Biochemical and Biophysical Research Communications **281,** 32–36 (2001)
doi:10.1006/bbrc.2001.4289, available online at http://www.idealibrary.com on IDEAL®

Water-Soluble ^{99m}Tc-Labeled Dendritic Novel Porphyrins Tumor Imaging and Diagnosis

Murugesan Subbarayan,*,† Shankar J. Shetty,‡ Tapeshwari S. Srivastava,‡ Oliver P. D. Noronha,* Aben M. Samuel,* and Hasan Mukhtar†,[1]

*Radiation Medicine Centre, Bhabha Atomic Research Centre, Tata Memorial Hospital Annexe, Parel, Mumbai 400 012, India; †Department of Chemistry, Indian Institute of Technology, Powai, Mumbai 400 076, India; and ‡Department of Dermatology, Case Western Reserve University, Cleveland, Ohio 44106

Received December 27, 2000

We have synthesized two water soluble dendritic porphyrins, termed DP1 and DP2 and have successfully radiolabeled them with ^{99m}Tc. These ^{99m}Tc-labeled porphyrins were administered to C_6-glioma tumor bearing Wistar rats and scintiimaging and biodistribution studies were carried out. Tumor to muscle ratios of DP1 and DP2 were 8.0 and 9.7, respectively. These molecules may have potential for tumor imaging and diagnosis and may even prove useful as photosensitizers in photodynamic therapy applications.

Key Words: **^{99m}Tc-labeled dendritic porphyrin; scintiimaging; photodynamic therapy; photodynamic diagnosis.**

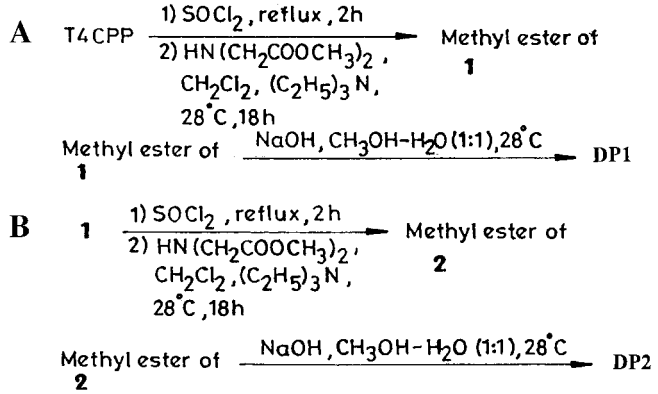
Photodynamic therapy (PDT), a new treatment modality, uses a combination of photosensitizing drug and visible/near infrared light for the management of a variety of solid malignancies and many nonmalignant disorders (1, 2). Most of the studies carried out on PDT have employed Photofrin as a photosensitizer. The fluorescence of porphyrins is also used for the diagnostic detection of tumor cells, such as early stage cancer of the skin, bladder, lung, and brain. This procedure, termed photodynamic diagnosis (PDD), helps to define poorly defined tumor borders before the use of invasive PDT treatment (1, 2). The fluorescence of δ-aminolevulinic acid biotransformed protoporphyrin IX is a commonly used PDD procedure for cutaneous lesions (2). Photofrin, despite of its proven efficacy, has many limitations limiting its widespread use in PDT protocols. The development of the effective second generation photosensitizers suitable for PDD and PDT is a continuing area of investigation (1, 2).

Over the last decade, there has been remarkable advances in the synthesis of dendrimers (3–8). This development has provided a new methodology for dendritic architecture to build up globular shaped hyperbranched macromolecules of nanoscopic size. Much of the earlier work in this area concentrated in the synthesis of dendrimers with few skeletons in high generation. In recent years there has been greater emphasis in the development of dendritic architectures incorporating specific functional moieties either on the surface or in the interior of dendrimers. The shape and structures of these dendrimers with three distinct environments, namely, core, branched shell and external surface, have been elegantly utilized to replicate or modulate processes known from biological system (4, 7, 8). The research activities on these lines have led to the most spectacular applications of dendrimers in magnetic resonance imaging as a contrast agent to visualise the blood-stream in the body for diagnostic purposes (9–13). Dendrimers have also shown promise as therapeutic agents in boron neutron capture therapy (14–16) as well as in gene therapy (17–19).

Dendrimers with multiple identical ligands are very attractive agents because these structures can exhibit amplified substrate binding (8, 20). We considered that it should be possible to synthesize dendrimers with porphyrin core which may have superior tumor accumulation than porphyrins. We have earlier reported the preparation of ^{99m}Tc-labeled 5,10,15,20-tetrakis[4-(carboxymethyleneoxy) phenyl] porphyrin (T4CPP) which accumulated preferentially in mammary tumor-bearing rats (21). The present study reports the synthesis of two dendrimers with T4CPP core, termed as DP1 and DP2 molecules. These two ^{99m}Tc-labeled dendrimers were found to be preferentially localized in tumors than in surrounding muscle in C_6-glioma tumor-bearing Wistar rats.

[1] To whom correspondence should be addressed at Department of Dermatology, Case Western Reserve University, 11100 Euclid Avenue, Cleveland, Ohio 44106. Fax: 216-368-0212. E-mail: hxm4@po.cwru.edu.

0006-291X/01 $35.00



SCHEME 1

MATERIALS AND METHODS

General

4-Hydroxybenzaldehyde (Fluka), pyrrole (Alrich) ethyl chloroacetate (Aldrich), iminediactic acid (Alrich) were used. Other chemicals used were of analytical grade. The solvents were purified and dried before use by the standard methods (22). $^{99m}TcO_4^-$ used in this study was obtained from ^{99}Mo and further processed by solvent extraction procedures (32).

Synthesis

The methyl esters of the first generation dendritic porphyrin DP1 and the second generation dendritic porphyrin DP2 have been prepared as described recently (23).

First Generation Dendritic Porphyrin (DP1)

To prepare the water soluble dendritic porphyrin (DP1), the methyl ester of 1 (500 mg) was hydrolyzed with NaOH in methanol-water ratio (24). The hydrolyzed product was neutralized by 2 N hydrochloric acid, and kept overnight at 10°C. The solid mass thus obtained was filtered, washed with small portions of ice-cold water, and methanol and finally dried (yield: 85%) IR(K Br):1610, 1640 {((C═O)} cm^{-1} 1H NMR (500 MHz, D_2O, DSS); 4.16 (s, 8H-NCH_2-protons), 4.21 (s, 8H-NCH_2-protons), 5.29 (s, 8H,-OCH_2-protons), 7.45–7.65 (m, 16 H, benzenoid protons), 8.90 (sb, 8H, pyrrole (H protons) ppm. UV/vis (H_2O): (max) 421 (5,00,000), 483 (3260), 519 (21,000), 546 (6050), 589 (5620), 651 (4830) nm. Analytically calculated for C68 H58 N8 O24: C, 59.56, H, 4.26; N, 8.17. Found C, 59.92; H, 4.32; N, 8.25. The structure of DP1 is shown in Scheme 1.

Second Generation Dendritic Porphyrin (DP2)

The water soluble second generation dendritic porphyrin (DP2) was prepared by following the procedure as given for 1 except the methyl ester of 2 (500 mg) was used in place of the methyl ester of 1. Final dried product was obtained in 30% yield. IR (KBr):1615, 1640 {v (C═O)} cm^{-1}. ^1H NMR (500 MHz, D2), DSS): (3.60–3.80 (sb, 32 H 2nd generation -NCH_2-protons), 3.90 (s, 8H, 1st generation -NCH_2-protons), 4.85 (s, 8H -OCH_2-protons), 7.65–8.10 (dd, 16H, benznoid protons), 8.50 (s, 8H, pyrrole (-H protons) ppm. UV/Vis (H_2O): (max) 422 (5,00,000), 482 (3800), 519 (21000) 546 (6050), 589 (5,200), 651 (4200) nm. Analytically calculated for C100 H98 N16 O48: C, 52.41; H, 4.31; N, 9.78 Found: C, 51.27; H, 4.82; N, 9.92. Its structure is shown in Scheme 1.

Radiolabeling

Radiolabeling of these photosensitisers was carried out with ^{99m}Tc using Sn^{2+} ion as a reducing agent. Two milligrams of these ligands was dissolved in 0.4 ml of physiological saline. This was treated with 20 μl (20 μg) of a solution of stannous chloride (2.0 mg of $SnCl_2 \cdot 2H_2O$ in 0.1 N HCl and made it to 2.0 ml with physiological saline) followed by the addition of 0.2 ml of $^{99m}TcO_4^-$ (74 MBq). The reaction mixture was incubated of room temperature for 15 min. The reaction product was then passed through 0.22 μm membrane filter to obtain the product free from colloidal particulates. The radiochemical purity was greater than 95% as determined by ascending paper chromatography using physiological saline as development solvent (R_f of labeled compound = 0.0; while for free TcO_4^- = 0.7–0.8).

Animal Tumor Model

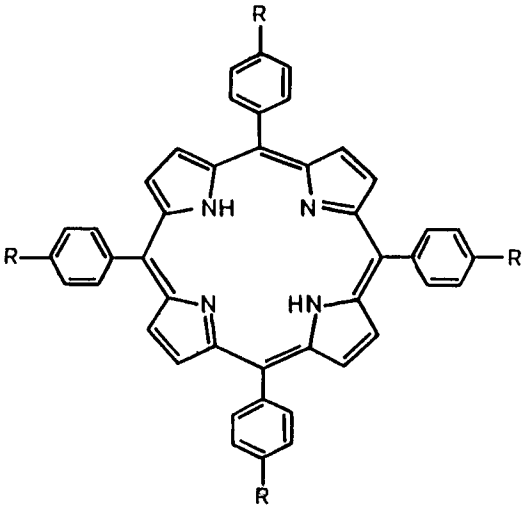
C_6-glioma tumor model. C_6-glioma cell line was obtained from National Institute of Tissue Culture Centre (Pune, India) and 5.0×10^6 cells were injected into newborn Wistar rat pups. Tumor size was monitored daily and was excised when it attained diameter between 10–12 mm. The tumors were fresh and nonnecrotic and were collected in sterile petri dishes. The tumor tissues were cut into fine pieces with sterile scissors in approximately 4.0 ml sterile phosphate buffer solution and filtered through a sterile wiremesh. The cell suspension (0.1 ml) was then injected into the newborn pups through subcutaneous injection on the back. These pups were periodically monitored for palpable tumors twice a week. When the tumor size reached a diameter 20–24 mm, these animals were subjected to scintigraphic imaging using gamma camera computer systems.

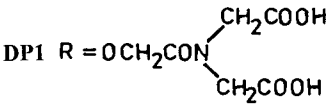
Scintiimaging

C_6-glioma tumor-bearing rats were anaesthetised by ether and 0.4 ml of ^{99m}Tc-dendritic porphyrins was injected intravenously through tail vein. *In vivo* scintiimaging studies were performed at 5.0 h post injection by acquiring 400 kilocounts on a 256 × 256 matrix using gamma camera with low energy all purpose collimator with a energy setting centered at 140 KeV and a 20% window. The accumulated tracer activity in tumor and other organs was determined by drawing regions of interest and counts obtained from these areas were normalised to same pixel counts to thigh muscle region of interest. Then tumor to muscle ratios were then determined.

RESULTS AND DISCUSSION

The strategy used for the synthesis of DP1 and DP2 is given in Scheme 1. The starting material for synthesis of DP1 is a tetraacid chloride of T4CPP. The latter compound was prepared by reaction of T4CPP with $SOCl_2$. Next the tetraacid chloride of T4CPP was coupled with *N*,*N*-di(carbomethoxymethylene)amine. The methyl ester of 1 thus obtained was hydrolyzed by NaOH in methanol-water (24) which resulted in the formation of DP1 (Scheme 1a). A repeat of this reaction sequence resulted in the generation of the methyl ester which on hydrolysis with NaOH in methanol-water gave DP2 (Scheme 1b). The compounds DP1 and DP2 show two C═O stretching frequencies suggesting two types of C═O groups. The ^1H NMR spectrum of DP1 shows peaks to both porphyrin moiety and *N*,*N*-di(carboxymethylene)amine groups. The peaks of porphyrin moiety was observed





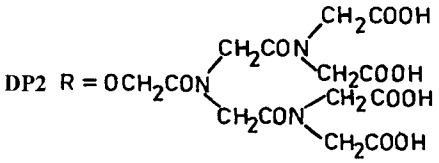


FIGURE 1

between 7.45 and 7.65 ppm (benzenoid protons) and at 8.90 ppm (pyrrole B-H protons). The peaks of side chains were observed at 4.16 (-NCH_2-protons) 4.21 (-NCH_2-protons) and 5.29 ppm (-OCH_2-protons). Similarly the ^{1}H NMR spectrum of DP2 showed peaks of porphyrin moiety at 7.65–8.10 ppm (benzenoid protons) and at 8.50 ppm (pyrrole (-H protons). Other peaks present due to side chains at 3.60–3.80 ppm (2nd generation-NCH_2-protons) 3.90, 4.00 ppm (1st generation-NCH_2-protons) and 4.85 ppm (-OCH_2-protons). The UV-Vis spectra of DP1 and DP2 exhibited typical porphyrin type with a Soret at 421–422 nm and four visible bands between 519–615 nm (23). The above spectral data on DP1 and DP2 support their structures as given in Fig. 1.

With a view to ascertain the efficacy of these dendritic porphyrins for detection of tumors, these molecules were labeled with ^{99m}Tc. Chromatography of these labeled complex showed a radiochemical purity of greater than 95% and the labeled product was found to be stable (*in vitro*) for more than 4 h at room temperature. Scintigraphic imaging studies were performed 5 h postinjection of the complex into the C_6-glioma tumor bearing rats using gamma camera computer system. The accumulation of the agent in the tumor is shown in Fig. 2. Tumor to muscle (T/M) ratio

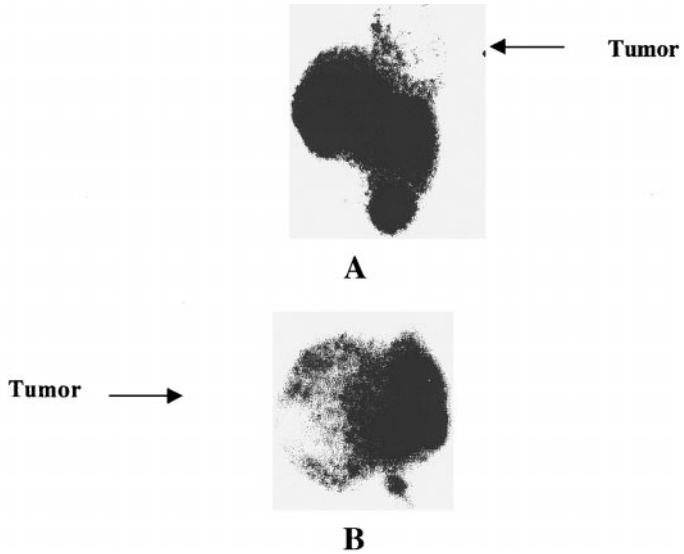


FIG. 2. Scintiimage of ^{99m}Tc-dendritic porphyrins of (A) DP1 in C_6-glioma tumor Wistar rat (B) DP1 in C_6-glioma tumor Wistar rat.

was calculated and the results obtained are shown in Table 1.

Wong earlier labeled hematoporphyrin derivative (HpD), the earliest employed photodynamic therapy photosensitizer, with ^{99m}Tc but was unsuccessful (28). The reason could be that the HpD/Photofrin II is a mixture of many oligomers and monomers with no chelating moieties. The dendritic porphyrins were designed to synthesis as a single molecule with iminodiacetic acid groups as chelating moieties for successful labeling with ^{99m}Tc. The labeling of these dendritic porphyrins is therefore unique and the results obtained were compared with published data with ^{14}C/^{3}H labeled HpD (Table 2). The T/M ratio obtained in the present study was significantly higher (DP1, 8; DP2, 9.7) as compared to that of ^{14}C/^{3}H labeled HpD (3.37; 3.43) validating the significance of these ligands for

TABLE 1

In Vivo Distribution of ^{99m}Tc Dendritic Porphyrins in C_6 Glioma Tumor-Bearing Wistar Rats

Organs	^{99m}Tc-1st generation dendritic porphyrin (DP1) $n = 8$	^{99m}Tc-2nd generation dendritic porphyrin (DP2) $n = 8$
Tumor	29.72 ± 1.95	30.22 ± 1.21
Liver	10.21 ± 1.02	9.58 ± 0.95
Kidneys	29.02 ± 1.75	31.77 ± 2.01
Muscle	3.71 ± 0.35	3.12 ± 0.25
T/M ratio[a]	8.0	9.7

Note. n, number of rats used for each study.
[a] Normalized with pixel counts.

TABLE 2

Comparison of ^{99m}Tc-Labeled Dendritic Porphyrins with That of ^{14}C-HpD and ^{3}H-HpD

Animal species	Tumor	Muscle	T/M ratio	Labeled ligands	Reference
C_3Hf/sed BH mice	3.31	0.98	3.4	^{14}C-HpD	Gomer and Doughertry 1979 (30)
DBA/2HaDD mice	5.35	1.56	3.4	^{3}H-HpD	Gomer and Doughertry 1979 (30)
C_6-glioma rats	29.72	3.71	8.0	^{99m}Tc-DP1	Present study
C_6-glioma rats	30.2	3.12	9.7	^{99m}Tc-DP2	Present study
NMU-induced mammary tumor rats	4.72	1.00	4.7	^{99m}Tc-T3,4BCPC	Murugesan *et al.* (in press) (31)

possible tumor localizing agents. The difference in T/M ratio could be due to species-specificity. An ideal agent, for tumor imaging and diagnosis, should accumulate in the target tissue with a target to nontarget ratio greater than 3.0 (29). The sufficiently high T/M ratio obtained in this study indicates that both radiolabeled dendritic porphyrins DP1 and DP2 have potentials for use in tumor imaging and diagnosis. Non-invasive detection of tumors using these radiolabeled photosensitisers could be a simple, viable and a practical method of tumor detection. Under optimized conditions, the measurement of accumulation of the porphyrins in different tissues/organs using this technique could even supersede the optical methods based on flurometry (26) or reflectance spectrophotometry (27). In addition to use of radiolabeled DP1 and DP2 for detection of tumors, these agents could be employed to monitor the progression or regression of tumors following many treatment protocol before, during and after chemotherapy or PDT. If these dendritic porphyrins DP1 and DP2 on light illumination are effective generators of singlet oxygen, then these could even be used as photosensitizers in PDT protocols. If these applications are possible then these DP1 and DP2 could have dual applications in detection (PDD) and treatment of cancer (PDT).

ACKNOWLEDGMENTS

We wish to thank Shri R. B. Patkar and Shri Girish L. Waghinkar for their help extended in carrying out the experiments. The efforts of Dr. Mukhtar were supported by USPHS Grants RO1 51802 and PO1 CA48735.

REFERENCES

1. Dougherty, T. J., Gomer, C. J., Henderson, B. W., Jori, G., Kessel, D., Korbelik, M., Mean, J., and Peng, Q. (1998) *J. Natl. Cancer Inst.* **90,** 889–905.
2. Kalka, K., Merk, H., and Mukhtar, H. (2000) *J. Amer. Acad. Dermatol.* **42,** 389–413.
3. Newkome, G. R., Moorefield, C. N., and Vogtle, F. (1996) Dendritic Molecules: Concepts, Synthesis and Perspectives, VCH, Weinheim, New York.
4. Zeng, F., and Zimmerman, S. C. (1997) *Chem. Rev.* **97,** 1681–1712.
5. Archut, A., and Vogtle, F. (1998) *Chem. Soc. Rev.* **27,** 233–240.
6. Smith, K. M., and Diederich, F. (1998) *Chem. Eur. J.* **4,** 1353–1361.
7. Fischer, M., and Vogtle, F. (1999) *Angew Chem. Int. Ed.* **38,** 885–905.
8. Bosman, A. W., Janssen, H. M., and Meijer, E. W. (1999) *Chem. Rev.* **99,** 1665–1688.
9. Wiener, E. C., Brechbiel, M. W., Brothers, H., Magin, R. L., Gansow, O. A., Tomalia, D. A., and Lauterbur, P. C. (1994) *Magn. Reson. Med.* **31,** 1–8.
10. Wiener, E. C., Auteri, F. P., Chen, J. W., Brechbiel, J. W., Gansow, M. W., Schneider, D. S., Belford, R. L., Clarksen, R. B., and Lauterbur, P. C. (1996) *J. Am. Chem. Soc.* **118,** 7774–7782.
11. Toth, E., Pubanz, D., Vauthey, S., Holm, L., and Merbach, A. E. (1996) *Chem. Eur. J.* **2,** 1607–1615.
12. Adams, G., Neuerberg, J., Spuntrup, E., Muhler, A., Scherer, K., and Gunther, R. W. (1994) *J. Magn. Reson. Imag.* **4,** 462–466.
13. Bourne, M. W., Margerun, L., Hylton, N., Champion, B., Lai, J. J., Derugin, N., and Higgins, C. B. (1996) *J. Magn. Reson. Imag.* **6,** 305–310.
14. Qualmann, B., Kessels, M. M., Musiol, H.-J., Sierralta, W. D., Junglelut, P. W., and Moroder, L. (1996) *Angew Chem. Int. Ed. Engl.* **35,** 909–911.
15. Qualmann, B., Kessels, M. M., Klobasa, F., Junglelut, P. W., and Sierralta, W. D. (1996) *J. Microsc.* **183,** 69–77.
16. Barth, R. F., Adams, D. M., Soloway, A. H., Alam, F., and Darley, M. V. (1994) *Bioconjug. Chem.* **5,** 58–66.
17. Haensler, J., and Szoka, F. C., Jr. (1993) *Bioconjug. Chem.* **4,** 372–379.
18. Kukowska Latallo, J., Bielinkska, A. U., Johnson, J., Spindler, R., Tomalia, D. A., and Baker, J. R., Jr. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 4897–4902.
19. Qin, L., Pahud, D. R., Ding, Y., Bielinska, A. U., Kukowska-Latallo, J. F., Baker, J. R., Jr., and Bromberg, J. S. (1998) *Human Gene Ther.* **9,** 553–560.
20. Mammen, M., Choi, S.-K., and Whitesides, G. M. (1998) *Angew. Chem. Int. Ed.* **37,** 2754–2794.
21. Chatterjee, S. R., Murugesan, S., Kamat, J. P., Shetty, S. J., Srivastava, T. S., Noronha, O. P. D., Samuel, A. M., and Devasagayan, T. P. A. (1997) *Arch. Biochem. Biophys.* **339,** 242–249.
22. Furniss, B. S., Hannaford, A. J., Smith, P. W. G., and Talchel, A. R. (1989) Vogel's Text Book of Practical Organic Chemistry, pp. 395–413, 5th ed., Longman, London.
23. Singh, B. P., Vijaya, R., Shetty, S. J., Kandaswamy, K., Puntambekar, P. N., and Srivastava, T. S. *J. Porphyrins Phthalocyanines*, in press.
24. Newkome, G. R., and Lin, X. (1991) *Macromolecules* **24,** 1443–1444.
25. Gouterman, M. (1959) *J. Chem. Phys.* **30,** 1139–1161.
26. Progio and Sarnaik, C. (1984) *in* Porphyrin Localization and

Treatment of Tumors (Doivon, D. R., and Gomer, C. J., Eds.), p. 163, Alan R. Liss, New York.

27. Patterson, M. S., Wilson, B. C., Feather, J. W., Burns, D. M., and Pushka, W. (1987) *Photochem. Photobiol.* **46,** 337–343.
28. Wong, D. W. (1983) *J. Label. Comp. Radiopharm.* **20,** 351–361.
29. Strauss, A. W., Nunn, A., and Linder, K. (1995) *J. Nucl. Cardiol.* **2,** 437–445.
30. Gomer, C. J., and Dougherty, T. J. (1979) *Cancer Res.* **39,** 146–151.
31. Murugesan, S., Shetty, S. J., Srivastava, T. S., Noronha, O. P. D., and Samuel, A. M. *J. Photochem. Photobiol. B: Biol.*, in press.
32. Noronha, O. P. D. (1985) IAEA Publication, pp. 223–232, Vienna.