

Synthesis and Application of New Gadolinium-Porphyrins as Potential MR Imaging Contrast Agents for Cancer Detection in Nude Mice

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ABSTRACT

Two new potential magnetic resonance imaging contrast agents, Gd-hematoporphyrin (Gd-H) and Gd-tetra-carboranyl-methoxyphenyl-porphyrin (Gd-TCP), were synthesized and applied to nude mice with human melanoma (MM-138) xenografts. These agents showed a high relaxivity because of their greater potential to coordinate water molecules. The reduction of T₁ relaxation times of 16 and 21% was observed in human melanoma tumors grafted in the nude mice 24 h after injection of Gd-TCP and Gd-H, respectively. The percent of injected Gd, that localized to the tumor and measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES), was approximately 21% for Gd-TCP and 28% for Gd-H. A higher concentration of Gd was achieved compared with control indicating selective delivery of Gd-porphyrins to the melanoma xenografts. The data indicate that Gd-TCP can be used as a dual probe for diagnosis in MR imaging and for therapy in boron neutron capture therapy (BNCT). *Iran. Biomed. J. 5 (2 & 3): 87-95, 2001*

Keywords: MR imaging, Contrast agent, Porphyrins, Melanoma, Cancer

INTRODUCTION

The use of contrast agents to shortening relaxation times following enhanced signal intensity may extend the potential of magnetic resonance (MR) imaging to diagnosis of tumors in the early stages. Paramagnetic chelates using the endogenous porphyrin ring as the chelating agents are a promising and interesting family group of potential MR imaging contrast agents [1]. Gd-porphyrins have been synthesized and are currently being investigated [2-4] and shown selective affinity for a variety of tumors [5]. By choosing gadolinium as the metal for incorporation into the carboranyl-methoxyphenyl-porphyrin (TCP) and hematoporphyrin, they can be used simultaneously as MR imaging contrast agents. For this reason, two new gadolinium complexes of porphyrins were synthesized. The synthetic porphyrin, 1, 6, 11, 16-tetra (3-*o*-carboranyl-methoxy) phenyl-porphyrin was produced by modification of the method of Miura *et al.* [6] and was inserted with Gd to yield Gd-tetra-carboranyl-methoxyphenyl-porphyrin (Gd-TCP). The naturally occurring porphyrin,

hematoporphyrin IX was also inserted with Gd to yield Gd-hematoporphyrin (Gd-H).

The porphyrins offer a stable chelate for the transportation of paramagnetic metals into the tumors and by attachment they could destroy the cancer cells. The synthetic porphyrin TCP is an example of this concept where boron atoms have been attached chemically to the porphyrin, thus offering the potential for boron neutron capture therapy (BNCT). The gadolinium complex of these two porphyrins is unknown and in this work the synthesis of these MR imaging contrast agents are investigated.

An animal study was performed for developing radiopharmaceuticals and pharmacokinetics of these contrast agents. In the present study, Gd-hematoporphyrin (Gd- [18, 13-bis (hydroxyethyl)-3, 7, 12, 17-tetramethyl-21H, 23 H porphin-2, 18-dipropionic acid]) and Gd-TCP (Gadolinium-tetra-carboranyl-methoxyphenyl-porphyrin) were targeted into the nude mice model with a human melanoma (MM-138) xenograft [7]. The bio-distribution, the T₁ relaxation times, and the signal enhancement of the contrast agents are presented and the results are compared. The gadolinium concentration of tissues

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was determined using an acid digestion method [8] by Inductivity Coupled Plasma Atomic Emission Spectroscopy (ICP-AES).

MATERIALS AND METHODS

Gd-H. Gadolinium (III) nitrate hexa-hydrate (0.30 g, 0.66 mmol) was dissolved in 2 ml of distilled water. Hematoporphyrin ([8, 13-bis (1-hydroxyethyl)-3, 7, 12, 17-tetramethyl-21H, 23H-porphine-2, 18-dipropionic acid]) (Sigma, Aldrich) powder (0.40 g, 0.66 mmol) was suspended in 2 ml of distilled water, added to the gadolinium solution and refluxed until the solution become homogeneous. The solution was allowed to cool at room temperature and then was concentrated to 1 ml under reduced pressure by heating. The resulting white solid was filtered, washed carefully with ice-cold water (2 × 0.5 ml) and dried in the oven at 80°C. The yield was 0.11 g (21%).

Gd-TCP. The experimental procedure for the synthesis of this new MR imaging contrast agent is as follows:

The synthesis of this compound involved the preparation of the known porphyrin, tetra-carboranylmethoxyphenyl-porphyrin (TCP-H₂) followed by insertion of the gadolinium ion into this porphyrin. The first part of this synthesis involved the formation of an aldehyde containing the *o*-carborane group. This aldehyde was reacted with pyrrole to form the porphyrin, TCP-H₂. The gadolinium ion was inserted into TCP-H₂ by adaptation of Miura's method [6] for the nickel complex. The synthetic route for the production of Gd-TCP is shown in Figure 1. The purity and identity of each compound was confirmed by low-resolution mass spectroscopy, LRMS (Shimadzu QP5000/GC17A), and NMR analysis, both of them are previously discussed.

(3-(*o*-carboranylmethoxy)phenyl)methanol. (3-(*o*-carboranylmethoxy)phenyl)-methyl ethanoate (5.2 g, 24.5 mmol) was refluxed in a 1:80 (v/v) conc. HCl: MeOH (80 ml) at 60°C for 1 hour. The solvent was removed under reduced pressure yielding a crimson solid. The yield was 5.8 g (85%).

MS (molecular ion, m/z (peak intensity))

Observed. 276.30 (2.3); 277.30 (9.1); 278.30 (28.9); 279.30 (65.8); 280.30 (100.0); 281.30 (93.8); 282.30 (43.4); 283.30 (4.8).

Calculated. 276.26 (1.6); 277.26 (8.0); 278.25 (27.3); 279.25 (64.2); 280.25 (100.0); 281.24 (94.3); 282.24 (43.3); 283.24 (3.7).

¹H NMR (CDCl₃). δ ppm 6.90 (d, 1H, *J*_(HH)2.1 Hz, ArH); 7.00 (d, 1H, *J*_(HH)7.4 Hz, ArH); 7.30 (t, 1H, *J*_(HH)7.4 Hz, ArH); 6.75 (dd, 1H, *J*_(HH)7.4, 2.1 Hz, ArH); 4.70 (s, 2H, B₁₀H₁₁C₂CH₂O-Ar); 4.40 (s, 2H, Ar-CH₂OH); 4.10 (br s, 1H, carborane CH); 1.5-3.3 (br m, 10H, B₁₀H₁₀).

¹³C NMR (CDCl₃). δ ppm 64.7 (Ar-CH₂OH); 142.9, 157.3 (ArCR); 129.9, 120.8, 113.8, 112.9 (ArCH); 69.1 (B₁₀H₁₁C₂CH₂O-Ar); 57.8 (RCH, carborane); 71.3 (CR, carborane).

¹¹B (CDCl₃). δ ppm 51.7, 49.9, 45.5, 42.9, 41.6.

3-*o*-carboranylmethoxybenzaldehyde. (3-(*o*-carboranylmethoxy)phenyl)-methanol (7.8 g, 27.7 mmol) was added to pyridiniumchlorochromate (9.0 g, 41.6 mmol) in dichloromethane (62 ml) at 0°C and was stirred for 2.5 hours. The combined solution was washed with water (50 ml × 3) and then extracted with dichloromethane (50 ml × 3). The combined extracts were dried with anhydrous sodium sulfate and the solvent was removed under reduced pressure. The resulting compound was purified using flash chromatography (silica, gel CH₂Cl₂ eluent) to give 6.2 g (80%) of a crimson crystalline solid.

MS (molecular ion, m/z (peak intensity))

Observed. 275.25 (3.1); 276.30 (10.7); 277.30 (21.1); 278.30 (30.4); 279.30 (29.0); 280.30 (13.6); 281.40 (1.7).

Calculated. 275.24 (2.4); 276.24 (8.3); 277.23 (19.5); 278.23 (30.4); 279.23 (28.7); 280.22 (13.2); 281.22 (1.1).

¹H NMR (CDCl₃). δ ppm 9.90 (s, 1H, CHO); 7.35 (dd, 1H, *J*_(HH) 1.1, 2.5 Hz, ArH); 7.14 (dt, 1H, *J*_(HH)7.7, 2.6 Hz, ArH); 7.48 (t, 1H, *J*_(HH)7.7 Hz, ArH); 7.53 (ddd, 1H, *J*_(HH)7.7, 2.6, 1.1 Hz, ArH); 4.50 (s, 2H, B₁₀H₁₁C₂CH₂O-Ar); 4.10 (s, 1H, carborane CH); 1.5-3.3 (br m, 10H, B₁₀H₁₀).

¹³C NMR (CDCl₃). δ ppm 191.4 (Ar-CHO); 137.9, 157.5 (ArC-); 130.6, 125.5, 121.8, 112.5 (ArC-H); 69.3 (B₁₀H₁₁C₂CH₂O-Ar); 57.8 (RCH, carborane); 71.3 (CR, carborane).

^{11}B (CDCl_3). δ ppm 51.9, 50.0, 45.6, 42.9, 41.5.

1, 6, 11, 16-tetra-[3-(carboranylmethoxy)phenyl]-porphyrin. 3-*o*-carboranyl-methoxy-benzaldehyde (4.2 g, 15 mmol) and pyrrole (1.04 ml, 15.0 mmol) were added to 1 L of distilled CH_2Cl_2 in a 2 L, three-neck, round-bottomed flask fitted with a reflux condenser and nitrogen inlet port, and stirred magnetically at room temperature. After 15 minutes, BF_3 etherate (0.4 ml of a 2.5 M, 37.5 mmol solution in CH_2Cl_2 , 10^{-3} M) was added and the reaction vessel was shielded from ambient lighting. After 1 hour, *p*-chloranil (2.8 g, 11.0 mmol) was added. The flask was immersed in a water bath and the solution was refluxed for 1 hour. The solution was then concentrated to approximately 50 ml under reduced pressure, and 10-12 g of Florisil was added. The slurry was further dried under reduced pressure to afford a damp dark powder, which was poured onto the top of a chromatography column (2.5 cm diameter) filled with Florisil (38 cm in height). The column was eluted with CH_2Cl_2 /petroleum ether (3:1, 500 ml). The product was then eluted from the column with CH_2Cl_2 (300-400 ml). The solvent was removed under reduced pressure and a crystalline purple solid was obtained. The yield was 2.1 g (43%).

^1H NMR (CDCl_3). δ ppm 2.85 (br s, 2H, NH); 1.4-3.2 (br s, 40H, $\text{B}_{10}\text{H}_{10}$); 4.12 (s, 4H, carborane CH); 4.48 (s, 8H, CH_2); 7.26 (m, 4H, benz. ArH); 7.76 (m, 8H, benz. ArH); 7.93 (m, 4H, benz. ArH); 8.93 (s, 8H, pyr.ArH).

^{13}C NMR (CDCl_3). δ ppm 131.0 (*meso*-carbons); 143.1, 156.1 (benz.ArC-); 129.2, 128.1, 121.0, 114.2 (benz.ArCH); 69.6 ($\text{CH}_2\text{O-Ar}$); 57.9 (RCH , carborane); 71.4 (CR , carborane); 128.5 (pyr.ArC-); 121.3 (pyr.ArCH).

^{11}B (CDCl_3). δ ppm 51.9, 50.0, 45.5, 43.0, 41.9.
UV-Vis. (CH_2Cl_2) λ_{max} nm: 417, 513, 548, 590, 649.

Gadolinium-1, 6, 11, 16-tetra-[3-carboranyl-methoxyphenyl]-porphyrin acetate. A 50-ml, three-neck, round-bottomed flask fitted with a reflux condenser and nitrogen inlet port was filled with 17 ml of distilled CH_2Cl_2 . Tetra-carboranylmethoxyphenyl-porphyrin (0.20 g, 0.16 mmol) was added and the solution was stirred magnetically for 10 minutes. To this solution, a Gadolinium (III) acetate hydrate (0.050 g, 0.16 mmol) in methanol (2 ml)

was added and the reaction vessel was shielded from ambient lighting. The flask was immersed in a water bath and the solution was allowed to reflux overnight. Insertion of gadolinium into the porphyrin was monitored by UV-visible (Shimatzu, UV-1601 PC) for completion. The solution was evaporated to dryness under reduced pressure to yield a purple crystalline solid, which was washed with water (2×2 ml). The yield was 0.23 g (100 %).

Observed % Gd 10.6 Calculated % Gd (based on $\text{Gd}(\text{C}_{56}\text{H}_{76}\text{B}_{40}\text{N}_4\text{O}_4)(\text{CH}_3\text{CO}_2^-)$) 10.4.
UV-Vis. (CH_2Cl_2) λ_{max} nm: 424, 548, 588.

Sample preparation. Solutions of different MR imaging contrast agents was prepared in the following procedures:

Solutions of GdCl_3 , Gd-DTPA, and Gd-H were prepared by accurately dissolving the required amount in 0.9% saline solution.

Gd-TCP (15 mg, 0.010 mmol) was dissolved in 1 ml of cremophor EL (CRM) and 2 ml of 1,2-propanediol. This solution was transferred into a 10 ml volumetric flask, and a 0.9% saline solution was added to the mark. This gave a final concentration of 1.0 mM.

Animal selection. The animal studies were performed with nude mice (nu/nu, BALB/c) of 6-8 week old with a mean weight of 20 g (Animal Resources, Western Australia). Animals were randomly divided into five groups of six. Each group was housed per cage in humidity and temperature controlled, isolated animal house at St. George Hospital, Sydney.

Tumor xenograft model. The human melanoma cells, MM-138 (St. George Hospital, Sydney), grown in tissue culture and originally derived from human malignant melanoma, were injected (2.5×10^6 cells) subcutaneously in the both flanks of nude mice.

Injected dose. Three to four weeks after tumor implantation, when the tumor diameter was 3-5 mm (mean weight of tumors was 200 mg), mice were injected with different contrast agent conjugates. All contrast agents were diluted in physiological saline to a final concentration as injected in bolus doses (10 mmol/kg of body weight). Two groups of six mice were injected each intraperitoneally (i.p.) with Gd-H and Gd-TCP. One group received Gd-DTPA and the fourth group received GdCl_3 . The

last group was a control group. The total injected volume was 200 μL . The animals were sacrificed by an over-dose of pentobarbital sodium 24 h post i.p. injection, followed by removal of critical organs (tumor, kidney, liver, spleen). These organs were minced for MR imaging and ICP-AES experiments [7]. The gadolinium concentration in tumor and various removed organs in *in vivo* measured using acid digestion method by ICP-AES [8].

In vivo Proton relaxation times (T_1) determination. All MR imaging measurements and spectra were obtained on a 300 MHz, 7.0 Tesla, Varian UNITY Plus (Varian Associated, Inc., CA) with a vertical Oxford Instruments magnet of bore size 89 mm using the 15 mm saddle coil (DOTY Scientific Instruments) resonator. The effect of contrast agents on proton relaxation times was measured in tumors and other organs using an inversion recovery (IR) pulse sequence technique.

The T_1 values and Gd concentration data for different contrast agent solutions were used to generate relaxivity rate constants, r_1 in reciprocal mmol seconds. This was accomplished via linear regression analyses of $1/T_1$ versus Gd concentration with r_1 calculated as the slope of the fitted lines for data collected different concentrations.

MR image signal intensity. The enhancement effect of these agents on MR imaging signal was investigated. All images were obtained using the T_1 -weighted imaging method using IR pulse sequence technique, with $T_E = 15$ msec, $T_R = 300$ msec, $T_1 = 200$ msec, 5 mm slice thickness, $3 \times 3 \text{ cm}^2$ field of view, and matrix size of 256×128 . The MR image signal intensity was performed by selecting voxels in the image transfer display. Five signal intensity measurements were randomly obtained for each voxel and an average of those was calculated as the signal intensity.

Gd concentration measurements. All samples (tissues) were frozen until used for ICP-AES measurements. The gadolinium content was measured based on an acid digestion procedure using ICP-AES (Applied Research Laboratory, UK) instrument according to the method of Tamat et al. [8]. The 342.249 nm atomic emission line of Gd was chosen for the ICP-AES analysis. The tissue uptake of the Gd was calculated as a percentage of the initial injected dose of contrast agent (% i.d.).

RESULTS AND DISCUSSION

Relaxivity. Specific targeting of MR imaging contrast agents demands a detailed knowledge of properties of the agent used. These details including the feasibility and the required dose of injection as well as uptake by the selected ligands. The efficacy of porphyrin based contrast agents was calculated by measuring their effect on proton relaxation times. Therefore, a relaxivity measurement was performed to detail investigation of the tissue-specific contrast agents as shown in Table 1. Relaxivity values of Gd-porphyrins are approximately 4 or 5 times higher than Gd-DTPA. This increase is due to its greater potential to coordinate water molecules. These results are consistent with reported relaxivity of porphyrin based contrast agents [2].

Gadolinium concentration. The Gd tissue uptake was calculated as a percentage of the injected contrast agent by organs and results are shown in Table 2 and Figure 2. As can be seen from Figure 2, for GdCl_3 and Gd-DTPA the Gd uptake by the tumor was 13% and 18%, respectively.

Tumor uptake of 21% and 28% of the injected gadolinium was recorded for Gd-TCP and Gd-H, respectively. This amount identified the potential of the porphyrin-based compounds as tumor-specific detection agents. Calculations of the concentration boron introduced into the tumor by Gd-TCP yield 24 .tg of boron concentrated into the cancer cells. This amount is in agreement with the values for related complexes studied by Miura *et al.* [6], is sufficient for BNCT and proves the potential application of the Gd-TCP in the dual roles as MR imaging agent and a boron delivery agent for BNCT.

Table 1. Relaxivity values of gadolinium concentrations in aqueous solutions of MR imaging contrast agents at room temperature (23°C).

Contrast agents	r_1 ($\text{mM}^{-1} \text{s}^{-1}$)	r_2 ($\text{mM}^{-1} \text{s}^{-1}$)
GdCl_3	12.3 ± 0.7	15.8 ± 1.1
Gd-DTPA	3.7 ± 0.1	4.8 ± 0.2
Gd-H	16.3 ± 1.4	20 ± 1.6
Gd-TCP	31.7 ± 0.3	38.2 ± 0.6

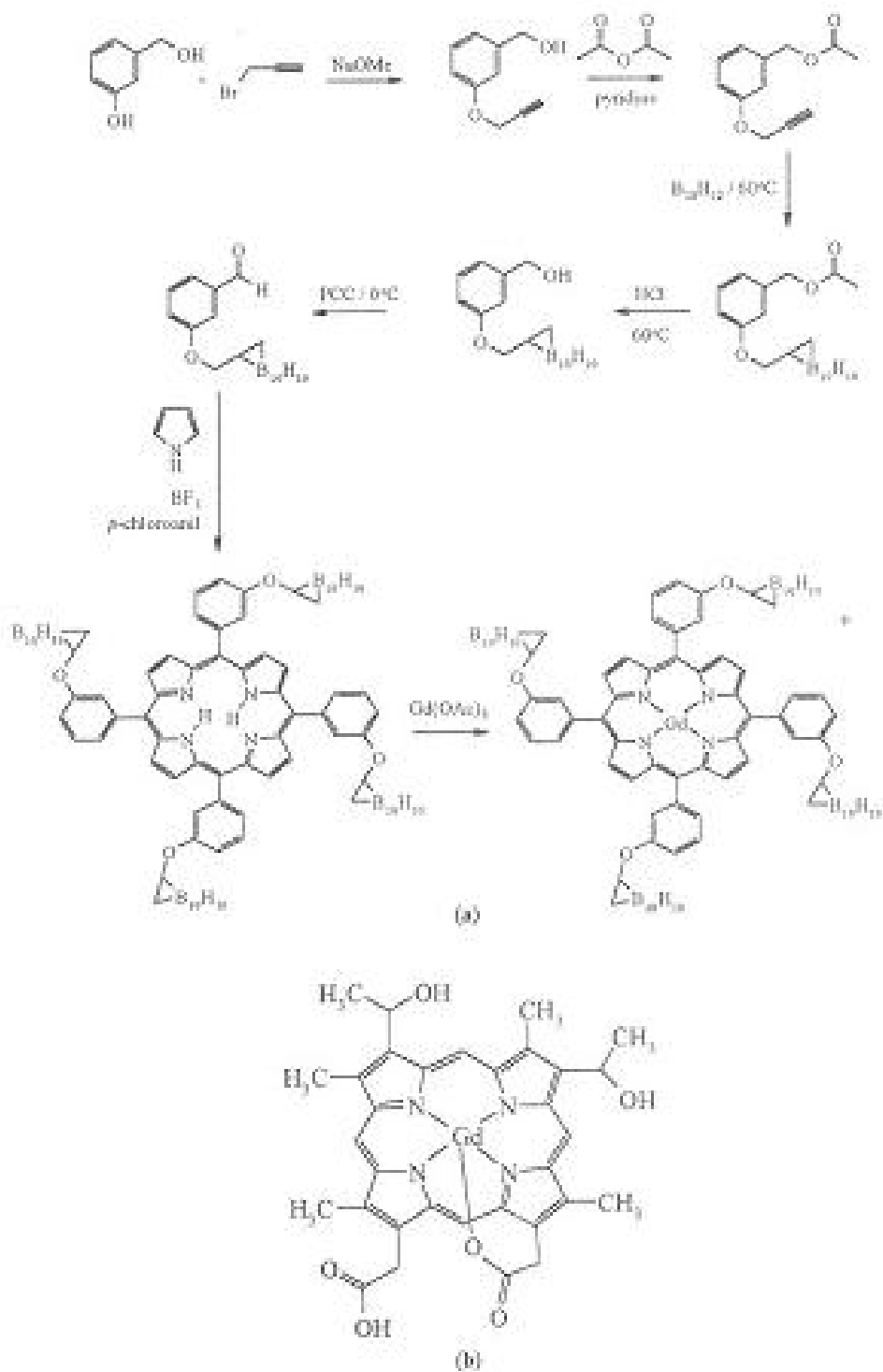


Fig. 1. (a) The schematic of synthesis of gadolinium-tetra-carboranyl-methoxyphenyl-porphyrin and (b) the chemical structure of Gd-H.

Table 2. T1 relaxation times(s) of different organs of nude mice xenografted with human melanoma cell line. Tissues were removed 24-h after injection of different contrast agents.

Compound/Sample	Tumor	Liver	Kidney	Spleen
GdCl ₃	1.43 ± 0.03	0.74 ± 0.02	0.85 ± 0.01	0.84 ± 0.03
Gd-DTPA	1.37 ± 0.02	0.77 ± 0.01	0.87 ± 0.02	0.88 ± 0.02
Gd-TCP	1.33 ± 0.02	0.78 ± 0.02	0.94 ± 0.02	0.83 ± 0.02
Gd-H	1.26 ± 0.01	0.74 ± 0.02	0.98 ± 0.03	0.71 ± 0.03
Control	1.59 ± 0.03	0.96 ± 0.01	0.99 ± 0.04	0.96 ± 0.02

Data are mean ± SEM of values obtained from an average of five mice.

As indicated in Table 2, liver retained the highest amount of gadolinium for both Gd-H and Gd-TCP. At 24-h post-injection, the gadolinium content of GdH and Gd-TCP was 34% and 32%, respectively. This amount was at least 2 times greater in liver than that of Gd-chelates. Some of the gadolinium found in the liver might represent gadolinium dissociated from the DTPA. It is known that free gadolinium accumulates in the liver and this may explain some of the high uptake in the reticuloendothelial organ [18]. These data were consistent with results of gadolinium content observed in the liver for other porphyrin-based agents [9]. Significant gadolinium accumulated in the liver, spleen and kidney probably reflects clearance and metabolism of the gadolinium complexes [10]. The amount of boron concentrated in the tumor by the uptake of Gd-TCP was 24 µg, verifying its potential application for BNCT.

A small amount of gadolinium was observed in the kidney at 24-h post injection. This is due to the properties of gadolinium-based contrast agents, which are hydrophilic and accumulated in the extracellular water of tissues and have rapid renal excretion [10].

T₁ relaxation times. T₁ values and standard deviations were calculated by using an inversion recovery pulse sequence. Table 2 shows the T₁ measurements of organs using different contrast agents and untreated mice (control).

The results showed T₁ relaxation time of tumor was significantly greater than that of normal tissues. The general theory that T₁ values are longer in tumors was confirmed by this animal study. This difference is reported to arise from an increase in water content and the large extracellular volumes

of the cancerous tissues and these abnormalities elevate the T₁ values [11].

As Table 2 illustrated, for GdCl₃, the T₁ relaxation time was reduced approximately 10% in the tumor compared to the control. For Gd-DTPA, the modification of about 14% was observed in T₁ values of the tumor. This standard clinical used contrast agent showed a similar reduction of T₁ relaxation time for both spleen and kidney. Both porphyrin-based contrast agents, Gd-TCP and Gd-H, showed a 16% and 21% decrease in the T₁ value for the tumor, respectively.

The decreases in the T₁ values of the contrast agents were in line with the concentrations of gadolinium absorbed by the tumors. These reductions in the T₁ relaxation times of the tumor upon administration of contrast agent are highly significant, even though this concentration of agents used in this study (0.01 mmol/kg) is lower than the doses of Gd-DTPA commonly used as a contrast agent in clinical MR imaging (0.1 mmol/kg).

MR image signal intensity. The values of echo time (T_E = 15 ms) and repetition time (T_R = 300 ms) were optimized for the tumor of the control mice and were used for all other tissue.

The graphs of MR image signal intensity for removed organs and different contrast agents are shown in Figure 3. In the control, the MR image signal intensity for tumors was lower than that recorded for the normal tissues under study. This may result from the T₁ relaxation time for the tumor being longer than the other normal tissues, which decreases MR image signal intensity and is consistent with T₁ values measured in this work (Table 2).

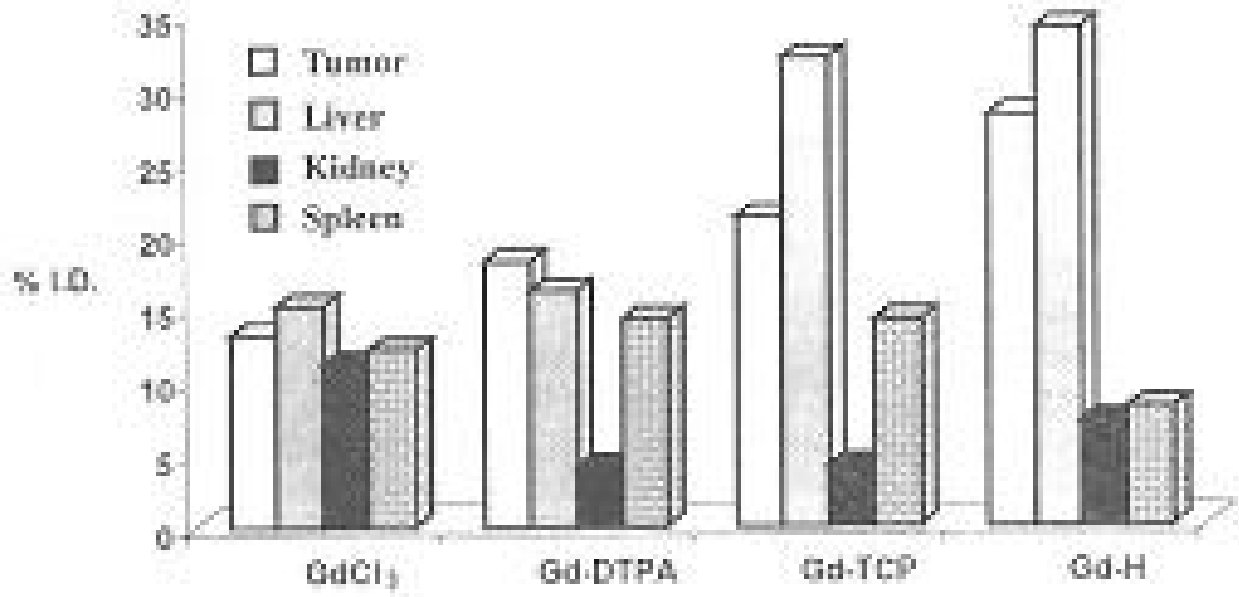


Fig. 2. Comparison of biodistribution of the gadolinium uptakes in melanoma grafted in nude mice for different compounds as MR imaging contrast agent.

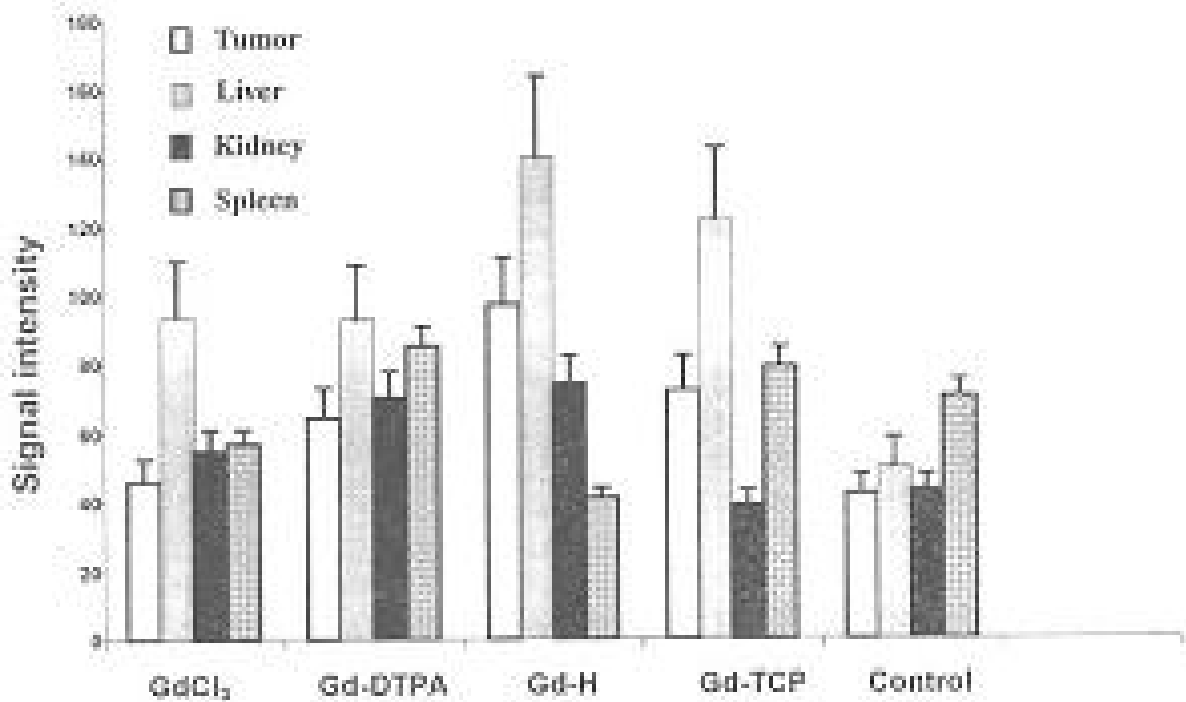


Fig. 3. MR image signal intensity imaging of tissues 24-h after injection of different gadolinium compounds (Data are mean \pm SEM of values obtained from an average of five mice).

The highest MR image signal intensity (120%) was observed for the tumor upon injection of Gd-H over the control. After Gd-H, the other porphyrin-based agent, Gd-TCP, showed good signal enhancement (70%) over the control. The signal enhancement of the porphyrin-based complexes in this study is in good agreement with that of reported previously by conjugation of Gd-DTPA with porphyrins under *in vivo* conditions in mice [12]. The results also showed the signal enhancement of Gd-DTPA and GdCl₃ was significant, but lower than those of porphyrin-based contrast agents.

These results demonstrate the MR contrast enhancing capabilities of Gd-TCP. This signal enhancement of Gd-TCP accompanying with the delivery of the number of boron atoms into the tumors may be serve the dual use of the compound, as it can enhance contrast between tumor and normal tissues in MR images and be potentially effective as an agent for BNCT.

The liver was the only organ that showed the greatest enhancement for both porphyrin-based agents and as stated previously, showed the accumulation of gadolinium complex. In spite of good accumulation of porphyrin-based agents into the tumor, the accumulation of these agents in the liver is problematic.

In conclusion, it is likely that tissue-specific agents will be required for MR imaging of tumors or lymph nodes. Methods using MR imaging and porphyrinbased contrast agents could offer the advantage of tissue contrast enhancement and precise anatomic localization of the tumors. Because of the ability of coordinate several water molecules in aqueous solution, porphyrin-based contrast agents showed higher relaxivity than that of Gd-DTPA. These materials showed significant enhancement effect in nude mice with melanoma. Further developments in MR imaging contrast agents, in combination with improved imaging techniques, may lead to novel applications in the diagnostic MR imaging. In this study, using Gd-porphyrins and melanoma tumor xenograft model, making possible quantitative studies of paramagnetic contrast agent uptake.

These findings showed the possible application of porphyrin-based contrast agents for the detection of tumor, in particular for melanoma. The findings also indicated that with the satisfactory Gd uptake by the tumor and low levels in kidney and spleen these agents are useful for tumor detection.

The outcome of this study may help the design of tumor-specific contrast and chemotherapeutic agents. The permeability of porphyrin-based contrast agents may provide tissue uptake information for both contrast and chemotherapeutic agents. With current debate on tumor-specific and therapeutic agents for tumor diagnosis and therapy, the permeability of macromolecules to tumor tissues is an essential question that needs to be answered.

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