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ORIGINAL ARTICLE

Synthesis of lactosylated piperazinyl porphyrins and their hepatocyte-selective targeting

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Abstract The aim of this work was the synthesis of a new family of lactosylated piperazinly porphyrins in which the galactoside piperazine moieties are linked to the tetra- and monophenyl ring by an amide bond. Two compounds were designed, synthesized, and characterized by ^{1}H-nuclear magnetic resonance (^{1}H-NMR), ultraviolet (UV)-visible, and matrix-assisted laser desorption/ionization (MALDI) mass spectra. The biological activity on cancer cells and the pharmacokinetics have also been evaluated, showing a very high liver-to-skin ratio and short retention time in tissues. It is suggested that such novel lactosylated piperazinyl porphyrins, as potential hepatocyte-selective targeting drugs, thus show promising activity in photodynamic therapy.

Keywords Hepatocyte-selective targeting drug · Lactosylated piperazinyl porphyrin · Photodynamic therapy · Synthesis

Introduction

Recently, promising porphyrin photosensitizers have been developed for photodynamic therapy (PDT) (Ali and van Lier, 1999; Allison et al., 2004; Venosa et al., 2006). Although a number of porphyrin-based photosensitizers have been approved for clinical trials, very little is known about their mode of action. In addition, they have demonstrated some obvious drawbacks. Because of their low selectivity, they enter almost every tissue, and their slow clearance leads to a long-lasting

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photosensitivity, causing skin sensitivity for several weeks (Sharman et al., 1999). Moreover, PDT treatment also was not suitable for therapy of cancer affecting deep tissues (Yuan and Jie, 1984). As a result, new photosensitizers exhibit high selectivity for neoplastic cells, fast elimination from healthy tissues, and strong light absorption in the red region of the visible spectrum.

Although the exact mechanism of sensitizer uptake by tumor cells is still undetermined, there is evidence that, besides the subtle balance between hydrophobicity and hydrophilicity, charge distribution, symmetry, and configuration of the molecules are important factors influencing the selective accumulation and subcellular distribution. The asialoglycoprotein receptor (ASGR) is known to be present only on hepatocytes at a high density of 500,000 receptors per cell, and retained on several human hepatoma cell lines. This receptor system not only can recognize terminal β-D-galactose or *N*-acetylgalactosamine residues, but can also internalize them within membrane-bound vesicles or endosomes (Kim et al., 2004). ASGR are considered as a particularly attractive target in many drug carrier studies. The use of such nature molecules with galactosylated or lactosylated residues in drug targeting carriers has resulted in significant targeting efficacy to the liver (Han et al., 2001). Galactose is known as a specific adhesive ligand to ASGR of hepatocyte. Lactobionic acid (LA), bearing a galactosyl group, is usually used as a recognition moiety for the hepatocyte-targeting carrier (Gao et al., 2003; Wang et al., 2006). Therefore, many amphiphilic porphyrins linked to sugar moieties have been synthesized, and notably porphyrins bearing one or two carbohydrate substituents gave promising results (Sylvian et al., 2002; Ahmed et al., 2004; Chen et al., 2004; Tome et al., 2005; Sol et al., 2006). Such conjugates increase the water solubility of the parent porphyrin, which contributes to their elimination from the organism after treatment.

As part of our research program on the synthesis of such compounds (Guo et al., 2003; Li, 2006), we are interested in the preparation and biological evaluation of lactosylated piperazinyl porphyrins obtained from synthetic precursors. In comparison with lactosylated porphyrins, lactosylated piperazinyl porphyrins could increase anticancer activity, because piperazine-containing compounds are widely used in the pharmaceutical industry; some substituted piperazine compounds especially have distinctive anticancer activity (Hagihara et al., 1999; Hepperle et al., 1999; Guo et al., 2003; Li, 2006). In the present article, the synthesis of two lactosylated piperazinyl porphyrins and an evaluation of their biological activity are reported.

Materials and Methods

Apparatus and reagents

Infrared spectra were recorded with a Nicolet Avator 360 spectrophotometer. ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectra was recorded with a Varian FT-80A spectrophotometer(400 MHz), and the chemical shifts was reported on the δ scale relative to trimethylsilane (TMS; solvent: $CDCl_3$). Elemental analyses were

obtained via a PE 2400. Mass spectra were obtained via a QP-5000 mass spectrophotometer. Ultraviolet (UV)-visible absorption spectra were measured with an UV 2100 spectrophotometer. All fluorescence measurements were made with a Hitachi M850 fluorescence spectrophotometer using a 1-cm quartz cell, and with both excitation slit and emission slit at 10 nm. All pH measurements were made with a digital pH and temperature meter 631 (Extech, Boston, MA).

Column chromatography was performed using alumina (200–300 mesh). Lactobionic acid was purchased from Fluka Chemical (Buchs, Switzerland). 5-Fluorouracil (5-Fu), *N,N′*-Dicyclohexylcarbodiimide (DCC) and 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Three human tumor cell lines—Bel-7404, MCG and HNE1—were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Science. The pyrrole and propionic acid were distilled before use. All other chemicals were used as received.

Synthesis of *N*-phenylpiperazine

A mixture of aniline hydrochloride (0.04 mol) and diethanolamine hydrochloride (0.04 mol) was heated at about 220–240°C for 6–8 h. After cooling, the gummy solid was treated with concentrated sodium hydroxide and the resulting oil was then distilled under vacuum at 10 mm Hg (b.p. 139–141°C). The light yellow oil was obtained (3.9 g, 60%). MS (*m/z*): 162. IR (KBr): 3200 (–NH–), 1600, 1500 (Ar C–H) cm^{-1}. ^1H NMR ($CDCl_3$): δ2.75–3.15 (m, 8H, NCH_2), 6.85–7.38 (m, 5H, ArH).

Synthesis of *N*-lactobionylamino-*N′*-phenylpiperazine

A mixture of *N*-phenylpiperazine (0.02 mol), lactobionic acid (0.05 mol), tetramethylethylene diamine solution of DCC (0.04 mol), and benzene (100 ml) was stirred at 28–40°C for 72 h. The solvent was removed and the product was purified by column chromatography (neutral alumina, 200–300 mesh; ethanol). Yield, 7.2 g, 70%. MS (*m/z*): 516. IR (KBr): 3400 (O–H), 1657 (C=O), 1605, 1500 (Ar C–H), 755 (C–H) cm^{-1}. ^1H NMR ($CDCl_3$): δ2.35–3.27 (m, 10H, NCH_2), 3.42–4.45 (m, 13H, lactobionyl), 6.65–7.57 (m, 5H, ArH).

Synthesis of *N*-lactobionylamino-*N′*-(4-formylphenyl)piperazine

N-Lactobionylamino-*N′*-phenylpiperazine (2.0 mmol) was dissolved in dimethylformamide (10 ml). The solution was cooled in an ice bath. Phosphoryl trichloride (2.0 mmol) was added in small portions and the temperature was kept below 0°C with an ice bath. The mixtures were continuously stirred for 30 min at 0°C, then stirred at 90°C for 1.5 h. The mixtures were poured into crushed ice (400 ml) with vigorous stirring, neutralized to pH 5 with 5 M NaOH solution, and stored in the refrigerator overnight. The mixtures were extracted with $CHCl_3$ and the organic layer was washed with water and dried with Na_2SO_4 overnight. The product was purified by column chromatography (neutral alumina, 200–300 mesh; ethanol). Yield, 0.816 g, 75%. MS (*m/z*): 544. IR (KBr): 1670 (C=O) cm^{-1}, ^1H NMR

($CDCl_3$), δ2.40–3.27 (m, 10H, CH_2N), 3.45–4.55 (m, 13H, lactobionyl), 6.83–7.67 (m, 4H, ArH), 9.76 (s, 1H, CHO).

Synthesis of 5,10,15,20-tetra[4-(4′-lactobionylaminopiperazinyl)phenyl]porphyrin (TLPP)

A mixture solution of *N*-lactobionyl-*N*′-(4-formylphenyl)piperazine (2.0 mmol), pyrrole (2.0 mmol), and propionic acid (100 ml) was refluxed for 30 min and then stored overnight at 0°C. The purple precipitate was obtained. The precipitate was purified via column chromatography (neutral alumina, 200–300 mesh; ethanol–acetone, 20/80) to obtain TLPP as a purple solid (156 mg, 13.5%). ^{1}H NMR ($CDCl_3$):δ8.62–8.66 (m, 8H, pyrrolic), 6.97–7.33 (m, 16H, ArH), 2.82–3.57 (m, 32H, N-CH_2), 4.22–4.54 (m, 52H, lactobionyl C–H), 2.08 (s, 32H, lactobionyl O–H), –2.46 (s, 2H, NH). UV-vis[λ_{max}, nm ($\varepsilon \times 10^{-3}$ cm^{-1} mol^{-1} L)] in CH_2Cl_2: 427 (204.9), 526 (17.6), 569 (22.1), 614 (19.2), 658 (10.8). MS (*m/z*): 2311(M^++1). Anal. calcd. for $C_{108}H_{142}N_{12}O_{44}$: C, 56.10; H, 6.15; N, 7.27; O, 30.48%; found: C, 56.08; H, 6.18; N, 7.25; O, 30.49%. IR (KBr): 3324, 1640, 1606, 1512 cm^{-1}.

Synthesis of 5-mono[4-(4′-lactobionylaminopiperazinyl)phenyl]-10,15,20-triphenyl- porphyrin (MLPP)

N-Lactobionyl -*N*′-(4-formylphenyl)piperazine (0.5 mmol), benzaldehyde (1.5 mmol) and pyrrole (2.0 mmol) gave MLPP (90.3 mg, 17.4%). ^{1}H NMR ($CDCl_3$):δ8.72–8.84 (m, 8H, pyrrolic), 7.03–7.34 (m, 19H, ArH), 2.85–3.59 (m, 8H, N-CH_2), 4.24–4.57 (m, 13H, lactobionyl C–H), 2.10 (s, 8H, lactobionyl O-H), –2.69 (s, 2H, NH). UV-vis[λ_{max}, nm ($\varepsilon \times 10^{-3}$ cm^{-1} mol^{-1} L)] in CH_2Cl_2: 424 (255.8), 524 (24.5), 562 (21.3), 617 (20.3), 662 (10.1). MS (*m/z*): 1039 (M^++1). Anal. calcd. for $C_{60}H_{58}N_6O_{11}$: C, 69.36; H,5.59; N, 8.09; O, 16.96%; found: C, 69.47; H, 5.54; N, 8.01; O, 16.98%. IR (KBr): 3332, 1639, 1605, 1514 cm^{-1}.

In vitro studies of TLPP

Irradiation. The light source used was that of a Kodak slide projector equipped with a 150-W lamp. The light was filtered through a 3-cm water layer to absorb the heat (Alvarez et al., 2000).

Cell survival assay. The cytotoxic effects of some compounds on Bel-7404, MCG, and HNE1 cells were determined via the MTT assay (Carystinos et al., 2001). Cells were planted in 100 μl of medium at a concentration of 1×10^3 cells per well in 96-well microtiter plates. Plates were then incubated for 18 h at 37°C under an atmosphere of air containing 5% CO_2. Medium (100 μl) containing the tested drug dissolved in appropriate solvent was added to quadruplicate wells and incubated for an additional 48 h. The medium was then removed from the wells and 200 μl of MTT (1 μg/ml in complete medium) was added to each well followed by a 3-h incubation. The formazan crystals were dissolved in 100 μl of dimethyl sulfoxide buffered with 25 μl of glycine–NaCl solution (0.1 M glycine, 0.1 M NaCl, pH 10.5). The absorbance was measured in an enzyme-linked immunoabsorbent assay plate

reader (Bio-Rad) at a wavelength of 570 nm. Based on concentration, 50% of cell death (ID_{50}) was determined for various compounds tested.

In vivo studies of PDT

Animal and tumor model. Female KM mice 6–8 weeks old (18–22 g) were maintained in standard cages with free access to tap water and normal dietary chow. When required, the animals were anesthetized with Ketalar (150 mg/kg, by i.p. injection). The experimental tumors LM-2, originally obtained from Xiangya Hospital (Changsha, China), were subcutaneously implanted by a sterile injection of *ca.* 10^6 cells in phosphate-buffered saline into the right foreleg. The pharmacokinetic and/or phototherapeutic experiments were performed on the sixth day after implantation, when the tumor was 0.6–0.7 cm in outer diameter. Spontaneous tumor necrosis was minimal or absent for these tumor sizes.

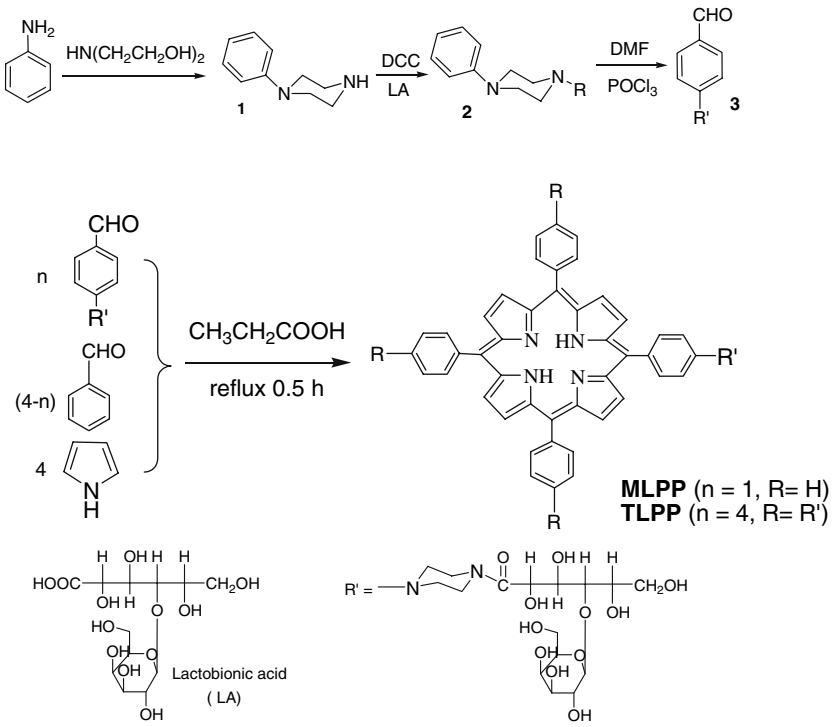
Pharmacokinetic studies. TLPP (10 mg/kg) was administered by i.p. injection. The animals were killed during different points of time (six mice at 24 h and three mice at other points of time), varying between 1 h and 1 week after administration. The skin, muscle, tumor, and the liver were recovered. About 200 mg of tissues were homogenized in THF. The homogenates were centrifuged at 3000 rpm for 15 min, and the fluorescence of the supernatants was measured, setting the excitation wavelength at 420 nm and recording the emission spectrum from 500 and 800 nm. Serum samples, isolated from the blood by centrifugation, were diluted with suitable volumes of 500 µl of tetrahydrofuran and the TLPP content was measured by fluorescence. In all the cases, TLPP amounts were determined via interpolation of emission intensity and TLPP concentration was plotted on a standard curve.

Results and Discussion

Synthesis and characterization of lactosylated piperazinyl porphyrins

An approach to porphyrin-based drugs makes use of a convenient reaction for converting an aldehyde and pyrrole to the corresponding meso-substituted porphyrin. The reaction provides a means for converting prefunctionalized benzaldehyde to the corresponding porphyrin. In this study, the functional groups selected were the drugs that are used in the treatment of cancers. We now report the synthesis and the biological activity studies of lactosylated piperazinyl porphyrins. The preparation of lactosylated piperazinyl porphyrins is summarized in Scheme 1. While this work was in progress, different piperazinylporphyrins (Guo et al., 2003; Li, 2006) have been reported.

The structures of lactosylated piperazinyl porphyrins were characterized by elementary analysis, mass spectroscopy (MS), ^{1}H-NMR, infrared (IR), and UV-vis. The ^{1}H NMR spectra at –2.46 to –2.69 ppm showed the characteristic single peak for each porphyrin. Mass spectrometry of porphyrins was performed using the matrix-assisted laser desorption ionization–time-of-flight (MALDI–TOF) technique. Positive ion mass spectra exhibited a base peak corresponding to the intact



Scheme 1 The synthesis of TLPP and MLPP

porphyrin and no fragment ions were detected. Analysis of the isotopic components indicated the presence of a protonated species $(M+H)^+$ with a minor contribution of the radical cation $M^{+\cdot}$, allowing the determination of the molecular mass with an accuracy generally around 0.001%. Its structure was further confirmed by the elementary analysis.

The key to the synthesis of lactosylated piperazinyl porphyrins is how to introduce lactosylated piperazine to the porphyrin ring. TLPP is a symmetrical lactosylated piperazinyl porphyrin, but MLPP is asymmetrical. We had tried to couple iodoporphyrin with various lactosylated piperazine to synthesize target molecules at room temperature according to the Buchwald method (Wolfe and Buchwald, 1997). However, the reaction did not proceed, even after stirring over an extended period of time. The coupling of aminophenylporphyrin with *N,N*-di(2-bromoethyl)lactosamide at room temperature can produce the corresponding lactosylated piperazinyl porphyrin, but the components of products are too complicated to purify. In this study, synthesis of lactosylated piperazinyl porphyrins was realized according to the method of Adler et al. (1967). The procedure consists of the condensation of pyrrole (4 equiv) with *N*-lactobionylamino-*N*′-(4-formylphenyl)piperazine (4 equiv) or *N*-lactobionylamino-*N*′-(4-formylphenyl)piperazine (1 equiv) and benzaldehyde (3 equiv). In all cases, these reagents were added to dry propionic acid as solvent at refluxed temperature, followed by flash chromatography

and purification on silica gel TLC, giving porphyrins TLPP and MLPP in 13.5% and 17.4% yields, respectively.

N-lactobionylamino-*N*′-(4-formylphenyl)piperazine was prepared according to the literature (Amaresh and Perumal, 1998). *N*-lactobionylamino-*N*′-phenylpiperazine was synthesized via the formation of amide bonds between the secondary amino group of the *N*-phenylpiperazine and the carboxylic acid group of lactobionic acid according to the method reported by Bernkop-Schnürch and Thaler (2000). As shown in reaction formula, the carboxylic moieties of lactobionic acid were activated by *N,N*′-dicyclohexylcarbodiimide (DCC), forming an intermediate product, which reacted with the secondary amino group of *N*-phenylpiperazine. The efficacy of the purification method for the resulting *N*-lactobionylamino-*N*′-phenylpiperazine could be verified by controls that were prepared in exactly the same way as the conjugate but omitting DCC during the coupling reaction, exhibiting a negligible amount of product. To optimize the synthesis of *N*-lactobionylamino-*N*′-phenylpiperazine, the influence of the coupling reaction time was evaluated. Results demonstrated that the yield increased with increasing reaction time, but when the reaction time lasted more than 72 h, the yield could not increase obviously, indicating that the coupling reaction already came to equilibrium. Other factors that could influence the yield, such as pH of the reaction mixtures and the weight ratio of *N*-phenylpiperazine to lactobionic acid, have already been studied. As far as pH of the coupling reaction was concerned, alkaline pH benefits the reaction to a greater extent than acidic pH. Therefore, a tetramethylethylene diamine solution was selected as the catalyst of the coupling reaction.

In vitro anticancer activity

It is well known that substituted tetraphenylporphyrin can be used as a sensitizer in PDT (Ali and van Lier, 1999; Allison et al., 2004; Venosa et al., 2006), and nitrogenous heterocycle porphyrins have better anticancer activity than the corresponding nitrogenous heterocycles in the absence of light (Guo et al., 2003; Li, 2006). To study whether these synthetic lactosylated piperazinyl porphyrins have anticancer activity, the cytotoxic effects of TLPP, MLPP, and a common anticancer drug, 5-fluorouracil (5-Fu), on three human tumor cell lines—Bel-7404 (a liver cancer cell), MCG (a stomach tumour cell), and HNE1 (a nasopharyngeal carcinoma cancer cell)—were determined via the MTT assay (Carystinos et al., 2001) with and without irradiated conditions. The results of the prescreenings are given in Fig. 1.

It can be seen from Fig. 1 that the death rates ranged from 24% to 19% for TLPP, 12% to 8% for MLPP, and 43% to 29% for 5-Fu when cells were incubated for 18 h at 37°C under an atmosphere of air containing 5% CO_2 without light. Thus TLPP and MLPP showed smaller cytotoxic effects than that of 5-Fu in this condition. However, the death rates ranged from 96% to 98% for TLPP and 75% to 78% for MLPP after 18 h incubation and 20 J/cm^2 irradiation (Fig. 1), indicating the presence of photosensitizing toxicity.

Structurally, TLPP and MLPP consist of two parts: tetraphenylporphyrin (TPP) and a lactosylated piperazine compound. TLPP, MLPP, and two corresponding

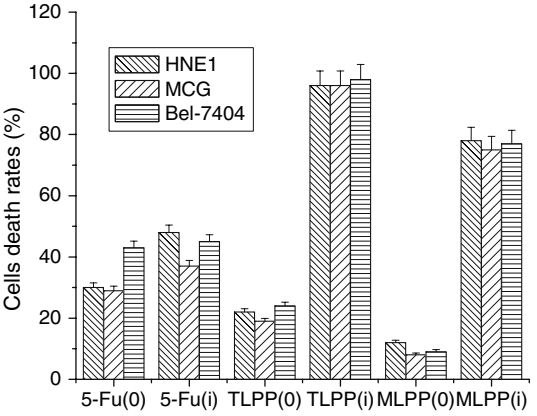


Fig. 1 Anticancer activity of 5-Fu, MLPP, and TLPP in cancer cells (Bel-7404, MCG, HNE1) determined via MTT assay. Cancer cells were incubated for 18 h in solution (6.25 μg/ml). (0), without irradiation; (i), represented with irradiation (at 650 nm and 20 J/cm^2)

Table 1 ID_{50} values (μg/ml) of TLPP and other compounds

Compound	Bel-7404	MCG	HNE1
TLPP	29	35	33
MLPP	43	48	46
TPP	>50	>50	>50
Lactobionylated piperazine	>50	>50	>50

structural parts, porphyrin TPP and lactosylated piperazine, were also tested *in vitro* against bel-7404 liver cancer cells via MTT (Carystinos et al., 2001) in the absence of light. The ID_{50} values of the bel-7404 liver cancer cell in response to these compounds are listed in Table 1.

The test results showed that TLPP and MLPP have smaller ID_{50} values than those of TPP and the corresponding lactobionylated piperazine. This indicates that TLPP and MLPP have better anticancer activity than that of either structure parts of lactobionylated piperazine porphyrins. At the same time, it can be seen in Table 1 that for TLPP and MLPP, when the numbers of substitution group lactobionylated piperazine increased, it increased the anticancer activity of the porphyrins. It is interesting that TPP, which is not an anticancer drug, would be a good carrier for toxic moieties to arrive at improved anticancer drugs. The possible reason might be that TPP with anticancer drugs can intercalate strongly into the base pairs of DNA. TLPP showed good anticancer activity toward cancer cells in the absence of light, and might be a promising agent in chemotherapy and PDT. The molecular mechanism for the anticancer activities of TLPP is being studied further.

Pharmacokinetics studies: biodistribution in tumor-bearing mice

The lipophilic and hydrophilic properties were characterized by the partition coefficient of the compound between the two nonmiscible solvents octanol and water. The octanol/water partition ratios were 18 for TLPP and 39 for MLPP,

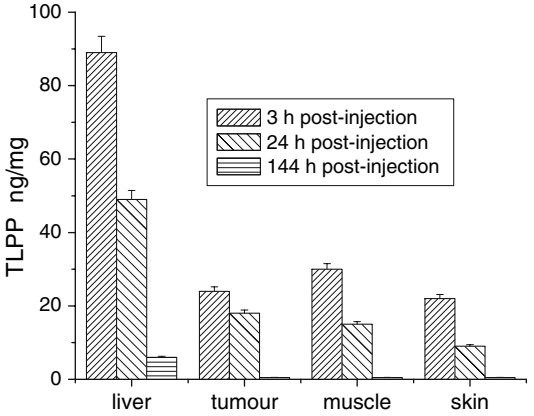


Fig. 2 Biodistribution of TLPP in tumor-bearing mice: Recoveries of TLPP from tumor-bearing female KM mice injected with 10 mg/kg of drug. Values represent the averages of three experiments

indicating that TLPP was more hydrophilic than MLPP, but TPP is insoluble in water. The distribution of TLPP was examined in different selected tissues of tumor-bearing female KM mice at 3-, 24-, and 144-h postinjection periods (Li, 2006). The results are shown in Fig. 2.

In vivo results indicate that TLPP is rapidly distributed in tissues and rapidly eliminated. The highest concentrations were observed in the liver at 3 h postinjection; the concentration of TLPP decreased rapidly in all tissues after 24 h, and very low sensitizer quantity was detected in these tissues after 144 h. The low quantity detected in the skin was important, as it would cause photosensitization. The shorter retention time of TLPP in tissues could decrease prolonged cutaneous photosensitivity. The higher amounts of TLPP accumulated in the liver could be explained by the presence of four lactosylated moieties that could recognize ASGR. Although the efficiency of TLPP incorporation in tumor tissue was not high in relation to other tissues, a high antitumor activity was observed when cells were excited by visible light, since a high percentage of cells in apoptosis appeared in tumors that were obtained from mice treated with TLPP and irradiated. In summary, the present results show that TLPP is a potential tumor photosensitizer. The further development of experimental protocols using a given sensitizer *in vitro* and *in vivo* can contribute to the understanding of the photokilling effect in basic oncological research and to assessment of the potential for clinical applications in the PDT of cancer, and more extensive biological studies will be reported in the near future.

Conclusion

We have developed a practical and efficient approach to synthesize lactosylated piperazinyl porphyrins based on the Adler method. Lactosylated piperazinyl porphyrins showed good anticancer activity toward in the presence of light, and would be promising agents in chemotherapy and PDT. The incorporation of lactosylated residues into porphyrin enhanced the liver targetability. Moreover, the results of intrahepatic distribution provided evidence that the recognition of

lactosylated residues of TLPP by ASGR on the surface of parenchymal cells accounted for the liver accumulation of the drug. These results suggested that porphyrin containing lactosylated residues could be a useful drug carrier system for hepatocyteselective targeting.

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