2-) which can then form the highly reactive hydroxyl radical ($OH^{\bullet}$). The excited photosensitizer can also react with superoxide radicals ($O_2^{\bullet}$) to produce superoxide anions (O_2-) which can then create the highly reactive hydroxyl radical ($OH^{\bullet}$). Collectively these reactions are classified as Type-I photoreactions and are characterized by a dependence on the target-substrate concentration, Fig 2.

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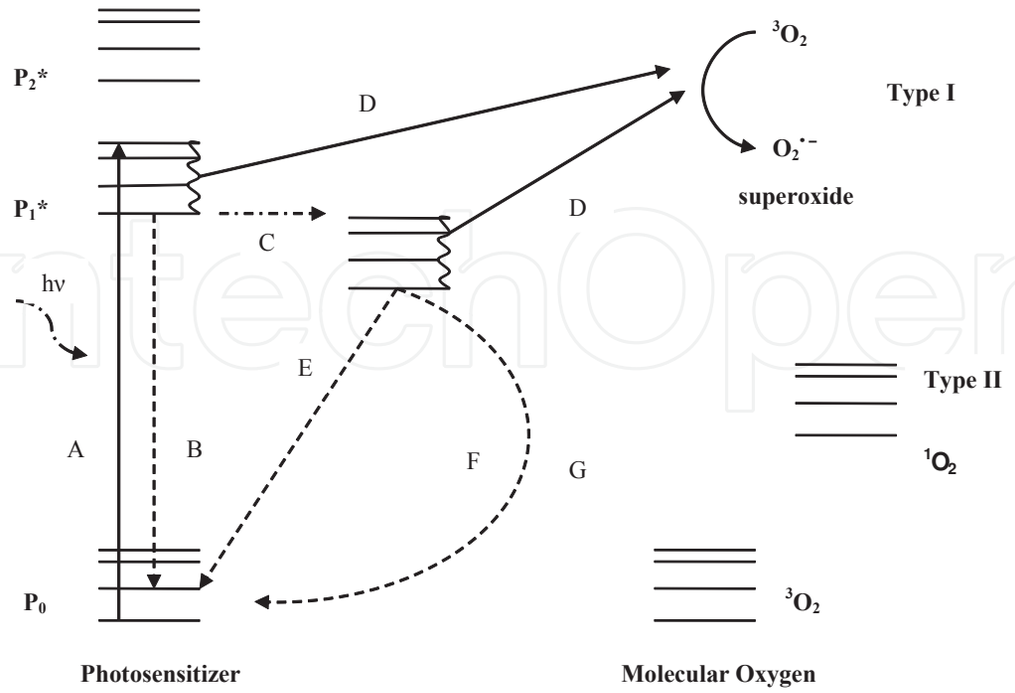

Figure 1. A diagram shows the various energy and electron transfer during PDT: (A) excitation; (B) fluorescence; (C) intersystem crossing; (D) electron transfer; (E) phosphorescence; (F) internal conversion; (G) non-radiated transfer of energy to singlet oxygen. P_0 is the ground state of photosensitizer, P_1* and P_2* are the excited state of photosensitizer, $^3O_2$ is the triplet ground state oxygen, $^1O_2$ is the singlet excited state oxygen

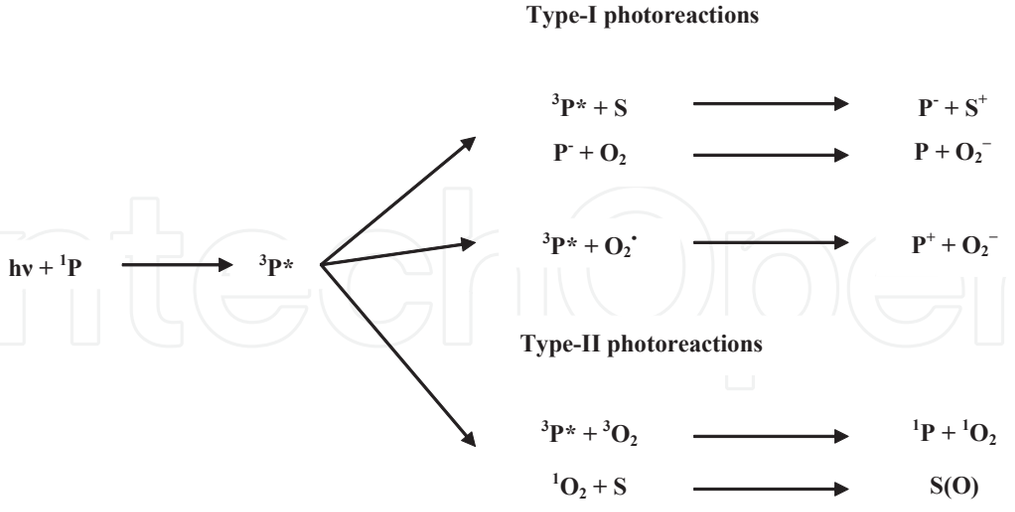

Figure 2. Type-I and type-II photoreactions, where ^1P is a photosensitizer in a singlet ground state, $^3P^*$ is a photosensitizer in a triplet excited state, S is a substrate molecule, P- is reduced photosensitizer molecule, S^+ is an oxidized substrate molecule, O_2 is molecule oxygen (triplet ground state), O_2- is the superoxide anion, $^3O_2$ is triplet ground-state oxygen, $^1O_2$ is singlet excited state, and S(O) is an oxygen adduct of a substrate.

The second mechanism also involves excitation of the photosensitizer with light but in this mechanism energy is transferred to the ground state of molecular oxygen resulting in excited singlet oxygen which goes on to destroy cellular function. [19] It was first identified that the cyto-toxic product of the photochemical reaction for PDT to be singlet oxygen by Weishaupt *et al*. in 1976. [20] The photosensitizer and oxygen interact through the triplet states because oxygen has a unique, triplet-ground state and low-lying excited states. The energy required for the triplet to singlet transition in oxygen is 22 kcal mol^{-1} which corresponds to the energy of a wavelength of 1274 nm (infrared light). [21] The energy needed to produce singlet oxygen is relatively low. Photochemical reactions of this type are known as Type-II photoreactions and are characterized by a dependence on oxygen concentration, Fig 2. [22] It is believed that the Type-II mechanism dominates during PDT. [23]

6. Photosensitizers

6.1. Development of photosensitizers and photofrin

Numerous attempts were made to treat tumor tissue with photosensitizing agents since the potential of photo treatment was discovered. Before the 1960s, scientists applied natural macrocycles to patients and tumor-bearing animals in an attempt to more accurately detect tumor tissue by florescence. [24-27] During the 1960s, Schwartz isolated a tumor localizing impurity from hematoporphyrin preparations that was later named hematoporphyrin derivative (HpD), Fig 3. Meanwhile, Lipson was investigating how to detect tumor tissue by fluorescence of hematoporphyrin. Unable to obtain reproducible results with hematoporphyrin, Lipson began experimenting with Schwartz's HpD. He used it as a tumor detection agent, [28] and first recognized that it could work as a photosensitizer to destroy tumor tissue. [29] In the 1970s, Dougherty's group discovered that fluorescein diacetate could photodynamically destroy TA-3 cells in vitro [30] and found that it could be used as a photosensitizer. [31] Following that, the group of Weishaupt identified that the cytotoxic product of photodynamic reaction to be singlet oxygen. [13] However, fluorescein has a low singlet oxygen quantum yield and a long wavelength absorption in the green portion of the electromagnetic spectrum that does not penetrate deeply into tissue. Macrocyclic photosensitizers were then examined as photosensitizers because they are efficient singlet oxygen generators and have absorption maxima in the red portion of the electromagnetic spectrum.

Eventually, Schwartz's Hpd was rediscovered by Dougherty, which by then was known to have a high singlet oxygen quantum yield, an absorption maximum in the red, and is selectively retained in tumor tissues. [30] After several years spent isolating and identifying the active fractions of HpD, a purified version named Photofrin®, was approved for use in the United States against early- and late-stage lung cancers and esophageal cancers and dysplasia with other indications pending. [30] This drug upon photoexcitation in the visible region (red) of the spectrum generates singlet oxygen from triplet oxygen through energy transfer (type II mechanism) resulting in cell death. [31]

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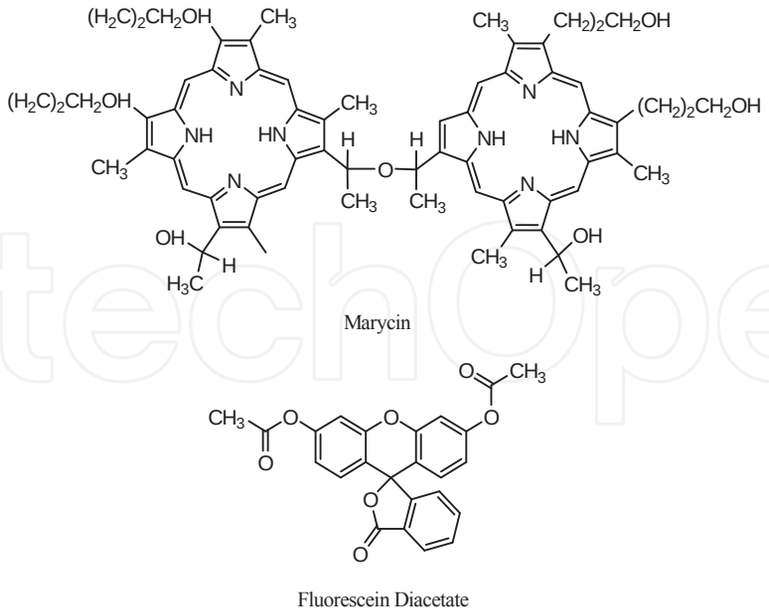

Figure 3. Marycin, an HpD isolated [28] in 1988 and Fluorescein diacetate

This chapter will focus on macrocycles (porphyrins and phthalocyanines) as potential photosensitizers for the treatment of melanoma by photodynamic therapy. There are a number of reviews of porphyrin and phthalocyanine photosensitizers and their use as PDT agents for a variety of cancers. [32-41]

7. Porphyrin Photosensitizers

7.1. Porphyrin background

Porphyrins have been extensively studied as potential photosensitizers in photodynamic therapy (PDT). [42]- [45] Their planar aromatic structure coupled with their photophysical properties and synthetic versatility has made them attractive components for PDT. [46] Although synthetic routes toward porphyrins leads to relatively low yields, their starting materials are typically inexpensive and their synthesis is generally straightforward, as illustrated for the porphyrin synthesis of *meso*-tetrapyridylporphyrin, Fig. 4. The synthesis typically requires reaction of pyrrole with the aldehyde of choice to give the appropriate substitution at the *meso*-positions. The *meso*-positions are indicated in Fig. 4 by the numbers 5, 10, 15, 20. Purification by column chromatography gives the desired porphyrin. As illustrated in the electronic spectra of Fig. 4 porphyrins display a very intense Soret band at approximately 410 nm and four lower energy Q-bands. The lowest energy Q-band, with absorptivities in the tens of thousands, at approximately 650 nm is the transition of interest for PDT. In addition, porphyrins have been associated with high affinity for tumor sites and efficient formation of ROS. [47] Specifically, cationic porphyrins have been studied primarily for their water solubility and their strong electrostatic interactions with negatively charged phosphate oxygen

atoms of the DNA backbone. [48] This section will focus on studies, performed in the past decade, involving porphyrins as photosensitizers for the treatment of melanoma.

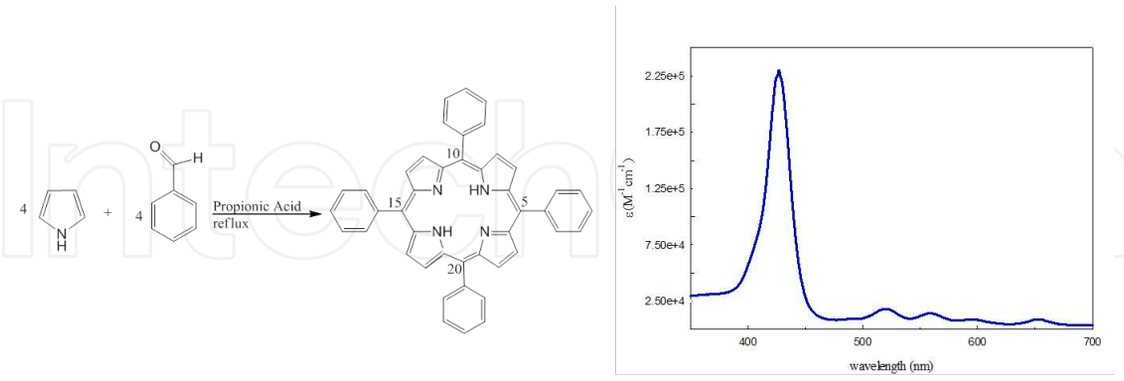

Figure 4. Synthesis and electronic spectra of *meso*-tetrapyridylporphyrin (H_2TPP).

8. Porphyrins, PDT and melanoma

In 1999 Busetti, Soncin and coworkers studied the effects of PDT on melanoma tumors in mice. [49] The inefficiency of Photofrin to treat highly pigmented melanoma has been related to the absorption of the wavelengths of light necessary to activate Photofrin by the melanin in the tumors. For this reason Busetti and coworkers chose a benzoporphyrin derivative monoacid ring A (BPD-MA), verteporfin, trade name visudyne, Fig. 5. Verteporfin has been used to eliminate abnormal blood vessels in the eye associated with macular degeneration.

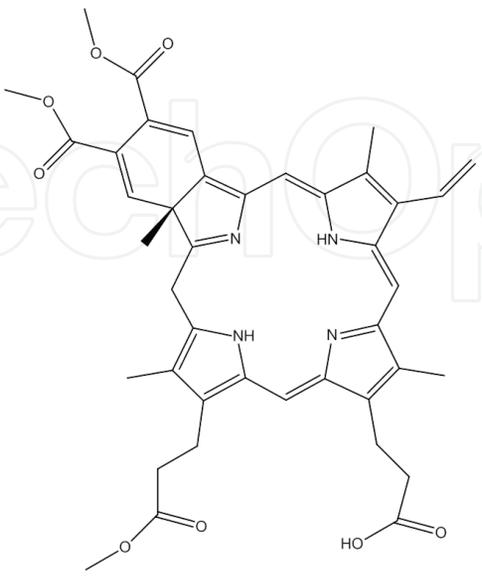

Figure 5. Structure of verteporfin.

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The studies were conducted on C57/BL6 mice implanted with heavily pigmented melanoma B16. Injection of the photosensitizer, 5.5 μmol/kg body weight, was allowed 3 h for a maximum concentration to be reached in the cells. The researchers noted limited selectivity of BPD-MA toward tumor cells and adjacent tissue. The majority of the BPD-MA was cleared through the bile-gut pathway within 24 h of injection, with small amounts found in the liver. Irradiation of the tumors involved an argon-pump dye laser at 690 nm, within the photodynamic window of 600-800 nm. Three hours after injection of the photosensitizer the tumors were irradiated with energy of 520 mJ cm^{-2}. Large necrotic areas of the tumor were observed at this time as well as reduction in tumor growth. The researchers noted that mice implanted with B16 tumors and irradiated after injection of the photosensitizer were tumor free for up to two weeks after treatment. The phototoxicity observed for BPD-MA treated mice was not observed in tumors irradiated in the absence of the photosensitizer.

In a subsequent study researchers investigated a porphyrin dimer as a potential PDT agent for the treatment of melanoma. The dimer, 10, 15, 20-tritolylporphyrin-5-(4-amidophenyl)-[5-(4-phenyl)-10, 15, 20-tritolyporphyrin] (T-D), Fig. 6, was synthesized by the Adler method of refluxing mixed aromatic aldehydes with pyrrole in propionic acid. [50] The dimer (T-D) was synthesized from the monomeric porphyrin units by a known literature method.

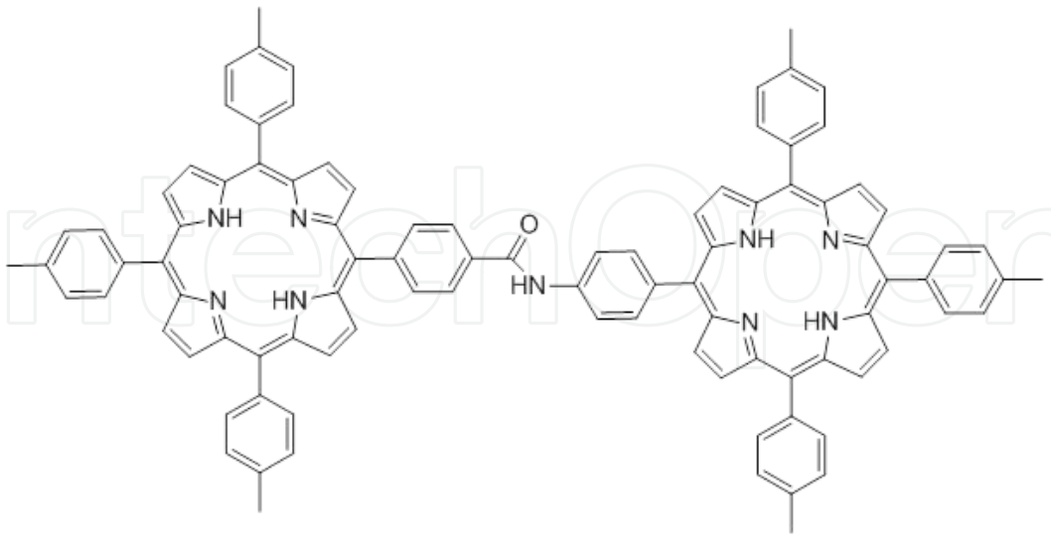

Figure 6. Structure of 10, 15, 20-tritolylporphyrin-5-(4-amidophenyl)-[5-(4-phenyl)-10, 15, 20-tritolyporphyrin] (T-D)

The electronic spectra of the dimer displayed an intense Soret band at 423 nm with less intense Q-bands at 517, 552, 592, and 649 nm in organic solvents. Photoexcitation for PDT treatment focused on the 649 band, although its molar absorptivity was relatively low (7550 M^{-1} cm^{-1}). Singlet oxygen generation for the excited dimer gave a very high quantum yield of 0.8. Due to the complexes insolubility in aqueous buffer solutions the dimer was dissolved in dimethyl sulfoxide (DMSO) and diluted to 10^{-8} to 10^{-5} M concentrations in aqueous phosphate buffer for the cell studies.

Photodynamic experiments were performed on human (SKMEL 188) melanoma cells and mouse (S91) melanoma cells. The melanoma cells were incubated with various concentrations of the T-D solutions for 24 h before irradiation. Melanoma cells incubated with 10^{-7} M T-D phosphate buffer solutions were irradiated with an LH313K lamp filtered to cut off wavelengths below 630 nm. Energy doses between 13.5 and 82 J cm^{-2} were used with irradiation times of 30 min. Cells were incubated for 36 h after irradiation before determining cell death. Both human and mouse melanoma cells showed a 3-fold decrease in size compared to cells irradiated without T-D at 81 J cm^{-2}. Although the dimer is insoluble in aqueous solutions and requires high energy irradiation for phototoxicity the authors point out that this complex has some distinct advantages over Photofrin for the treatment of pigmented melanoma, namely, chemical homogeneity, low aggregation (which can lead to excited state quenching), and good solubility in hydrophobic base with relatively long-lived triplet excited states, leading to significant singlet oxygen production.

9. Halogenated porphyrins

Synthetically enhancing porphyrins as PDT agents has been achieved through incorporation of halogens into the porphyrin structure. [51-54] Halogens covalently bound to phenyl groups at the *meso*-positions promote intersystem crossing, increasing singlet oxygen production; for example, halogenated tetraaryl porphyrins combined through a diarylethyne linker (where the halogens are chloro and fluoro substituted phenyl groups), Fig 7, had significantly longer excited state lifetimes when compared to their non-halogenated analogs. [51]

In addition, PDT agents with fluorophenyl substituents have been efficiently converted to porphyrin-saccharide conjugates to enhance their uptake in cancer cells, while in a separate study water soluble fluorinated porphyrins have shown more efficient PDT activity than their non-fluorinated counterparts. [53,54]

Researchers in 2007 looked at a water soluble porphyrin incorporating chloro groups. [55] The porphyrin, 5, 10, 15, 20-tetrakis-(2-chloro-3-sulfophenyl)porphyrin ($TCPPSO_3H$), Fig. 8, was synthesized by reacting 2-chlorobenzaldeyde with pyrrole by the method of Adler and Longo, followed by sulfonation. The electronic spectrum of the complex is typical of porphyrins with an intense Soret band and four lower energy lower intensity Q-bands. The lowest energy Q-band at 633 nm displayed a molar absorptivity of 504 $M^{-1}cm^{-1}$ in aqueous pH 7 phosphate buffer. Singlet oxygen quantum yields were determined to be 0.74, significantly higher than the non-halogenated analog.

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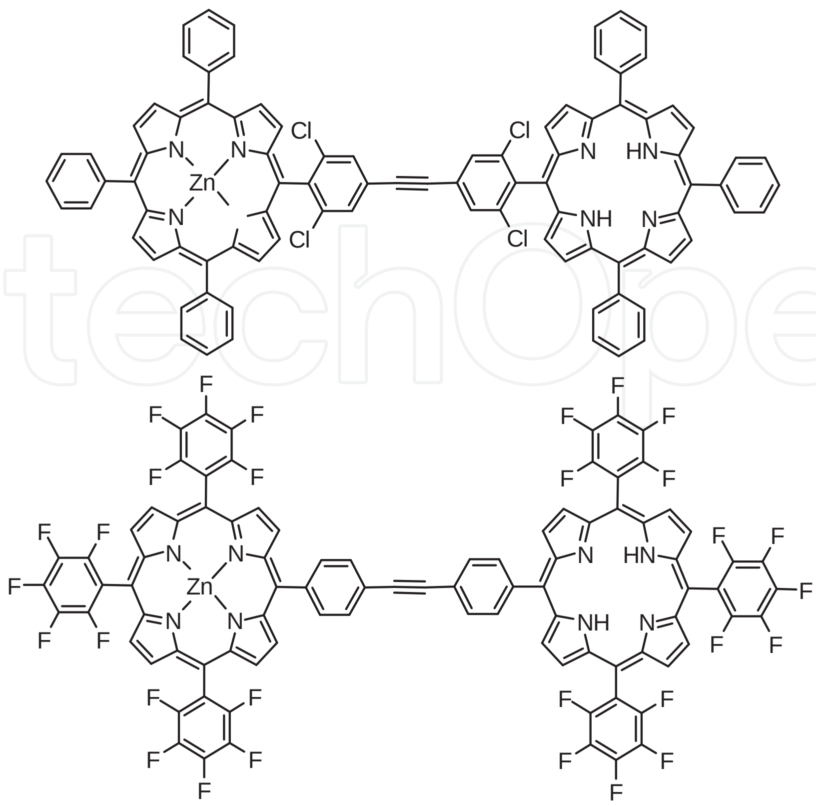


Figure 7. Structures of the halogenated tetraaryl porphyrins combined through a diarylethyne linker.

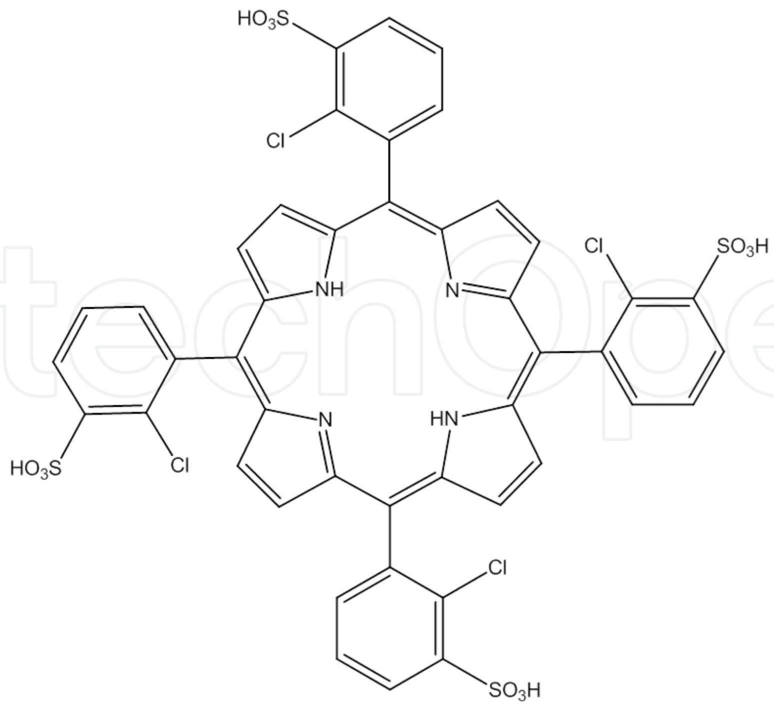
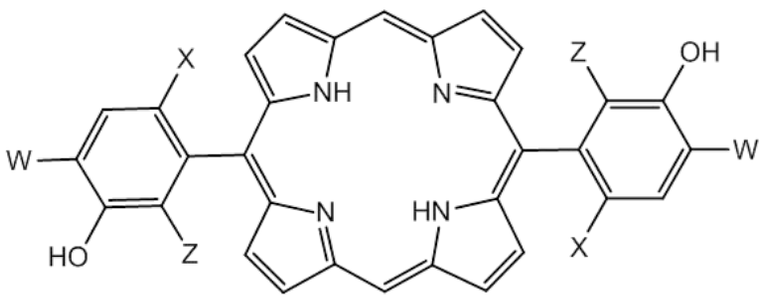


Figure 8. Structure of 5, 10, 15, 20-tetrakis-(2-chloro-3-sulfophenyl)porphyrin (TCPPSO$_3$H).

The melanoma cells studied were mouse (S91) and human (SKEML 188). At concentrations of $TCPPSO_3H$ of 2 x 10^{-4} M or greater dark toxicity in S91 melanoma cells was observed. The S91 melanoma cells were determined to be more sensitive to the photosensitizer in the dark than the human SKMEL 188 cells; therefore, concentrations of 2 x 10^{-5} M buffered solutions of $TCPPSO_3H$ were used for PDT experiments on both cell lines. Cellular uptake of $TCPPSO_3H$ buffered solutions reached a peak after two hours with the S91 cells being five times greater than the SKMEL 188 cells. Thirty minute irradiation with 4 J cm^{-2} or higher led to 90% cell toxicity for both types of cells. S91 cells were still dead 24 h post PDT treatment with 20 μM $TCPPSO_3H$ and 6.2 J cm^{-2} light doses.

In a more recent study halogenated porphyrin photosensitizers were studied as PDT agents, Fig. 9, against A375 melanoma cells. [56] It has been noted that halogenated structures can interfere with the activity of P-glycoprotein (P-gp). This can enhance drug therapy by preventing the function of the P-gp to eliminate these drugs from the cells. Complexes 2-5, Fig. 9, (the halogenated porphyrins) led to the highest quantum yields for singlet oxygen formation compared to the non-halogenated complex, 1 Fig. 9. Photobleaching after 24 h of irradiation was minimal for complexes 1-5. In addition, no dark toxicity was observed for the complexes in the presence of melanoma cells.



1. X = Z = W = H
2. X = Br, Z =W = H
3. X = Z = H, W = I
4. X = W = H, Z = I
5. Z = Cl, Z = W = H

Figure 9. Structure of halogenated porphyrins.

For the PDT experiments melanoma cells were incubated for 24 h with concentrations of complexes 1-5 ranging from 50 nM to 10 μM. Irradiation of the cells after incubation with the complexes, performed with 10 J cm^{-2}, was evaluated 24 h post PDT treatment. Surprisingly, all of the complexes, including the non-halogenated photosensitizer, showed thirty times the efficiency for photokilling than Photofrin against A375 melanoma cells; however, no appreciable difference was noted between the halogenated and non-halogenated complexes. Further studies are needed to determine the effect of the halogenated porphyrins on P-gp inhibition.

Apart from production of singlet oxygen another mechanism of PDT action, as stated earlier, is the formation of reactive oxygen species (ROS) within the cell. ROS, for example, hydrogen

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peroxide, hydroxyl radicals, or superoxide anions are highly reactive leading to oxidative damage of cellular biomolecules. Cells are equipped with enzymes capable of counter acting a limited amount of ROS; however, PDT can lead to an overproduction of ROS which cellular mechanisms are unable to handle. To this end, researchers have synthesized and investigated a series of water soluble porphyrins containing Zn(II), Pd(II) metal centers, as well as, the free-base porphyrin. [57] The sulfonated porphyrin analogs of *meso*-tetrakis-(4-sulfonatophenyl)porphyrin ($TPPS_4$) are illustrated in Fig. 10. Fluorescence probes were used to determine the production of hydrogen peroxide after PDT treatment in human melanoma (G361) cells. Production of hydrogen peroxide by PDT leads, through a series of reactions, to the formation of hydroxyl radicals, the most reactive and deadly form of ROS. The photosensitizers in this study were noted to be homogeneously distributed throughout the cells, excluding the nucleus. The most effective of the photosensitizers in this study was the Zn(II)$TPPS_4$ complex, generating the highest concentration of ROS in the melanoma cells (G361) at 100 μM concentrations and light doses of 5 J cm^{-2}; however, the optimal concentration leading to photocytotoxicity was determined to be 10 μM. Cell viability was linked to light dose, photosensitizer concentration, and the type of photosensitizer.

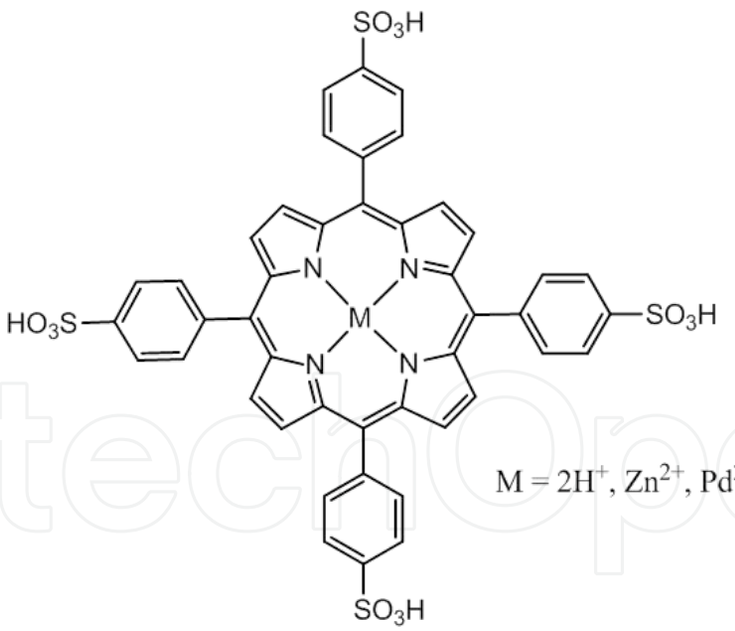

Figure 10. Structure of *meso*-tetrakis-(4-sulfonatophenyl)porphyrin ($TPPS_4$)

In a unique application, researchers have combined PDT with boron neutron capture therapy (BNCT). [58] The porphyrin photosensitizer in this study was appended with 36 boron atoms, *meso*-tetra-(4-nido-carboranylphenyl)porphyrin (H_2TCP), as illustrated in Fig. 11.

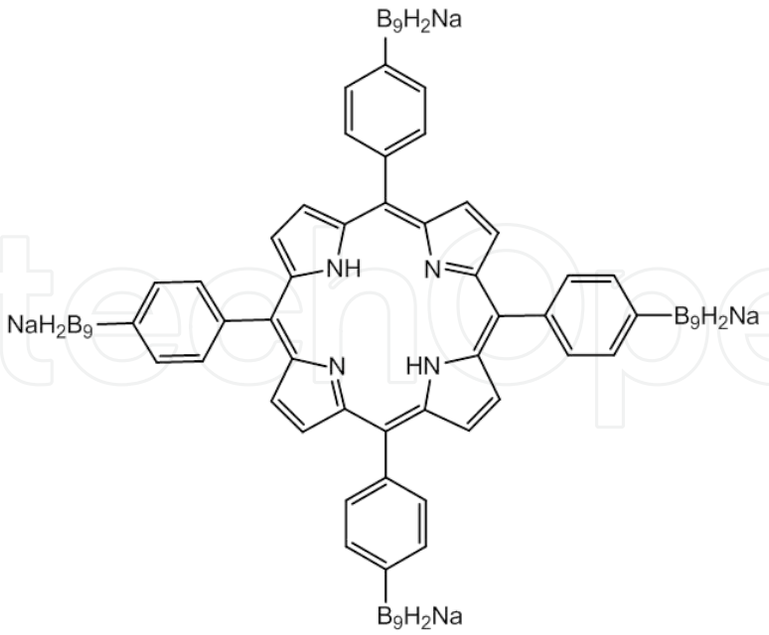

Figure 11. Structure of *meso*-tetra-(4-nido-carboranylphenyl)porphyrin (H_2TCP).

As a PDT agent H_2TCP was measured to generate singlet oxygen in aqueous solutions with a quantum yield of 0.44. Boron neutron capture therapy (BNCT) is used for cancer treatment by injection of a non-toxic radio-sensitizing agent. Irradiation of ^{10}B-enriched tumor lesions with low energy neutrons results in the release of highly toxic $^4He^{2+}$ and $^7Li^{3+}$ causing severe damage to biological molecules and eventually leading to cell death. Like PDT, BNCT is a targeted treatment modality which the authors anticipated could be used in a synergistic fashion to give maximum tumor killing with minimal collateral damage to healthy cells.

For this study pigmented melanoma (B16F1) cells were subcutaneously transplanted into mice through injection. After seven days tumors were measured at 0.6 cm in diameter, at this point treatment was initiated. The photosensitizer H_2TCP showed no photobleaching after 20 min irradiation indicating its stability under these conditions. The appropriate solution for PDT treatment was obtained when H_2TCP was dissolved in 20% dimethylsulfoxide – 30% polyethyleneglycol – 50% water. Distribution of the photosensitizer was observed in melanoma cells after 24 h incubation. Irradiation with 600-700 nm light for 10 min led to complete cell death at 20 μM concentrations of H_2TCP. There was no dark toxicity toward the melanoma cells at concentrations as high as 50 μM H_2TCP. Fluorescence experiments of the cell studies suggest that the predominant pathway to cell death was necrotic. Mouse studies indicated that injections of H_2TCP of 5 μg/kg were almost completely eliminated from the plasma within 3 h post injection. In addition, maximum tumor accumulation of the photosensitizer was achieved at 3 h post injection with little change after 24 h. Unfortunately poor tumor selectivity was also observed with equal amounts of the photosensitizer being recovered from the skin as well as the tumor tissue. Before PDT and BNCT studies are conducted the authors feel it is important to further investigate other methods of photosensitizer transport to enhance its

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localization in the tumor cells. This complex does offer the hope of a combined targeted therapy for the treatment of melanoma.

10. Ruthenium complexes and ruthenated porphyrins

Although Photofrin® was approved by U.S. Food and Drug Administration (FDA) for use in the United States, it suffers from dark toxicity and purification difficulties. In order to overcome these, another class of compounds which have received a great deal of attention as potential PDT agents are ruthenium complexes containing polypyridyl ligands. [59-62] For example, excitation of the Metal to Ligand Charge Transfer (MLCT) state of a Ru(II) polypyridyl complex, Fig 12, has been shown to lead to the formation of ROS resulting in efficient cleavage of supercoiled DNA. [63] The Ru(II) polypyridyl complexes have intense, overlapping MLCT transitions in the visible region of the spectrum for acceptor ligand and ligand-based $\pi \rightarrow \pi^*$ transitions in the UV region. The complexes efficiently absorb light throughout the UV and visible allowing for efficient excitation. The MLCT emissions of these Ru complexes are quenched by oxygen to produce $^1O_2$.

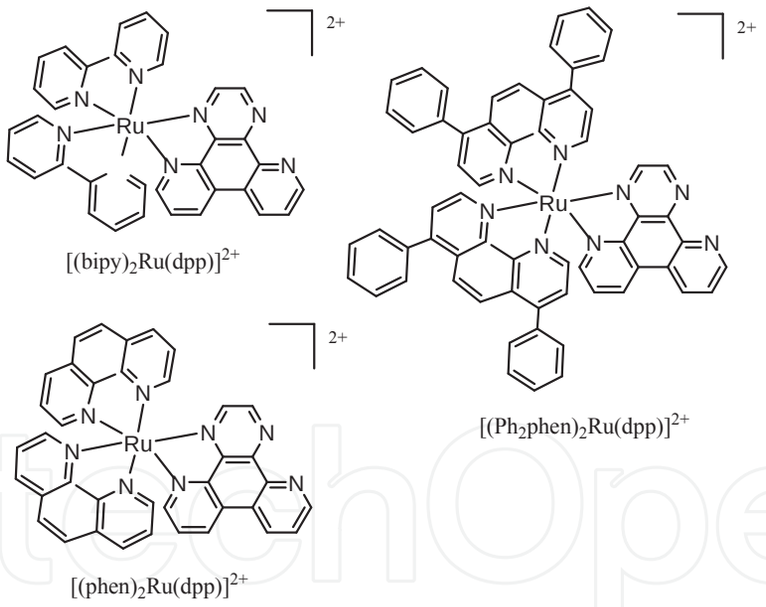

Figure 12. DNA photocleavage agents [(TL)2Ru(dpp)]Cl2 with TL = 2,20-bipyridine (bpy), 1,10-phenanthroline (phen) or 4,7-diphenyl- 1,10-phenanthroline (Ph2phen), with the polyazine bridging ligand 2,3-bis(2-pyridyl)pyrazine (dpp).

Ruthenium complexes coordinated to the periphery of porphyrin molecules have also been shown to interact with DNA. [64-71] In one study a mono-ruthenated porphyrin, Fig 13, caused single strand breaks of circular plasmid DNA when irradiated with UV light. It was suggested that the mechanism of photocleavage was related to the formation of radical cations of guanine. [66] A separate study of a tetra-ruthenated porphyrin, Fig 13, suggested electrostatic binding to DNA and photocleavage of circular plasmid DNA through formation of singlet oxygen. [69]

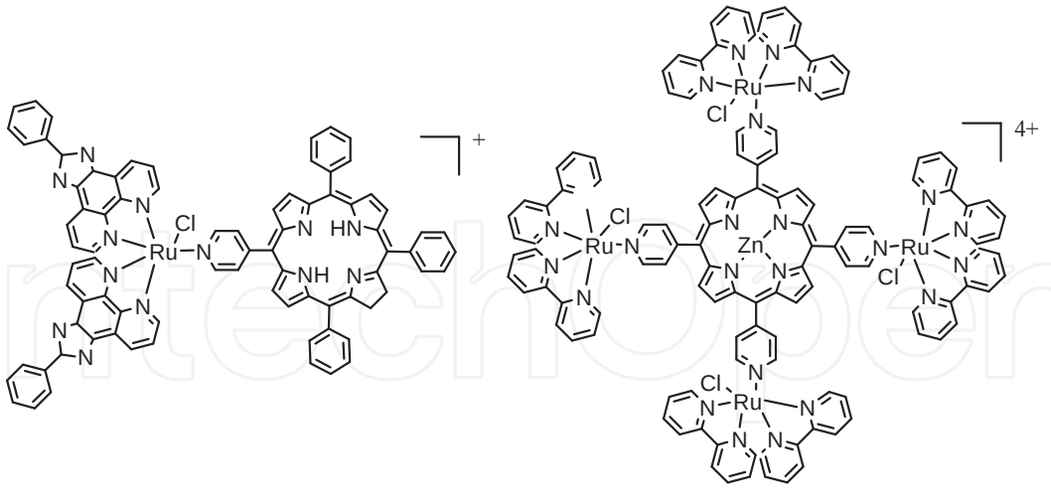

Figure 13. The structure of the mono-ruthenated porphyrin: $[MPyTPPARu(pip)2Cl]^{+}$ and the structure of tetra-ruthenated porphyrin: μ-{meso-5,10,15,20-tetra(4-pyridyl)porphyrin}- tetrakis-{bis-(bipyridine)chlororuthenium(II)}$^{4+}$.

11. Melanoma and ruthenium porphyrins

Reactions of Ru(η^6-arene)(μ-Cl) complexes with 5, 10, 15, 20-(4-pyridyl)porphyrin (TPP) in refluxing methanol gave tetranuclear ruthenium porphyrins in excellent yield, Fig. 14. [72]

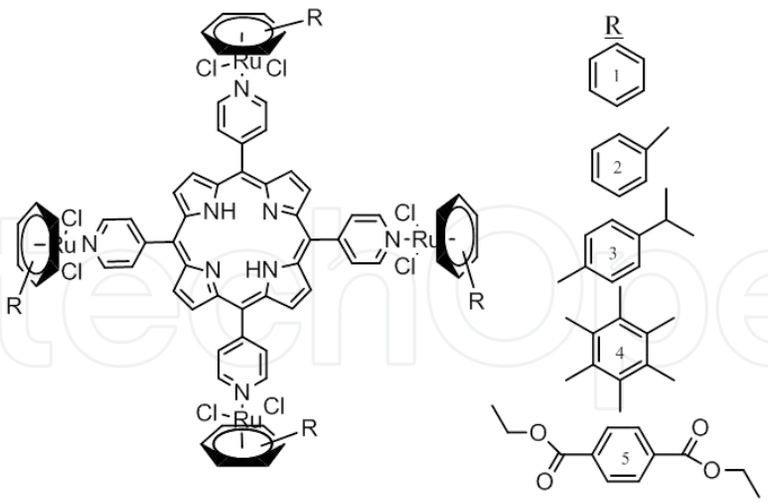

Figure 14. Structures of ruthenated tetraphenyl porphyrins.

The complexes in this study were used to investigate growth inhibition of human Me300 melanoma cells. The melanoma cells were incubated with various concentrations of complexes 1-5 over a 24 h period. Cell survival was determined by accepted protocol (MTT assay). Dark toxicity experiments revealed that complexes 3 and 4 were moderately toxic while complexes

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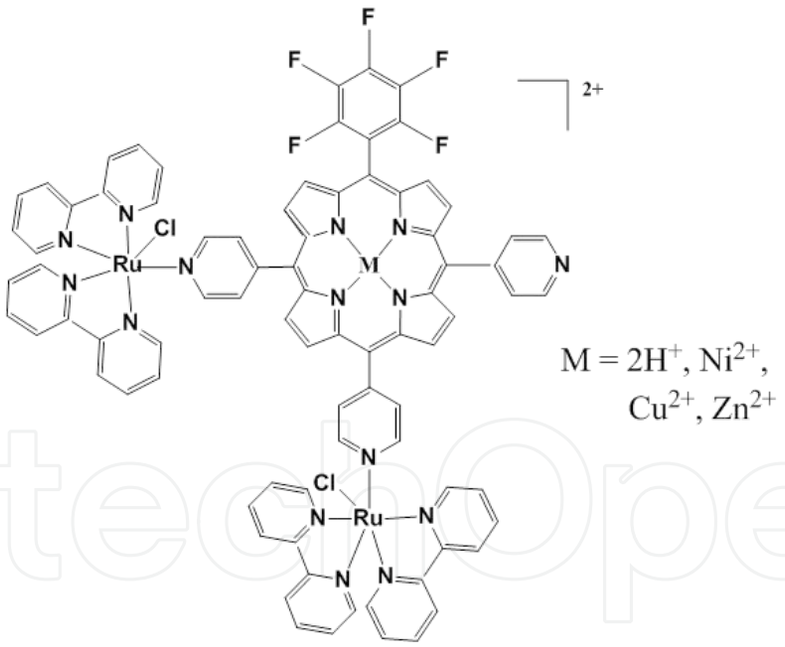
1, 2, and 5 were less toxic to the cells. Localization of complex 2 in the cells cytoplasm was determined by fluorescence measurements; however, nuclear localization was not observed.

Melanoma cells incubated with 10 μM concentrations of complexes 1-5 for 24 h were irradiated using a red laser at 652 nm, with a light dosage range of 5-30 J cm^{-2}. After irradiation 24 h was allowed to lapse before cell cytotoxicity was measured. In the absence of the photosensitizers, 1-5, irradiation of the melanoma cells was determined to be ineffective at cell killing. Light doses as low as 5 J cm^{-2} in the presence of the complexes indicated 60-80% melanoma cell killing. Little difference in phototoxicity was observed for the complexes after PDT treatment under the same conditions. Therrien and coworkers have studied a variety of interesting ruthenium substituted porphyrin photosensitizers for their effects as PDT agents against pigmented melanoma. [73-75]

In an attempt to combine the tumor affinity of porphyrins with the enhanced excited state lifetimes afforded by halogens and the added benefits of coordinated ruthenium polyazine groups we have investigated the synergistic effects of these substituents as PDT agents for the treatment of melanoma. [76,77] In our most recent study we synthesized a series of ruthenated pyridyl porphyrins containing one pentafluorophenyl group at the *meso*-position combined with a series of transition metal ions, Fig. 15. [77]



I-IV

Figure 15. Transition metal and free base ruthenium porphyrin analogs.

Early experiments of the free base porphyrin showed extraordinary ability to photocleave circular plasmid DNA when irradiated with light above 400 nm. When various transition metal ions (Ni(II), Cu(II), and Zn(II)) were inserted into the porphyrin the photocleavage ability was markedly reduced. Cell studies were performed on human dermal skin fibroblast cells and

malignant melanoma cells obtained from a 53 year old male. Normal and melanoma cells were incubated for 24 h at 37 ºC in the dark with complex concentrations of 5 and 10 μM. The complexes (I-IV) did not show any dark toxicity toward either normal fibroblast or melanoma cells. PDT experiments were performed by irradiating the cultured cells (containing the photosensitizers) for either 30 or 60 min intervals with a 60 W tungsten lamp. In the case of the free base porphyrin (I) irradiation of 30 min at concentrations as low as 3 μM indicated complete cell killing of both normal fibroblast and melanoma cells. In contrast, irradiation times of 60 min at concentrations of the Ni(II) and Cu(II) complexes (II and III) of 10 μM showed no cell damage for either the normal fibroblast or melanoma cells. The Zn(II) complex (IV) showed the greatest potential as a PDT agent. Irradiation for 30 min at concentrations of the Zn(II) porphyrin of 5 and 10 μM showed minimal cell damage of the normal fibroblast cells but induced complete cell killing of the melanoma cells. Further studies are needed to determine the effect of the central metal ions on the phototoxicity of these complexes.

12. Phthalocyanine photosensitizers – Background

Phthalocyanines represent another form of macrocyle, similar in many ways to porphyrins. Synthetic routes to phthalocyanines vary; however, one common method involves reacting o-cyanobenzamide with phthalimide to give the tetrabenzoporphyrazin, more commonly named, phthalocyanince (Pc), Fig. 16.

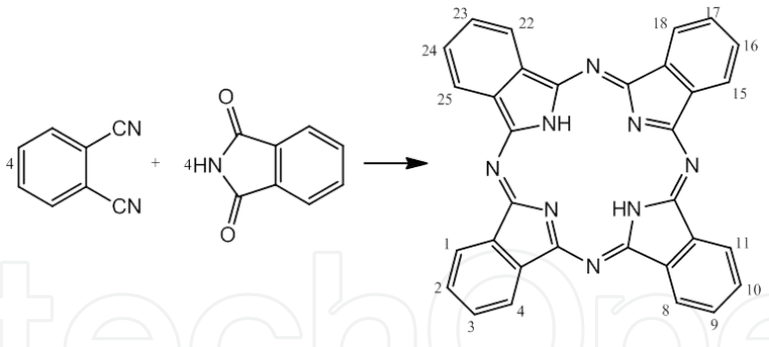


Figure 16. Synthesis of phthalocyanine.

Phthalocyanines have the ability, like porphyrins, to coordinate metal ions within their nitrogen core, they are a tetradentate ligand. This has offered numerous ways to synthetically alter their physical properties. If not at the metal center, substitutions of Pc's is typically made at the benzo-periphery (positions 1-4, 8-11, 15-18, and 22-25, Fig. 16). Two advantages Pc's seem to have over porphyrins, as potential PDT agents, is their comparatively high yields and their spectroscopic properties. Like porphyrins, Pc's have a Soret band at higher energy ca. 400 nm and Q-bands at lower energy; however, in the case of Pc's the low energy Q-bands (650-750 nm) are typically much more intense than the Soret bands, opposite of porphyrins. These intense absorptions fit very nicely within the photodynamic window needed for PDT.

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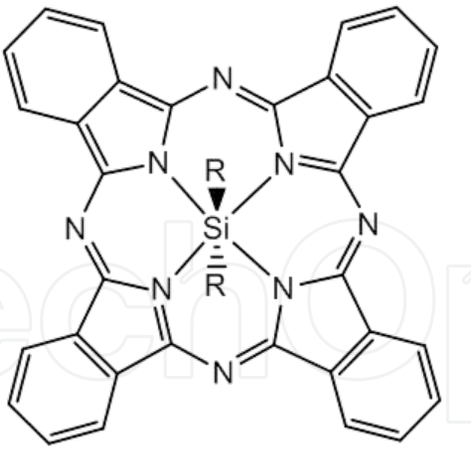
One major drawback is that Pc's lack the specificity toward tumor cells that porphyrins possess, this has led to numerous synthetic manipulations to try and find ways to get the Pc's into the tumor.

13. Phthalocyanines, PDT and melanoma

Researchers in 1999 compared Photofrin (HpD), Zn(II) phthalocyanine (ZnPc), Zn(II) naphthalocyanine (ZnNc), and a newly synthesized tetrabenzamido-substituted Zn(II) phthalocyanine (ZnNcA) as PDT agents against B16 pigmented melanoma. Pathogen-free male C57B1/6 mice between 6 and 8 weeks of age were transplanted with 1 mm^3 pieces of melanoma tumor tissue. Six days post-transplant, tumor diameters were between 3 and 4 mm, at this point PDT treatment was initiated. [78] Tumor infected mice were injected with the photosensitizers and irradiated 24 h after injection with wavelengths between 630-780 nm. Twenty one days after PDT treatment HpD and ZnPc treated mice showed no effect on the tumors. Some tumor growth delay was observed 15 days post PDT treatment with the ZnNc photosensitizer. Higher photosensitizer concentrations and increased irradiation energy did not produce increased phototherapeutic effects for these photosensitizers. In the case of the ZnNcA photosensitizer treated mice pronounced tumor necrosis post irradiation was observed with tumor diameters held at 4 mm up to 19 days after treatment. ZnNcA shows singlet oxygen generation quantum yields of 0.33 with good accumulation in the tumor which has been linked to its success against these particular tumors.

Aggregation is a common issue with macrocyclic complexes. To address this two new silicon(IV) centered phthalocyanines have been synthesized, Fig. 17. [79]

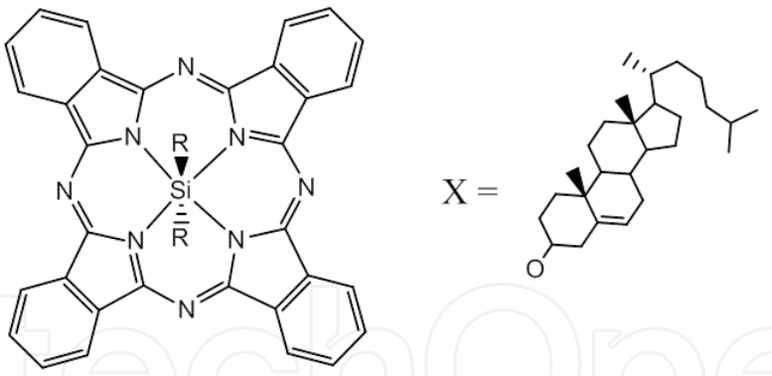
Both of the complexes in Fig. 17 have electronic spectra displaying intense Q-bands at 668 nm. In this study M6 achromic melanoma cells were incubated with the desired photosensitizer for 1 h prior to irradiation. Irradiation experiments involved a 250 W tungsten-halogen lamp containing a filter to cut off wavelengths below 480 nm. After the cells were irradiated they were washed with PBS buffer solutions and grown in a fresh culture for 6 h. Due to low water solubility of HexSiPc, this photosensitizer was incorporated in EYL (egg yolk lecithin) lisosomes. Under these conditions the maximum concentration of Cl_2SiPc in M6 cells was 2.18 ng and 15.3 ng for HexSiPc, obtained after 90 min of incubation. No evidence of dark toxicity was observed for concentrations of Cl_2SiPc between 10^{-10} and 10^{-8} M or for concentrations of HexSiPc between 10^{-10} and 10^{-5} M. The Cl_2SiPc complex showed little M6 killing after 1 h of incubation and 20 min irradiation with a 250 W tungsten-halogen lamp. The HexSiPc photosensitizer, on the other hand, (entrapped in EYL) gave LD_{90} of 3×10^{-7} M after 20 min of irradiation and 2×10^{-9} M after 2 h of irradiation with a 250 W tungsten-halogen lamp. It was concluded that the superior photokilling observed for HexSiPc was linked to lipid peroxidation.



R = Cl (Cl_2SiPc)
R = O-Si(n-hex)$_3$ (HexSiPc)

Figure 17. Structure of (Cl_2SiPc) and (HexSiPc).

Another study aimed at decreasing Pc aggregation while increasing its lipophilicity included a series of SiPc complexes containing various substituents coordinated to the silicon metal center, one of which is illustrated in Fig. 18. [80]



Chol-O-SiPc

Figure 18. Structure of bis(cholesteryloxy) derivative of SiPc.

This study involved human pigmented melanoma cells SKMEL-2. The photosensitizers were dissolved in tetrahydrofuran (THF) and entrapped in liposomes to increase tumor affinity. Irradiation experiments were performed using a 250 W tungsten-halogen lamp filtered to cut off wavelengths below 480 nm. The melanoma cells were incubated with the substituted SiPc liposome entrapped complexes prior to irradiation for 20 min. Of the six Si-substituted Pc's in this study the best photokilling was observed for the complex illustrated in Fig. 18. Photokilling efficacy of Chol-O-SiPc, in vitro, was seven to nine times greater than the known reference

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chloro-aluminum phthalocyanine with LD_{50} = 6-8 x 10^{-9} M. The mechanism of photokilling of this complex was evaluated by kinetic studies indicating that faster mitochondrial mediated apoptosis was occurring by PDT with this complex as compared to the reference Pc.

As noted earlier generation of ROS leads to cell death and blood vessel damage; crucial to tumor regression. In a study of G361 human melanoma cells with a disulfonated chloroaluminum phthalocyanine ($ClAlPcS_2$) photosensitizer, researchers were interested in looking at the generation of ROS and hydrogen peroxide after PDT treatment. The complex, $ClAlPcS_2$, was made water soluble by conversion to its ammonia salt. [81] The electronic spectra of this complex displayed an intense Q-band at 670 nm. PDT experiments were performed by irradiation with a semiconductor laser at 675 nm and energies of 10 and 20 J cm^{-2}. At lower light doses of energy (10 J cm^{-2}) and higher concentrations of Pc, 7.5 and 75 μg/mL, increased generation of ROS was observed; however, at light doses of 20 J cm^{-2} and concentrations of Pc of 0.75 μg/mL larger amounts of ROS were generated as compared to higher concentrations and higher light doses. The most ROS generated were at a concentration of 75 μg/mL and light dose of 10 J cm^{-2}. G361 melanoma cell viability studies indicated that the optimal phototoxicity could be obtained with a light dose of 25 J cm^{-2} and concentrations of photosensitizer of 5 μg/mL and 10 μg/mL.

Phthalocyanines, like porphyrins, have also been combined with boron isotopes in an attempt to obtain a synergistic treatment method involving PDT and BNCT (boron neutron capture therapy). The ZnPc coupled to boron, in this study, is illustrated in Fig. 19. [82]

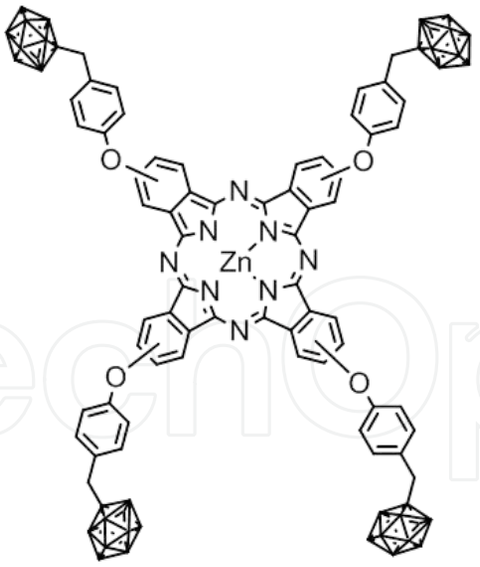

Figure 19. Structure of ZnB_4Pc.

Due to low water solubility the ZnB_4Pc complex was incorporated into liposomes. The complex was stable for one week in liposomal aqueous suspensions. The photosensitizer-liposome suspension was used to study B16F1 melanotic melanoma cells. After 18 h of incubation with the melanoma cells the peak concentration of ZnB_4Pc complex obtained was 7 μm, with no

dark toxicity observed. Red light irradiation of B16F1 cells incubated for 1 h with 7 μM ZnB_4Pc-liposomes resulted in > 95% cell death. No regrowth of melanotic melanoma cells after several days post-irradiation was observed.

Pigmented melanoma cells were transplanted into C57BL/6 mice subcutaneously. The best results for PDT treatment of melanoma transplanted mice after injection of ZnB_4Pc was observed with irradiation 3 h post injection with wavelengths between 600-700 nm. PDT experiments conducted 24 and 48 h post injection were ineffective in shrinking the tumors, leading the authors to conclude that the PDT treatment was affecting the blood vessels associated with the tumor. Moreover, significant tumor growth delay was observed when BNCT treatment was conducted 24 h post injection of the ZnB_4Pc complex suggesting that this complex offers a dual pathway toward melanoma treatment.

Various synthetic routes have been taken to reduce aggregation of phthalocyanines while increasing their water solubility. A recent study looked at coordination of N-methylated piperazine substituents at the axial positions of a silicon(IV) phthalocyanine. [83] N-methylation lends a cationic charge to the complex making it water soluble while the bulky Si-coordinated substituents prevent aggregation, Fig. 20.

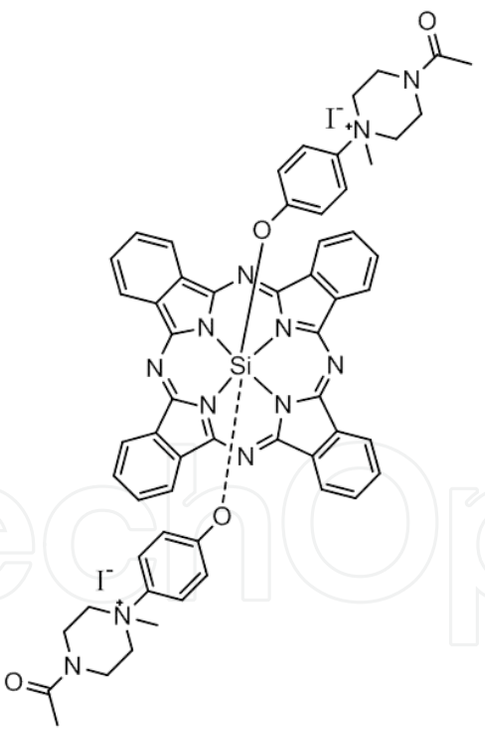

Figure 20. Structure of bis(4-(4-acetylpiperazine)phenoxy)phthalocyanoto silicon(IV) N-methyl derivative.

Suprisingly, this structurally complicated phthalocyanine was synthesized with a yield of 73%. Electronic absorption spectra of the complex reveals an intense Q-band at 685 nm in N,N-dimethylformamide (DMF). In aqueous solution the Q-band shifts to lower energy, 690 nm, while obeying the Beer-Lambert law, indicating no aggregation at the concentrations studied.

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Investigations of singlet oxygen production gave a quantum yield of 0.49. To enhance biocompatibility and selectivity the phthalocyanine was complexed with bovine serum albumin (BSA), a common protein carrier for anticancer drugs. Studies were conducted on B16 melanoma cells using 0.1 μM concentrations of photosensitizer. At this concentration no dark toxicity was observed. PDT treatment was conducted with red light greater than 610 nm at 60 J cm^{-2} resulting in complete killing of the B16 melanoma cells.

There are a limited number of studies in which phthalocyanines have been combined with ruthenium complexes for the purpose of PDT. Researchers in 2009 synthesized a Tb(III) phthalocyanine capable of hydrogen bonding to a polypyridyl Ru(II) complex in aqueous solution, Fig. 21. [84] The idea was to incorporate the photosensitizing ability of the phthalocyanine to generate singlet oxygen under oxygen rich conditions, when irradiated with low energy light and photolytic release of nitric oxide from the ruthenium complex under hypoxic conditions. This would give dual capabilities for photodynamic damage since nitric oxide has been linked to antitumor activity.

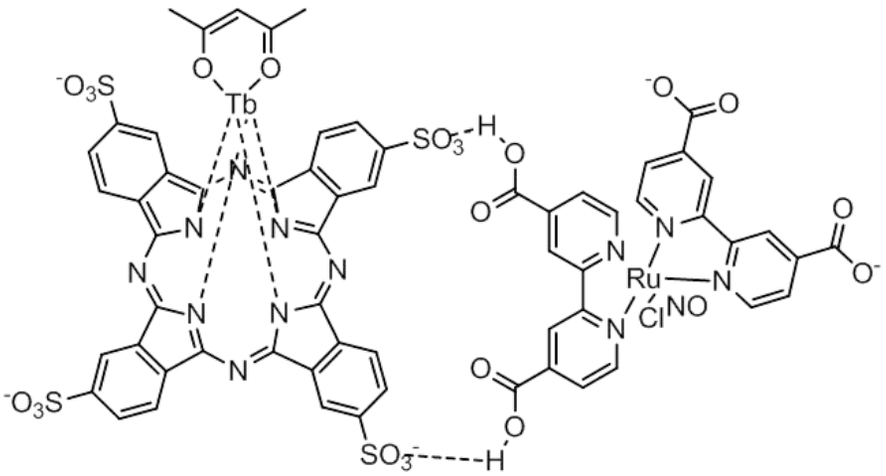

Figure 21. Structure of *cis*-[Ru(H-dcbpy-)$_2$(Cl)(NO)][Na_4(Tb(TsPc)(acac)]

Although the complex was not isolated it was assumed to form hydrogen bonds in aqueous solution. The electronic spectrum of a one to one mixture of the complexes in aqueous solution reveals two intense absorption bands at 642 nm and 682 nm. PDT studies were performed on B16F10 pigmented murine melanoma cells. When irradiated at 691 nm the quantum yield for singlet oxygen production was measured to be 0.41. Some dark toxicity was observed; however, upon irradiation, 80% of the melanoma cells had undergone photocytotoxicity. In the absence of the phthalocyanine there was reduced cell viability which was linked to the release of nitric oxide upon irradiation of the ruthenium complex.

Studies have indicated that nanoparticles enhance tumor targeting. To take advantage of this property researchers have combined phthalocyanines with gold nanoparticles dispersed in an emulsion, Fig. 22. Electronic spectra of the free C_{11}Pc and C_{11}Pc-Np display an intense Q-band at 696 nm. [85]

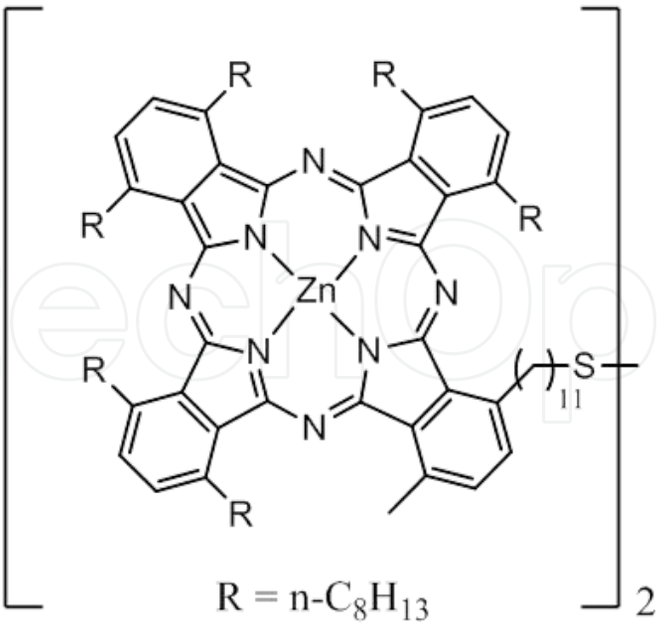

Figure 22. Structure of $C_{11}Pc$.

Mice (C57) transplanted with B78H1 amelonotic clone of murine melanoma were used in this study. At 10 to 15 days post melanoma transplantation (tumor external diameters of 0.4 to 0.6 cm) the $C_{11}Pc$ and $C_{11}Pc$-Np emulsions were injected at a dose of 1.5 μmol/kg body weight. The highest accumulation of photosensitizer found in the tumor was observed at 24 h post injection, while at 3 h post injection significant amounts of photosensitizer was found in the serum. Irradiation studies were performed at 3 h and 24 h post injection with wavelengths of light between 600 nm and 700 nm. It was noted that untreated mice died within 3 weeks of melanoma transplant. Suprisingly, little effect on tumor growth was observed when tumors were irradiated 24 after photosensitizer injection, when the photosensitizer was at a maximum concentration within the tumor. However, irradiation 3 h post injection led to significant tumor growth delay for both $C_{11}Pc$ and $C_{11}Pc$-Np. The authors indicate that the observed growth delay was most likely due to vascular damage to the tumor cells leading to low blood flow to the cells.

14. Other macrocycles for PDT of melanoma and concluding remarks

We would be remiss if we did not acknowledge the great deal of research devoted to photosensitizer macrocycles other than porphyrins and phthalocyanines for the treatment of melanoma. Although this chapter has focused primarily on porphyrins and phthalocyanines there are other similar complexes under investigation as PDT agents for melanoma. One of the more promising photosensitizers being studied is based on the macrocycle texaphyrin. The lutetium texaphyrin in this study, Fig. 23, showed water solubility and intense absorbance between 700 nm and 760 nm. [86]

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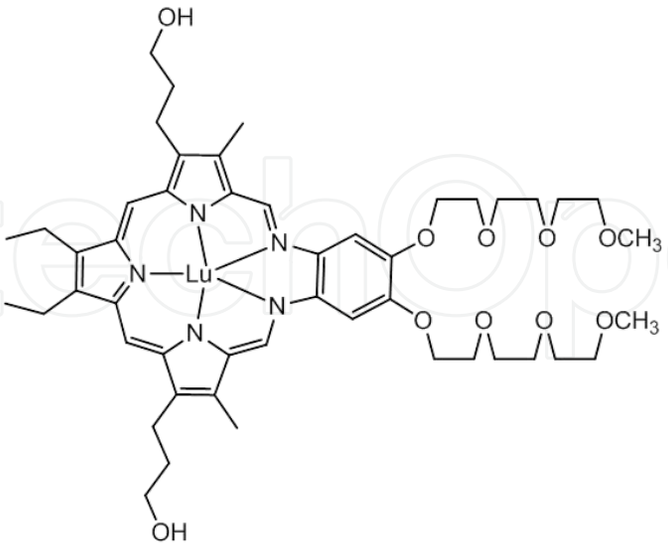

Figure 23. Structure of Lu-texaphyrin (PCI-0123).

PCI-0123 was studied in C57BL/6 mice transplanted with the pigmented murine melanoma B16F10. PDT studies of the tumor transplanted mice injected with PCI-0123 irradiated with 732 nm light showed delay in tumor growth and increased life spans. The interested reader is encouraged to review the following references for further information on other macrocycles being studied as PDT agents for the treatment of melanoma. [87-93]

It is clear that a great deal of research is ongoing toward finding new PDT agents for the treatment of melanoma. This trend will certainly increase as the incidence of melanoma continues to increase throughout the world. There are many challenges yet to be overcome; however, photosensitizers capable of treating melanoma by PDT are continually being developed. Two major hurdles which must be addressed are, first, overcoming the ability of pigmented melanoma to absorb wavelengths needed to activate the photosensitizer and second, the metastatic nature of melanoma. Great strides have been made to develop photosensitizers capable of phototoxicity in pigmented melanoma. Unfortunately, it is unlikely that PDT will be a viable option (on its own) for the treatment of advanced stage melanoma which has begun to metastasize. In combination with traditional melanoma treatments PDT is becoming a more accepted regimen for the treatment of this most deadly form of skin cancer.

Author details

Shawn Swavey and Matthew Tran

Department of Chemistry, University of Dayton, Dayton, Ohio, USA

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