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Perspective

Current Clinical and Preclinical Photosensitizers for Use in Photodynamic Therapy

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A little over 100 years ago, Raab reported the killing of the microorganism paramecia with the combination of acridine and light. The importance of oxygen in the process was recognized a few years later by von Tappeiner, who expanded these initial observations to the use of eosin (**1**, Chart 1) and light in the treatment of basal cell carcinoma.[1] This marked the beginning of photodynamic therapy (PDT), a treatment modality that uses the combination of light and a photosensitizer to affect a biological outcome. Little of significance developed in the clinical area until the early 1960s where hematoporphyrin derivative (**2**, HpD, Chart 1)[2] was used as a diagnostic tool.[3,4] Since 1961, thousands of papers have been published relating to photodynamic therapy using myriad photosensitizers. In 1993, regulatory approval was first granted in Canada for the use of PDT in the clinic.

In this Perspective, we examine the various classes of photosensitizers either currently in clinical use or in various stages of development in vitro and in vivo with respect to chemical structure and properties and with respect to mechanisms of biological action. Prior to discussing the individual photosensitizers, we provide a general description of the uses of PDT in the clinic and the general properties of a good photosensitizer for PDT.

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Clinical Uses of Photodynamic Therapy. Over the past 30 years, clinical protocols for numerous cancers have been developed including cancers of the lung,[5] gastrointestinal tract,[6] the head and neck region,[7−9] bladder,[10] prostate,[11] and nonmelanoma skin cancers and actinic keratosis.[12−18] Photodynamic therapy produces a complete response in a very high percentage of patients, and the frequency of follow-up treatments for recurrences is no greater than found with other treatment modalities. Ex vivo procedures have also been examined for treating leukemia patients' bone marrow and hematopoietic stem cell grafts.[19−26]

PDT is employed as a treatment for noncancerous conditions such as psoriasis[27,28] and age-related macular degeneration (ARMD).[29] Until recently, laser photocoagulation was the only viable treatment to reduce the risk of vision loss from ARMD but was suitable for only about 15% of patients. PDT has been found to be a well-tolerated treatment that stabilizes or slows visual acuity loss in adult patients and is a suitable treatment for 30−40% of all cases.

Successful treatment of atherosclerosis or arterial plaque with PDT has been reported.[30−33] The combination of the photosensitizer and a blood vessel catheter to deliver the light gives photoangioplasty that appears to be safe and well-tolerated with minimal damage to the blood vessel walls in treated regions.

PDT has also been shown to have some efficacy against local viral diseases such as herpes.[34] PDT has

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also been used to purge blood and blood products of viral and bacterial pathogens.[35−40]

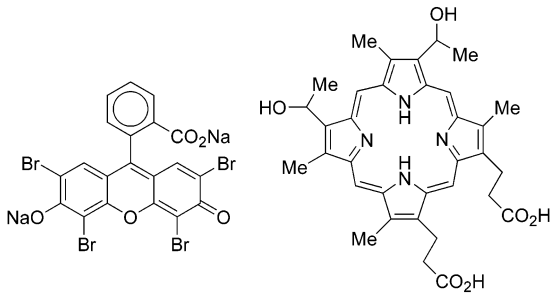
Properties of a Good Photosensitizer. To the medicinal chemist, the critical element in all of these procedures is the photosensitizer, which must be localized at the site of photodynamic action, absorb appropriate wavelengths of light, and produce the desired biological response. For the treatment of cancer, the ideal photosensitizer should meet the following requirements: (1) be chemically pure and of known composition, (2) have minimal dark toxicity and only be cytotoxic in the presence of light, (3) be preferentially retained by the target tissue, (4) be rapidly excreted from the body to provide low systemic toxicity, (5) have a high quantum yield for the photochemical event, which is often the generation of singlet oxygen ($^1O_2$) or superoxide (O_2^-), and (6) have strong absorbance with a high extinction coefficient in the 600−800 nm range where tissue penetration of light is at a maximum and where the wavelengths of light are still energetic enough to produce singlet oxygen.[41] Treatable tumors include those tumors that can be irradiated externally or internally (endoscopically) through the use of optical fibers.

One limitation of PDT is the optical properties of tissue, which influence the penetration depth of activating light as well as the intensity of light that can be tolerated to produce only nonthermal effects.[42] Light penetration into tissue is limited by light scattering and absorption, the former of which dominates except in highly pigmented tissues.[43] The penetration of visible and near-infrared light as a function of wavelength of light through bovine muscle has been described in terms of the 1/e depth (approximately 37% of the incident light density).[43] The depth of penetration doubles from the 4 mm observed between 500 and 600 nm to 8 mm at 800 nm, which defines the 600−800 nm range where light penetration is most effective. Biological effects can still be observed at 2−3 times the 1/e depths, which correspond to 3−10% of the incident light intensity and a depth of penetration of nearly 2.5 cm.

The photophysics associated with photosensitization involve (1) excitation of the photosensitizer ground state by photon energy in the 600−800 nm range to give the excited singlet state of the photosensitizer and (2) intersystem crossing from the excited-state singlet to the triplet state.[44] The interactions of the photosensitizer excited states with endogenous oxygen in the target cells or the surrounding target tissues provide the cytotoxic effects. Electron transfer from the photosensitizer excited state to ground-state oxygen produces a superoxide radical anion (O_2^-), while a spin-allowed interaction of the triplet-state photosensitizer with ground-state oxygen, which is also a triplet, produces $^1O_2$. Nearly all of the photodynamic action described in this Perspective involves the production of either $^1O_2$ or O_2^-. However, phototherapy of severely hypoxic tissues would necessitate different mechanisms of action such as direct photoinduced charge transfer from photosensitizer to biological tissue or photochemical bond formation such as [2 + 2] cycloaddition reactions.

Molecular oxygen must be present at sufficient levels throughout irradiation of target tissues or cells to produce $^1O_2$ continuously.[43,44] Depletion of molecular oxygen in tissue during high-fluence-rate irradiation has been reported to counteract the effectiveness of PDT. Light delivered at high fluence rates generates $^1O_2$ at a rate that depletes molecular oxygen more quickly than it can be resupplied, which limits the cellular and tissue damage derived from $^1O_2$. This complication has gained much attention, and numerous methods have been developed to circumvent this problem. Addition of oxygen enhancers such as fluosol DA prior to irradiation have met with minimal success, while fractionation of the exciting light to allow for resupply of oxygen during dark periods has been shown to increase the effectiveness of PDT under some conditions.[45] Thus, a combination of photosensitizer at optimal concentration, abundant molecular oxygen, and delivery of light at a fluence rate that allows for resupply of molecular oxygen within the target tissue are necessary for successful application of PDT.

Chart 1



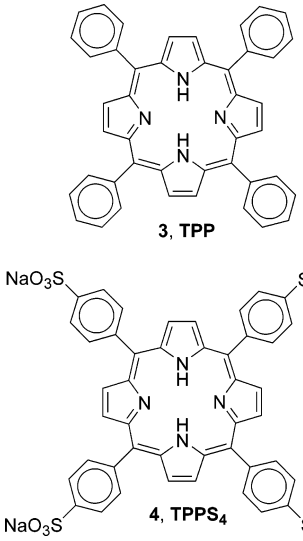
1, Eosin **2**, Hematoporphyrin

In the following sections, we shall examine porphyrins and related molecules and several classes of nonporphyrin photosensitizers including various cationic dyes and neutral dyes such as the merocyanines with respect to clinical or preclinical status and current research on the development of related or novel structures. For all of the molecules described herein, the generation of $^1O_2$ and/or O_2^- is presumed to be the critical photochemical event. With respect to porphyrins and related molecules, various naturally occurring molecules have often provided lead molecules from which clinically viable materials have been developed. The natural products have often been followed by synthetic molecules of simpler structure with improved performance.

A. Porphyrin-Related Structures

Hematoporphyrin Derivative and Other Porphyrin Photosensitizers. HpD (**2**, Chart 1) was the first photosensitizer to receive regulatory approval in Canada in 1993 and has received subsequent approval in the U.S., Europe, and Japan for a number of indications including cervical cancer, endobronchial cancer, esophageal cancer, bladder cancer, and gastric cancers.[46] As successful as HpD has been, it still suffers from many drawbacks, which have stimulated subsequent research for better photosensitizers. HpD is a mixture of compounds that include the hematoporphyrin monomer, dimer, and oligomers.[47] Partial purification of this mixture gives the commercial product porfimer sodium or Photofrin (Axcan Pharma, Montreal, Canada).[48] This "purified" product still consists of many porphyrin-containing compounds (about 60), which makes repro-

Chart 2



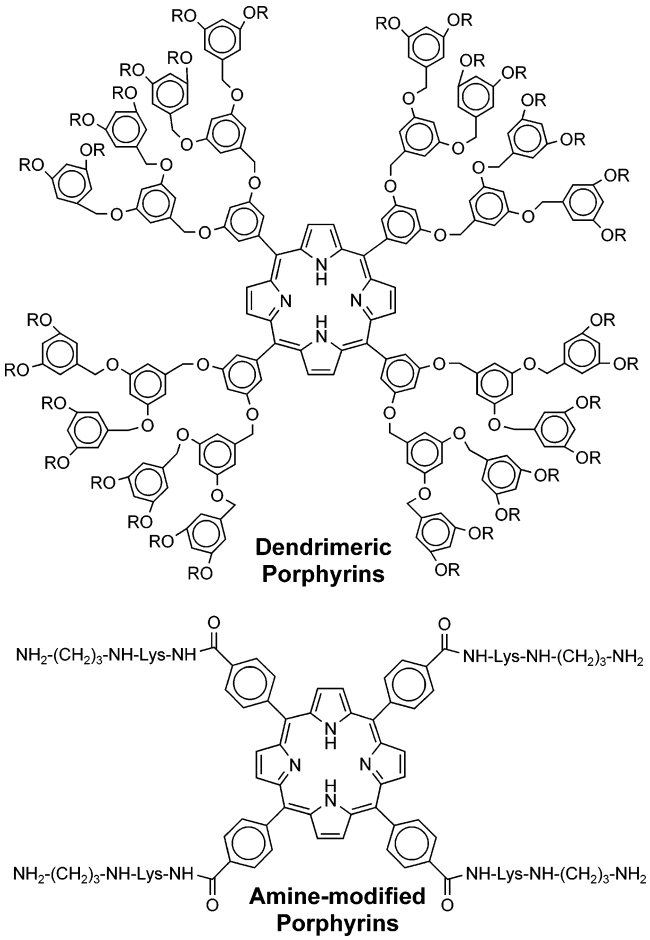
ducibility difficult in their manufacture. Photofrin and other porphyrin-related sensitizers have weak absorbance in the red region of the spectrum (≥600 nm where penetration of light in tissue is optimal) and induce long-lasting skin photosensitivity (4−6 weeks) through retention in cutaneous tissue.[41,49] The band I absorption maximum of Photofrin is at 630 nm with a weak molar extinction coefficient, ϵ, of 1170 M^{-1} cm^{-1}.

The quest for HpD-related photosensitizers has focused on derivatives that are synthetically accessible in pure form and on derivatives that have longer wavelengths of absorption.[50] Porphyrin-related chromophores all tend to be efficient producers of $^1O_2$, which makes them additionally attractive as photosensitizers.

The tetraarylporphyrins were the first easily prepared, easily purified porphyrins to be evaluated as photosensitizers. Tetraphenylporphyrin (**3**, TPP, Chart 2) has a band I absorption maximum of 630 nm and is an efficient generator of $^1O_2$ but has limited solubility. Sulfonation of TPP gives the tetrasulfonate $TPPS_4$ (**4**, Chart 2), which remains an excellent producer of $^1O_2$ [quantum yield for $^1O_2$ generation, $\phi(^1O_2)$, of 0.71],[51] has excellent water solubility, and was once viewed as a promising photosensitizer for PDT.[52] $TPPS_4$ is membrane-permeable, displays lysosomal accumulation in cells, accumulates in tumors, and is effective both in vitro and in vivo. However, clinical ambitions for $TPPS_4$ ended after reported neurotoxicity in mice exposed to high doses of $TPPS_4$.[53,54]

Recent work with porphyrin derivatives suggests some novel approaches to porphyrin delivery. Dendrimeric porphyrin derivatives with a porphyrin ring as the dendrimer core (Chart 3) have been prepared.[55] The porphyrin chromophore is buried inside a hydrophobic shell. While these molecules can look protein-like, surface modifications of the dendrimer periphery have provided cationic and anionic surfaces for zinc porphyrins with up to 32 charged groups that have been touted as effective photosensitizers in vitro.[56] Tetraphenylporphyrin derivatives bearing amino acids, peptides, or diamines (Chart 3) have demonstrated excellent surface recognition for potassium channels.[57] These two different classes of porphyrin molecules suggest different

Chart 3



ways of targeting porphyrin-based photosensitizers, which may be developed in the future.

Core-Modified Porphyrins. Substituent changes in the meso positions of the porphyrins have little impact on the wavelengths of absorption of the porphyrin chromophore. While this allows tailoring of biological properties without a major change in absorption maximum, it limits the absorption maxima of porphyrin molecules to the shorter wavelength region of the biological window for PDT. Longer-wavelength-absorbing porphyrins (band I absorption maxima of ∼665 nm) have been prepared by substituting a chalcogen atom (S or Se) for an NH at the 21-position of the porphyrin ring.[58,59] Such molecules are called core-modified porphyrins, and sulfonated analogues of the 21-thia- and 21-selenaporphyrins (Chart 4) have been evaluated as photosensitizers for PDT.[60−62] The substitution of a chalcogen atom for N has little if any impact on singlet-oxygen generation [$\phi(^1O_2) > 0.75$].[62] The sulfonated 21-thia- and 21-selenaporphyrins **5** and **6**, respectively (Chart 4), are reported to be comparable to chlorin e_6 (described below) for efficacy in vivo with BFS1 sarcoma-bearing mice, and the 21-selenaporphyrins show no skin photosensitization in animals irradiated 24 h following injection.[60,61]

N-confused 21-thia core-modified porphyrins have recently been described (Chart 4).[63] These molecules have band I absorption maxima near 730 nm with values of ϵ between 8×10^3 and 1.8×10^4 M^{-1} cm^{-1}. However, their use as photosensitizers has yet to be reported.

Chart 4

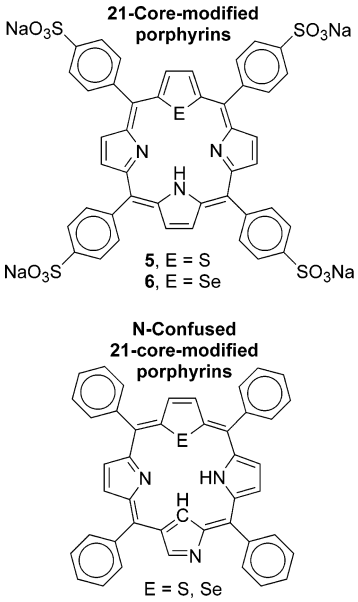
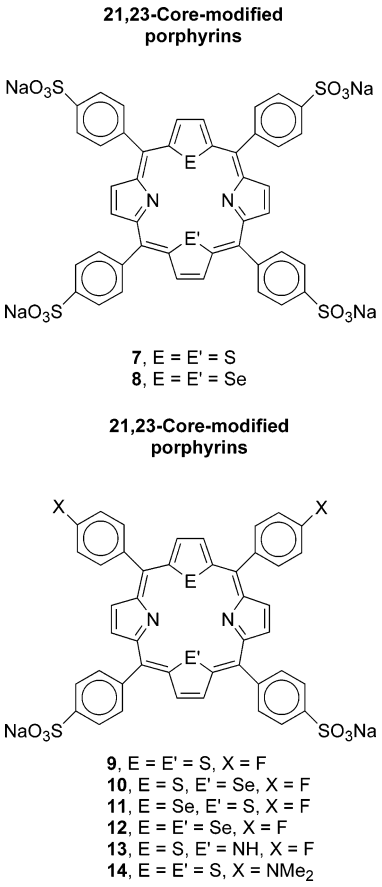
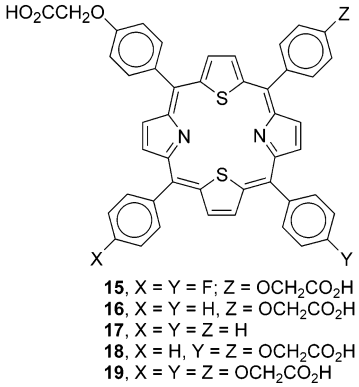


Chart 5



Replacing a second core heteroatom with S or Se gives 21,23-core-modified porphyrins with even longer wavelength band I absorption maxima (~695−700 nm).[64,65] Sulfonated derivatives **7** and **8** are easily prepared (Chart 5). In these two molecules, the chalcogen atom substitution does impact singlet-oxygen generation with $\phi(^1O_2)$ of 0.50 for **7** and 0.17 for **8**.[62,66] However, **7** is more effective than $TPPS_4$ (**4**, Chart 2) in vitro and also shows activity in vivo.[62,66]

Chart 6

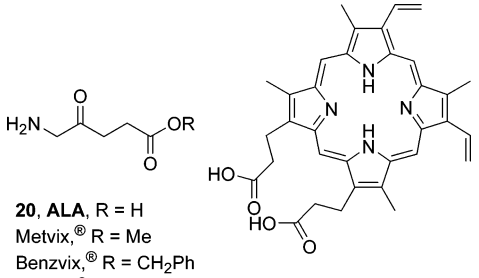


In the porphyrin series, analogues of $TPPS_4$ have been prepared that have allowed a limited structure−activity relationship (SAR) to be developed.[52,67] Molecules with two sulfonatoaryl substituents at the 5- and 10-position of the porphyrin core and with other substituents at the 15- and 20-position have shown optimal properties for both uptake and distribution. Studies with 21,23-core-modified porphyrins confirmed these observations with sulfonatoaryl groups at the 5- and 10-position relative to sulfonatoaryl substituents at all four meso positions (the 5-, 10-, 15-, and 20-position).[62,66] Compounds **9**−**14** (Chart 5) with sulfonatophenyl substituents at the 5- and 10-position give 50% cell kill with 4 J cm^{-2} of 590−800-nm light at concentrations of 0.64−7.9 μM with Colo-26 cells in vitro.[62] In contrast, dithiaporphyrin **7** gives 50% cell kill at 30 μM and $TPPS_4$ gives 50% cell kill at 125 μM under identical conditions. Dithiaporphyrin **9** (Chart 5) at 0.125 mg kg^{-1} is comparable in efficacy to Photofrin at 2.5 mg kg^{-1} in BALB/c mice bearing Colo-26 tumors.[62] Compounds **9** and **12** also show greatly reduced skin photosensitization relative to Photofrin.

The photophysical properties of **9**−**13** were little affected by heteroatom substitution in the core. All five compounds were efficient generators of singlet oxygen with $\phi(^1O_2)$ in the range 0.55−0.78. The band I absorption maxima are all very similar (λ_{max} = 691−703 nm) including 21-thiaprophyrin **13**. Dithiaporphyrin **14** with two 4-dimethylaminophenyl substituents has a longer wavelength band I absorption maximum (λ_{max} = 716 nm) with a higher molar absorptivity (ϵ = 7500 M^{-1} cm^{-1}) than compounds **9**−**13**.[62]

The sulfonic acid groups have very low pK_a values and remain in the anionic form throughout the physiological pH range. Replacing the sulfonato groups with carboxylic acid groups gives core-modified porphyrins that can achieve both neutral and anionic states at physiological pH. Core-modified porphyrin **15** (Chart 6) with two 4-fluorophenyl substituents at the 5- and 10-position and two carboxylic acid residues on substituents at the 15- and 20-position gives 50% cell kill with 4 J cm^{-2} of 590−800-nm light at 0.43 μM with Colo-26 cells in vitro.[62] Diphenyl derivative **16** (Chart 6) was even more efficacious with 50% cell kill with 0.15 μM photosensitizer under identical conditions for irradiation.[62] For comparison purposes, Photofrin gives 50% cell kill with 4 J cm^{-2} of 590−800 nm light at 10 μM with Colo-26 cells, while the chlorin derivative HPPH (described below) gives 50% cell kill at 1 μM.[68]

Chart 7



20, **ALA**, R = H
Metvix,® R = Me
Benzvix,® R = CH_2Ph
Hexvix,® R = *n*-C_6H_{13}

21, **Protoporphyrin IX**

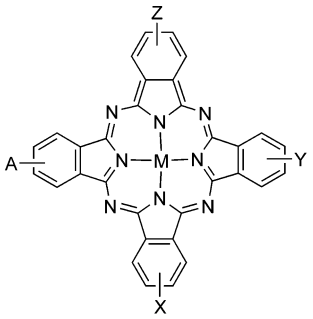
The carboxylic acid substituted core-modified porphyrins display an SAR similar to that of the sulfonated derivatives with respect to uptake and efficacy.[68] Compound **16** with two carboxylic acid groups shows greater cellular uptake and greater efficacy than compounds **17**−**19** with one, three, and four carboxylic acid groups, respectively, in R3230AC rat mammary adenocarcinoma cells. The meso-substituent changes have essentially no impact on either band I absorption maxima or values of $\phi(^1O_2)$. However, the number of carboxylic acids has a significant impact on the *n*-octanol/water partition coefficient (log *P*) with log *P* near −1.0 for **18** and **19**, near 0.0 for **16**, and >3.5 for **17**.[68]

5-Aminolevulinic Acid (20) and Protoporphyrin IX (21). 5-Aminolevulinic acid (**20**, ALA, Chart 7) is a naturally occurring amino acid that is a precursor to protoporphyrin IX (**21**, PpIX, Chart 7). PpIX is actively under investigation as a photosensitizer for PDT. PpIX is converted into heme through the action of the enzyme ferrochelatase. The exogenous administration of ALA can overwhelm the somewhat limited capacity of ferrochelatase. Furthermore, ferrochelatase activity is lower in certain tumors relative to normal tissues.[69] Both of these factors allow a selective buildup of PpIX in tumor relative to surrounding normal tissues, and PpIX concentrations in tumor reach a maximum 1−6 h following administration of ALA.[70]

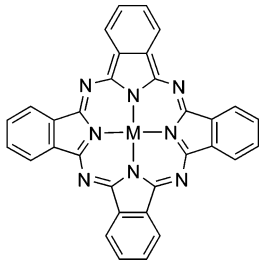
PDT using PpIX as the photosensitizer has received approval in Europe for the treatment of actinic keratosis and basal cell carcinoma[16,18] and has been found to be effective against Derier's disease (keratosis follicularis).[71] Skin photosensitization with PpIX is limited to 1−2 days, which offers a distinct advantage relative to PDT with Photofrin. As a photosensitizer, PpIX has a band I absorption maximum of 635 nm with $\epsilon < 5000$ M^{-1} cm^{-1}, which offers little improvement relative to Photofrin and other porphyrin photosensitizers.

Drug development with PpIX has focused on the ALA precursors. While ALA itself is quite hydrophilic, various alkyl esters of ALA penetrate cells much more readily than ALA.[72] ALA (Levulan, Dusa Pharma, Toronto, Canada) and its methyl ester (Metvix, PhotoCure ASA, Oslo, Norway) have been approved for use in Europe. while the benzyl ester (Benzvix, PhotoCure ASA, Oslo, Norway) and hexyl ester (Hexvix, PhotoCure ASA, Oslo, Norway) are registered indications for the treatment of gastrointestinal cancers and the diagnosis of bladder tumors, respectively.[46] The topical administration of ALA and derivatives has also been found to be effective at producing PpIX on various surface lesions.[72,73]

Chart 8



22, **ZnPc**, M = Zn
23, **AlPcS$_2$**, M = Al, A = X = SO_3^-
24, **AlPcS$_3$**, M = Al, A = X = Y = SO_3^-
25, **AlPcS$_4$**, M = Al , A = X = Y = Z = SO_3^-



26, **Pc 4**
M = $Si(OH)OSi(CH_3)_2CH_2CH_2CH_2NMe_2$

PpIX binds tightly to a variety of proteins including the mitochondrial peripheral benzodiazepine receptor (PBR)[74] and the heme proteins Hb and myoglobin,[75] which suggests sites of action and possible interactions in PDT. PpIX can be conjugated to various agonists and antagonists to provide greater selectivity and efficacy. PpIX has successfully been conjugated to gonadotropin-releasing hormone (GnRH) analogues.[76] The PpIX conjugates were more phototoxic toward pituitary gonadotrope αT3-1 cells than PpIX alone, and the conjugates showed more than an order of magnitude greater selectivity than PpIX alone.

Phthalocyanines and Related Compounds. Phthalocyanines (such as **22**−**26** in Chart 8) absorb very strongly in the red region of the spectrum with absorption maxima in the 670−780-nm window and values of ϵ greater than 100 000 M^{-1} cm^{-1}. These molecules are easily prepared, and water-soluble derivatives are prepared by sulfonation of the phthalocyanine core.[77] The zinc (**22**), aluminum (**23**−**25**), and silicon (**26**) phthalocyanines are efficient generators of singlet oxygen with long-lived triplet states and have been found to be useful photosensitizers for PDT.[77−81] The silicon phthalocyanine Pc 4 (**26**, $\lambda_{max} = 670$ nm, Chart 8) is in various stages of clinical evaluation for cutaneous and subcutaneous lesions from diverse solid tumor origins.[46]

The phthalocyanines, porphyrins, and core-modified porphyrins share a common SAR. Uptake and efficacy are directly related to the number of hydrophilic groups.[77] In the aluminum phthalocyanine series (Chart 8), $AlPcS_2$ (**23**) with two sulfonate groups shows greater uptake and phototoxicity than $AlPcS_3$ (**24**) with three sulfonate groups, which in turn shows greater uptake and efficacy than $AlPcS_4$ (**25**) with four sulfonate groups.

Chart 9

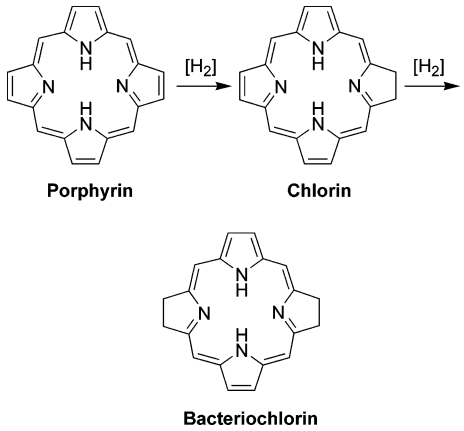
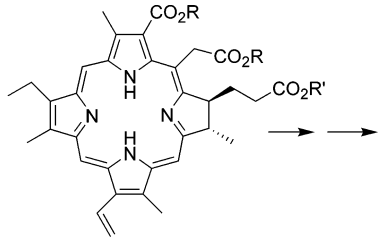
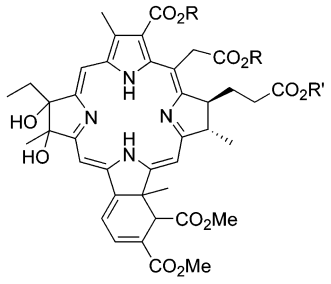


Chart 10



27, **Chlorin e_6**, R = R' = H
28, **mono-*L*-aspartyl Chlorin e_6**, R = H, R' = (*L*)-NHCH(CO_2H)CH_2CO_2H
29, **Chlorin e_6 Trimethyl Ester**, R = R' = Me



30

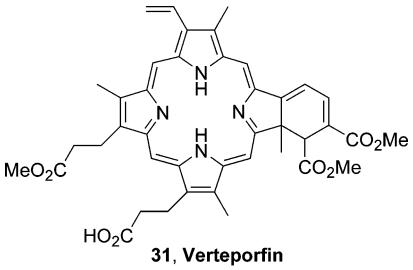
In BALB/c mice bearing EMT-6 tumors, $AlPcS_2$ is 10 times more phototoxic than $AlPcS_4$.[81]

Chlorin and Bacteriochlorin Derivatives from Naturally Occurring Sources. The porphyrin and phthalocyanine cores are oxidatively stable, which has allowed numerous derivatives to be prepared and examined.[50] However, the porphyrin core absorbs wavelengths of light too short for optimal penetration in tissue. Reduction of a pyrrole double bond on the porphyrin periphery gives the chlorin core, and further reduction of a second pyrrole double bond on the chlorin periphery gives the bacteriochlorins (Chart 9). Both of these classes of molecules have band I absorption maxima at longer wavelengths (λ_{max} = 650−670 nm for chlorins and λ_{max} = 730−800 nm for bacteriochlorins) than the porphyrins and yet still remain efficient generators of $^1O_2$.[82] Several chlorins and bacteriochlorins are in various stages of evaluation for PDT.

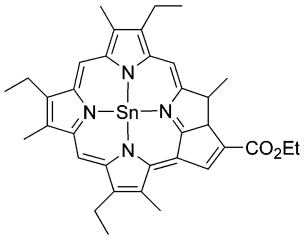
One of the first chlorins to be evaluated was the naturally occurring chlorin e_6 (**27**, Chart 10). Chlorin e_6 is derived from oxidation of chlorophyll *a* and has a band I absorption maximum of 654 nm with a value of ϵ near 40 000 M^{-1} cm^{-1}. Unfortunately, chlorin e_6 shows long-term skin photosensitization[41] and requires high doses to be effective. Alkyl esters of chlorin e_6 and derivatives such as mono-L-aspartyl chlorin e_6 (**28**, Chart 10) have been found to be more bioavailable and more effective at lower doses and are being evaluated for topical delivery.[17]

Osmium-catalyzed *cis*-dihydroxylation of chlorin e_6 derivatives such as the trimethyl ester (**29**, Chart 10) gives bacteriochlorin derivatives.[83] Subsequent Diels−Alder reactions of the vinyl group at C-8 and the adjoining pyrrole give benzobacteriochlorin derivatives such as **30** (Chart 10) with band I absorption maxima between 737 and 805 nm. Several of these compounds show limited skin photosensitization and are effective at 5 μmol kg^{-1} with 135 J cm^{-2} of light in mice bearing RIF tumors.[83]

Chart 11



31, **Verteporfin**



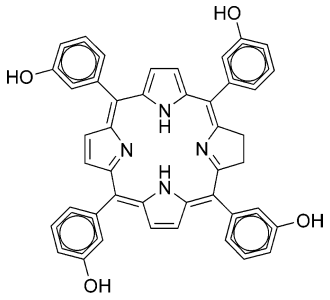
32, **SnET2**

Cycloaddition of PpIX with dimethyl acetylenedicarboxylate provides the benzoporphyrin derivative (**31**, Verteporfin or Visudyne, Chart 11), which has a band I absorption maximum of 690 nm and ϵ of 35 000 M^{-1} cm^{-1}.[41] Verteporfin shows limited skin photosensitivity (3−5 days) and rapid clearance from tissues. Verteporfin is undergoing clinical trials for the treatment of basal cell carcinoma[46] but has also had clinical success in the treatment of ARMD.[29]

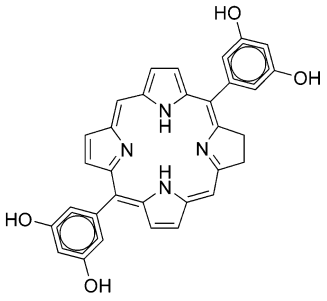
Metalated chlorin and bacteriochlorin cores have also given useful photosensitizers. Tin etiopurpurin (**32**, SnET2, Chart 11) is a metal chlorin derivative that is currently being evaluated for the treatment of cutaneous metastatic breast cancer and basal cell carcinoma.[46] SnET2 has a band I absorption maximum of 660 nm with ϵ of 28 000 M^{-1} cm^{-1}.[41] However, SnET2 treatment also imparts long-term skin photosensitization, which limits its general utility.[41]

One of the complicating side reactions of the bacteriochlorins is reoxidation to the corresponding chlorin derivative or even to the corresponding porphyrin. The introduction of palladium to the bacteriopheophorbide core gives Tookad, a bacteriochlorin derivative that is stable to oxidation back to the chlorin core. Tookad has absorption maxima at 762 and 538 nm and shows

Chart 12

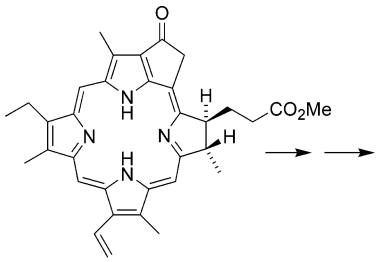


33, *m*THPC

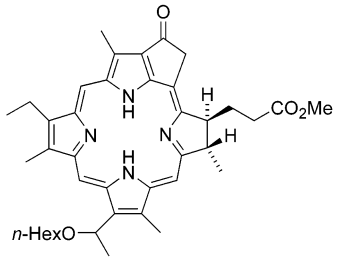


34

Chart 13



35, Pyropheophorbide-*a* Methyl Ester



36, HPPH

promise against diseases involving proliferation of neovessels[84] and against prostate cancer.[85]

Synthetic Chlorins. The synthetic chlorin 5,10,15, 20-tetra(3-hydroxyphenyl)-2,3-dihydroporphyrin (**33**, *m*THPC, Chart 12) is perhaps the most useful photosensitizer of the synthetic chlorins. *m*THPC (Foscan, Biolitec Pharma, Scotland, U.K.) has been approved in Europe for use against head and neck cancer, and additional indications have been filed for prostate and pancreatic tumors.[46] *m*THPC has also been used effectively in recurrent breast cancer for chest wall lesions with patients receiving 0.10 mg kg^{-1} and 5 J cm^{-2} of 652 nm light.[86] However, *m*THPC shows long-term skin photosensitization of up to 6 weeks, which is one drawback to its use.

*m*THPC has a band I absorption maximum of 652 nm with ϵ of 30 000 M^{-1} cm^{-1}. *m*THPC has four phenolic hydroxyl groups with pK_a values higher than the that of the carboxylic acid functionality on other chlorin photosensitizers. However, the cellular uptake of *m*THPC and its porphyrin analogues are unaffected by pH in the range 6.5−8.0.[87]

Unlike with many photosensitizers, the mitochondria do not appear to be the primary cellular target for *m*THPC. Instead, the Golgi apparatus and the endoplasmic reticulum are inactivated by *m*THPC-mediated PDT. The mitochondrial marker enzymes cytochrome *c* oxidase and the dehydrogenases are unaffected.[88] Other studies show that there is no significant correlation between tumor drug levels and PDT response in vivo, which suggests that PDT effects are most likely associated with vascular damage.[89] *m*THPC-mediated PDT may also be useful against multidrug-resistant tumors because no difference in uptake is observed between wild-type MCF-7 breast cancer cells and MCF-7/DXR cells, which express P-glycoprotein (P-gp).[90]

Other structures related to *m*THPC have also shown activity as photosensitizers. 2,3-Dihydro-5,15-di(3,5-dihydroxyphenyl)porphyrin (**34**, Chart 12) is comparable to *m*THPC with respect to $\phi(^1O_2)$, band I absorption maximum (λ_{max} = 650 nm, ϵ = 13 000 M^{-1} cm^{-1}), and efficacy in vitro and in vivo.[91,92] Chlorin **34** appears to have a more rapid uptake in tumors and more rapid clearance from tissues than *m*THPC.

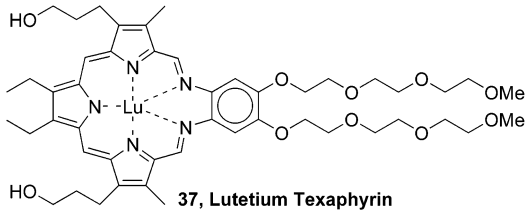
The pharmacokinetics of *m*THPC appear to be improved by the preparation of poly(ethylene glycol) conjugates (PEG−*m*THPC). PEG−*m*THPC conjugates give increased selectivity in rat liver and rat ovarian tumor models[93] and reduced PDT damage to normal tissues.[94] However, in certain mouse xenografts, PEG−*m*THPC is less effective than free *m*THPC.[95] Presumably production of $^1O_2$ by PEG−*m*THPC is about 30% that of *m*THPC, which is most likely due to the inactivaton of $^1O_2$ by the PEG groups.

Another *m*THPC-related structure, glucoconjugated tri(3-hydroxyphenyl)chlorin, has been shown to have higher phototoxicity and greater mitochondrial affinity than *m*THPC.[96] A 4-fold higher concentration of *m*THPC is required for the same level of phototoxicity even though cellular uptake of glucoconjugated tri(3-hydroxyphenyl)chlorin is 30% lower than *m*THPC.

The formal Markovnikov addition of various alcohols across the vinyl group of pyropheophorbide-*a* methyl ester (**35**) allowed a series of alkyl ethers to be prepared from which the hexyl ether HPPH (**36**, Chart 13) emerged as a potent photosensitizer in vivo.[97] The synthetic chlorin derivative HPPH (Photochlor) has a band I absorption maximum of 665 nm and is currently undergoing clinical trials for the treatment of basal cell carcinoma.[46] One intriguing aspect of the studies of HPPH and closely related molecules is the apparent correlation of very large values of the octanol/water partition coeficient (log P > 10) with uptake and efficacy.[97]

Core-Expanded Porphyrins. Another class of synthetic porphyrin-related molecules is the core-expanded porphyrins or texaphyrins.[98] These molecules have five N atoms in the expanded core, which can accommodate metals with larger ionic radii such as lutetium and

Chart 14



37, Lutetium Texaphyrin

gadolinium. The metalated texaphyrins have unusual biological properties, which make them of interest for PDT and radiation therapy.[99]

Lutetium texaphyrin (**37**, Lutex or Motexafin Lutetium, Chart 14) is registered for the treatment of cervical, prostate, and brain tumors. Lutetium texaphyrin has also been evaluated in phase II clinical trials for the treatment of recurrent breast cancer.[41] Currently, lutetium texaphyrin is being evaluated for the treatment of coronary atherosclerosis and vulnerable plaque via photoangioplasty.[31,33] The lutetium texaphyrin accumulates in plaque where subsequent catheter-delivered irradiation removes the plaque without damage to the normal blood vessel wall. Lutetium texaphyrin has also been evaluated for the treatment of ARMD and for fluorescence imaging of ocular disorders.[93]

As a photosensitizer, lutetium texaphyrin has a long-wavelength absorption maximum of 732 nm with ϵ of 42 000 M^{-1} cm^{-1} and is an efficient generator of $^1O_2$.[41] Lutetium texaphyrin is taken up rapidly by cells and clears rapidly, which leads to minimal skin photosensitization but also to a narrow treatment window 4−6 h after injection.[41] Some concerns have been raised over the specificity of gadolinum texaphyrin,[100] which has been used as a radiation sensitizer.[99]

B. Biology and Photobiology of Porphyrins and Porphyrin-Related Photosensitizers for PDT

The six desirable features of a good photosensitizer are described above. These have developed over time, but three critical features for successful photodynamic action were derived from early descriptions of photosensitization with porphyrins. A photosensitizer must be present in a sensitive location within a target tissue or cell such that photodamage occurs upon irradiation and must rapidly clear from or be localized in a nonsensitive region within normal tissue so as not to induce damage upon exposure to light. The exciting light must be of an appropriate wavelength to be absorbed by the photosensitizer and must be able to penetrate the target tissue deep enough to elicit damage throughout the affected region. Molecular oxygen must be present in sufficient quantities to allow toxic levels of $^1O_2$ to be generated during the entire period of irradiation. Numerous studies have investigated each of these three integral components of PDT.

Studies of the interaction of light with biological tissue have demonstrated that visible light at wavelengths greater than 600 nm but less than that of the infrared harmonics of water, which absorb between 900 and 1000 nm, is ideal because tissue penetration is optimal in this region of the spectrum.[42,43] The photodamage produced by PDT upon irradiation of Photofrin or other porphyrin-related materials with these wavelengths appears to be from a combination of extracellular damage to vasculature and lymphatic structures[44,101] and direct cell killing through both necrotic and apoptotic pathways.[102]

Porphyrins and chemically related compounds such as phthalocyanines and chlorins have been studied extensively since the early reports of Figge,[103] Lipson,[3,4] and Diamond,[104] which demonstrated that the basic porphyrin structure had desirable properties such as light absorbance in the red region of the spectrum, affinity for malignant tissue, and the ability to produce $^1O_2$ during exposure to light. What is somewhat surprising, in view of the long-standing recognition of the suitability of porphyrins and porphyrin-related structures, is the paucity of systematic SAR studies of porphyrins[1,67] and core-modified porphyrins,[62,68] chlorins and bacteriochlorins,[83,97] and phthalocyanines[77,78,81] by single investigators. What is most often the case is the report of the chemical, physical, photophysical, and photobiological properties of a single compound. Subsequent comparison of results of several compounds in a class among various investigators is made difficult by the differences among cell lines, tumor models, and light sources used.

Despite being a class of photosensitizer that works, the porphyrins have drawbacks that seriously impact their usefulness as photosensitzers for PDT. Many of the synthetic porphyrins have long retention times in the skin, resulting in prolonged skin photosensitivity.[41] The affinity of porphyrins for malignant tissue is not as specific as originally believed, and the mechanisms of localization are not well understood. The lack of specificity can be circumvented somewhat by utilizing lasers that can be focused more precisely on the targets of interest. The apparent preferential uptake or retention by malignancies is most likely due to the poor vascularization in these tissues, which may impede blood flow and trap porphyrin molecules or aggregates of porphyrins allowing for extended periods when they can be taken up by the tumor cells.[44] The hydrophobicity displayed by many porphyrin compounds has impacted their ability to be formulated in biologically acceptable solvents. Additionally, porphyrin photosensitization might cause nonspecific damage within a target tissue. Investigators have demonstrated that many porphyrin photosensitizers act via damage to the vasculature of malignant tissue[44,101] and by direct cellular toxicity.[102] Although, in many cases this combination may be beneficial to treatment, the tumor vasculature may be damaged during PDT light treatment, occluding vessels and shutting off further circulation of the photosensitizer and molecular oxygen. The lack of a specific target is of concern for investigators attempting to design future-generation porphyrin-related photosensitizers. In an ideal situation there would be a single site or sites where the photosensitizer is sequestered or bound so that structure−activity relationships might be addressed.

In reality, a variety of organelles[88] are targeted by porphryin-related PDT with the mitochondria as perhaps the most important organelle target.[62,68,105,106] Within the mitochondria, the peripheral benzodiazepine receptor (PBR), an 18 kDa protein on the outer mitochondrial membrane that interacts with the voltage-dependent anion channel, is thought to be a target for some photosensitizers.[74,83,107−109] The small mitochon-

drial protein Bcl-2 is also thought to be a target for the phthalocyanine photosensitizer Pc 4 (**26**, Chart 8), although Pc 4 appears to be bound to several mitochondrial targets.[110]

Because of the less than optimal properties of porphyrins, investigators have focused on designing systems such as immunoconjugates of porphyrins to effect greater selectivity and specificity[76,111] or nano(micro)-particle-bound photosensitizers[112] to enhance cellular uptake. Each of these approaches has shown some promise in initial studies. However, antibody-targeted photosensitization using antibody-conjugated porphyrins has limitations due to the diversity of tumor types and their cellular makeup. Antibodies may be too specific and only target antigens on a single or severely restricted cell phenotype, leaving others that lack the specific antigen to continue to multiply following treatment. In a study of immunoconjugated chlorin e_6 (**27**, Chart 10), Goff et al.[111] conclude that there is ample room for improvement in tumor uptake of their ovarian cancer-specific antibody OC125 and that more specific antibodies such as products of oncogenes may improve the selectivity of PDT. In a separate study, PpIX (**21**, Chart 7) was conjugated to either a peptide agonist or a peptide antagonist of the gonadotropin-releasing hormone (GnRH) receptor.[76] The conjugates are more phototoxic toward the pituitary gonadotrope αT3-1 cell line than is unconjugated PpIX. Furthermore, the phototoxicity of the conjugates is alleviated by co-incubation with the parent peptide from which the conjugates were prepared. This suggests that the phototoxicity is receptor-mediated. However, even with advances in antibody specificity, there still remains the heterogeneity of cells within a tumor that could largely limit the effectiveness of antibody-conjugated photosensitizers.

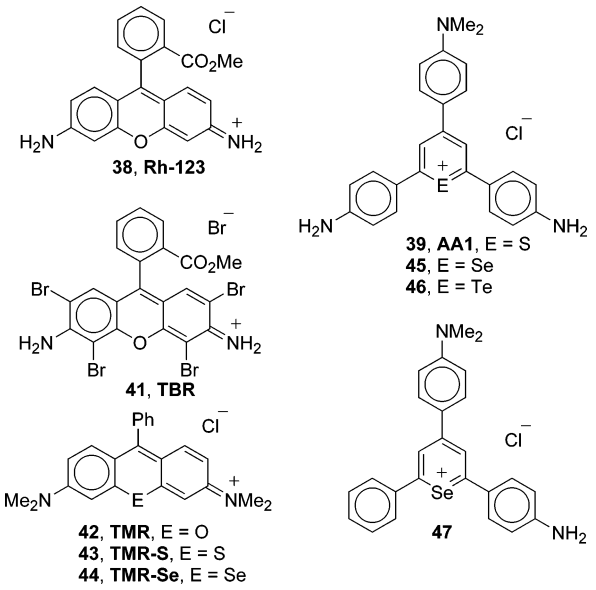
Nanoparticle-bound photosensitizers[112] also have drawbacks. Although binding or adhering photosensitizers to synthetic substrates may increase cellular uptake of a particular photosensitizer, the possibility that this may occur in normal tissue may be just as great as in malignant lesions. Dendrimeric porphyrins have been prepared in which the photosensitizer is the core of the dendrimer.[55,56] The porphyrin photosensitizer is no longer on the surface but is buried in the center of the dendrimer. Taken together, the drawbacks and limitations of the porphyrin photosensitizers have led to investigations into new classes of photosensitizers with greater potential for targeting.

Several groups have investigated the water-soluble, core-modified porphyrins described above, whose structures are shown in Charts 4−6.[60−62,66,68] The evolution of these compounds as photosensitizers illustrates the fine-tuning of a basic structure to give optimal performance. Initially, 21-thio and 21-seleno core-modified, tetrasulfonated porphyrins **5** and **6** (Chart 4) were prepared followed by the 21,23-dithio- and 21,23-diseleno core-modified porphyirns **7** and **8** (Chart 5). Surprisingly, generation of $^1O_2$ was negatively impacted by the presence of S or Se atoms in the tetrasulfonated core-modified porphyrins **5**−**8** relative to the parent $TPPS_4$.[62,66] It was speculated that the nonplanarity of the central core contributed to the reduced yields for the production of $^1O_2$.[66] The sulfonate hydrophilic groups increase water solubility and facilitate formulation for introduction into biological systems. Disrupting the symmetry of **5**−**8** gave longer wavelengths of absorption and higher values of $\phi(^1O_2)$ in diseleno, selenothio, and dithio core-modified porphyrins **9**−**14** (Chart 5) bearing two aryl substituents and two sulfonatophenyl substituents compared to the parent compound $TPPS_4$. Furthermore, these compounds demonstrated comparable photosensitization of Colo-26 and R3230AC cells in culture relative to that obtained with Photofrin. The dithio and diseleno core-modified porphyrins **9** and **13**, respectively, were also as effective as Photofrin in delaying the growth of subcutaneously implanted Colo 26 tumors after PDT.[62]

The dithiodicarboxylic acid derivatives **15** and **16** (Chart 6) of these core-modified porphyrins, although not as water-soluble, are more effective photosensitizers in vitro than Photofrin, HPPH (**36**, Chart 13), or the sulfonate derivatives **9**−**14**.[62,68] From these studies, the dithiodicarboxylic acid derivative **16** was selected as the parent compound for extended QSAR studies. These comparative studies include core-modified porphyrin **16** with two carboxylic acid groups and **17**−**19** (Chart 6) with one, three, and four carboxylic acid groups, respectively.[68] The dithiodicarboxylic acid containing porphyrin **16** is the most effective photosensitizer in vitro against R3230AC cells compared to the other carboxylic acid containing derivatives. Compound **16** is also more effective against Colo-26 cells in vitro than derivatives **17**−**19**, Photofrin, HPPH (**36**, Chart 13), and the disulfonates **9**−**14**.

In an effort to determine subcellular localization and possible mechanisms of action for this unique class of core-modified porphyrins, the photosensitized inhibition of the mitochondrial enzyme cytochrome *c* oxidase in cultured R3230AC cells was used as an endpoint. This enzyme is critical to mitochondrial function because it is the final step in the electron-transport chain.[105,106] The core-modified dithioporphyrins **17** and **16** with one and two carboxylic acid groups, respectively, and the core-modified porphyrins **9**−**14** with two sulfonate groups are potent inhibitors of whole-cell mitochondrial cytochrome *c* oxidase.[62,68] The dithiodicarboxylic acid derivative **16** is, under the conditions employed, the most potent photosensitizer of this group against mitochondrial enzyme activity in the dark and in light exposed cells. These data demonstrate that the porphyrin structure, after careful, methodical modification, can be altered to fit the criteria thought necessary for the design of an effective photosensitizer. The core-modified porphyrins demonstrate cellular uptake in the 10−100 femtomole per cell range, dark toxicity only at concentrations greater than 1 μM, significant phototoxicity toward cultured cells at 0.1−0.4 μM, localization in Colo-26 tumors, effective photosensitization of tumors in vivo, and limited skin photosensitization.[62,68] Continued QSAR studies are warranted for this class of core-modified porphyrin photosensitizers because they possess many of the characteristics of an "ideal photosensitizer." As stated above, for successful PDT, one would prefer either a single cellular site or a known, small number of sites such as the PBR or Bcl-2 proteins where the photosensitizer is sequestered or bound so that structure−activity relationships could be addressed.

Chart 15



C. Chemistry and Biology of Non-Porphyrin Photosenstizers

Mitochondrial Localization of the Cationic Dyes Rhodamine 123 and AA1. Much of our current knowledge about the localization of cationic dyes in cells comes from studies with the xanthylium dye rhodamine 123 (**38**, Rh-123, Chart 15).[113] Relative to the concentration in culture media, cytoplasmic concentrations of Rh-123 and presumably other cationic dyes may be 10-fold higher while mitochondrial concentrations may be up to 10000-fold higher. Rh-123 accumulates selectively in the mitochondria of certain cancer and muscle cells relative to other cell types. Rh-123 is selectively toxic to certain cancer cell lines[114] and gives prolonged survival in vivo in tumor-bearing animals.[115] Unfortunately, Rh-123 is a poor photosensitizer for PDT because of its low triplet yield, which limits its ability to produce $^1O_2$. Consequently, irradiation of Rh-123-treated cancer cells in vitro gives little if any added phototoxicity, and irradiation of Rh-123-treated, tumor-bearing animals gives no further increase in post-treatment survival.

The cationic thiopyrylium dye AA1 (**39**, Chart 15) is related in structure to Rh-123. The amino substituents of both Rh-123 and AA1 delocalize the positive charge on the molecule, and both Rh-123 and AA1 target the mitochondria of cancer cells.[116] AA1 inhibits mitochondrial ATP-ase activity, inhibits growth of the human colon carcinoma cell line CX-1 in vitro, and gives prolonged survival in mice implanted with several different tumor lines.[116] As in studies with Rh-123, irradiation of either AA1-treated cancer cells in vitro or AA1-treated tumor-bearing animals gives no increase in either toxicity or survival.

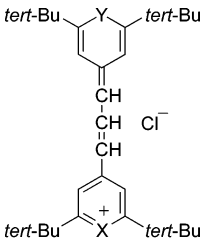
The affinity of the cationic dyes for the mitochondria is a driving force for investigating such molecules as photosensitizers for PDT. Presumably, the affinity of the cationic dyes for the mitochondria would be much greater than that of the generally neutral porphyrins and related molecules. Why would mitochondria be a desirable target for photosensitization? The mitochondria are the energy center of the cell, creating all the ATP necessary to support cellular functions.[105] Through electron transport in the respiratory chain, which is tightly coupled to oxidative phosphorylation of ADP producing ATP, the mitochondria generate cellular energy and a potential across their inner membranes. Interruption of these processes causes serious cellular malfunction, and if an insult is severe enough or prolonged, cell death ensues. Damage to any one of the processes of ATP synthesis, electron transport, membrane transport for ions or proteins, or interruption of the membrane potential could result in dire consequences for the whole cell.

Designing agents to disrupt specific mitochondrial functions has been the goal of many therapeutic studies. Photosensitizers designed specifically to be localized in the mitochondria and to inhibit particular functions might dramatically increase the specificity of PDT. Positively charged photosensitizers might be advantageous because of their attraction to the mitochondria due to the membrane potential.[113] A positive charge might also increase the selectivity of photosensitizers for cancer cells because in general cancer cells have increased metabolism and a higher membrane potential.[114,115]

An interesting study was published 14 years after Chen's pioneering work[113] when a synthetic monocationic porphyrin was tested as a photosensitizer toward cells in culture and the RIF tumor in vivo.[117] The goal of this study was to determine whether a positively charged porphyrin would show increased uptake by mitochondria and enhanced effectiveness as a photosensitizer for PDT. This cationic porphyrin apparently retained more porphyrin characteristics than it gained in properties of a lipophilic cation. The cationic porphyrin was localized in the membranous structures of the cells, especially the plasma membrane, and although it was a potent photosensitizer, its damage occurred at the plasma membrane. Neither was it localized in mitochondria nor did it damage mitochondria as expected. Inhibition of tumor growth in vivo was mainly due to vascular damage and not due to direct cell kill as might be expected for a cationic compound. This study demonstrates the impact that the porphyrin macrocycle has on cellular uptake and localization, which can supersede the mitochondrial preference of the positive charge.

Heavy-Atom Analogues of Cationic Dyes with Improved Photophysical Properties. Substitution of heavy atoms such as sulfur, selenium, or tellurium for oxygen and nitrogen in the cationic dye chromophore should increase the $\phi(^1O_2)$ yield of photosensitizers through spin−orbit effects. As their initial entry into cationic photosensitizers, Detty and colleagues investigated a series of chalcogenapyrylium dyes **40** (Chart 16) for their photophysical properties and for their photosensitizing capabilities in vitro.[118] As the chalcogen atoms increase in atomic weight, absorption maxima shift to longer wavelengths and values of $\phi(^1O_2)$ increase. Dyes where only oxygen and sulfur atoms are present give very little production of $^1O_2$. Incorporation of one or two selenium atoms increases $\phi(^1O_2)$ to 0.005−0.01, while incorporation of tellurium atoms increases $\phi(^1O_2)$ to 0.05−0.12. Electron microscopy studies demonstrated that **40**(Te,Se) entered cells and disrupted the inner mitochondrial membrane in the dark and to a

Chart 16



40(X,Y)
X, Y = O, S, Se, Te

much greater extent after exposure to light. The activity of cytochrome *c* oxidase was inhibited in suspensions of isolated rat mammary tumor mitochondria by Se−S [**40**(Se,S)] and Te−Se [**40**(Te,Se)] analogues of these chalcogenopyrylium dyes upon exposure to broad-band white light. The enzyme inhibition was dependent on the light dose and dye dose, and through the addition of imidazole, an $^1O_2$ trap, or reduction in the oxygen concentration in the mitochondrial suspension, it was demonstrated that $^1O_2$ was the primary toxic agent involved.

Although telluropyrylium dye **40**(Te,Se) targets the mitochondria in vitro, it is too lipophilic for general circulation following intravenous injection, which severely limits any utility for this molecule as a photosensitizer. However, intralesional injection of **40**(Te,Se) followed by irradiation gives prolonged survival in tumor-bearing animals.[119] Studies with **40**(Te,Se) also demonstrate that pH differences between tumor and normal tissues can be exploited to retain the photosensitizer selectively in tumor.[119] The higher pH of the normal tissue surrounding a tumor gives more rapid hydrolysis to essentially colorless products in the normal tissue relative to the more acidic tumor.

Heavy-Atom Analogues of Rh-123 and AA1. The studies with chalcogenopyrylium dyes **40** were the first to correlate systematically higher triplet yields and, consequently, higher values of $\phi(^1O_2)$ directly with the atomic weight of the heteroatom. This approach has subsequently been exploited in several classes of photosensitizers as described below and has provided viable photosensitizers in both the rhodamine and AA1 classes of dyes.

Bromination of the xanthylium core has also been utilized to introduce heavy atoms. The four bromine atoms of tetrabromorhodamine 123 (**41**, TBR, Chart 15)[120,121] impart a much higher value of $\phi(^1O_2)$ than for Rh-123, but the absorption maximum is little affected by this type of heavy-atom substitution ($\lambda_{max} = 516$ nm, $\epsilon = 91\,000$ M^{-1} cm^{-1} for TBR; $\lambda_{max} = 514$ nm, $\epsilon = 101\,000$ M^{-1} cm^{-1} for Rh-123). The addition of the four bromine atoms also alters the intracellular localization of TBR to some locus other than the mitochondria.[121] TBR is currently being evaluated as an ex vivo photosensitizer for the treatment of chronic myeloid leukemia.[41] TBR has also been used to treat multidrug-resistant cells[120] and has been found to trigger apoptosis following treatment of cells in vitro.[121] Unfortunately, the four bromine atoms do not red-shift the absorption maximum sufficiently to make TBR a useful photosensitizer for work in vivo.

The substitution of the heavier chalcogen atoms S and Se into the xanthylium core gives chalcogenoxanthylium analogues with higher values of $\phi(^1O_2)$ and with significantly longer wavelengths of absorption.[122] In the tetramethylrosamine series, the xanthylium analogue TMR (**42**, Chart 15) has a λ_{max} of 552 nm with a $\phi(^1O_2)$ of 0.08. The thioxanthylium analogue TMR-S (**43**) has a $\phi(^1O_2)$ of 0.21 and a λ_{max} of 571 nm, while the selenoxanthylium analogue TMR-Se (**44**) has a $\phi(^1O_2)$ of 0.87 and a λ_{max} of 582 nm.

TMR-S and TMR-Se are both effective photosensitizers against R3230AC rat mammary adenocarcinoma cells in vitro.[122] With 5 J cm^{-2} of filtered 350−750 nm light, TMR-Se is phototoxic to R3230AC cells at a concentration of 50 nM. Studies of whole-cell cytochrome *c* oxidase activity suggest that the mitochondria are targets for these dyes. Irradiation of R3230AC cells treated with either TMR-S or TMR-Se gives light-fluence-dependent inhibition of cytochrome *c* oxidase activity. Although both TMR-S and TMR-Se absorb significantly longer wavelengths of light than TBR, these two dyes still have minimal absorbance at wavelengths above 600 nm, which may limit their value as photosensitizers in vivo.

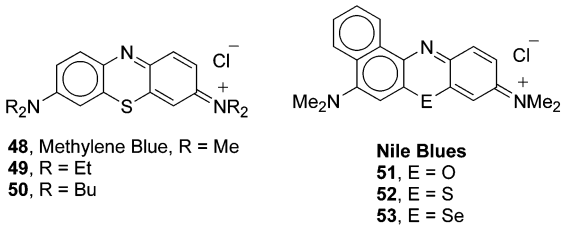
The thiopyrylium dye AA1 (**39**, Chart 15) has a λ_{max} of 581 nm. Heavier chalcogen analogues of AA1 have also been prepared with longer wavelengths of absorption and higher values of $\phi(^1O_2)$.[123,124] The selenopyrylium analogue **45** has a λ_{max} of 610 nm, while the telluropyrylium analogue **46** has a λ_{max} of 620 nm.[123] The selenopyrylium AA1 analogue **47** has a 2-phenyl substituent replacing the 2-(4-aminophenyl) substituent[124] and has an absorption maximum of 651 nm. Compounds **45**−**47** all produce measurable quantities of $^1O_2$ relative to AA1, although the solution yields are <0.10, which is low relative to those of TMR-S and TMR-Se.

AA1 gives 90% inhibition of whole cell cytochrome *c* oxidase activity in the dark.[123] In contrast, dyes **45**−**47** do not inhibit whole cell mitochondrial cytochrome *c* oxidase in the dark in mitochondrial suspensions prepared from R3230AC rat mammary adenocarcinomas, but they inhibit this enzyme in a fluence-dependent manner when the cells are irradiated with filtered 350−800 nm light. Mechanistic studies using **45**−**47** used mitochondrial suspensions exposed to 100 mW cm^{-2} of broad-band (530−750 nm) light in the presence of each of these dyes. The dye photosensitized inhibition of cytochrome *c* oxidase does not occur in a reduced oxygen environment (0−2% oxygen), is reduced by 75% with the addition of 0.08 M imidazole, a singlet oxygen trap, and is unaffected by the addition of 30 U of superoxide dismutase. Compounds **45**−**47** all demonstrate efficacy in vitro upon irradiation of dye-treated Colo-26 or R3230AC cells.[123,124] These results, taken together, demonstrate that AA1 and analogues **45**−**47** target the mitochondria, that **45**−**47** are photosensitizers, and that the primary toxic agent is probably $^1O_2$.

Compound **47** has demonstrated efficacy in vivo in BALB/c mice bearing implanted Colo-26 tumors that is comparable to that of Photofrin with respect to treatment dose, prolonged survival, and cured animals.[124] Unlike Photofrin, compound **47** shows no long-term skin photosensitization.[124]

Methylene Blue and Related Compounds. The phenothiazinium dye methylene blue (**48**, Chart 17) has

Chart 17



48, Methylene Blue, R = Me
49, R = Et
50, R = Bu

Nile Blues
51, E = O
52, E = S
53, E = Se

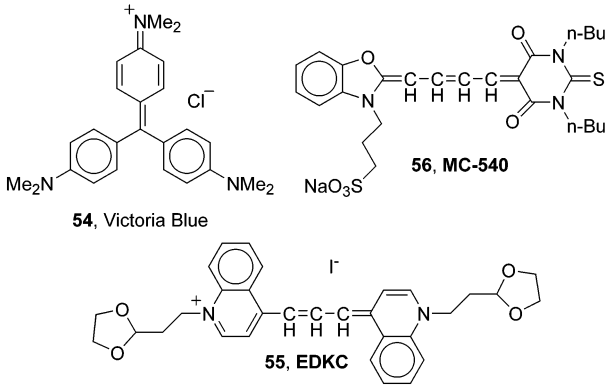
been used as a photosensitizer (λ_{max} = 620 nm) for the inactivation of various pathogens contained in blood plasma and is currently being used for the decontamination of freshly frozen plasma units.[39,41,125] One concern with analogues of Rh-123 and AA1 is the low therapeutic ratio in vivo between the PDT-effective dose and the toxic dose. In contrast, methylene blue has low toxicity toward humans and has been used for the treatment of methaemoglobinaemia.[125] One strength of methylene blue and related phenothiazinium structures is their ease of synthesis.[37] One limitation of methylene blue is its ease of reduction to the neutral amine, which is colorless and ineffective as a photosensitizer.[39]

Like Rh-123 and AA1, methylene blue was thought to target the mitochondria and to induce cell death through apoptosis.[126] However, more recent studies suggest that localization of phenothiaznium derivatives is very dependent on values of log *P* for the individual dyes.[127] Studies in vitro suggest that all of the *N*,*N*,*N*′,*N*″-tetraalkyl derivatives initially are localizes in the lysosomes and then are relocated following irradiation. For the tetramethyl analogue, the parent methylene blue with log *P* = −1, irradiation gives relocalization primarily to the nucleus.[127] For the tetraethyl analogue **49** (Chart 17), with log *P* = 0.3, no relocalization was observed and the dye remained primarily in the lysosome. For higher tetraalkyl analogues such as the tetrabutyl analogue **50** (Chart 17) with log *P* > 1.0, irradiation gives relocalization to the mitochondria.

The benzophenoxazines **51** and benzophenothiazines **52** (nile blues, Chart 17) are methylene blue related compounds that are not as readily reduced as methylene blue.[128,129] These molecules absorb light in the 600−650-nm window, are efficient generators of $^1O_2$, and are reported to accumulate in the lysosomes of cancer cells. Structurally, the nile blues have an extra fused benzo ring, which increases values of log *P*. Like the methylene blue derivatives with longer alkyl chains on the amino groups,[127] irradiation of the nile blue derivatives leads to relocalization from the lysosomes to the mitochondria.[128] A selenium analogue **53** of the nile blues has been prepared that absorbs at slightly longer wavelengths than either the benzophenoxazine **51** or benzophenothiazine analogues **52**.[129] Compounds **51**−**53** are also all active photosensitizers in vivo against selected carcinoma implants.

Other Cationic Dyes. The victoria blues (**54**, λ_{max} = 610−650 nm) also target the mitochondria of various cancer cells (Chart 18).[130] The victoria blues are triarylmethane cations that have been used as photosensitizers against normal cancer cell lines[130] and against multidrug-resistant cancer cell lines.[131] Again, the therapeutic ratios for the victoria blues and related structures limit their applicability for use in vivo.

Chart 18



54, Victoria Blue

56, **MC-540**

55, **EDKC**

Kryptocyanine[132,133] and rhodacyanine[134,135] derivatives have also shown therapeutic activity as photosensitizers. The kryptocyanine dye, EDKC (**55**, Chart 18), is localized in the mitochondria of cultured bladder carcinoma cells and is an efficient photosensitizer of this cell line.[132] Complex **I** of the mitochondrial electron transport chain was found to be the primary target of EDKC photosensitization in isolated liver mitochondria. However, EDKC demonstrated little dark or light-induced toxicity toward F_1F_0 ATP synthetase in contrast to the effects on this enzyme observed with Rh-123.[133] EDKC was one of the first dyes for which a specific photosensitive intracellular site was identified.

The rhodacyanines also target the mitochondria and disrupt electron transport upon irradiation.[134] The rhodacyanine dye Rhodac is a potent photosensitizer toward HeLa cells and induces caspase-3 activation and apoptosis following irradiation.[135] The phototoxicity is oxygen-dependent, which is consistent with the production of $^1O_2$ or O_2^-. Interestingly, Rhodac is far less phototoxic toward multidrug-resistant cells relative to normal cancer cell lines.

Merocyanine 540 and Related Structures. Merocyanine 540 (**56**, MC-540, Chart 18) is an uncharged dye with a λ_{max} of 555 nm and a ϵ of 16 000 M^{-1} cm^{-1}. MC-540 has been used in preclinical models and in a phase I clinical trial for the ex vivo purging of autologous bone marrow grafts contaminated with leukemia or lymphoma.[20] In the treatment of a patient afflicted with either of these conditions, the patient's bone marrow can be treated with MC-540-based PDT prior to reintroduction into the patient following high-intensity chemotherapy. The same approach can be used to remove breast cancer cells in autologous stem cell grafts using MC-540 alone or in combination with other agents such as crystal violet[23] or the alkyllysophospholipid edelfosine.[22,24] The effects of temperature (hyper- and hypothermia) after irradiation of MC-540 have also been examined.[19,21]

MC-540 appears to be a substrate for P-gp in multidrug-resistant cells,[20] allowing treatment of cultures of multidrug-resistant cells with MC-540-based PDT. Unfortunately, the λ_{max} of 555 nm for MC-540 limits its utility for PDT in vivo. Blood purging of HIV-1 using photochemically "preactivated" MC-540 has also been described.[136]

D. Use of Photosensitizers for Pathogen Reduction in Red Blood Cells.

Optimal Photosensitizer Properties. During the past 15 years, a number of photosensitizers have been studied in efforts to reduce or eliminate the infectivity of those rare units of blood that are collected during the "window period", or early in infection when infectious-disease tests fail to detect the presence of a pathogen's nucleic acid, antigens, or an individual's antibodies against the organism. In addition, pathogen reduction methods hope to reduce the risk of known but untested agents as well as unknown agents. Optimal properties of a photosensitizer for use in ex vivo red cell decontamination are somewhat different from those used in procedures in vivo and include selection of dyes that (1) permeate both cell and viral membranes to inactivate both intracellular and extracellular pathogens, (2) adhere to nucleic acid with little affinity for red cells in order to facilitate pathogen photoinactivation with limited damage to annucleate red cells, (3) absorb red light to avoid light attenuation by hemoglobin absorption, and (4) display little dark toxicity to either red cells or transfusion recipients.

Hematoporphyrin, Benzoporphyrin, and Merocyanine 540. Early efforts in pathogen reduction in red cells included studies evaluating the usefulness of hematoporphyrin derivative (**2**, HpD, Chart 1), benzoporphryin derivative (**31**, veteporfin, Chart 11), and merocyanine 540 (**56**, MC-540, Chart 18). Studies with HpD demonstrated 4.3 $\log_{10}$ inactivation of extracellular and intracellular HIV-1 using 10 or 20 μg/L of the HpD mixture and 5 J cm^{-2} of red light, with no immediate impact on selected plasma and red cell properties.[137] However, no storage studies were performed. Benzoporphyrin derivative has been used in concentrations of 2−4 μg/mL with 57.6 J cm^{-2} of broad-band light to produce up to 7 $\log_{10}$ inactivation of vesicular stomatitis virus (VSV), a model single-stranded RNA virus, with little immediate hemolysis,[138] although later red cell survival studies demonstrated that phototreatment resulted in excessive red cell damage. Studies using MC-540 demonstrated inactivation of extracellular virus and, to a lesser extent, of intracellular virus.[139] However, the spectrum of MC-540 overlaps with that of hemoglobin, necessitating treatment of low-hematocrit (15%) red cells. In addition, ATP levels decreased and hemolysis considerably increased in MC-540-phototreated red cells during routine storage.[140]

Phthalocyanines as Photosensitizers. Studies by Horowitz, Ben-Hur, and colleagues with aluminum phthalocyanine tetrasulfonate (**25**, Chart 8) and red light established that phototreatment resulted in 3 $\log_{10}$ to ≥4 $\log_{10}$ inactivation of intracellular and extracellular forms of VSV and extracellular, but not intracellular, forms of HIV.[35,141] A more potent phthalocyanine, Pc 4 (**27**, Chart 8), was utilized to inactivate >5.5 $\log_{10}$ of intracellular and extracellular HIV, >6.3 $\log_{10}$ of VSV, and >5 $\log_{10}$ of PRV and BVDV and also inactivated the parasites *T. cruzi* and *P. falciparum*.[36,38] The sensitizer was formulated in liposomes to maximize delivery to viruses with minimum distribution to red cells.[38] In addition, the quenchers tocopherol succinate, carnitine, and cysteine were added to diminish the extensive hemolysis, potassium efflux, and binding of IgG to phototreated red cells that would occur in their absence.[38,40] With quenchers, hemolysis was limited to ∼0.6% after 21 days of refrigerated storage. Red cells are typically stored for 42 days, and regulations limit hemolysis to <1% at the end of storage.

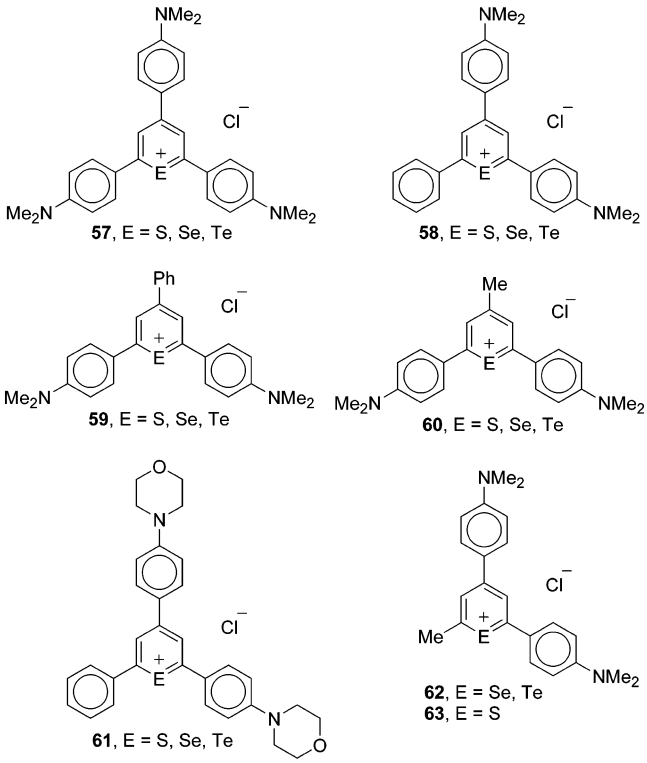
Anionic Core-Modified Porphyrins. Some unpublished preliminary studies were conducted by S. J. Wagner and colleagues with three anionic, core-modified porphyrins (see Chart 6, for example, compound **15**). Inactivation of VSV by >6 $\log_{10}$ is observed using 10 μM **15** in conjunction with 1.1 J cm^{-2} red light. Rapid potassium efflux is observed following phototreatment, and approximately 50% of red cells were hemolyzed within 1 week of storage. This rapid and extensive hemolysis is much greater than that which is typically observed with other photosensitizers under virucidal conditions. With the demonstration that Pc 4 binds to the benzodiazepine receptor,[110] it is interesting to speculate whether the cause for rapid potassium efflux and photoinduced hemolysis that are observed with core-modified porphyrins is due to an association of the sensitizer to another benzodiazepine binding site, which has been previously discovered on the plasma membranes of erythrocytes.[141]

Methylene Blue and Related Compounds. Methylene blue (**48**, Chart 17) is currently being used as a photosensitizer for the decontamination of freshly frozen plasma units.[39,41,125] However, in the presence of red cells, the phenothiazines as a class have some issues. Methylene blue, methylene violet, and 1,9-dimethylmethylene blue have been extensively investigated for pathogen reduction in red cell suspensions.[125] One advantage of these dyes is their association with nucleic acids,[142] suggesting that these dyes would be more specific for pathogen reduction and cause less damage to red cells. However, up to one-half of an added phenothiazine is associated with red cells, and it is this bound form of the dye that is responsible for much of the photoinduced hemolysis.[125] Some fraction of red-cell-associated dye is probably localized by charge interactions to the negatively charged lipid interface of the plasma membrane, while a large proportion of dye is trapped on red cells by reduction to the dye's leuco form and does not participate in photochemical reactions.[39]

One disadvantage with methylene blue is its inability to inactivate intracellular virus presumably because the permanent positive charge of the hydrophilic dye prevents permeation through the plasma membrane of some cells.[143] The phenothiazine methylene violet, an uncharged dye, and dimethylmethlylene blue, a more hydrophobic dye with permanent positive charge, overcome this deficiency and can inactivate intracellular virus.[144] However, these more hydrophobic dyes have greater potential for hemolysis under more stringent virucidal conditions.[144,145]

The mechanism for hemolysis seems to be colloidal osmotic in origin. Phototreated red cells exhibited high rates of potassium efflux, indicative of membrane damage that produces small pores permeable to ions but not hemoglobin. At ionic equilibrium, the internal osmotic pressure in ion permeable red cells is greater than the external osmotic pressure because hemoglobin contributes as an osmoticum. This imbalance in pressure leads to water influx, cell swelling, and ultimately lysis.[146]

Chart 19



After 42 days of storage, hemolysis can be as extensive as 25% in traditional red cell storage media but can be reduced to approximately 2% in an experimental medium that contains citrate, an impermeable ion, which at appropriate concentrations produces an osmotic pressure that equals the osmotic pressure contribution from hemoglobin.[145] Red cell hemolysis from dimethylmethylene blue phototreatment can be further reduced to 1.2% by the addition of a molecule with similar structure, quinicrine, that functions as a competitive inhibitor of dye binding to red cells.[147] This residual hemolysis in citrate containing medium and in the presence of quinacrine is postulated to be generated from red cell damage that is noncolloidal in nature and is caused by reactive oxygen species generated by free rather than bound dye.

Pyrylium-Based Photosensitizers. In unpublished work, a series of 21 chalcogenopyrylium sensitizers[123,124] (**42**−**44**, Chart 15, and **57**−**63**, Chart 19) have been screened by S. J. Wagner and colleagues for virucidal and hemolytic activities. Pyrylium sensitizers cannot efficiently generate singlet oxygen in the unbound state because internal conversion via bond rotation in the flexible dye diminishes the lifetime of the excited singlet state, reducing intersystem crossing to the triplet state.[118] However, the dye may better generate singlet oxygen if rigidly bound to a substrate in a planar orientation. Of the 21 dyes screened, 10 displayed adequate extracellular virucidal activity (>6 $\log_{10}$ of VSV), yet only one produced <1% hemolysis during 42 days of storage. Interestingly, this dye, 2,4-bis(dimethylaminophenyl)-6-methylthiopyrylium chloride (**63**, Chart 19), has been shown to intercalate into nucleic acid and specifically stain DNA in organisms.[148] Use of 160 μM of **63** and 1.1 J cm^{-2} of red light resulted in >7 $\log_{10}$ inactivation of extracellular VSV, >5 $\log_{10}$ inactivation of intracellular VSV, and 4 $\log_{10}$ to 8 $\log_{10}$ inactivation of six bacterial strains.[149] Under these conditions, 0.6% hemolysis is observed if phototreated cells are stored in a medium that protects against colloidal osmotic hemolysis. Hemolysis is further reduced to 0.3% if a competitive inhibitor for dye binding to red cells is added.

Chalcogenoxanthylium Dyes. Another promising class of cationic photosensitizing dyes that have been studied for pathogen reduction in red cell suspensions is the chalcogenoxanthylium class.[122] The photosensitizer TMR-Se (**44**, Chart 15) is extremely virucidal in red cell suspensions. Only 1 μM compound and 2 J cm^{-2} light were required to inactivate >7.4 $\log_{10}$ of extracellular VSV in 20% hematocrit red cells. With 45% hematocrit red cells, >7.4 $\log_{10}$ of extracellular VSV was inactivated with 5 μM and 2 J cm^{-2} light. Under these conditions, there was little hemolysis (0.22% and 0.36% hemolysis, respectively) during the 42-day storage. Unfortunately, no inactivation of intracellular virus was observed under these conditions. Derivatives of TMR-Se are currently being investigated with better potential for intracellular inactivation because of improved permeation across cell membranes.

Perspective

Photodynamic therapy is becoming a common treatment modality in the clinic for various forms of cancer and for age-related macular degeneration. In ex vivo procedures, PDT is being used for the purging of viral and bacterial pathogens from whole blood and blood components and for the purging of leukemia cells from bone marrow and stem cells prior to their reintroduction into the patient. For procedures done in vivo, nonspecificity is an issue. Investigators have demonstrated that many porphyrin and porphyrin-related photosensitizers act via damage to the vasculature feeding the tumor[44,101] and by direct cellular toxicity.[102] Cationic dyes target the mitochondria and the lysosomes. In an ideal situation there would be a single site or sites where the photosensitizer is sequestered or bound so that structure−activity relationships might be addressed to improve binding to a single site. Tetraarylporphyrins bearing amino acids, peptides, or diamines (Chart 3) have demonstrated excellent surface recognition for potassium channels.[57] This type of combinatorial approach could be applied to optimize photosensitizer binding and performance at other sites. Antibody-conjugated porphyrins have been described[76,111] and offer promise for increased selectivity. However, this approach has limitations because of the diversity of tumor types and their cellular makeup. Antibodies may be too specific and only target antigens on a single or severely restricted cell phenotype, leaving others that lack the specific antigen to continue to multiply following treatment. Advances in "recognition" chemistry associated with the photosensitizer is an open area for PDT research.

Improved uptake in tumors (or around tumors if the neovasculature is targeted) can be realized by slight structural modifications. PEG−*m*THPC (**33**, Chart 12) conjugates give increased selectivity in rat liver and rat ovarian tumor models[93] and reduced PDT damage to normal tissues.[94] The number of solubilizing groups influences the uptake of porphyrin,[67] core-modified

porphyrin,[62,68] and phthalocyanine photosensitizers.[78,79] In HPPH-related structures (**36**, Chart 13), very large values of log *P* appear to be important for uptake in tumor.[97] Continued SAR studies should also give improved uptake and localization of photosensitizers in or around the tumor.

Increased cellular uptake has been described for nanoparticle-bound photosensitizers.[112] This approach will probably be applied to many different classes of photosensitizer. However, the nanoparticles may need to be tailored to provide appropriate selectivity. Although binding or adhering photosensitizers to synthetic substrates may increase cellular uptake of a particular photosensitizer, the possibility that this may occur in normal tissue may be just as great as in malignant lesions. Furthermore, the nanoparticle-bound photosensitizer will not be able to recognize specific subcellular sites.

PDT appears to be an excellent treatment modality against multidrug-resistant tumors[90,120,131] and against recurrent breast cancer,[41,46,86] and one might expect the use of PDT for these diseases to be expanded in the future. Photosensitizers that are not substrates for P-gp or the multidrug-resistance (MDR) protein will be effective against both drug-responsive and drug-resistant phenotypes. Photosensitizers that are substrates for P-gp or the MDR protein may damage these proteins upon irradiation, which may then allow conventional chemotherapeutic drugs to do their work. Combination therapies may prove to be effective. Recurring lesions following breast cancer surgery can be treated with a combination of PDT and chemotherapy without further surgery.

Future work on the development of photosensitizers for ex vivo procedures will also focus on improvements in specificity as well as on analogues with longer wavelengths of absorption. In particular, the purging of bacterial and viral pathogens in red cells would be more effective at wavelengths well removed from the absorption of hemoglobin.

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Biographies

Michael R. Detty obtained his B.Sc. degree in Chemistry from Bowling Green State University, Bowling Green, OH, and his Ph.D. in Organic Chemistry from The Ohio State University, Columbus, OH. After 17 years in the Research Laboratories of the Eastman Kodak Company, Rochester, NY, he joined the Department of Medicinal Chemistry in the School of Pharmacy at the University at Buffalo, The State University of New York, as an Associate Professor in 1995 and is currently a Professor in the Medicinal Chemistry Division of the Department of Chemistry at the University at Buffalo. His areas of interest include near-infrared-absorbing dyes as photosensitizers for photodynamic therapy and organochalcogen compounds as catalysts for the activation of hydrogen peroxide.

Scott L. Gibson received his B.Sc. degree in Biology from Hobart College, Geneva, NY, and his M.Sc. in Environmental Studies from the University of Rochester, Rochester, NY. After serving as a research technician and medical specialist for 4 years in the Army, he joined the University of Rochester in 1973 as a research biochemist in hormone research related to breast cancer. Since 1980, he has investigated mechanism of action of photodynamic therapy. He has over 70 publications in peer-reviewed scientific journals.

Stephen J. Wagner received his B.Sc. degree in Chemistry from the University of Maryland, College Park, and M.Sc. and Ph.D. degrees in Biophysics from the Pennsylvania State University, University Park. After a postdoctoral fellowship at Frederick Cancer Research Center, he joined Zetachron, Inc., where he worked as a Staff Scientist for 5 years. He joined the Product Development Department of the American Red Cross Holland Laboratory for the Biomedical Sciences in 1989 and has initiated research programs investigating virus inactivation of cellular blood components, transfusion-associated bacterial sepsis, and the isolation and culture of mononuclear cells. Dr. Wagner is a recipient of the American Red Cross Tiffany Award for technical excellence and serves on the editorial board of the journal *Transfusion*. He currently directs the blood processing section of the Blood Component Development Department of the American Red Cross.

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