JOURNAL OF BIOMEDICAL OPTICS 4(3), 276–285 (JULY 1999)

# NAPHTHALOCYANINE COMPLEXES AS POTENTIAL PHOTOSENSITIZERS FOR PHOTODYNAMIC THERAPY OF TUMORS

**M. Shopova,[†] D. Woehrle,[‡] V. Mantareva,[†] and S. Mueller[‡]**
[†]Institute of Organic Chemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev str, bl. 9, 1113 Sofia, Bulgaria; [‡]Institute of Organic and Macromolecular Chemistry, University of Bremen, Leobener str/NW 2, D-28334 Bremen, Germany

(Paper PT-002 received Sep. 7, 1997; revised manuscript received June 1, 1999; accepted for publication June 1, 1999. )

## ABSTRACT

In the present paper information about the synthesis and results on the pharmacokinetic and experimental photodynamic therapy (PDT) of naphthalocyanines are given. The photodynamic activity of differently substituted zinc(II)- and silicon(IV)-naphthalocyanines using liposomes or Cremophor EL as drug-delivery systems is shown on different tumor models. For the evaluation of the phototherapeutic effect different assessment criteria were used, including light and electron microscopy observations. The main conclusions which can be arrived at on the basis of our findings are the following: silicon(IV)-naphthalocyanine seems to be not a very effective tumor sensitizer, especially in the treatment of pigmented melanoma, while zinc(II)-naphthalocyanines appear to be very promising for PDT of tumors. Their selective targeting and slow clearance from tumor tissue, fast clearance from skin and pronounced phototherapeutic effect on different tumor models and especially at melanotic tumors, even after application of low drug doses, make this group of photosensitizers very attractive for successful PDT of cancer. © *1999 Society of Photo-Optical Instrumentation Engineers.* [S1083-3668(99)01203-4]

**Keywords** naphthalocyanine; photosensitizers; tumors; drug-delivery systems; lasers.

## 1 INTRODUCTION

Photodynamic therapy (PDT) is based on visible light activation of tumor-localized photosensitizers (PS) for destruction of solid tumors. The method is capable of long-term control of many early stage tumors and may be used for palliative purposes of advanced tumors.[1–4]

An efficient PDT activity must be expected with naphthalocyanines since they display a large molar extinction coefficient in a spectral region which is characterized by a deep light penetration into most mammalian tissues ($\varepsilon \geqslant 5 \times 10^5\ M^{-1}\,cm^{-1}$, around 780 nm) and several naphthalocyanines coordinated with closed d shell metal ions are known to be efficient photogenerators of hyperactive oxygen derivatives such as singlet oxygen.[5,6] We previously reported on the photophysical properties (steady state absorption fluorescence and phosphorescence yield, fluorescence decay times and singlet oxygen quantum yield of Zn(II)- and Si(IV)-naphthalocyanines.[7,8] In the present work we present the synthesis, pharmacokinetic and PDT studies of some Zn(II)-naphthalocyanines and Si(IV)-naphthalocyanine. Using liposomes or Cremophor EL as drug delivery systems, the photodynamic therapy is shown at different tumor models in order to demonstrate the excellent properties of some of these compounds as phototherapeutic agents.

## 2 MATERIALS AND METHODS

### 2.1 CHEMICALS AND INSTRUMENTS

DPPC over 98% pure and Cremophor EL were purchased from Sigma Chemicals Co. (Deisenhafen, Germany). All other chemicals were analytical grad reagents. Fourier transform infrared (FTIR) spectra were recorded with a Biorad-SPC-3200. Thin layer chromatography (TLC) was carried out using Macherey–Nagel's (Düren, Germany) Polygram 0.25 mm SIL $G/UV_{254}$ precoated plastic sheets. Matrex Silica Si chromatography medium (60 Å, 35–70 $\mu$m particle size) was used for flash chromatography (Amicon, Witten, Germany). Mass spectra (MS) were recorded on a Finnigan-MAT CH7-A and a Finnigan-MAT 8200 instrument. $^1$H-nuclear magnetic resonance (NMR) spectra were recorded on a Bruker WH 360 (360 MHz) instrument using tetramethylsilane as internal standard. The optical absorption spectra of the synthesized ZnNcs and

Address all correspondence to M. Shopova. Tel: +359 2 7334231; Fax: +359 2 700 225; E-mail maria_shopova@hotmail.com

SiNc were recorded on a Shimadzu UV-3000 double-beam spectrophotometer and a Perkin-Elmer Lambda 9 instrument.

### 2.2 SYNTHESIS

In this section is discribed both the synthesis of unsubstituted zinc (II)-2,3-naphtalocyanine (ZiNc 1) and of three tetrasubstituted derivatives (ZnNc 2–4) as well as the synthesis of Si(IV)-naphthalocyanine (SiNc).

#### 2.2.1 ZnNc 1–3

Tetranaphtho(2,3-*b*:2′,3′-*g*:2″,3″-1:2‴,3‴-*q*)porphyrazinatozinc (ZnNc 1) was prepared and purified as described previously.[9] Tetraacetamidotetranaphtho(2,3-*b*:2′,3′-*g*:2″,3″-1:2‴,3‴-*q*) porphyrazinatozinc (ZnNc 2) and tetraaminotetranaphtho(2,3-*b*:2′,3′*g*:2″,3″-1:2‴,3‴-*q*) porphyrazinatozinc (ZnNc 3) were prepared and purified following a known procedure.[10] The purity of 1–3 was controlled by mass spectra, quantitative UV-visible spectra and TLC.

#### 2.2.2 Tetramethoxytetranaphtho(2,3-*b*:2′,3′-*g*:2″,3″-*l*:2‴,3‴-*q*) porphyrazinatozinc (ZnNC 4)

ZnNC 4 was prepared by a six step procedure. The first four steps leading to 6-hydroxy-2,3-dicyanonaphthalene were carried out by a modification of a described method[11]).

*4-Benzoyloxy-1,2-dimethylbenzene.* 3,4-dimethylphenol (6.5 g, 50 mmol) was dissolved in 40 ml of aqueous 2 M KOH. While the solution was stirred at −5 °C, benzoylchloride (5.8 ml, 50 mmol) was added dropwise. The white, crystalline crude product was filtered off and recrystallized from ether-petrolether (1:1). Yield 8.8 g (78%); melting point (mp) 64 °C; IR (KBr): 3058, 2980, 2917, 1729 (C=0), 1602, 1497, 1257 (C–O), 1236, 1152, 1061 (C–O), 716 $cm^{-1}$; MS (70 eV): *m* / *z* 226 $M^{\cdot +}$), 105 ($C_7H_5O^+$, 100%), 77 ($C_6H_5^+$), 51; $^1$H-NMR ($CDCl_3$): $\delta$ 8.2 (*m*, 2H), 7.65 (*m*, 1H), 7.5 (*m*, 2H), 7.2 (*d*, 1H), 7.0 (*d*, 1H), 6.95 (*dv*. *d*, 1H), 2.25 (*d*, 6H).

*4-Benzoyloxy-ω-tetrabromo-1,2-dimethylbenzene.* 4-Benzoyloxy-1,2-dimethylbenzene (5.66 g, 25 mmol) was dissolved in 30 ml of $CCl_4$. N-bromosuccinimide (17.8 g, 100 mmol) and azoisobutyronitrile (0.14 g) were added. The reaction mixture was heated under reflux for 18 h and illuminated with a 500 W UV lamp. The byproduct, succinimide, was filtered off. The filtrate was evaporated and the crude product was recrystallized from petrolether. Yield 11.4 g (84%); mp 131 °C; IR (KBr): 3064, 1736 (C=O), 1602, 1497, 1244 and 1061 (C–O), 709, 660 (C–B) $cm^{-1}$; MS (70 eV): *m* / *z* 542 ($M^{\cdot +}$), 463 and 461 ($C_{15}H_{10}O_2Br_3^+$), 105 ($C_7H_5O^+$, 100%), 77, 51; $^1$H-NMR ($CDCl_3$); $\delta$ 8.2 (*m*, 2H), 7.75 (*s*, 1H), 7.65 (*m*, 1H), 7.6 (*s*, 1H), 7.55 (*m*, 2H), 7.35 (*m*, 1H), 7.1 (*m*, 2H).

*6-Benzoyloxy-2,3-dicyanonaphthalene.* To a solution of 4-Benzoyloxy-ω-tetrabromo-1,2-dimethylbenzene (3.25 g, 6 mmol) in dry N,N-dimethylformamide (DMF) (40 ml), fumarodinitrile (0.5 g, 30 mmol) and anhydrous sodium iodide (5 g, 30 mmol) were added. The mixture was heated to 70–80 °C for 12 h. The dark reaction mixture was slowly added to a solution of sodium thiosulfate (5.5 g, 30 mmol) in water (200 ml). The beige precipitate was filtered off and purified by recrystallization from acetone. Yield 1.6 g (90%); mp 238 °C; IR (KBr); 3079, 2235 (C≡N), 1736 (C=O), 1623, 1497, 1265 and 1061 (C–O), 920, 695 $cm^{-1}$; MS (70 eV): *m* / *z* 298 ($M^{\cdot +}$), 194 ($C_{12}H_6ON_2^+$), 165 ($C_{11}H_5N_2^+$), 138 ($C_{10}H_4N^+$), 105 ($C_7H_5O^+$, 100%), 77, 51.

*6-Hydroxy-2,3-dicyanonaphthalene.* To a suspension of 6-benzoyloxy-2,3-dicyanonaphthalene (2.6 g, 8.8 mmol) in methanol (20 ml), NaOH (2 g, 50 mmol) was added. The mixture was stirred for 10 min at room temperature. After filtration, the solution was diluted with water (40 ml) and acidified with HCl to *p*H 2. The residue was filtered off and dried at 70 °C in vacuum. Yield 1.2 g (70%): mp 300 °C (decomp.): TLC on silica gel with toluene-ethylacetate (3:1): $R_f$ = 0.28; IR (KBr): 3346 (O–H), 3095, 2230 (C≡N), 1623, 1518, 1398, 1230, 1202, 920, 470 $cm^{-1}$; MS (70 eV): *m* / *z* 194 ($M^{\cdot +}$, 100%), 165 ($C_{11}H_5N_2^+$), 139 ($C_{10}H_5N^+$), 112 ($C_9H_4^+$); $^1$H-NMR [dimethylsulfoxide-$d_6$(DMSO-$d_6$)]: $\delta$ 10.9 (*s*, 1H), 8.7 (*s*, 1H) 8.6 (*s*, 1H), 8.05 (*d*, 1H), 7.45 (*dv*. *d*, 1H), 7.35 (*d*, 1H).

*6-Methoxy-2,3-dicyanonaphthalene.* In a dry apparatus 6-hydroxy-2,3-dicyanonaphthalene (0.2 g, 1 mmol), an excess of methyl iodide (160 μl, 2.5 mmol) and anhydrous potassium carbonate (0.346 g, 2.5 mmol) were dissolved in 20 ml of dry acetone. The reaction mixture was heated under reflux. After two days the hot mixture was filtrated. The residue was washed twice with boiling acetone. The reaction and washing filtrates were combined and evaporated. The crude product was recrystallized from acetone and dried at 70 °C in vacuum. Yield 0.19 g (90%); mp 218 °C; TLC on silica gel with toluene-ethylacetate (3:1): $R_f$ = 0.63; IR (KBr): 3079, 2952, 2235 (C≡N), 1623, 1602, 1499, 1256 (C–O–C), 1211 $cm^{-1}$; MS (70 eV): *m* / *z* 208 (100%, $M^{\cdot +}$), 178 ($C_{12}H_6N_2^+$), 165, 138; $^1$H-NMR ($CDCl_3$): $\delta$ 8.4 (*s*, 1H), 7.9 (*d*, 1H), 7.4 (*d*, 1H), 7.3 (*s*, 1H), 4.0 (*s*, 3H).

*Tetramethoxytetranaptho*(2,3-*b*:2′,3′-*g*:2″,3″-*l*:2‴,3‴-*q*)*porphyrazinatozinc (ZnNc 4).* Some 6-Methoxy-2,3-dicyanonaphthalene (0.1 g, 0.43 mmol), urea (0.1 g, 1.6 mmol), zinc acetate dihydrate (0.03 g, 0.13 mmol) and a catalytic amount of ammonium molybdate were mixed and placed in a glass tube. After flushing with nitrogen the evacuated tube was sealed and heated for 2 h at 200 °C. The product was washed with water, aqueous 1.5 M HCl, aqueous 1 M NaOH and again twice with water. The crude product was extracted with methanol in a

Soxhlet apparatus, until the solvent remained colorless. The greenish black product was purified by flash column chromatography using toluene/tetrahydrofuran (THF) (1:1) as eluting solvent and dried at 70 °C in vacuum. Yield 45 mg (50%); TLC on silica gel with toluene-THF (1:1): $R_f = 0.80$; IR (KBr): 3065, 2966, 2931, 2833, 1616, 1553, 1511, 1469, 1356, 1244 (C–O–C), 1152, 1026 $cm^{-1}$; UV visible (DMF): 759, 677, 326 nm; MS [DCI (direct chemical ionization) negative, $NH_3$, 8 MA $s^{-1}$): $m/z$ 896 ($M^{\cdot +}$), isotopic pattern corresponds to that calculated: $^1$H-NMR (DMSO-$d_6$): $\delta$ 9.3 ($m$, 8H), 8.4 ($m$, 4H), 7.8 ($m$, 4H), 7.4 ($m$, 4H), 4.1 ($m$, 12H).

### 2.2.3 Synthesis of Bis(methyloxyethyleneoxy) (2,3-naphthalocyaninato)silicon (SiNc)

To 3 ml of dry ethyleneglycolmonomethylether 30 mg of sodium was added. Then 1 ml of this solution containing 0.4 mmol of the glycolate was added under dry inert gas to a suspension of 81.1 mg (0.1 mmol) bis(chloro) (2,3-naphthalocyaninato)-silicon in 5 ml of dry N,N-dimethylformamide (DMF). The mixture was heated for 1.5 h under reflux. After washing the solvent and the excess of the ethyleneglycolmonomethylether were removed under vacuum. The residue was washed with hot water and purified by flash chromatography on silica Si 60 in chloroform. Yield 46 mg (52%) of a green powder; IR (KBr), $\nu$ ($cm^{-1}$): 3069, 2963, 2930, 2884, 1615, 1509, 1474, 1428, 1336, 1289, 1262, 1120 (C–O–C), 1080, 911, 735, 573; MS (DCI negative, $NH_3$): $m/z$ 892 (22%), 891 (55%), 890 ($M,^{\cdot +}$ 83%), 815 ($M^{\cdot -}$—$OCH_2CH_2OCH_3$, 100%), 784 (815—$OCH_3$); $^1$H NMR (360 MHz, $CDCl_3$): $\delta = 10.10$ ppm ($s$, 8H), 8.65 ppm ($m$, 8H), 1.80 ppm ($s$, 6H), 0.70 ppm ($t$, 4H), $-1.05$ ppm ($t$, 4H); UV visible ($CHCl_3$): 778, 739, 691, 347 nm; UV visible (DMF): 782 nm ($\varepsilon = 5.8$ 10-5 M-1 $cm^{-1}$).

## 2.3 DRUG DELIVERY SYSTEMS

### 2.3.1 Liposome Preparation

Liposomes were prepared essentially as described in Ref. 12. A solution of ZnNc 1–4 (0.5 mg) dissolved in THF (600 $\mu$l), together with DPPC (60 mg) dissolved in chloroform (12 ml), was dried under reduced pressure by a rotary evaporator. The phospholipid film thus obtained was suspended in phosphate buffer (10 ml). The suspension was sonicated and centrifuged. ZnNc 1–4 were incorporated into liposomal vesicles in monomeric form. This is suggested by the position of the absorption maxima and the presence of fine structure in the red absorption region. The concentrations of ZnNc in liposomal dispersions were calculated by diluting the system with an excess of DMF and measuring the absorbance at $\lambda_{max}$: ZnNc 1, 756 nm ($\varepsilon = 1.7\times10^5$ l $mol^{-1}$ $cm^{-1}$); ZnNc 2, 768 nm ($\varepsilon = 0.26\times10^5$ l $mol^{-1}$ $cm^1$); ZnNc 3, 768 nm ($\varepsilon = 1.2\times10^5$ l $mol^{-1}$ $cm^{-1}$); ZnNc 4, 759 nm ($\varepsilon = 0.5\times10^5$ l $mol^{-1}$ $cm^{-1}$).

### 2.3.2 Cremophor El Formulation

For the Cremophor emulsion drug formulation the procedure of Soncin et al.[13] with some modifications was followed. Typically 1.5 mg of SiNc was added with 0.3 ml of Cremophor EL and sonicated until the drug was completely dissolved; resonication after adding 0.09 ml absolute ethanol; temperature control of the sonicating solution; dilution of the suspension to a volume of 7.5 ml by addition of physiological solution; filtration through a 0.45 $\mu$m filter. Dye concentration in Cremophor emulsion was estimated upon dilution in DMF by measuring the absorbance at 782 nm.

## 2.4 ANIMALS AND TUMOR MODELS

Induced 7,12-dimethylbenz(a)anthracene (DMBA) rhabdomiosarcoma[14] in golden hamsters aged 2–3 months with body weights of about 110 g was used as a transplanted first-generation tumor model. For transplantation the tumor tissue was cut into pieces of 0.5–0.7 cm in saline and injected percutaneously (pc) in the right scapula region. Before PDT irradiation the animals were anesthetized by intraperitoneal (ip) injection of Thiopental (2.5 mg $kg^{-1}$).

Pathogen free male C57Bl/6 mice, eight weeks of age, were purchased from the Experimental Animal Production Area (National Oncology Center, Sofia). The studied tumor models were Lewis lung carcinoma and B 16 pigmented melanoma. Lewis lung carcinoma cells were injected subcutaneously into the right hind leg of the mice with 0.2 ml of sterile suspension containing at least $2\times10^6$ viable cells per ml. Seven days after implantation the tumor reached an outer diameter between 0.3 and 0.5 cm. B16 pigmented melanoma was transplanted in mice as follows. A 1 $mm^3$ piece of tumor tissue in saline was transplanted to the right hind flank. On the sixth day after transplantation the external tumor diameter was between 0.3 and 0.5 cm.

## 2.5 PHARMACOKINETIC STUDIES

### 2.5.1 Study of Induced 7,12-dimethylbenz(a)antracene (DMBA) Rhabdomiosarcoma

ZnNc 1 incorporated into D, L-alpha-dipalmitoylphosphatidylcholine (DPPC) liposomes was injected intraperitoneally, at a dose of 0.15 mg $kg^{-1}$ b.w., when the tumor diameter was in the range 0.3–0.5 cm. At different times after injection the animals were killed (three animals at each time). Several tissues (tumor, muscle, liver, lung, spleen and kidney) were removed, washed with saline and frozen until analyses for ZnNc 1 content were performed. For analysis tissue samples were homogenized in 2% aqueous SDS as described previously.[6] The homogenate was kept at 37 °C for 1 h and pe-

riodically stirred. The suspension thus obtained was centrifuged at 3500 $rpm^{-1}$ for 15 min. The supernatant was collected and its ZnNc 1 content (milligram of drug per milliliter of solution) was analyzed by fluorescence measurements with a Perkin-Elmer LS-5 apparatus. Results of ZnNc 1 recovery were referred to 1 g of tissue. The fluorescence excited at $\lambda = 673$ nm was recorded in the 700–800 nm spectral interval. The fluorescence intensity data were converted into ZnNc 1 concentration by interpolation with a calibration plot obtained from known amounts of ZnNc 1 in 2% SDS. The ZnNc 1 fluorescence obtained from tissue extracts was corrected for the contribution of a 673 nm excitable background fluorescence as observed in tissue extracts from control animals. No significant fluorescence was detected after additional extraction of the different tissues.

#### 2.5.2 Study of Lewis Lung Carcinoma and B16 Pigmented Melanoma

Seven days after the transplantation the respective tumor-bearing mice were intraperitoneally (ip) injected with 0.5 mg $kg^{-1}$ b.w. SiNc, incorporated into Cremophor EL emulsion. At fixed times after dosing the mice were sacrificed and tumor, peritumoral skin, liver and brain were collected and the SiNc concentration in each specimen was determined through fluorescence spectroscopy measurements[15] using a Perkin-Elmer MPF-5 apparatus (exitation at 695 nm, emission in the 720–800 nm interval).

### 2.6 PHOTODYNAMIC THERAPY STUDIES

For experimental PDT studies with tumor transplanted golden hamsters (with mean tumor diameter between 3 and 4 mm) 0.3 mg $kg^{-1}$ b.w. liposome-bound ZnNc 1-4 were used. During the investigation of SiNc at both studied tumor models (Lewis lung carcinoma and B16 Pigmented melanoma—mean tumor diameter 3–5 mm), tumor-bearing mice were injected ip with 0.5 mg $kg^{-1}$ b.w. SiNc incorporated in Cremophor EL emulsion.

In all cases the tumors were illuminated at 24 h postdrug injection at the $\lambda_{max}$ for the respective sensitizer using argon pumped dye laser (Spectra Physics, USA). The irradiation parameters for the treatment of hamsters with ZnNcs were a fluence rate of 250 or 180 mW $cm^{-2}$ and a fluence of 450 J $cm^{-2}$ with exposure time about 30 min. In the investigations with SiNc in all cases the fluence rate was selected to be 370 mW $cm^{-2}$ for a fluence of 360 J $cm^{-2}$. Because of possible melanin-promoted photoeffects, a group of B16 pigmented melanoma bearing mice was irradiated by the same protocol but without sensitization.

The effectiveness of the treatment was evaluated by comparing the mean tumor diameter and the tumor growth of the treated (drug-injected and irradiated) groups of animals with those of the control groups (without drug and irradiation). In the case of hamsters treatment the antitumor effect was also evaluated by the percentage of cured animals and histological analysis. In the additional study the PDT effect of different generations of sensitizers was comparatively evaluated on the treatment of B16 pigmented melanoma in mice.

## 3 RESULTS

### 3.1 SYNTHESIS

The 2,3-naphthalocyanine-zinc complex ZnNc 1–4 were successfully prepared by a multistep procedure, starting from unsubstituted or substituted o-xylenes. The general procedure was introduced by Mikhalenko and Lukyanets.[9] After side-chain alkylation with N-bromosuccinimide and an analogous Diels–Ader reaction with fumarodinitrile, the resulting 2,3-dicyanonaphthalenes were reacted with zinc acetate in a sealed glass tube.

The procedure for synthesis of known compound ZnNc 4 started from commercially available 4-hydroxy-1,2-dimethylbenzene. For the reaction with N-bromosuccinimide and fumarodinitrile the hydroxyl group had to be protected by formation of a benzylester. Afterwards the ester linkage was cleaved in alkaline medium. The 6-hydroxy-2,3-dicyanonaphthalene was alkylated with methyl iodide and 6-methoxy-2,3-dicyanonaphthalene was reacted in a sealed glass tube to yield ZnNc 4. The crude product was extracted with methanol and purified by flash column chromatography on silica gel, yielding a compound eluting as a single spot on TLC. Constitutional isomers are possible, but TLC gave no indication of whether isomers existed. All ZnNc complexes are soluble in DMSO or DMF and insoluble in water. After incorporation into DPPC liposome vesicles ZnNc 1–4 exist in water in monomeric form.

The bismethyloxyethyleneoxy substituted silicon naphthalocyanine (SiNc) was obtained by the reaction of the corresponding bischloro substituted naphthalocyanine with an excess of the sodium alcoholate. Purification was carried out by column chromatography. The SiNc is soluble in polar organic solvents like DMF or $CHCl_3$ and insoluble in water. Solubility in water can be achieved by incorporation of the SiNc in Cremophor EL followed by dilution with water. The dissolved naphthalocyanine derivative exhibits the longest wavelength absorption at $\div 780$ nm with $\varepsilon > 5 \times 10^5$ M 1 $cm^{-1}$ (Figure 1).

### 3.2 PHARMACOKINETIC STUDIES

In Figure 2 the concentration of ZnNc 1 in three selected tissues after ip administration versus time is shown. ZnNc 1 recoveries were estimated for the tumor, muscle, i.e., the tissue, where the tumor is implanted and liver, i.e., the tissue which usually

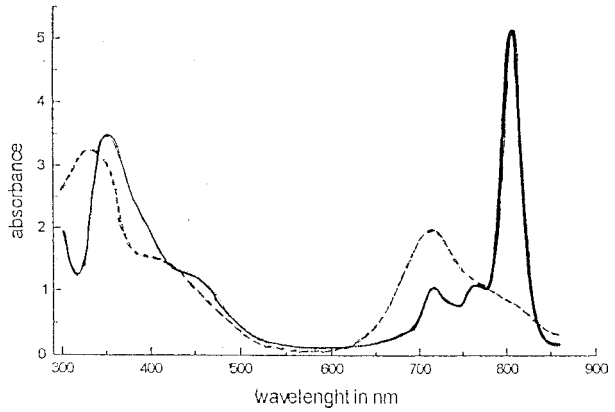


**Fig. 1** Visible spectrum of Nc in monomeric and aggregated form.

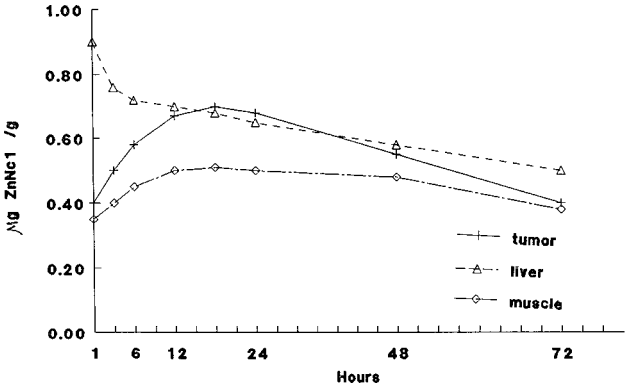


**Fig. 2** Time-dependent concentration of ZnNc 1 in three selected tissues of rhabdomyosarcoma-bearing hamsters at different times after injection of 0.15 mg ZnNc 1 incorporated into DPPC per kilogram of b.w.

shows a large uptake of systematically injected photosensitizers. Table 1 shows the results for all studied organs. The data represent the average of the recoveries from three different hamsters. Significant concentrations of sensitizer of the order of 0.7 $\mu$g per gram of tissue were accumulated and retained by the tumor. The peritoneal tissue (i.e., muscle) accumulated smaller amounts of ZnNc 1. Large amounts of ZnNc 1 were recovered from the liver, lung and spleen and they were present even 72 h after administration.

The biodistribution of SiNc in the two tumor models—Lewis lung carcinoma and B16 pigmented melanoma—showed significant differences (Figure 3) with constantly higher naphthalocyanine levels at LLC. The SiNc reached maximal concentration in LLC (up to 0.70 $\mu g\ g^{-1}$ tissue) at 24 h postinjection, as compared with maximal values of 0.15 $\mu g\ g^{-1}$ in the melanotic tumor. However, the amount and time dependency of photosensitizer accumulation from several normal tissues, including liver, skin and brain, were fairly comparable; as a consequence, there are significant differences in the selectivity of tumor targeting by the naphthalocyanine photosensitizer, especially as regards the ratio between the SiNc concentration in the tumor and the skin (peritumoral tissue). The time dependency of this ratio is shown for the two tumor models in Figure 4.

### 3.3 PHOTOTHERAPEUTIC STUDIES

Table 2 shows the percentage of cured animals after PDT treatment with ZnNc 1–4. ZnNc 2 and 4 possess the highest effectiveness. Rhabdomyosarcoma-bearing hamsters without treatment did not survive in the observation time.

The tumor diameter of the uncured animals was followed during the observation period. Here again ZnNc 2 and 4 show the best effects: at the end of the observation period (29 days), the mean tumor diameter for ZnNc 2 was 11.4±5.1 mm and for ZnNc 4 was 12.5±6.4 mm, whereas ZnNc 1-treated rhabdomyosarcoma had a mean tumor diameter of 20.5±8.3 mm and ZnNc 3-treated rhabdomyosarcoma had a mean tumor diameter of 26.7±3.4 mm (largest). In the control group (untreated animals) no animal survived.

The extent of tumor necrosis, expressed as percentage of the necrotized tumor area versus the total area of tumor slice, 24 h after PDT treatment (sensitizers ZnNc 1–4) is summarized in Figure 5.

**Table 1** Recovery of ZnNc 1 from tumor-bearing hamsters at different times after injection of 0.15 mg sensitizer per kilogram of b.w. Data are expressed in micrograms of ZnNc 1 per gram of tissue.

| Tissue | 1 h | 3 h | 6 h | 12 h | 16 h | 24 h | 32 h | 48 h | 72 h |
|---|---|---|---|---|---|---|---|---|---|
| Tumor | 0.40 | 0.50 | 0.58 | 0.67 | 0.70 | 0.70 | 0.65 | 0.55 | 0.40 |
| Muscle | 0.35 | 0.40 | 0.45 | 0.50 | 0.51 | 0.50 | 0.50 | 0.48 | 0.38 |
| Liver | 0.90 | 0.76 | 0.72 | 0.70 | 0.68 | 0.65 | 0.60 | 0.58 | 0.50 |
| Lung | 0.76 | 0.58 | 0.55 | 0.51 | 0.48 | 0.45 | 0.45 | 0.40 | 0.40 |
| Spleen | 0.94 | 0.90 | 0.90 | 0.65 | 0.60 | 0.87 | 0.57 | 0.57 | 0.40 |
| Kidney | 0.57 | 0.76 | 0.76 | 0.68 | 0.76 | 0.80 | 0.58 | 0.58 | 0.40 |

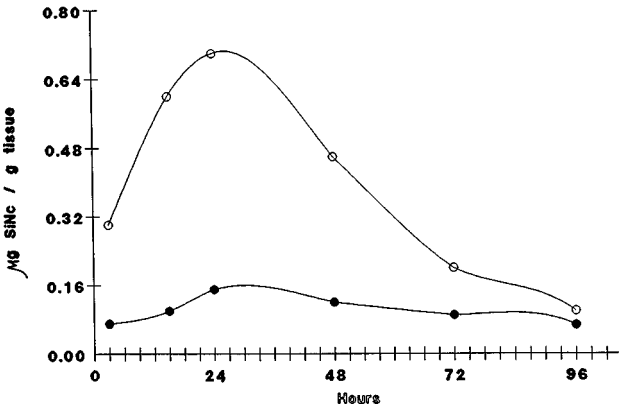


**Fig. 3** Levels of SiNc in LLC (-○-○-) and B16 pigmented melanoma (-●-●-) of mice at various times following intraperitoneal injection of SiNc in Cremophor EL (0.5 mg $kg^{-1}$ b.w.). Each point is the mean of three animals with SD from ±0.05 to ±0.13. Values for controls (uninjected mice) were subtracted from the individual points.

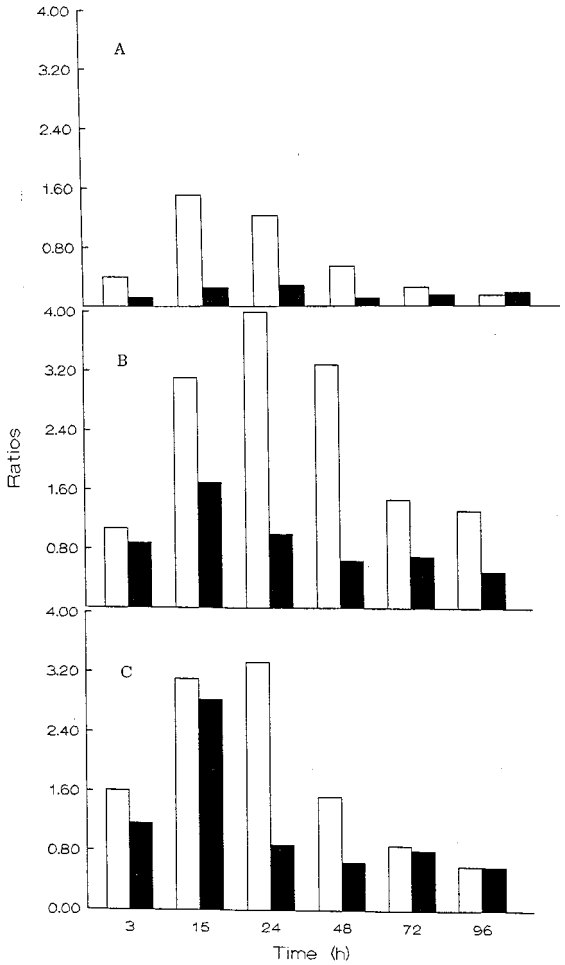


**Fig. 4** Tumor/tissue ratios for SiNc in Lewis lung carcinoma (empty columns) and B16 pigmented melanoma (black columns)-bearing mice as a function of time after ip injection of 0.5 mg $kg^{-1}$ b.w. dye formulated in Cremophor emulsion. Ratios: tumor/liver (A); tumor/skin (B); tumor/brain (C).

**Table 2** Photodynamic therapy of rhabdomyosarcoma in hamsters after administration of 0.3 mg sensitizer per kilogram b.w. Percentage of cured animals at the end of the observation period (29 days).

| Sensitizer | Cured animals (%) |
|---|---|
| ZnNc 1 | 10 |
| ZnNc 2 | 50 |
| ZnNc 3 | 0 |
| ZnNc 4 | 40 |
| Control | 0 |

The standard deviations in the histograms are 3.5% for ZnNc 4. Interestingly, after treatment with ZnNc 4 only 20% of the total area was necrotic. This may be related to a different mechanism of tumor necrosis. Again ZnNc 1 and 3 had little effect. A very well expressed vessel change (stasis and microtrombi) for the animal groups treated with ZnNc 1 and 2 was also established.

Figure 6 presents the tumor regression (mean tumor diameter) in LLC (A) and B16 pigmented melanoma (B) after sensitization with 0.5 mg kg-1 b.w. SiNc formulated in Cremophor emulsion. The exitation was performed at λ max of SiNc (782 nm), 24 h after its ip administration. As it is seen the PDT efficiency increased significantly in the case of LLC [Fig. 6(A)]. The mean tumor diameter keeps almost constant after the seven day posttreatment to the end of observation and it is two times lower towards that of the control groups of animals. In the case of B16 pigmented melanoma treatment the phototherapeutic effect was very low. The difference between the mean tumor diameter for the treated and untreated (including the irradiated) control groups was nearly 2 mm [Figure 6(B)].

In all cases, the intratumoral temperature during irradiation (as measured by a thermocouple in-


**Fig. 5** Extent of tumor necrosis expressed as the percentage of necrotized tumor cells vs the total area of the slices. Probes were taken 24 h after PDT of transplanted rhabdomyosarcoma in hamsters with liposome-administered ZnNc 1–4 as photosensitizers.

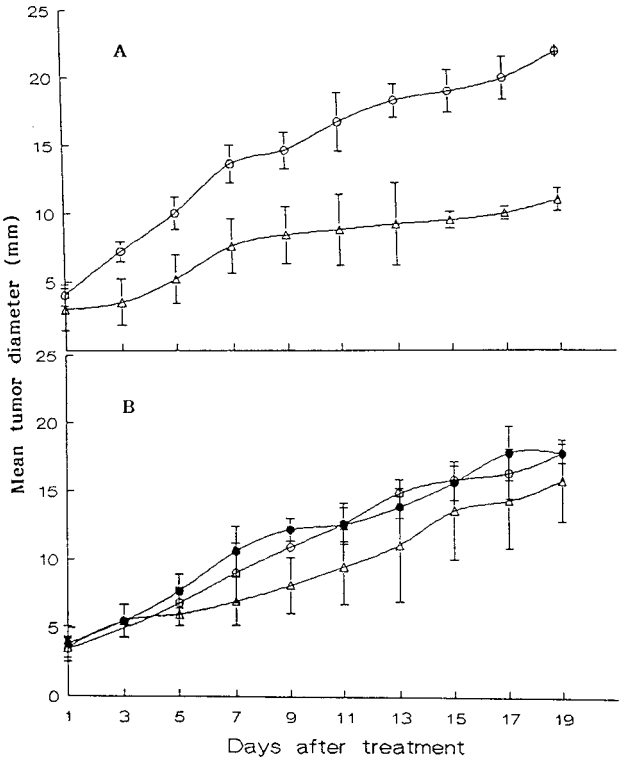


**Fig. 6** Photodynamic therapy of LLC (A) and B16 pigmented melanoma (B) bearing mice with SiNc (0.5 mg kg$^{1}$ b.w.) complexed with Cremophor EL. Treated and irradiated with 370 mW cm$^{2}$ and 360 J cm$^{2}$ groups (-▽-▽-); untreated and unirradiated control groups (-○-○-); untreated but irradiated with the same fluence rate and fluence as the treated tumor-bearing mice (-●-●-).

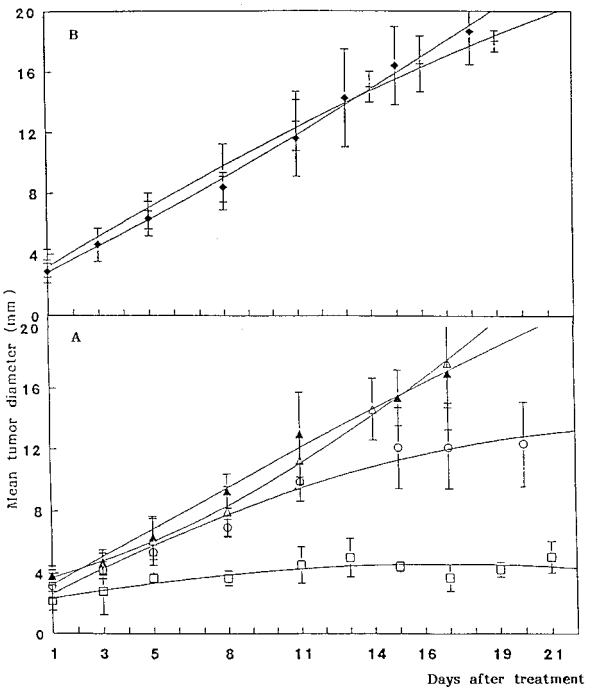


**Fig. 7** Comparison of the B16 pigmented melanoma growth rate in mice. Irradiation (370 mW cm$^{-2}$ and 360 J cm$^{-2}$): A: treatment with H*p*D (–▽–), ZnNc (–▲–), ZnNc (–○–) and ZnNcA—equal to ZnNc 2 (–□–); B: untreated and not irradiated mice (–+–) and mice exposed only to light (–◆–).

serted into a hypodermic needle) never exceeded 38.7±0.6 °C, i.e., about 6 °C above the basal temperature of an anaesthetized mouse.[16] This temperature is not considered to yield significant hyperthermia.[17] The regrowth delay caused by the PDT for the two tumor models is given in Table 3.

Figure 7 presents the effect of photodynamic treatment on B16 pigmented melanoma in C57Bl/6 mice with hematoporphyrin derivative (H*p*D), zinc(II)-phthalocyanine (ZnPc), unsubstituted zinc(II)-naphthalocyanine (ZnNc) and tetrabenzamido-substituted zinc(II)naphthalocyanine (ZnNcA) incorporated into DPPC liposomes and irradiated with 370 mW/cm$^2$ and 360 J/cm$^2$ of respective nm light ($\lambda_{abs\ max}$). As can be seen [Figure 7(A)], to the end of observation period (21 days after PDT) there was no PDT effect at all after H*p*D and ZnPc photosensitization (the mean tumor diameter reached 20 mm). To some degree tumor growth delay is observed 15 days after ZnNc phototreatment. The phototherapeutic effect is, however, very pronounced after ZnNcA phototherapy. In the first days after PDT the mean tumor diameter increases very slowly to a maximum of 4.9 mm, after which it decreases. The application of higher drug concentration and irradiation with 520 and 260 J/cm$^2$ [similar to the investigation of B16 pigmented melanoma with silicon(IV)-naphthalocyanine] does not cause any macroscopically or microscopically detected photonecrosis. In Figure 7(B) the rates of tumor growth in untreated and unirradiated mice, and in untreated mice but exposed to light (370 mW/cm$^2$ and 360 J/cm$^2$) are shown. As is seen the rate of tumor growth for the irradiated control group is the same as that for the untreated and nonirradiated one. Similar to the observation in Ref. 18 the temperature measurements at the irradiated control group indicated rapid (only in the first minute of irradiation) increase of the tumor tissue temperature from a basal value of 29°C±1 °C to a plateau value of 39°C±1 °C being

**Table 3** PDT effect on the tumor regrowth delay for the Lewis lung carcinoma and the B16 pigmented melanoma.

| Tumor model | Growth time[a] (days) | Regrowth delay[b] (days) |
|---|---|---|
| LLC | 9.2±2.48 | 4.6±1.54 |
| Control | 4.6±0.9 | |
| B16 | 6.6±2.58 | 1.27±1.66 |
| Control | 5.33±0.91 | |

[a] The time interval (±SD) for the tumor to grow to a mean diameter of 0.7 cm, from the diameter at the time of irradiation (0.3–0.5 cm).

[b] Difference between the growth time for treated and control mice.

constant for the whole period of irradiation. Very fast recovery (less than 20 s after irradiation) of the initial temperature (about 24 °C) is observed.

## 4 DISCUSSION

The method of Mikhalenko and Lukyanets[9] allows the synthesis of tetrasubstituted 2,3-naphthalocyanines in high yields which are easily monomerized in liposome vesicles for use in aqueous solution. A Si(IV)-naphthalocyanine bearing two methoxyethyleneglycol axial ligands to the centrally coordinated metal ion was obtained by chemical synthesis and successfully formulated in Cremophor EL emulsion before being assayed for phototherapeutic activity.

Photokinetic data were obtained for ZnNc 1, because it is the fundamental structure of the studied ZnNcs. After ip injection of only 0.15 mg $kg^{-1}$, significant concentrations of ZnNc 1 were accumulated and slowly eliminated by the examined tumor model (Table 1, Figure 2). The poor lymphatic drainage, which is typical of tumor tissues, offers one possible explanation for the slow release of photosensitizing drugs from tumors.[19] According to Reddi et al.[20] the slow clearance of photosensitizers may also depend on the low accessibility of the drug binding sites to the protein carriers. The slow clearance of ZnNc 1 by the tumor also suggests the possibility of multiple phototreatments following a single drug injection. The surrounding muscle accumulated smaller amounts of ZnNc 1. This provides a lower risk of damage to healthy tissues adjacent to the tumor during PDT. Large amounts of the naphthalocyanine sensitizer were present in liver, spleen and kidneys, even 72 h after administration (Table 1). The suitability of the liposome carrier system employed for ZnNc 1 administration and transport is underlined by the high degree of selective tumor targeting: 24 h after injection similar or larger amounts of ZnNc 1 were found in the tumor compared with the liver.

Although SiNc is accumulated by LLC in relatively high amounts (0.70 $\mu g\, g^{-1}$ of tumor tissue at 24 h after ip injection of 0.5 $\mu g\, g^{-1}$), it appears to be a less efficient tumor localizer than the corresponding unsubstituted Zn(II)-napthalocyanine, which is recovered at a concentration of 1.4 $\mu g\, g^{-1}$ at 24 h after ip injection of 0.3 mg $kg^{-1}$ in the same tumor/animal model. The lower tumor targeting efficiency of SiNc may be due to the hydrophilic character of the two methoxy (polyethyleneglycol) axial ligands; as known,[21] the affinity of porphyrin derivatives for neoplastic tissues increases with increasing hydrophobicity. Actually, hydrophobic photosensitizers, especially when incorporated into liposomal vesicles or oil emulsions, are preferentially released to serum lipoproteins, and in particular to the low-density lipoproteins,[22] which in turn develop a specific interaction with malignant cells through receptor-mediated endocytosis.[23] Thus the SiNc also shows a smaller selectivity of tumor targeting than ZnNc as compared to skin, which might affect safety of the phototherapeutic treatment.

The comparative PDT experiments show the best therapeutic effect with ZnNc 2 and the lowest with ZnNc 3. The extent of tumor photonecrosis expressed as a percentage versus the total area of the histological section, is highest using ZnNc 2 with 91%. The lowest percentage of photonecrosis (3%) was found 24 h after PDT with ZnNc 3. The high degree of necrosis determined in ZnNc 2-treated tumor slices is probably due to the observed changes in the blood vessels (stasis and microtrombi) of the tumor. It can be assumed that ZnNc 2 can also influence endothelial cells. For ZnNc 1 the same changes in tumor vessels were observed, but interestingly ZnNc 1 did not show the same high overall PDT effectiveness. The influence of naphthalocyanine sensitizers on the endothelial cells of blood vessels is the object of futher investigations. ZnNc 4 showed a much smaller percentage of necrotic tumor area (20%) 24 h after PDT than ZnNc 2, but the long-term PDT effectiveness of ZnNc 4 was nearly as high as that of ZnNc 2.

It is supposed that the increase in the hydrophobic character of the naphthalocyanines—highest in ZnNc 2—will result in better accumulation, distribution or retention. The effect of the naphthalocyanine substituent on the phototherapeutic activity is the object of further investigations. The percentage of cured animals at the end of the observation period (Table 2) and the development of the mean tumor diameter with time showed the same order of overall PDT effectiveness for the studied sensitizers: ZnNc 2>ZnNc 4>ZnNc 1>ZnNc 3.

According to all the used assessment criteria among the compared different generation sensitizers (H*p*D, ZnPc, ZnNc and tetrabenzamido-substituted zinc(II)-2,3-naphthalocyanine (ZnNc 2, or ZnNcA), the last one again showed the best antitumor effect. The lypophilic nature and the degree of steric hindrance of this compound looks to be very suitable for PDT of tumors. It is able to generate deep red optical absorbance and the applied liposome delivery system contributes for a longer wavelength absorbing maximum (774 nm) and for its good accumulation in tumor tissue.

The PDT efficiency of SiNc towards LLC in mice appears to be superior to that of ZnNc since it induces an essential identical growth delay of this tumor after irradiation under comparable experimental conditions in spite of the lower intratumoral concentration. Conversely, SiNc appears to be very poorly photoactive against the B16 pigmented melanoma. Such inefficiency is probably related to the low amount of naphthalocyanine accumulated by this tumor (Figure 3). As a consequence, in spite of the high molar extinction coefficient this photosensitizer cannot favorably compete with melanin pigments for the absorption of the incident light: although the absorbance of melanin monotonically

decreases with increasing wavelength, this compound still exhibits a residual significant absorbance in the 750–800 nm spectral region.[24] Our interpretation receives some support by recent observations[15] that a SiNc-bearing alkyl-type axial ligand, which was accumulated by the B16 pigmented melanoma with fourfold larger efficiency, induces an extensive photosensitized necrosis. Since the skin levels of SiNc in the C57 mice were almost indentical for the two types of malignant lesions studied in the present investigation, the drop in selectivity of melanoma targeting must be ascribed to a markedly lower affinity of SiNc for this particular tumor.

An exhaustive explanation of the differences in SiNc accumulation by LLC and B16 pigmented melanoma cannot be provided only on the basis of our findings. It is likely that a major role is performed by the less developed degree of vascularization which is typical of the melanoma:[25] as mentioned above, Cremophor-delivered photosensitizers are transferred to serum lipoproteins, which then reach the tumor compartments by extravasation through the largely fenestrated blood capillaries. As a consequence, a lower vascularization would imply a lower tumor uptake of the sensitizer. These findings underline the need to carry out detailed pharmacokinetic/phototherapeutic investigations on different tumor models in order to define PDT protocols which are tailored to the biological, optical and biochemical characteristics of any given tumor.

Liposome-delivered Zn(II)-2,3-naphthalocyanines appear to be promising sensitizers for PDT, due to selective targeting and slow clearance from tumor tissue, fast clearance from the skin and efficient photosensitization of tumor necrosis even on injection of very low doses.

Our findings suggest that other than the tumor nature, the photophysical properties (especially $\lambda_{abs\ max}$) and the chemical structure of the respective sensitizer can also be important factors for an effective photodynamic therapy of malignant tumors including a melanotic one.

## Acknowledgments

Financial support by the Deusche Forshungsgemeinschaft, Volkswagen Foundation (Germany), Bulgarian Academy of Sciences and National Foundation ''Scientific Investigations'' (Bulgaria) is gratefully acknowledged.

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