Photodynamic therapy of cancer: second and third generations of photosensitizers

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Photodynamic therapy of cancer (PDT) at early stages of various types of cancer is based on the use of the phototoxicity of photosensitizers (PS) which appears upon their excitation by light in tumor tissues. The review concentrates mainly on the data for the second and third generations of photosensitizers: the structure of PS, their photophysical and photochemical properties, and some results of their *in vitro* and *in vivo* application. A carefully designed PS should exhibit the following properties: long-wave absorption, good singlet oxygen quantum yield, an intramolecular polarity axis, and low *in vivo* (photooxidation) stability. Uncharged PS that are lipophilic, positively charged, and capable of binding with monoclonal antibodies are discussed as an example.

Key words: photosensitizers, naphthalocyanines, phthalocyanines, porphyrins; photodynamic therapy of cancer.

Introduction

As a phototherapeutic method, the photodynamic therapy of cancer (PDT) is based on the use of the phototoxicity of photosensitizers (PS), which appears after they are excited by light, with the aim of local destruction of various cancer tumors at their early and middle stages. The PS used for this purpose are diverse derivatives of porphyrin and related macrocycles. The method is applicable for long-term control of many early stages of cancer and may be used as a palliative for cancer at advanced stages (see Refs. 1—4 for reviews). The treatment of a patient includes several steps.

1. First, a solution of a suitable PS (0.5—5.0 mg per 1 kg body weight) is injected systematically (intraveneously, intraperitoneally; for skin cancer, local administration is also possible), and then time is allowed for clearance of serum and accumulation of the photodrug in the tumor. After ~10—30 h, the concentration of the PS in the tumor is often higher than that in peritumoral tissues. The fluorescence of the PS in the ultra-violet or visible light region may be detected after the irradiation with low light intensities of shorter wavelenght and can be used as a diagnostic means to probe the localization of the tumor

2. Irradiation with an appropriate dose of visible or near infrared light (in the longest-wavelength absorption region of the PS) results in the *in situ* activation of the photodrug and subsequent photoreactions with components of the tumor tissue. Cell disintegration is completed within the first hours after photodynamic treatment. Necrosis and apoptosis are considered as the main cell death mechanisms.[5]

3. Profound vascular and cellular effects occur within 12 to 18 h, causing a number of processes. After 2—3 weeks, hemorrhagic necrosis of the tumor tissues takes place.

The photosensitizers should exhibit good phototoxicity and low dark toxicity. In summary, the phototoxicity of a PS is determined by the following main factors: its photophysical and photochemical properties, its structure and charge, its intracellular concentration, and its subcellular localization.[5] Negatively charged and very polar PS are trapped *via* pinocytosis and are thought to be accumulated mainly in cytoplasmic liposomes. Cationic PS penetrate through hydrophobic cellular membranes due to the fact that the membranes are charged negatively on the inside surface. The PS which are delivered by liposomes and then transported with low density lipoproteins (LDL) mainly use the receptor-mediated endocytoxic pathway. After a number of steps, this pathway ends up in lysosomes. The intracellular localization of PS depends on the pathway of their trapping. For example, hematoporphyrin derivatives HpD/PII (see below) have been detected mainly in membranes (in cellular, nuclear, and mitochondrial membranes and in endoplasmic reticulum). Other, more lipophilic anionic PS are also generally localized in

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Table 1. Photosensitizers studied for clinical applications

Photosensitizer	Producer	
	Company, institute	City (country)
Al phthalocyanines (Photosens)	Research Institute of Organic Semiproducts and Dyes	Moscow (Russia)
Zn naphthalocyanines[a]	Bulgarian Academy of Sciences	Sofia (Bulgaria)
m-THPC	Scotia Pharmceut	Guildford (Great Britain)
Porphycenes	Cytopharm/partner	Menlo Park, CA (USA)
Monoaspartylchlorines (MACE)	Nippon Petrochemical	Tokyo (Japan)
Purpurins, Cetiopurpurin	PDT Inc.	Santa Barbara, CA (USA)
Texaphyrins	Pharmacyclis	Palo Alto, CA (USA)
δ-Aminolevulinic acid	DUSA	Toronto (Canada)
δ-Aminolevulinic acid	Medac GmbH	Hamburg (Germany)

[a] In preparation for clinical trials.

membrane structures, whereas hydrophilic PS seem to accumulate in lysosomes. Certain cationic PS accumulate preferentially in mitochondria. Hydrophobic PS delivered by liposomes or LDL have been found in membranes, mitochondria, endoplasmic and rough reticulum, and in endothelium (in smaller amounts).

The practical use of photosensitizers requires that their mutagenic and carcinogenic properties be taken into consideration. No statistically significant mutagenic potential was found for HpD/PII or sulfonated aluminum phthalocyanines.[1] It should, however, be taken into account that only small amounts of PS are injected into an organism. It is also interesting to note that the role of metabolism in removal of these compounds is either small or its contribution is not detected at all;[1a] PS are removed from the organism unchanged.

Clinical application of PDT and related fields of therapy

For *in vivo* applications of PDT, photosensitizers with absorption in the wavelength range from 600 to 850 nm are employed. In this spectral region, the maximum penetration depth of light into a tissue can be obtained (up to 2—3 cm under favorable circumstances). The majority of clinical applications of PDT have been performed using a mixture of hematoporphyrin derivatives (HpD) as the photosensitizing agent. Partially purified HpD named Photofrin II (PII; American Cyanamid/Lederle Laboratories, USA) or similar compounds (Photosan, Photohem, Photocarcinomin) are employed. In a number of countries (Canada, USA, Netherlands, Japan), these compounds are extensively used in clinical applications against some tumors. Because these compounds exhibit some disadvantages (in particular, light with a wavelength of ~630 nm is required), new photosensitizers with absorption shifted toward the red region (675 to 800 nm range) have been developed. Some of these PS are under clinical phase I/II trials (Table 1).

For treatment of cutaneous lesions, PDT treatment is as easy as illuminating an area of the skin. For treatment of internal cancers, the light is delivered *via* optical fibers. In modern photodynamic treatment, lasers are used as the light source because of their ability to create a small coherent light beam, which can be transferred efficiently to a distance through various fibers 200 to 400 μm in diameter. Since the light delivery method and light dose in PDT are determined by the kind and size of the tumor, much experience is required for a successful treatment. Gas and solid-state lasers as well as dye lasers pumped by Ar laser and Nd-YAG laser are used most commonly. In the future, semiconductor lasers that are less expensive and more easy to handle will acquire greater importance. The total dose of light delivered to neoplastic areas is usually varied from 80 to 600 J cm^{-2} in order to reduce the effects of hyperthermia.

The application of PS for the inactivation of viruses in blood is currently the subject of intense interest. Excellent virus killing is achieved with PS such as benzoporphyrins and sulfonated phthalocyanines, whose absorption is red-shifted relative to hemoglobin. The treatment of diseases caused by cytomegalovirus, herpes simplex virus (HSV), and human immunodificiency virus (HIV) is under investigation.[6]

Administration of δ-aminolevulinic acid (δ-ALA, $NH_2CH_2COCH_2CH_2COOH$) induces the biosynthesis of photosensitizing concentrations of endogenous protoporphyrin IX. Due to the advantages of this method, such as tissue specificity, rapid removal, and possibility of topical administration, δ-ALA is subject to extensive preclinical and clinical study by photodynamic therapy as a possible replacement for the usual PS (Medac GmbH, Hamburg, Germany).[7]

Photosensitizers in PDT

Photosensitizers used in therapy should exhibit the following properties:

(1) high quantum yield of the triplet state ($\varphi_T > 0.4$) combined with high triplet lifetime ($\tau_T > 100$ μs) because photochemical reactions predominantly occur in the excited triplet state;

(2) absorption in the long-wave region ($\lambda > 630$ nm, preferably $\lambda > 680$ nm) with high extinction coefficients ($\varepsilon > 50000$ L mol^{-1} cm^{-1}) is required to increase the penetration depth of light into the tissue and the number of photons absorbed, since light flux in the tissue decreases exponentially with distance;[1a]

(3) limited *in vivo* stability for rapid removal from the tumor and peritumoral tissues;

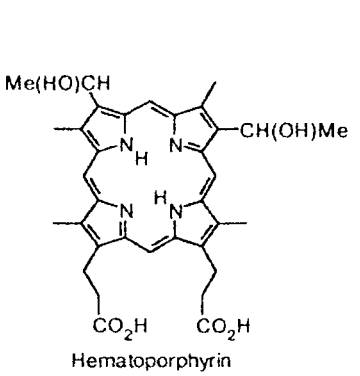
(4) selective transport of the PS to the tumor in order to reduce side effects in peritumoral tissues;

(5) low dark toxicity of the PS and their degradation products;

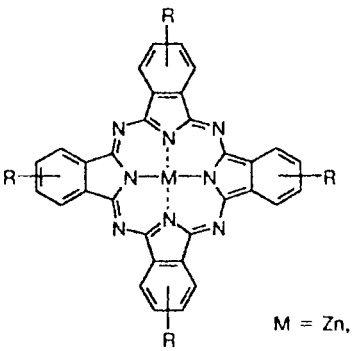
(6) availability.

Information on the PS used for PDT is shown in Tables 1 and 2. The structural formulas of certain PS are presented below.

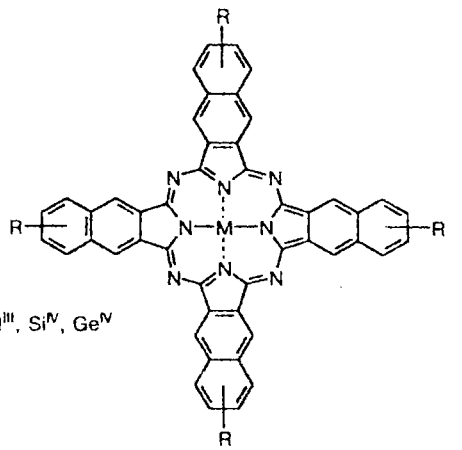
The principal cause of photodamage in PDT involves the following processes.[5] The excitation of a PS occurs *via* an intersystem crossing from the excited singlet state (^{1}PS*) to the triplet state (^{3}PS*) (Eq. (1)). The so-called type I reactions correspond to radical reactions with photoinduced electron transfer (redox reactions) and occur either as reductive quenching by biomolecules (Eq. (2)) or as oxidative quenching by O_2 (Eq. (3)). Type II reactions involve photoinduced energy transfer from the triplet state of the PS (^{3}PS*) to triplet oxygen ($^3O_2$, $^3\Sigma_g^-$) with formation of singlet oxygen ($^1O_2$, $^1\Delta_g$) followed by oxidation (Eq. (4)). It is generally agreed



Hematoporphyrin

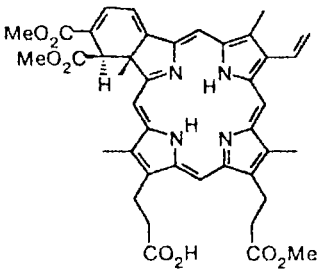


Phthalocyanine (Pc)

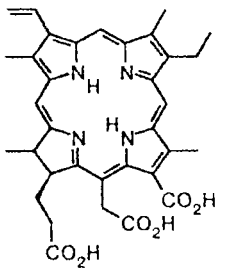


M = Zn, Al^{III}, Si^{IV}, Ge^{IV}

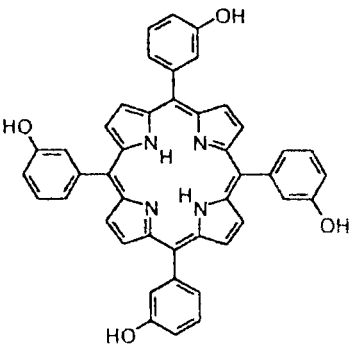
Naphthalocyanine (Nc)



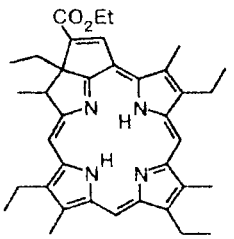
Benzoporphyrin (monoacid)



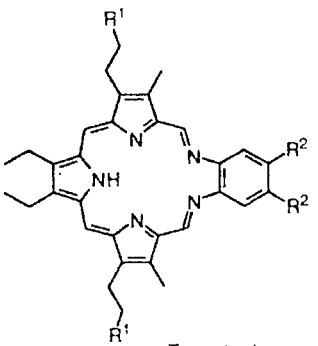
Chlorine e_6



m-THPC



Etiopurpurin (used as a Sn-complex)



Texaphyrin

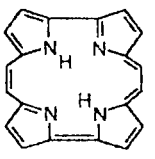

Porphycene

Table 2. Some photophysical properties of first- and second-generation photosensitizers

Photosensitizer	ε /L mol^{-1} cm^{-1}	λ_{max} /nm	φ_T [a]	φ_Δ [b]
Hematoporphyrin[c]	3500	630	0.83	0.65
Photofrin II (PII)[c]	~3000	~630		~0.2
Zn phthalocyanine[c]	150000	675	0.6	0.59
Al phthalocyanine-tetrasulfonic acid[d]	105000	675	0.38	0.38
Zn phthalocyanine-tetrasulfonic acid[d]	115000	672	0.56	0.52
Zn naphthalocyanine[c]	160000	764		0.45
Benzoporphyrin[c]	118000	685		0.60
Bacteriochlorine[c]	150000	785	0.32	0.32
Zn etiopurpurin[c]	~70000	~690	0.83	0.57
Porphycene[c]	52000	630	0.42	0.30

[a] Quantum yield of triplet state.
[b] Quantum yield of $^1O_2$.
[c] The parameters were determined in an organic solvent.
[d] The parameters were determined in water.

that singlet oxygen is the key agent in cellular damage.[8] In addition, photothermal effects due to nonradiating deactivation of excited states also leads to photosensitized cell death (Eq. (5)).[2,9]

$$PS + h\nu \rightarrow {}^1PS^* \rightarrow {}^3PS^* \quad (1)$$

$${}^3PS^* + RSH \text{ or } RNR'_2 \rightarrow \rightarrow PS^{\cdot -} + (RSH)_{ox} \text{ or } (RNR'_2)_{ox} \quad (2)$$

$${}^3PS^* + O_2 \rightarrow PS^{\cdot +} + O_2^{\cdot -} \rightarrow \rightarrow \text{subsequent reactions of cellular targets} \quad (3)$$

$${}^3PS^* + {}^3O_2 \rightarrow PS + {}^1O_2 \rightarrow \rightarrow \text{subsequent oxidation of cellular targets} \quad (4)$$

$${}^1PS^* \text{ or } {}^3PS^* \rightarrow PS + Q \rightarrow \text{cell death} \quad (5)$$

The photosensitizers employed in PDT or investigated for this purpose can be divided into three generations:

— *First generation*: HpD, PII (mixtures of PS); no selective accumulation in the tumor.

— *Second generation*: structurally distinct compounds, which in most cases display long-wave absorption; no selective accumulation in the tumor.

— *Third generation*: second-generation PS bound to carriers for selective accumulation in the tumor.

The first generation of photosensitizers

Hematoporphyrin derivatives (HpD, PII, Photohem, *etc.*) belong to the first generation of PS and are mostly used in clinical applications. They are obtained by treatment of hematoporphyrin with 5% H_2SO_4 in acetic acid at room temperature.[8] In order to prepare solutions for injection, HpD is treated with an aqueous base and then neutralized. Hematoporphyrin derivatives (HpD) consist of a mixture of several monomeric and oligomeric compounds.[4,10] The monomeric fraction is removed using HPLC or GPC. The HpD photosensitizer and its commercial variants exhibit four main disadvantages.

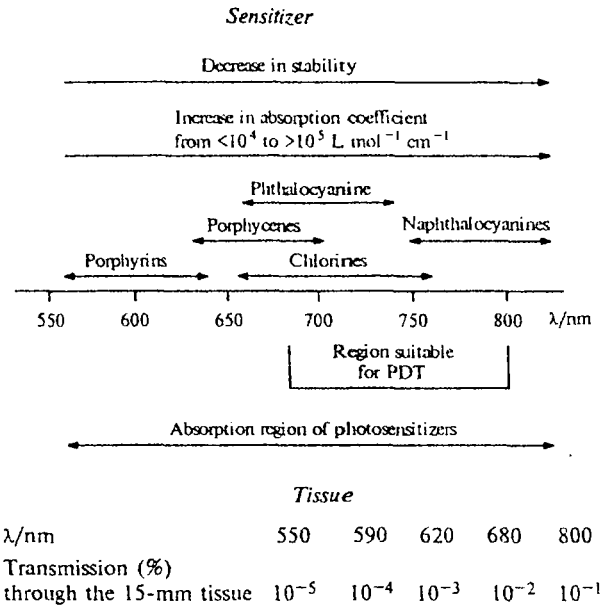
1. The longest-wave absorption band appears at 630 nm (with a low extinction coefficient); the biological effects after irradiation at this wavelength occur only to a tissue depth of ~5 mm (Scheme 1, Table 2).

2. Nonselective accumulation in the tumor results in distribution of PS between the tumor and the skin, with tumor/skin ratios of ~2 : 1. The amount of sensitizers in various organs decreases in the series: liver > urinary bladder > pancreas, kidney, spleen > stomach, bone, lung, heart > skin > muscle ≫ brain. Only ~0.1—3% of the injected PS amount is found in the tumor tissue.

3. The sensitizer is retained in cutaneous tissues for 2—3 months (after 75 days, 61% of PII in the spleen and 30% in the lung is still present), which requires that the patient avoids bright light.

4. The complex and difficultly separable mixture of compounds does not allow one to use only its active components.

Scheme 1



The second generation of photosensitizers

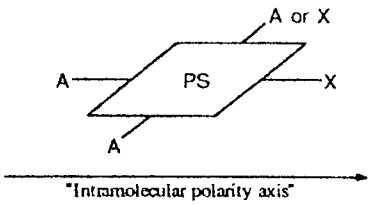
Photophysical and photochemical properties. The second generation of PS, which are in some cases used in phase I or phase II clinical trials, has absorption mainly from 675 to 800 nm. Light with this wavelength penetrates into tissues to a depth of up to 2—3 cm (see Scheme 1, Table 2). The interpretation of dose—response relationships for these PS (phthalocyanines,[4,11] naphthalocyanines,[12] benzoporphyrins,[13a,b] chlorines,[4] purpu-

rine,[13c] texaphyrins,[13d] porphycenes[13e]) is easier because they are not mixtures of compounds. In order to have good photophysical properties, these PS should either be metal-free or contain metal ions with closed d-electron shell configurations (Zn^{II}, Al^{III}, Si^{IV}, Ge^{IV}). A potential photosensitizer should be discussed in terms of its photophysical and photochemical properties, structure (hydrophobicity/hydrophilicity), and stability. Most PS of the second generation absorb at $\lambda > 675$ nm with $\varepsilon > 50000$ L mol^{-1} cm^{-1}. Singlet oxygen quantum yields (φ_Δ) are higher than 0.3. Since the diffusion distance of singlet oxygen in cells during its lifetime is less than 100 nm, local damage can be expected at its sites of origin.[14a] Therefore, in principle, the photodynamic activity of the aforementioned PS directly depends not on their $^1O_2$ quantum yields but on their different uptake mechanism and subsequent intracellular concentration and localization.[14b] In addition, as noted above, oxidative or reductive quenching *via* photoinduced electron transfer is possible.

Photosensitizers with hydrophilic groups. The skeletons of porphyrin and phthalocyanine are hydrophobic. In order to obtain water solubility required for injection, various polar hydrophilic substituents are introduced in photosensitizers: sulfonic, carboxy, hydroxy, quaternary ammonium, or pyridinium groups. Of 5,10,15,20-tetraphenylporphyrins bearing hydroxy groups at the phenyl substituent, the 3-hydroxy and 3,4-dihydroxy derivatives are ~30 times as potent as HpD in sensitizing tumors.[4,15] Chlorines (*e.g.*, chlorine e_6) and bacteriochlorines (like bacteriochlorophyll *a* and bacteriopheophorbide) contain hydrophilic or polar carboxylic acid groups. These compounds are well accumulated in tumors and display photodynamic activity. The photochemistry and photobiology of sulfonated Al^{III} phthalocyanines (Pc) have been studied intensely.[3,9] In the case of sulfonated Pc, the photodynamic properties are significantly affected by the degree of sulfonation. Mono- and disulfonated AlPc, GaPc, and ZnPc were found to be the most efficient PS in tissue culture, whereas tri- and tetrasulfonated Pc were less efficient: $AlPc(SO_3H)_2 \approx ZnPc(SO_3H) > AlPc(SO_3H) > AlPc(SO_3H)_3 \approx ZnPc(SO_3H)_2 > AlPc(SO_3H)_4 > ZnPc(SO_3H)_4$. Despite the fact that large aromatic dyes of the Pc type have a tendency to aggregate (particularly in water), low-sulfonated Pc display uniform cytoplasmatic fluorescence and good photodynamic activity, indicating their monomolecular state in the cell. Disulfonated Pc with substituents located at adjacent benzoid rings of the Pc nucleus were found to accumulate more efficiently. Subcellular localization was suggested to be responsible for the different phototoxicity of different sulfonated Pc. It should be mentioned that accumulation of all these hydrophilic Pc in the tumor is not selective but is enhanced compared to HpD (and similar mixtures). For example, the tumor/skin ratios in various tumors vary from 2 : 1 to 10 : 1 for partially sulfonated AlPc.

From these results it becomes evident that localization of PS in the tumor is improved if these molecules

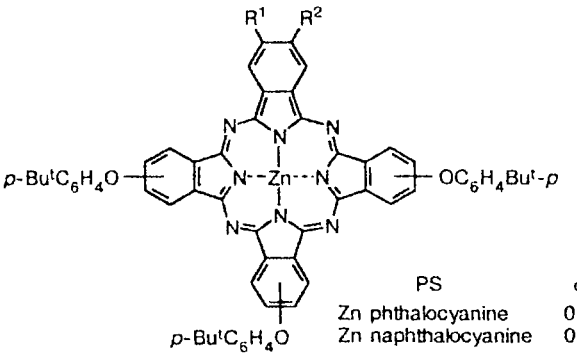
Scheme 2



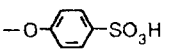
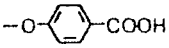
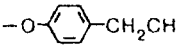
possess an "intramolecular polarity axis" owing to a suitable combination of hydrophilic and hydrophobic substituents or structural elements. The configuration of an optimized PS (A is an H atom or nonpolar lipophilic groups, X is a polar or active substituent) is shown in Scheme 2: one or two polar groups X form an "intramolecular polarity axis." One of these polar groups X (—COOH, —OH, $—NH_2$) can be used for linking to carriers. These structurally optimized PS are oriented in a certain way on the cell membrane to allow better accumulation in the tumor in specific cell compartments. The structures of some new PS with several polar reactive groups or positive charges are shown below.

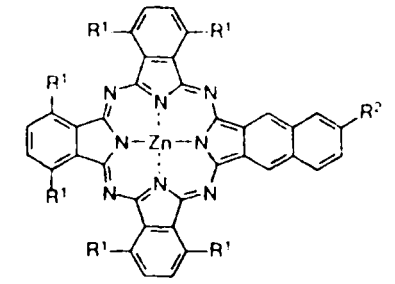
Positively charged porphyrins and phthalocyanines substituted with methylated pyridinium groups also cause significant tumor necrosis or exhibit *in vitro* activity.[18] Photosensitizers can be ordered according to the relative uptake and cell killing efficacy as follows: cationic > neutral > anionic compounds.[18b] Cationic PS are preferable due to the negative membrane potential.

Porphyrazines bearing from one to eight positively charged centers were obtained by statistical cyclotetramerization of pyridyloxy-substituted 1,2-benzene-

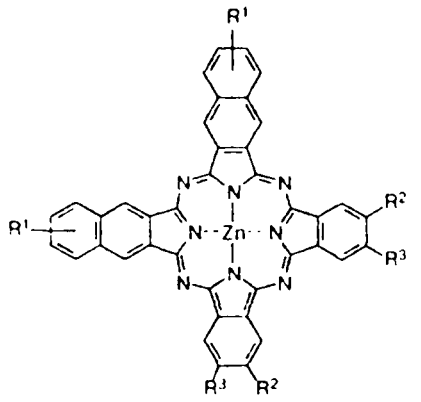


PS	φ_Δ
Zn phthalocyanine	0.56
Zn naphthalocyanine	0.45

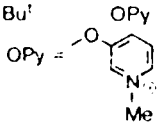
R^1	R^2	λ_{max} (DMF)	φ_Δ
	H	678	0.57
	H	675	0.50
	H	681	0.40



R^1	R^2	λ_{max} (DMF)	φ_Δ
OC_6H_{13}	COOH	749	0.49
OC_6H_{13}	OH	751	0.21



R^1	R^2	R^3	λ_{max} (DMF)	φ_Δ
Bu^t	H	OPy	716	0.43
Bu^t	OPy	OPy	716	0.39



dicarbonitriles and 6-*tert*-butyl-2,3-naphthalenedicarbonitrile.[18d] The mixture of porphyrazines with different pyridyloxy substituents was separated by the usual flash chromatography and converted into positively charged PS by methylation with CH_3I. *In vitro* experiments with 10 μM solutions of the PS containing $5 \cdot 10^5$ HeLa cells in 10% aqueous fetal bovine serum (FBS) show, after incubation for 18 h, that the uptake of PS increases with increasing number of positively charged centers and is 3%, 4.2%, and 5.4% for three, four, and eight positive charges, respectively.* However, cell phototoxicity is highest for the PS with three positive charges (shown in Fig. 1 as an example), because the singlet oxygen quantum yield ($\varphi_\Delta = 0.64$) is highest (for other PS, $\varphi_\Delta < 0.49$).

Hydrophobic photosensitizers. The photosensitizers which are too hydrophobic to be water soluble, such as unsubstituted phthalocyanines, naphthalocyanines, or their derivatives with alkyl, aryl, ether, thioether, amino, aminoalkyl, and amido substituents, have to be dissolved in a suitable delivery system before injection. Unilamellar liposomes, *e.g.*, those based on dipalmitoyl phosphatidylcholine (DPPC), or oil emulsions like Cremophor were used as delivery systems.[11,12,19] This method provides an easy way to make good PS available for PDT. Even strongly aggregating naphthalocyanines can be dissolved in monomeric form in aqueous DPPC.[12] The hydrophobic PS are located in the monomeric state in the lipophilic part of the double layers of liposomes (~50—100 nm in diameter). After injection, the PS are preferably transferred to low-density lipoproteins (LDL) in the blood serum. The plasma LDL consists of a core with hydrophobic lipids surrounded by polar lipids and apoproteins (22 nm in diameter). One LDL can incorporate more than 150 porphyrin molecules in the lipid core due to hydrophobic interactions. The targeted delivery of LDL carrying PS to the tumor is achieved due to the unique features of receptor-mediated preferential endocytosis of LDL by malignant cells. Hence, a large amount of LDL-associated PS can be transported. *In vivo*

* U. Michelsen, T. Nishisaka, I. Okura, and D. Wöhrle, unpublished results

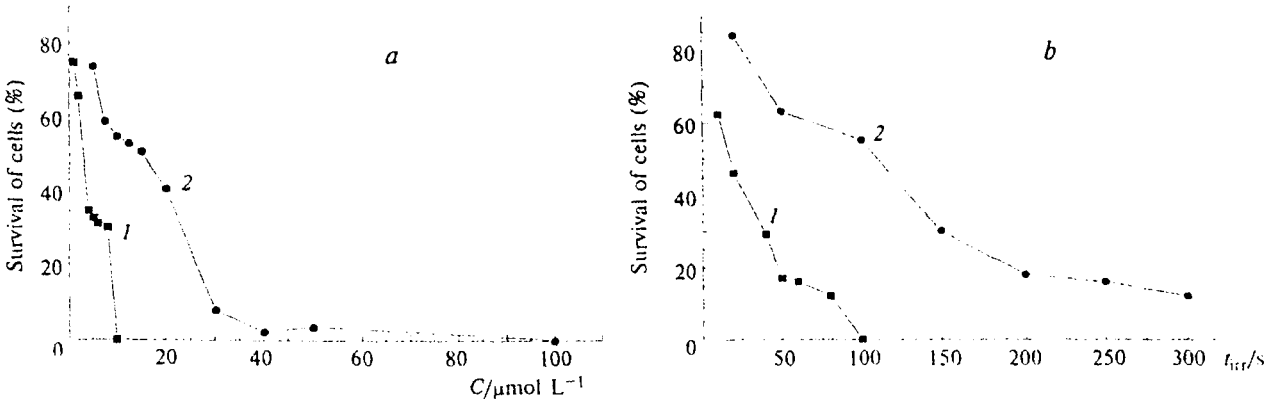


Fig. 1. Phototoxicity of Zn porphyrazines with three (*1*) and four (*2*) positive charges in HeLa cells (incubation of 10 μmol of PS with $5 \cdot 10^5$ HeLa cells in 10% FBS): *a*, concentration dependence (illumination power 50 mW cm^{-2}), *b*, dependence on irradiation time (50 mW cm^{-2})

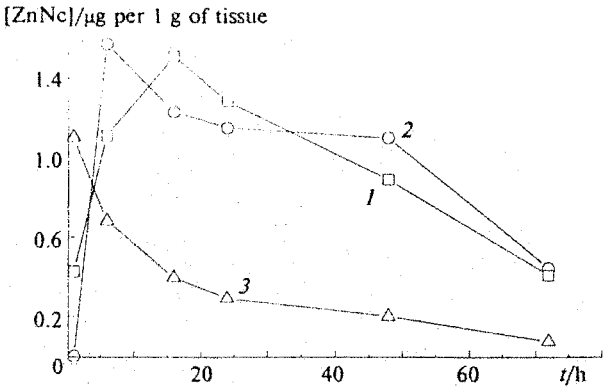


Fig. 2. Pharmacokinetics of unsubstituted Zn naphthalocyanine in black mice C57 with Lewis lung carcinoma (size ~5 mm) transplanted into a right leg (0.25 mg of PS in DPPC liposome per 1 kg body weight, intraperitoneal injection). Curves: *1*, tumor; *2*, liver; *3*, skin.

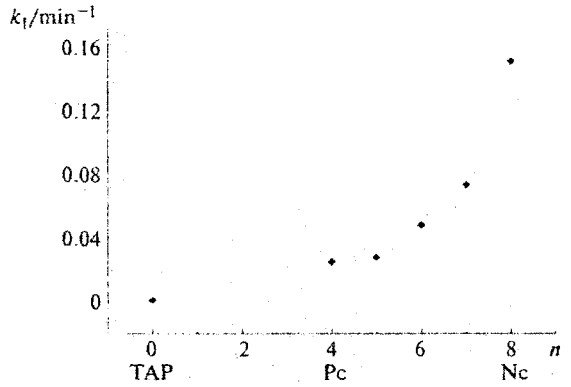


Fig. 3. Photostability of various unsubstituted and substituted Zn^{II} tetraazaporphyrin chelates (10^{-5} mol L^{-1}) measured in DMF upon light illumination at λ = 350—850 nm and a power of 20 mW cm^{-2} (*n* is the number of linearly annelated benzene rings; TAP is tetraazaporphyrin; Pc is phthalocyanine; Nc is naphthalocyanine). The measurements were carried out in the air.

pharmacokinetic experiments with mice bearing Lewis lung carcinoma 16 h after administration of Zn-naphthalocyanines dissolved in liposomes show a PS tumor/skin ratio of ~4 : 1 (Fig. 2).[12] LDL can also be used as a delivery system for PS.

Stability of photosensitizers. Attention should be given to the *in vivo* stability of photosensitizers. As mentioned above, HpD/PII, which have low selectivity for accumulation in the tumor, are retained in cutaneous tissues for 2 to 3 months. Nonselective second-generation PS, such as sulfonated Al phthalocyanines, can stay for several weeks in tissues others than the tumor[1a] (skin), causing light sensitivity of the patient. On the other hand, if photobleaching or decomposition of the PS occur before *in vivo* reactions with biomolecules are initiated, no tissue damage is incurred. Therefore, the following requirements are imposed on PS. The total

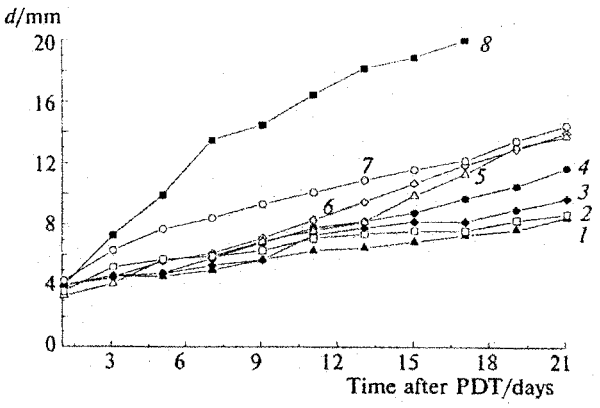


Fig. 4. Comparison of the growth rate of Lewis lung carcinoma (*d* is the mean tumor diameter) transplanted into a left leg of black mice C57 and treated with Zn naphthalocyanines with various substituents R (0.25 mg of Zn naphthalocyanines dissolved in DPPC liposomes per 1 kg body weight): R = NHCOPh (*1*), $NHCOC_5H_{11}$ (*2*), NHCOMe (*3*), H (*4*), $NHCOC_6H_4OMe$ (*5*), NH_2 (*6*), $NHCOC_{11}H_{23}$ (*7*); control (without PS) (*8*). The illumination dose was 450 J cm^{-2}, 20 h after intraperitoneal injection of PS.

in vivo stability should not exceed 2—3 days, since second generation PS reach maximum tumor/other tissue ratios in one day. The photostability should be less than 2—3 days. On the one hand, it should allow *in vivo* photoreactions. On the other hand, however, self-shielding of PS with high extinction coefficients is known to occur. Hence, decreased photostability increases the therapeutic depth of laser light penetration into the tissue, because PS molecules in the upper cellular layers photobleach during illumination faster than those in deeper layers. Therefore, the absorption of the already dead cells and photobleached PS molecules allows deeper light penetration during photodynamic treatment.

The photostability of various Zn complexes of tetraazaporphyrins in DMF upon irradiation in the air was determined. The measured decrease in intensity (*A*) of the longest-wave absorption (*Q* band) of the complex (and hence its concentration) obeys first-order kinetics: $\ln(A_0/A_t) = k_1 t$.[20a] It is seen that the photostability decreases with an increase in the number of annelated benzene rings at tetraazaporphyrins (Fig. 3).[20b,c] Tetraazaporphyrins are too stable for the use in PDT. It was shown for zinc naphthalocyanines that the skin is almost completely cleared after ~72 h (see Fig. 2), which makes long-term skin sensitization unlikely. Photodynamic *in vivo* experiments with mice bearing Lewis lung carcinoma treated with very low amounts of differently substituted zinc naphthalocyanines can cause a pronounced delay in tumor growth depending on the substituent (Fig. 4).[12] A reliable increase in the average survival time of the mice was achieved.

Comparison of the first and second generations of PS. Figure 5 compares the effect of photodynamic treatment for different generations of PS (change in the

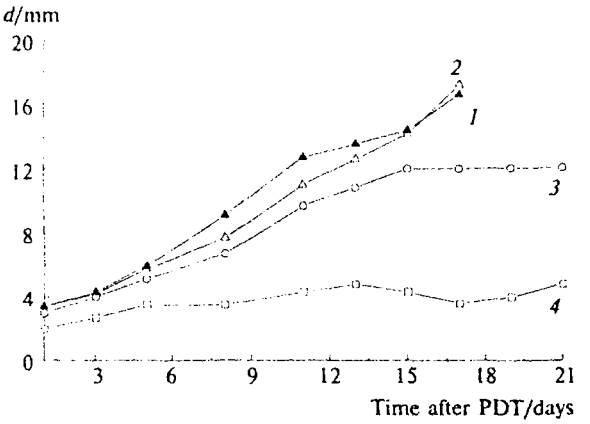


Fig. 5. Comparison of the growth rate of B16 pigmented melanoma (*d* is the mean tumor diameter) in mice under exposure to light (the dose was 360 J cm^{-2}) in the region of photosensitizer absorption: *1*, HPD (5 mg per 1 kg of body weight, illumination at λ = 630 nm); *2*, ZnPc (0.3 mg per 1 kg of body weight, illumination at λ = 673 nm); *3*, ZnNc (R = H) (0.3 mg per 1 kg of body weight, illumination at λ = 756 nm); *4*, NHCOPh (0.5 mg per 1 kg of body weight, illumination at λ = 774 nm).

main tumor diameter of B16 pigmented melanoma).[21] This skin cancer is quite common in some countries, *e.g.*, in Australia, due to the increase in UV radiation on the Earth. At the end of the observation period (21 days after PDT), the PDT effect was absent for the HpD PS and for ZnPc (as well as in control groups without PS). Substituted zinc naphthalocyanines are the only known PS that are active against pigmented melanoma. Of zinc naphthalocyanines, the derivative with R = NHCOPh is most active and is now under preclinical study.

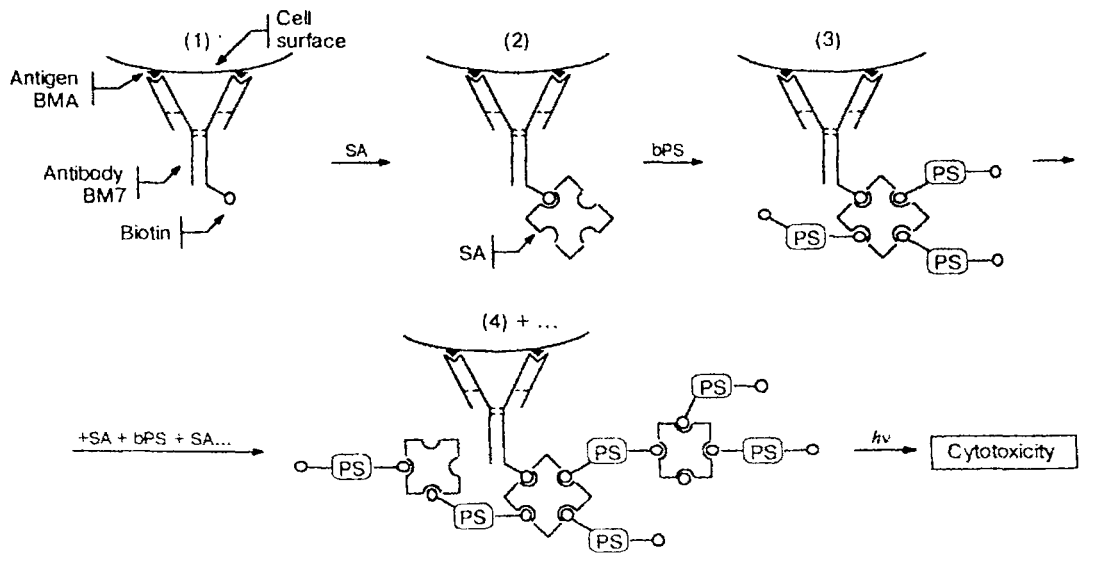
Third generation of photosensitizers

In addition to the characteristics of the second-generation PS, photosensitizers of the third generation should have properties providing selective delivery of PS to the tumor tissue, *e.g.*, by conjugation to biomolecules such as monoclonal antibodies (mAB). Malignant tumor cells have cell surface antigens which are different from those of normal cells. Monoclonal antibodies directed toward tumor cell antigens are able to couple to photosensitizers. The mAB—PS conjugate will bind specifically to the tumor tissue and sensitize its photokilling without damaging normal tissues.[22a] This difference in affinity to tumor and normal tissues is favorable for immunotherapy by mAB—PS conjugates. A large amount of research has been done in the last years on the use of antibodies in the diagnosis and treatment of cancer.[22b] Various toxic agents have been conjugated to antibodies, including radioisotopes, microbial toxins, and PS.[22c] After coupling of PS molecules to the antibody, it is important to keep the binding affinity of the immunoglobulins toward tumor-associated antigens.

Three strategies for binding PS are presently being studied: (1) direct binding of PS to the constant part of mAB (disadvantages: binding often also occurs to variable parts of mAB; accumulation of too small amounts of PS); (2) binding of PS to polymers (*e.g.*, dextran) and covalent linking of the polymer to mAB (disadvantages: binding often also occurs to variable parts of mAB; shielding of the mAB by the polymer); (3) multiplicative effect, which uses polyphasic tumor therapy to accumulate 10^6—10^8 PS molecules (assuming 10^4—10^5 antigen binding sites).[23]

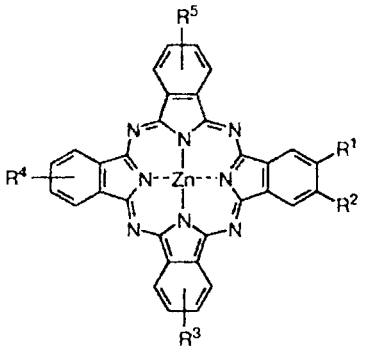
Let us demonstrate some recent results of the latter strategy.[24] To create a multiplicative system, the accu-

Scheme 3

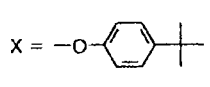


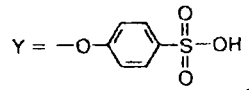
mulating properties of the avidin-biotin system were used for targeted delivery of PS specifically onto labeled tumor cells. Scheme 3 shows multiple binding of avidin and biotinylated PS to biotinylated mAB linked to the surface antigens of the tumor cell:[23] (1) cells with tumor marker are treated with biotinylated mAB; (2) after the excess mAB is removed, avidin or streptavidin is added (binding of avidin to biotin linked to mAB); (3) after the excess avidin is removed, biotinylated PS (bPS) are added (binding to various free sites of the mAB—avidin conjugate); (4) multiple addition of avidin and then bPS provides a high accumulation of PS on the surface of tumor cells. The experimental results presented below demonstrate the results of this method.

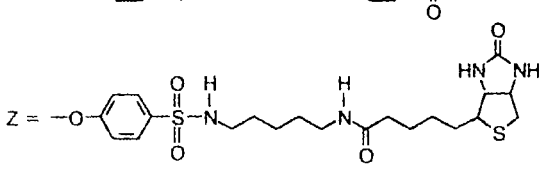
Several biotinylated bPS were prepared by the reaction of sulfochlorides of Zn phthalocyanines with biotinylated cadaverin.



bPS	R^1	R^2	R^3	R^4	R^5
bPS-1	Z	H	X	X	X
bPS-2	Z	Z	X	X	X
bPS-3	Z	H	Z	Y	Y







At first, the binding of bPS to streptavidin-modified mAB was studied by a modified enzyme-linked immunosorbent assay method (ELISA). Perforated plates were coated with the Brust-Mucin Antigen (BMA) and then treated with the biotinylated antibody BM7. Afterwards, they were incubated with streptavidin (10^{-8} g) for 15 min. The bPS ($5 \cdot 10^{-7}$—$5 \cdot 10^{-12}$ g) was added after that. After each step, the treated plates were washed with pH 7.2 buffer solutions. In order to estimate the number of non-biotinylated antibody binding sites, a test with the biotinylated peroxidase—*o*-phenylenediamine system was carried out. It was found that, depending on the kind of bPS, the avidin binding sites were saturated by

Table 3. Comparison of characteristics of first- (I), second- (II), and third-generation (III) photosensitizers

Compared parameter	Effect		
	I	II	III
Synthesis			
Relatively cheap, available	Yes (+)*	In most cases (+)	No
Structural homogeneity	No	Yes (+)	Yes (+)
Properties in solution			
Long-wave absorption	No	Yes (+)	Yes (+)
High extinction coefficient	No	Yes (+)	Yes (+)
High photosensitizing activity	Yes (+)	Yes (+)	Yes (+)
High stability:			
photooxidative	Yes	Yes and no (+)	Yes and no (+)
in the dark	Yes	Yes (+) and no	Yes (+) and ?
Properties in vitro and in vivo			
Rapid accumulation in tumor	Yes (+)	Yes (+)	?
Selective accumulation in tumor	No	No	Yes (+)
Photodynamic activity	Yes (+)	Yes (+)	Yes (?)
Stability *in vivo*	Yes	Yes and no (+)	?
Low dark toxicity	No (+)	In most cases no (+)	?
Wide spectrum of indications	Yes (+)	Yes (+)	?
Reduction of metastases	?	Yes (+) and ?	?

* The (+) sign indicates a positive effect.

$5 \cdot 10^{-8}$ g of bPS-1 and only $5 \cdot 10^{-10}$ g of bPS-2 or bPS-3. Thus, excellent binding of bPS was achieved.

After that, the dark toxicity and phototoxicity of the bPS were studied. Breast carcinoma cells (10^6 T47d cells) were treated with $4 \cdot 10^{-6}$ g of bPS. No dark toxicity was detected. Under irradiation with only 5 J cm^{-2} dose, the cells were photodynamically destroyed: the cell lysis was ~80 % with bPS-1 or bPS-2 and ~60% with bPS-3. Finally, steps (1)—(3) (see Scheme 3) were carried out with two cell lines. Breast carcinoma cells (10^6 T47d cells with BMA) and colon carcinoma cells (SW 1222 cells without BMA) were treated with biotinylated antibody BM7. After washing, 10^{-8} g of streptavidin was added, and after one more washing, $4 \cdot 10^{-6}$ g of bPS-1 was added. Irradiation with a 5 J cm^{-2} dose resulted in ~90% lysis of T47d cells and ~45% lysis of SW1222 cells. These first experiments indicate specific binding and phototoxicity of the bPS. Further work along this direction is in progress.

* * *

Table 3 summarizes the present situation with different generations of photosensitizers for PDT. The second generation satisfies the conditions required for PDT better than the first generation. PS of the third generation are still in the initial stages of research.

The future of PDT depends greatly on financial support and easier approval procedures for clinical testing, since a large number of suitable PS are available now.

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