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A novel chlorin–PEG–folate conjugate with higher water solubility, lower cytotoxicity, better tumor targeting and photodynamic activity

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ABSTRACT

Techniques to enhance tumor targeting and to improve the aqueous solubility of anticancer drugs and photosensitizers have recently been the focus of much research. In this study, a folate–PEG-conjugated chlorin was synthesized and characterized. Because of the use of PEG as a linker, the new chlorin displayed increased aqueous solubility, with a solubility of 40.1 mg/mL in PBS, and showed lower aggregation and cytotoxicity than its precursor, chlorin. Meanwhile, the introduction of folic acid to the new chlorin resulted in increased selectivity for folate-receptor-positive tumor cells (HeLa and Hep-2 cells); the cellular uptake of the new chlorin by HeLa and Hep-2 cells was strikingly higher than that of the precursor chlorin, and the photocytotoxicities of the new chlorin to HeLa and Hep-2 cells were 2.5 and 3.5 times greater than that of folate-free conjugate chlorin. During photodynamic therapy mediated by the new chlorin, both type I and type II reactions occur simultaneously.

1. Introduction

Photodynamic therapy (PDT) is emerging as a viable treatment for many cancers and for inactivation of bacteria and viruses. PDT is based on light-induced activation of a photosensitizer that results in subsequent in situ production of reactive oxygen species (ROS), a lethal cytotoxic species that directly destroys cells that have accumulated the photosensitizer [1].

Photofrin®, an FDA-approved photosensitizer, is a complex mixture of several partially unidentified porphyrins. Its main drawbacks include poor tumor selectivity, weak absorption in the near-infrared region where light is therapeutically useful, and a high accumulation rate in the skin, which induces prolonged cutaneous photosensitivity post-PDT [2]. Chlorin, a second generation photosensitizer, has been attracting wide attention due to its high phototoxic potential and its relatively strong absorption in the near-infrared region, which leads to the destruction of diseased tissue in deeper tissue [3–5]. Despite these promising results, chlorins leave much to be desired. The main drawbacks are a lack of selectivity [6,7] and poor water solubility, which results in aggregation in aqueous solution and a subsequent reduction in photodynamic activity against tumor cells [8,9]. A number of advances have been made to improve the targeting of these drugs by conjugating the photosensitizer to tumor-seeking molecules, such as epidermal growth factor, monoclonal antibodies, carrier proteins, carbohydrates, and hydrophilic polymers [10–13].

Because of folate receptor is frequently overexpressed on the surface of malignant cells, but largely absent from normal cells, there has been much interest in exploiting this natural cellular uptake mechanism for the targeted delivery of drugs [14,15]. Over the past decade, many anticancer drugs have been conjugated with folic acid (FA) either directly or by the use of a carrier [16–20]. However, because the aqueous solubility of folic acid is very poor and porphyrin-like or chlorin-like photosensitizers are usually liposoluble, the water solubility of targeting photosensitizers obtained from directly modifying porphyrins or chlorins with folic acid is unsatisfactory [21]. Although the tumor targeting of a photosensitizer could be improved by using a targeting carrier, other factors such as the drug-loading ability and the kinetics of drug release of the carrier still affect the photodynamic activity of a photosensitizer. Accordingly, we aimed to design a photosensitizer that possesses tumor targeting ability and high water solubility.

Polyethelene glycol (PEG) is a hydrophobic polymer approved by the FDA that has been widely used in the pharmaceutical industry for several decades. PEGylation increases the size and molecu-

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lar weight of conjugated biomolecules and improves their pharmacokinetics and pharmacodynamics by increasing water solubility, protecting from enzymatic degradation, reducing renal clearance and limiting immunogenic and antigenic reactions [22–24]. In this study, we propose that a chlorin molecule and a folic acid molecule could be linked by PEG and that this conjugation could be used to increase the water solubility and selectivity of chlorin for cancer cells. Therefore, this work aimed to determine (1) whether the aqueous solubility of the photosensitizer chlorin could be improved by PEGylation and how much it could be improved, (2) whether the aggregation of this conjugated molecule could be reduced and its tumor targeting could be enhanced, (3) what the photochemical properties, cytotoxicity and the photodynamic activity of this conjugate are and (4) what the mechanism of PDT mediated by this conjugate is. The results reported herein indicate that conjugate **1a** is a potent photo-therapeutic agent for tumors.

2. Experimental section

2.1. Materials and methods

2.1.1. Chemistry

Folic acid, N-hydroxyl-succinimide (NHS) and NH_2-PEG-NH_2 (Mw: 3350) were purchased from Sigma–Aldrich. t-BocHN-PEG-NH_2 (Mw: 3500) was purchased from Jiankai Co. (China). Pyrrole was distilled under reduced pressure before use. THF was distilled from sodium/benzophenone; DMSO and DMF were distilled under reduced pressure from anhydrous sodium sulfate before use. Other reagents were of commercial quality and were used without further purification. Silica gel 60 (230 × 400 mesh, LIANGCHEN, China) was used for column chromatography. DEAE ion exchange resin (TOSOH, Japan) and reverse phase silica gel (YMC, Japan) were used for the purification of the water-soluble compounds. Analytical thin-layer chromatography (TLC) was carried out using silica gel plates (G-60, 200 μm, LIANGCHEN, China). All reactions were performed under an argon atmosphere. Melting points were determined on an X-5 Micro-Melting Point Apparatus (Shanghai, China) and are uncorrected. Infrared spectra were recorded with a Varian 640 FT-IR spectrometer (USA, KBr pellets). Absorption spectra were measured on a BECKMAN DU-800 spectrophotometer (USA). ^{1}HNMR spectra were recorded on a Mercuryplus-400 MHz NMR spectrometer (Varian, USA). Fluorescence emission spectra were measured using a commercial FLS920 spectrofluorimeter (Edinburgh Instruments Ltd., UK), which was equipped with a 450-W xenon arc lamp. All samples, which contained 2.5 mL of solution, were held in a quartz cuvette mounted on a VARIOMAG magnetic stirrer unit (MINI, H + P Labortechnik AG, Bayern, Germany). This allowed the samples to be continuously stirred with a magnetic stirring bar and kept the source-sample-detector geometry constant between experiments. The detection system used to directly measure singlet oxygen ($^1O_2$) luminescence has been described previously [25]. Briefly, the $^1O_2$ luminescence was detected by the near-infrared (NIR) -PMT at an operating voltage of −900 V. Three NIR bandpass filters (1230, 1270, 1310 nm) were placed sequentially in front of the PMT to sample the $^1O_2$ luminescence spectrum. $^1O_2$ luminescence measurements were performed continuously while irradiating the sample by sampling each of the 1230, 1270 and 1310 nm filters in turn. In order to achieve a sufficient signal-to-noise ratio (SNR), the $^1O_2$ luminescence counts at each wavelength were totaled over 285, 000 laser pulses. Mass spectra were obtained on a TRACE GC/MS instrument (FINNIGAN, USA). MALDI-TOF mass spectra were obtained using an AXIMA Resonance spectrometer (Shimadzu, Japan). The HPLC assay was conducted on an Agilent 1200 series liquid chromatograph (Agilent, USA).

2.1.2. Biology

Cell culture materials were purchased from Costar (Dutscher, Brumath, France). Fetal bovine serum, penicillin, streptomycin, RPMI-1640, DMEM and DPBS were purchased from Hyclone (Logan, Utah, USA). Trypsinase, MTT, DMSO and folic acid were purchased from Sigma–Aldrich. Folate-free RPMI 1640 was purchased from GIBCO (USA). Human laryngeal carcinoma Hep-2 cells, human cervical carcinoma HeLa cells and human alveolar basal epithelial A549 cells were obtained from the Chinese Academy of Science Shanghai Cell Library. Confocal images were obtained with a Leica TCS SP2 confocal laser-scanning microscope (Germany). Culture media supplemented with 10% heat-inactivated fetal calf serum and penicillin (100 μ/mL)-streptomycin (100 μ/mL) is referred to as "complete media". Hep-2 cells were cultured in RPMI-1640 complete media, and HeLa and A459 cells were grown in DMEM complete media. Cell lines were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Irradiation was carried out with a KDH150B Red-light therapy instrument at a wavelength of 600–700 nm (KEDIAN Co., Beijing, China). The energy density of the red light was 100 mW/cm^2, and the irradiation light spot was 80 mm in diameter. During irradiation, the temperature never exceeded 25 ± 2 °C.

2.2. Chemical synthesis

2.2.1. Synthesis of 5, 10, 15-tris (3-hydroxyphenyl)-20-(4-methylbenzoate) porphyrin (**2**)

3-hydroxybenzaldehyde (7.32 g, 0.06 mol) and 4-formylbenzoate (3.28 g, 0.02 mol) were added to 100 mL of propionic acid, and then 6 mL of freshly distilled pyrrole (in 50 mL of propionic acid) was added to the mixture under reflux. The mixture was stirred at 145 °C for 55 min, the solvent was removed under vacuum, and the residue was purified by column chromatography (SiO_2, $CHCl_3$: AcOEt = 5:1), yielding **2** (1.13 g, 7.9%) as a purple solid. mp > 300 °C. ^{1}H NMR (400 MHz, CD_3COCD_3): δ = 9.10–8.69 (m, 8 H, β-H), 8.28–8.26 (d, J = 8 Hz, 2 H, ArH), 8.13–8.11 (d, J = 8 Hz, 2 H, ArH), 7.66–7.63 (b, 3 H, ArH), 7.57 (s, 3 H, ArH), 7.54–7.49 (m, 3 H, ArH), 7.26–7.22 (m, 3H, ArH), 4.06 (s, 3 H, —CH_3), −2.79 (s, 2 H, —NH); IR (KBr) ν/cm^{-1}:3383 (N—H), 3321 (O—H), 2950, 2850 (CH_3, CH_2), 1701 (—C═O), 1606 (Ar C═C); UV–vis (MeOH) λ_{max}/nm: 415, 510, 545, 586, 643; MS (ESI) m/z: 719 [M-1]$^+$

2.2.2. Synthesis of 5, 10, 15-tris (3-hydroxyphenyl)-20- (4-carboxyphenyl) porphyrin (**3**)

Compound **2** (1.44 g, 2 mmol) and KOH (1.12 g, 20 mmol) were suspended in MeOH/THF/pyridine (20:10:1, 46 mL). The reaction mixture was stirred at 60 °C overnight. After cooling to room temperature and neutralization with 0.76 mL of formic acid, the solvent was removed under vacuum. Purification of the crude material was performed using a silica gel column with 5% MeOH in $CHCl_3$, and compound **3** was obtained as a purple solid with a yield of 97.7% (1.38 g). mp > 300 °C. ^{1}H NMR (400 MHz, CD_3COCD_3): δ = 9.08–8.55 (m, 8 H, β-H), 8.27–8.25 (d, J = 8 Hz, 2 H, ArH), 8.07–8.05 (d, J = 8 Hz, 2 H, ArH), 7.62 (s, 3 H, ArH), 7.56–7.52 (m, 3 H, ArH), 7.49–7.43 (m, 3H, ArH), 7.21–7.20 (d, J = 4 Hz, 3 H, ArH), −2.79 (s, 2 H, —NH); IR (KBr) ν/cm^{-1}: 3317 (O—H), 2925, (═CH—), 1697 (—C═O), 1593 (Ar C═C); UV–vis (MeOH) λ_{max}/nm: 413, 511, 545, 586, 643; MS (ESI) m/z: 705 [M—H]$^+$

2.2.3. Synthesis of 5, 10, 15-tris (3-hydroxyphenyl)-20- (4-carboxyphenyl) chlorin (**4**)

In the dark, compound **3** (141 mg, 0.2 mmol), anhydrous K_2CO_3 (276 mg, 2 mmol) and KOH (45 mg, 0.8 mmol) were added to DMF (5 mL). Hydrazine hydrate (80%, 30 μL) was then added, and the mixture was stirred at 110 °C. Further quantities of hydrazine hydrate (30 μL) were added after 1, 2, 3, 4, 5 and 6 h, and the reaction