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Synthesis and photocytotoxic activity of new chlorin–polyamine conjugates

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Abstract—This paper reports the synthesis of new chlorin–polyamine conjugates designed to improve the targeting of cancer cells. Photocytotoxic activity of these photosensitizers was tested against human chronic myelogenous leukemia cells (K562) and compared to the effects of Photofrin II® and chlorin e_6.

Photodynamic therapy (PDT) is a promising cancer treatment based upon systemic or local administration of a photosensitizing drug followed by illumination of the tumor with visible or near-infrared light.[1,2] Photosensitizer molecules that accumulate into cancer cells are subjected to light activation; two types of photoreaction mechanisms are invoked to explain photosensitizer action: light-activated photosensitizer in its triplet state can generate free radicals by electron or proton transfer (type I photochemical reactions) or singlet oxygen ($^1O_2$) is produced by energy transfer (type II reactions). Singlet oxygen seems to be the major mediator of photochemical cell damage,[3] yet the mechanism of action is not well understood.[4] Photofrin II® is the first generation photosensitizer which has been the most extensively studied for the treatment of a variety of cancers.[5]

The ability to enhance penetration depth in tissues with longer wavelengths of light (>630 nm) and the introduction of lasers that emit light from 660 to 800 nm have stimulated the development of compounds absorbing in this region of the electromagnetic spectrum. Such compounds mainly include chlorins, bacteriochlorins, benzoporphyrins, and phthalocyanines. For example, *meta*-tetrahydroxyphenylchlorin (*m*-THPC trade name Foscan®) is a second generation photosensitizer that appears to be one of the most efficient photosensitizing compounds studied to date. It received regulatory approval in 2002 in the European Union for the palliative treatment of head and neck cancer.[6] Benzoporphyrin derivative monoacid ring A (BPD-MA. trade name Visudyne®) is being used for the treatment of age-related macular degeneration.[7] Monoaspartyl chlorin e_6 (MACE) developed under the acronym NPe6 is being used against breast adenocarcinoma, basal cell carcinoma, and squamous cell carcinoma.[8]

The poor selectivity of photosensitizing drugs frequently leads to necrosis of surrounding healthy tissues along with a cutaneous photosensitivity that may last several weeks after treatment. Thus, targeting of photosensitizers to cancer cells appears as a viable means to circumvent these problems and to increase efficiency of PDT treatment. Many approaches have been developed by several teams to target tumoral cells,[9–11] and one of them consists in covalently binding polyamines such as spermidine or spermine.[12,13]

Polyamines are ubiquitous low molecular weight cations, which are required for cell proliferation[14] and whose concentrations are especially elevated, in rapidly proliferating tumor cells.[15] A large number of eukaryotic cells harbor a hyperactive polyamine uptake system.[16] Most importantly, the structural requirements of polyamine uptake systems are not stringent; for instance, N^1 and N^8 primary amines of spermidine are the most

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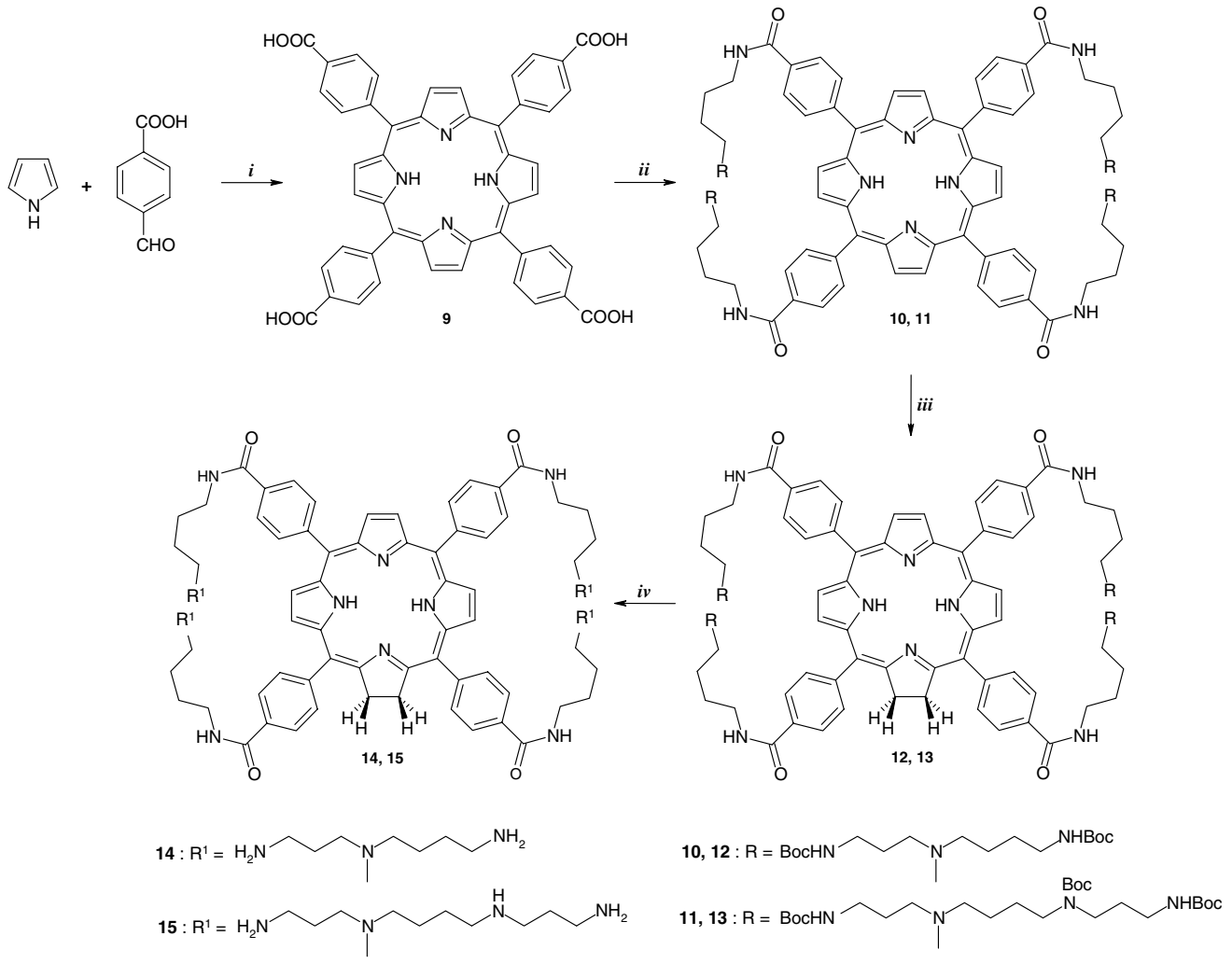
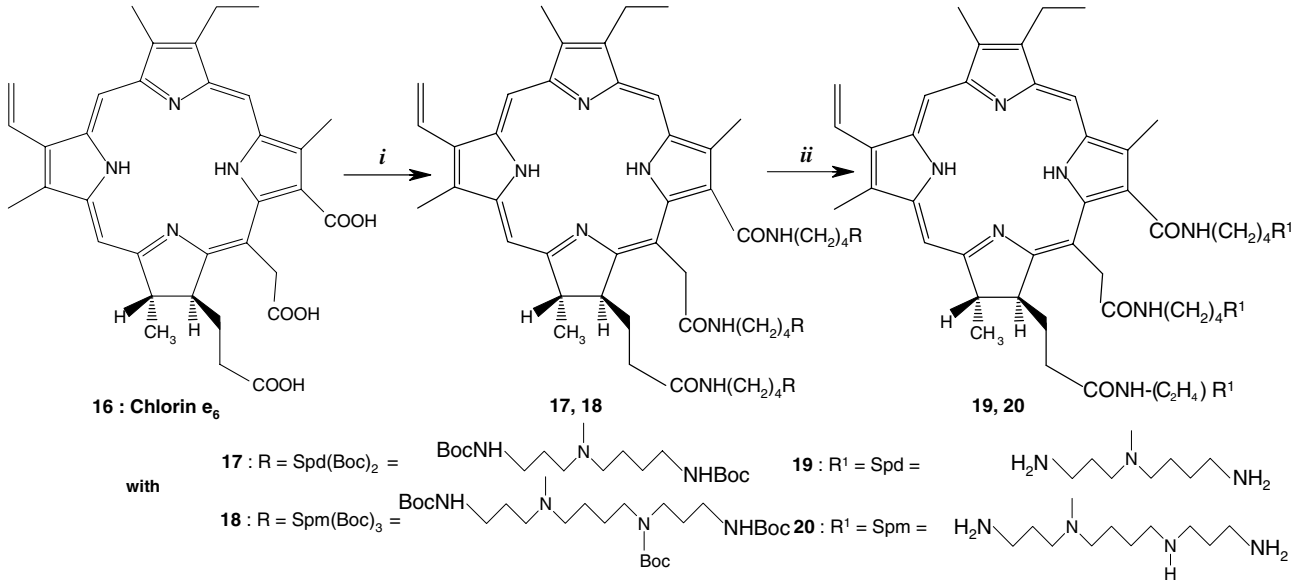
critical determinants for molecular recognition by the transport system, leaving the central N^4 secondary amine for possible derivatization.[17] Polyamine conjugation to enhance the conveying of cytotoxic drugs (acridine–carborane, chlorambucil, and nitroimidazole) to rapidly growing cells is well documented in the literature.[18]

In connection with our research program on photosensitizers and their use in PDT, it occurred to us that porphyrin-based sensitizers in combination with an intracellular recognition element might acquire 'dual action' capabilities.[10,19] To this end, we wanted to generate a suitable hybrid chlorin grafted to polyamine moities. In this paper, we report the synthesis of chlorin derivatives **14** and **15** used from mesoarylporphyrins bearing four polyamine units (spermidine or spermine) (Scheme 2) and **19** and **20** issued from natural chlorin e_6 (Scheme 3), bearing three molecules of polyamines. In all cases, spermine and spermidine analogues **7** and **8** have been attached to the macrocycle core by a spacer arm and an amide linkages.

Synthesis of chlorin–polyamines requires selective protections of spermidine **1** and spermine **2** (see Supplementary Scheme 1). We have used the Boc-protective group because it can be selectively removed in high yields in acid medium.[20] $SpermidineBoc_2$ **3** and $spermineBoc_3$ **4** were obtained in 83% and 72% yields, respectively, in one step from spermidine and spermine using 2-(*tert*-butoxycarbonyloxiimino)-2-phenylacetonitrile (Boc-ON) in dry THF.[18,21] Condensation of protected polyamines **3** and **4** in the presence of *N*-(4-bromobutyl)phthalimide and K_2CO_3 in dry CH_3CN under reflux for 18 h gave polyamine derivatives **5** and **6**, each in 90% yield. Deprotection of the phthalimide group was realized by addition of a large excess of hydrazine monohydrate in THF/EtOH (8:2) to lead to the polyamine derivative **7** or **8**.[22] Reaction was stirred at 90 °C for 5 h and then at 50 °C for 18 h. After treatment and purification by flash chromatography on silica gel, compounds **7** and **8** were obtained in 95% and 86% yield, respectively.[23] The synthesis of meso-tetrakis(4-carboxyphenyl)porphyrin **9** (TCPP) is shown in



Scheme 2. Reagents and conditions: (i) Propionic acid, reflux, 90 min, 16%; (ii) **7** or **8** (4.4 equiv), DCC (4.4 equiv), HOBt (4.4 equiv), DMF, rt, 18 h, 23% **10**, 41% **11**; (iii) K_2CO_3 (10 equiv), toluene-4-sulfonohydrazide, dry pyridine, 104 °C, Ar, 8 h, 42% **12** and **13**; (iv) CF_3COOH/CH_2Cl_2 (1:1), Ar, rt, 2 h, quantitative yields **14** and **15**.



Scheme 3. Reagents and conditions: (i) **7** or **8** (20 equiv), DCC (20 equiv), HOBt (20 equiv), DMF, rt, 96 h, 71% **17**, 72% **18**; (ii) CF_3COOH/CH_2Cl_2 (1:1), Ar, rt, 2 h, quantitative yields **19** and **20**.

Scheme 2. It was synthesized by the Little method, condensation of pyrrole with *para*-carboxybenzaldehyde in stoichiometric quantity in propionic acid and gave the expected porphyrin in 16% yield.[24] Condensation of TCPP **9** with polyamine derivative **7** or **8** was realized in the presence of *N*,*N*′-dicyclocarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) in dry DMF, at room temperature for 18 h. After purification by TLC ($CHCl_3$/EtOH 7:3 + 2% Et_3N), protected polyamine porphyrin conjugates **10** and **11** were obtained in 42% and 51% yield, respectively. Porphyrins **10** and **11** were reduced with di-imide using the method of Whitlock.[25] Porphyrin, anhydrous K_2CO_3, and dry pyridine were stirred by argon flushing. The mixture was heated at 104 °C and further quantities of *p*-toluenesulfonohydrazide (standard di-imide precursor) were added every 2 h for 8 h.[26] After 24 h, the reaction mixture purified with normal workup and compounds **12** and **13** were obtained both in 42% yield. Finally chlorin derivatives **14** and **15** were obtained in quantitative yields after deprotection by treatment with TFA/CH_2Cl_2 (1:1) at room temperature for 2 h.[27] Linkage of chlorin e_6 with polyamine derivatives **7** and **8** was realized as described in the example above (Scheme 3). Then, chlorin e_6–spermidine **17** and chlorin e_6–spermine **18** were obtained in 71% and 72% yields, respectively, and protecting group (Boc) was removed in quantitative yields with TFA in CH_2Cl_2 at room temperature (2 h).

^{1}H NMR (400 MHz) spectra of final compounds showed the expected signals. Mass spectrometry of all chlorin derivatives was performed using the MALDI-TOF technique and for all synthesized chlorins, spectra gave the quasi-molecular peaks $[M+H]^+$. Furthermore, these chlorins show characteristic absorption spectra with a Soret band near 400 nm and a Q (I) band near 660 nm (see Supplementary spectroscopic data).

In order to determine the photosensitizing properties of chlorins **14**, **15**, **19**, and **20**, trapping reactions of $^1O_2$ with ergosterol acetate were carried out.[28] Reference experiments with eosin, rose bengal or hematoporphyrin (HP), known singlet oxygen producers, gave ergosterol acetate epidioxide with nearly quantitative yields. In the same experimental conditions, synthesized chlorins showed the same $^1O_2$ production efficiency as HP.

In medicinal chemistry, lipophilicity has proven an important molecular descriptor that often is well-correlated with the bioactivity of drugs. Lipophilicity is indicated, for example, by the logarithm of a partition coefficient, log *P*, which reflects the equilibrium partitioning of a molecule between a nonpolar and a polar phase, such as the 1-octanol/water system.[29] In this work, we have determined log *P* of chlorin–tetrapolyamine **14** and **15** and chlorin e_6–polyamine conjugates **19** and **20** as $\log([\text{chlorin}]_{1\text{-octanol}}/[\text{chlorin}]_{\text{water}})$ (see Supplementary measurement of log *P* protocol). Results indicate that compounds **14** and **15** are more hydrophilic than **19** and **20** (Table 1).

Chlorin–polyamine conjugates (**14**, **15**, **19**, and **20**) were evaluated for their photocytotoxicity against human chronic myelogenous leukemia cell line (K562) and compared to Photofrin II® and chlorin e_6 at the same concentration (see Supplementary cell culture method). Cells were incubated for 3 h in a RPMI medium in the

Table 1. Partition coefficient of chlorin–polyamine conjugates (Determinations were repeated three times)

Derivatives	**14**	**15**	**19**	**20**
log *P*	−1.87	−1.98	−0.45	−0.83

presence of 2×10^{-6} M chlorins and irradiated with a broad band light source (570–670 nm) (PhotoCure™ Lamp (CURElight)—PhotoCure ASA, Oslo-Norway—COSMEDICO-Medizintechnik) and with a fluence rate of 5–75 J/cm^2 (fluence measured with a red light measuring equipment RLM-1 COSMEDICO-Medizintechnik). Dead cells were identified as propidium iodide (PI) permeable ones and were detected by flow cytometry immediately after irradiation or after a further 24 h incubation in the dark. Cells illuminated without chlorins and cells kept in the dark in the presence of chlorins, used as controls in each experiment, did not present any loss of viability.

The chlorin e_6–polyamine conjugates (**19** and **20**) tested induce significant cell death either just after irradiation or after 24 h in the dark (Fig. 1). Chlorin e_6 shows a less photocytotoxicity and Photofrin II® displays a feeble activity. After 24 h of incubation, a decrease of dead cell percentage was specifically observed in wells containing chlorin e_6–polyamine conjugates (**19** and **20**); this apparent increase is misleading and is most likely the consequence of a massive lysis of nonviable cells during the additional 24 h incubation: a microscopic examination of the well contents revealed the presence of a large amount of cell debris and a very small number of remaining cells among which the percentage of viable ones was actually higher than before this additional 24 h incubation in the dark. This massive lysis and this huge drop in cell count that were not observed in wells containing chlorin e_6 could then be attributed to the presence of polyamine groups on the same side of the chlorin e_6.

For chlorin–tetrapolyamine conjugates **14** and **15**, biological assays have showed a less photocytotoxicity (Fig. 1). Thus, the efficacy of the photoactivity is influenced by the hydrophilic/lipophilic character of the compounds; the presence of three polyamine units on the same side of the macrocycle (**19** and **20**) increases in parallel amphilic character and phototoxicity of these molecules.

On the other hand, the nature of polyamine units does not seem to be important; indeed, chlorin conjugates bearing spermine and spermidine units showed the same activity. Further biological evaluation of compounds **19** and **20** is currently in progress in our laboratory and will be reported elsewhere.

A series of new polyamine–chlorin conjugates have been designed, synthesized, and characterized. Preliminary in vitro tests confirm previous observations

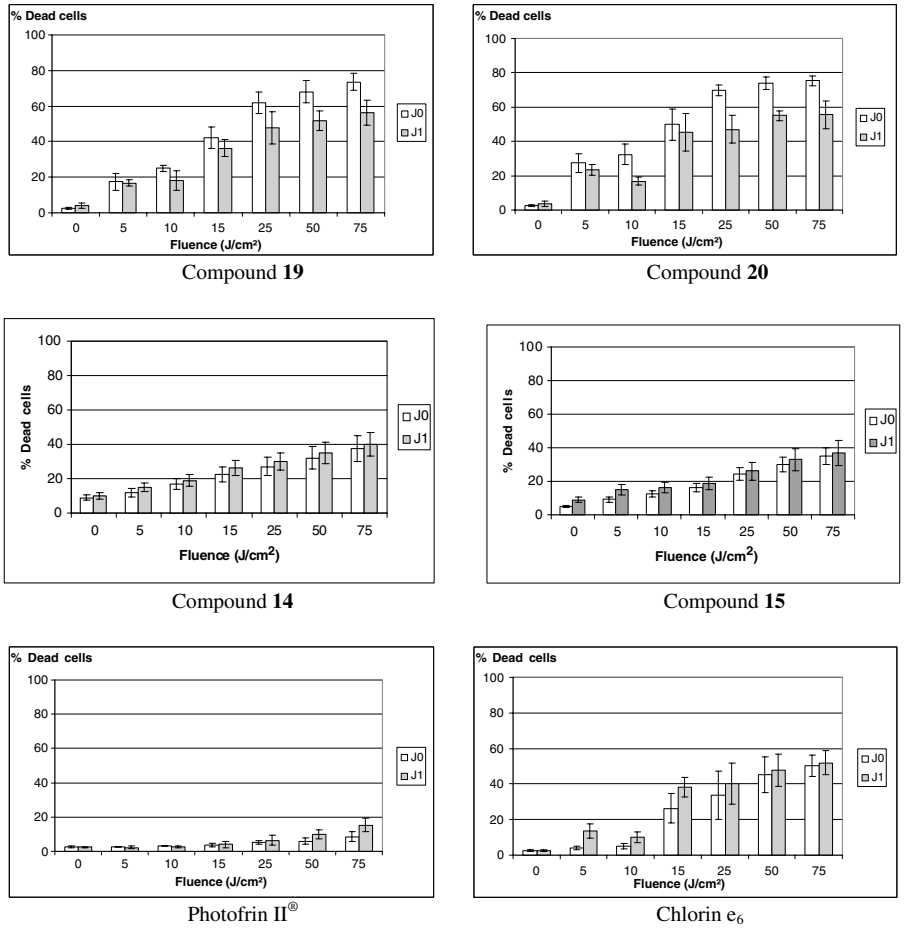


Figure 1. Percentage of propidium iodide (PI)-stained cells versus fluence. Cell suspensions were incubated with 2 μM of the indicated photosensitizer during 3 h prior to irradiation. Open bars: dead cell count after indicated irradiation time. Solid bars: dead cell count after a further 24 h incubation in the dark. Each histogram represents the average of three independent experiments (±standard deviation).

suggesting the requirement of amphiphilicity for efficient photodynamic activity. The grafting of three polyamine units is obviously a good means for bringing a efficient balance between hydrophilicity and hydrophobicity. Owing to the high photosensitizing potential of these new conjugates, the specific influences of polyamine moieties on cellular uptake and binding to specific cellular targets deserve additional studies that are currently underway, along with interactions with nucleic acids and their subsequent photocleavage.

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Supplementary data

Scheme of synthesis of polyamines **3**–**8**, spectroscopic data (Rf, UV, ^{1}H NMR, MALDI, HRMS, and microanalytical data) of photosensitizers **12**–**15**, **17**–**20**, and experimental information concerning partition coefficient measurement and cell culture method is available in Supplementary data. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.03.044.

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