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ORIGINAL RESEARCH

Chemo-Enzymatic Synthesis of Glycolyl-Ester-Linked Taxol-Monosaccharide Conjugate and Its Drug Delivery System Using Hepatitis B Virus Envelope L Bio-Nanocapsules

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Abstract: Chemo-enzymatic synthesis of glycolyl-ester-linked taxol-glucose conjugate, ie, 7-glycolyltaxol 2″-*O*-α-D-glucoside, was achieved by using α-glucosidase as a biocatalyst. The water-solubility of 7-glycolyltaxol 2″-*O*-α-D-glucoside (21 μM) was 53 fold higher than that of taxol. The hepatitis B virus envelope L particles (bio-nanocapsules) are effective for delivering 7-glycolyltaxol 2″-*O*-α-D-glucoside to human hepatocellular carcinoma NuE cells.

Keywords: chemo-enzymatic synthesis, 7-glycolyltaxol 2″-*O*-α-D-glycoside, drug delivery system

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Introduction

Taxol is one of the most potent anticancer agents used in the treatment of breast and ovarian cancers. It presents disadvantages such as low water-solubility and toxicity toward normal tissues. To date many efforts have been made to modify taxol chemically in order to create a more soluble and more easily delivered drug.[1–4] Taxol derivatives, that incorporate acids, have attracted much attention, because an ester linkage improves the solubility of taxol and can be hydrolyzed by hydrolytic enzymes to release taxol.[1–4] However, little attention has been paid to chemo-enzymatic synthesis of ester-linked taxol-glycoside conjugates as water-soluble taxol derivatives, which employ enzymatic cleavage by hydrolytic enzymes including esterases and glycosidases as their mode of activation. On the other hand, convenient drug delivery system (DDS) using hepatitis B virus surface antigen L particles has been recently reported.[5] However, this DDS system is not effective for delivering taxol, because the present nano-particles can incorporate only soluble drugs.

On continuing the study to develope the technology for delivering taxol, we report the chemo-enzymatic synthesis of glycolyl-ester-linked taxol-sugar conjugate, ie, 7-glycolyltaxol 2″-*O*-α-D-glucoside, and its new delivery system using hepatitis B virus envelope L particles to human hepatocellular carcinomas.

Experimental

General

Taxol was a gift from Ensuiko Sugar Refining Co., Ltd. The ^{1}H and ^{13}C nuclear magnetic resonance (NMR), H-H correlation spectroscopy (COSY), C-H COSY, and heteronuclear multiple-bond correlation (HMBC) spectra were recorded in CD_3OD using a Varian XL-400 spectrometer (Varian Inc). The chemical shifts were expressed in δ (ppm) referring to tetramethylsilane. The fast atom bombardment mass spectrometry (FABMS) spectra were measured using a JEOLMStation JMS-700 spectrometer (JEOL Ltd.). High performance liquid chromatography (HPLC) was carried out on Crestpak C18S column (4.6 × 150 mm, JASCO) [solvent: MeOH-H_2O (2:3, v/v); detection: UV (228 nm); flow rate: 1.0 mL/min].

Synthesis of 7-glycolyltaxol 2″-*O*-α-D-glucoside

Glycolic acid was glucosylated by α-glucosidase as follows.[6] To a solution of maltose (0.2 mol) and glycolic acid (0.02 mol) in DMSO-H_2O was added α-glucosidase (500 U). The mixture was stirred for 24 h at 40 °C and then was extracted with *n*-butanol. The organic layer was concentrated and purified by column chromatography on silica gel to afford carboxymethyl α-D-glucopyranoside (**1a**).

Synthesis of 7-glycolyltaxol 2″-*O*-α-D-glucoside was carried out as follows. To a solution of BnBr/NaH (0.15 mol) in DMF was added carboxymethyl α-D-glucopyranoside (**1a**). The mixture was stirred at rt for 12 h, followed by stirring with aq. KOH (1.5 equiv.). The reaction mixture was quenched with saturated aq. $NaHCO_3$ and extracted with ethyl acetate. The ethyl acetate layer was concentrated in vacuo and purified by silica gel column chromatography to give carboxymethyl 2,3,4,6-tetra-*O*-benzyl-α-D-gluctopyranoside (**2a**). To a solution of taxol (0.03 mol) and imidazole (0.12 mmol) in dry DMF was added chlorotriethylsilane (0.1 mol) dropwise at rt. The reaction mixture was stirred at rt for 2 h and diluted with ethyl acetate. The mixture was washed with water and brine, dried over $MgSO_4$, and concentrated in vacuo. Column chromatography of the residue on silica gel gave 2′-TES ester of taxol. To a mixture of 2′-TES ester of taxol (0.015 mol) in the presence of EDCI/DMAP (0.022 mol) in CH_2Cl_2 (10 mL) was added **2a** (1.2 equiv). The mixture was stirred at rt for 12 h. The reaction mixture was extracted with ethyl acetate. The organic layer was concentrated in vacuo and purified by column chromatography on silica gel to give **3a**. To a solution of Pd black (0.001 mol) in HOAc-H_2O (9:1, v/v) was added **3a**. The suspension was stirred at room temperature for 24 h. Extraction of the reaction mixture with *n*-butanol followed by column chromatography on silica gel yielded 7-glycolyltaxol 2″-*O*-α-D-glucoside (**4**).

Spectral data of 7-glycolyltaxol 2″-*O*-α-D-glucoside are as follows.

7-Glycolyltaxol 2″-*O*-α-D-glucopyranoside (**4**): HRFABMS: calcd for $C_{55}H_{63}NO_{21}Na$ $[M+Na]^+$ *m/z* 1096.3032, found 1096.3050; ^{1}H NMR (400 MHz, CD_3OD, δ in ppm): δ 1.09 (3H, s, H-16), 1.15 (3H, s, H-17), 1.78 (3H, s, H-19), 1.81 (1H, m, H-6β), 1.87

(3H, s, H-18), 2.00 (1H, dd, J = 15.6, 9.2 Hz, H-14a), 2.15 (3H, s, CH_3 in 10Ac), 2.23 (1H, dd, J = 15.6, 9.2 Hz, H-14b), 2.37 (3H, s, CH_3 in 4Ac), 2.58 (1H, m, H-6α), 3.29–3.78 (8H, m, H-2″, 2a, 3a, 4a, 5a, 6a), 3.90 (1H, d, J = 7.2 Hz, H-3), 4.18 (3H, m, H-7, 20), 4.75 (1H, d, J = 5.2 Hz, H-2′), 4.95 (1H, d, J = 3.2 Hz, H-1a), 5.01 (1H, d, J = 9.2 Hz, H-5), 5.63 (2H, m, H-2, 3′), 6.15 (1H, t, J = 9.2 Hz, H-13), 6.21 (1H, s, H-10), 7.28 (1H, t, J = 7.6 Hz, *p*-H in Ph), 7.39–7.58 (9H, m, *m*-H in NBz, *p*-H in NBz, *m*-H in OBz, *o*-H in Ph, *m*-H in Ph), 7.65 (1H, t, J = 7.6 Hz, *p*-H in OBz), 7.85 (2H, d, J = 8.0 Hz, *o*-H in NBz), 8.10 (2H, d, J = 8.0 Hz, *o*-H in OBz); ^{13}C NMR (100 MHz, CD_3OD, δ in ppm): δ 11.3 (C-19), 14.7 (C-18), 20.6 (CH_3 in 10Ac), 22.1 (C-16), 23.1 (CH_3 in 4Ac), 26.7 (C-17), 34.1 (C-6), 36.3 (C-14), 44.5 (C-3, C-15), 57.2 (C-3′), 57.7 (C-8), 62.4 (C-6a), 65.9 (C-2″), 71.4 (C-7, C-13), 72.1 (C-4a), 73.6 (C-5a), 74.1 (C-2a), 74.8 (C-2′), 75.1 (C-3a), 75.7 (C-2), 76.6 (C-10), 77.2 (C-20), 78.8 (C-1), 81.8 (C-4), 85.0 (C-5), 100.6 (C-1a), 128.3 (*o*-C in NBz, *o*-C in Ph), 128.9 (*p*-C in NBz), 129.5 (*m*-C in OBz, *m*-C in Ph), 131.1 (*m*-C in NBz, q-C in OBz), 132.7 (*o*-C in OBz, *p*-C in Ph), 134.1 (C-11), 134.5 (q-C in Ph), 135.4 (*p*-C in OBz), 139.8 (q-C in NBz), 142.1 (C-12), 167.4 (C=O in OBz), 170.1 (C=O in NBz), 170.9 (C-1″), 171.3 (C=O in 4Ac), 171.9 (C=O in 10Ac), 174.3 (C-1′), 203.2 (C-9).

Water-solubility of 7-glycolyltaxol 2″-*O*-α-D-glucoside

Water-solubility of 7-glycolyltaxol 2″-*O*-α-D-glucoside was examined as follows. The compound was stirred in water for 24 h at 25 °C. The mixture was centrifuged at 100000 g for 30 min at 25 °C. The concentration of test compounds was estimated on the basis of their peak areas using calibration curves prepared by HPLC analyses of authentic samples.

Preparation of hepatitis B virus surface antigen L particles

The hepatitis B virus surface antigen L particles were prepared according to the previously reported procedures.[5] The L particles were overexpressed in Saccharomyces cerevisiae AH22R$^-$ carrying hepatitis B virus envelope L expression plasmid pGLDLIIP39-RcT and purified by precipitation with polyethylene glycol 6000, CsCl isopycnic ultracentrifugation and sucrose density gradient ultracentrifugation. About 4 mg of purified L particles were obtained from wet wt. 20 g of the yeast cells. The solution including purified L particles was concentrated by Vinapspin Concentrator-1000,000 MWCO (Vivascience Ltd.) to 200 ng/mL.

Cytotoxicity assay in vitro

The sensitivity of human hepatocellular carcinoma NuE cells to 7-glycolyltaxol 2″-*O*-α-D-glucoside or 7-glycolyltaxol 2″-*O*-α-D-glucoside electroporated into L particles was determined according to the previously reported method.[5] Cells were diluted with culture medium to the seeding density (10^5 cells/mL), suspended in 96-well tissue culture plates (100 µL/well), preincubated at 37 °C for 4 h, and then treated for 24 h with 7-glycolyltaxol 2″-*O*-α-D-glucoside or 7-glycolyltaxol 2″-*O*-α-D-glucoside electroporated into L particles at various concentrations to obtain a dose–response curve for each compound. After incubation, 20 µL MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, yellow tetrazole) solution (2.5 mg/mL) was added to each well and the plates were further incubated for 4 h. Absorbance at 570 nm was measured with a microplate reader model 450 (BIO-RAD). Dose-response curves were plotted on a semi-log scale as percentage of the cell numbers in control cultures not exposed to test compounds.

Results and Discussions

The water soluble taxol derivative, ie, 7-glycolyltaxol 2″-*O*-α-D-glucoside (**4**), was synthesized from taxol by chemo-enzymatic procedures as shown in Figure 1. First, the 2″-hydroxyl group of taxol was protected with triethylsilyl (TES) group to give 2″-TES ester of taxol. Incubation of the reaction mixture including glycolic acid, α-glucosidase, and maltose in DMSO-H_2O at 40 °C for 24 h gave carboxymethyl α-D-glucoside. The carboxymethyl α-D-glucoside was benzylated with BnBr/NaH in DMF at room temperature for 12 h, followed by stirring with KOH (1.5 equiv.) to give carboxymethyl 2,3,4,6-tetra-*O*-benzyl-α-D-glucoside. The coupling of 2″-TES ester of taxol with carboxymethyl 2,3,4,6-tetra-*O*-benzyl-α-D-glucoside (1.2 equiv.) in the presence of EDCI/DMAP in CH_2Cl_2 at room temperature for 12 h

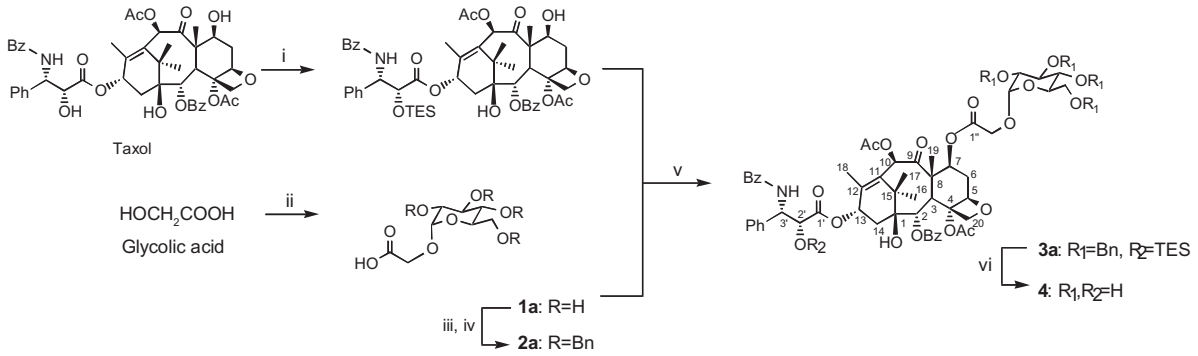

Figure 1. Synthesis of 7-glycolyltaxol 2″-*O*-α-D-glucopyranoside (**4**).
Notes: Reagents and conditions: (i) TESCl, imidazole, DMAP; (ii) α-glucosidase; (iii) BnBr, NaH, DMF; (iv) KOH; (v) EDCI, DMAP, CH_2Cl_2; (vi) H_2, Pd black, HOAc-H_2O (9:1, v/v).

afforded 2″-TES-7-glycolyltaxol 2‴,3‴,4‴,6‴-tetra-*O*-benzyl-2″-O-α-D-glucoside. The deprotection of both TES and benzyl groups with Pd black in HOAc-H_2O (9:1, v/v) yielded 7-glycolyltaxol 2″-*O*-α-D-glucoside (**4**).

The water-solubility of 7-glycolyltaxol 2″-*O*-α-D-glucoside (**4**) was examined (Table 1). The water-solubility of 7-glycolyltaxol 2″-*O*-α-D-glucoside (**4**) was 21 μM, which was 53-fold higher than that of taxol (0.4 μM). The glucosyl conjugation effectively improved the water-solubility of taxol.

Hepatitis B virus is a human liver-specific virus, the genome of which harbors three overlapping envelope genes in a single open reading frame, encoding small, medium, and large proteins. Recently, hepatitis B virus envelope large (L) protein was produced in yeast cells as hollow particles with no hepatitis B genome inside and the particle was used as immunogens in hepatitis B vaccines that were proven safe for humans. The N-terminal amino acid residues 108–119 of the L protein displayed on the surface of L particles functions as the specific ligand for receptor binding on human hepatocytes and are crucial for hepatitis B virus infectivity. The L particles could be used as a safe vehicle for delivering drugs with high targetting specificity to human hepatocyte-derived cells. The hepatitis B virus surface antigen L particles were prepared according to the previously reported procedures.[5] Taxol-prodrug, 7-glycolyltaxol 2″-O-β-D-glycoside, was electroporated into L particles with a Gene Pulser II electroporation system (Bio-Rad Laboratories Inc.). The mixture of taxol-prodrug (final cencentration of 2.1, 4.2, 8.3, 17, and 33 μg/mL) and 500 μL of L particles solution (100 ng of protein) was electroporated in a 4-mm gap cuvette at 220 V and 950 μF for 20 min. To clarify the efficient incorporation of taxol-prodrug in the L particles, the filtrate of L particles solution after centifugation by Vinapspin Concentrator-1000,000 MWCO was analyzed by HPLC and no taxol-prodrug was detected. After adding the same volume of water as the filtrate, the cytotoxic activity of taxol derivative **4** incorporated in L particles toward human hepatocellular carcinoma NuE cells was examined as follows. Human hepatocellular carcinoma NuE cells were diluted with RPMI/10% FBS (10^5 cells/mL), suspended in 96-well tissue culture plates (100 μL/well), preincubated at 37 °C for 12 h, and then treated for 3 d with 7-glycolyltaxol 2″-*O*-α-D-glucoside (**4**) and 7-glycolyltaxol 2″-*O*-α-D-glucoside (**4**) incorporated in L particles. After incubation, 20 μL MTT solution (5 mg/mL) was added to each well and the plates were further incubated for 5 h. Absorbance at 570 nm was measured with a microplate reader model 450 (BIO-RAD Laboratories Inc). The Dose–response curves were plotted in Figure 2. The cytotoxicity of both 7-glycolyltaxol 2″-*O*-α-D-glucoside (**4**) and 7-glycolyltaxol 2″-*O*-α-D-glucoside (**4**) incorporated in L particles was increased dose-dependently.

Table 1. Water-solubility of 7-glycolyltaxol 2″-*O*-α-D-glucoside.

Compound	Water-solubility (μM)[a]	Fold
Taxol	0.4	1
7-glycolyltaxol 2″-*O*-α-D-glucoside	21	53

Note: [a]Water-solubility was measured at 25 °C.

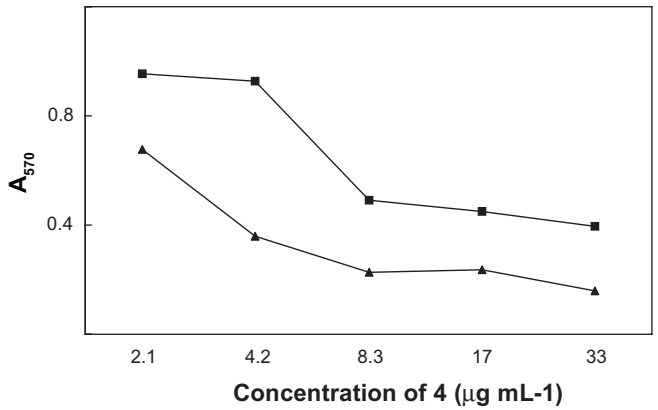

Figure 2. Dose-response curves in MTT assay of 7-glycolyltaxol 2″-*O*-α-D-glucoside (■) and 7-glycolyltaxol 2″-*O*-α-D-glucoside incorporated in hepatitis B virus envelope L particles (▲).

The cytotoxic activity of 7-glycolyltaxol 2″-*O*-α-D-glucoside (**4**) incorporated in L particles was higher than 7-glycolyltaxol 2″-*O*-α-D-glucoside (**4**) itself at each concentration tested.

In summary, a water-soluble taxol derivative, ie, 7-glycolyltaxol 2″-*O*-α-D-glucoside, was successfully synthesized by chemo-enzymatic procedures. The drug delivery system using hepatitis B virus surface antigen L particles was effective for delivering 7-glycolyltaxol 2″-*O*-α-D-glucoside to human hepatocellular carcinoma NuE cells. Further studies on in vivo thrapeutic values of taxol derivative, that is incorporated in L particles, are now in progress.

Author Contributions

KS, MH, MS, TM, and HH were responsible for data collection/entry/analysis and assistance with manuscript preparation. HH was responsible for the study design and preparation of the manuscript. All authors read and approved the final manuscript.

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Competing Interests

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