SYNTHESIS OF DOXORUBICIN-CYCLODEXTRIN CONJUGATES

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Doxorubicin-γ-cyclodextrin conjugates have been synthesized by the coupling of 14-bromodaunomycin with mono half-ester compounds linked to a 6-hydroxyl group of γ-cyclodextrin. Release of drug from the conjugates in saline phosphate buffer solution and *in vitro* antitumor activity against L1210 leukemia cells were also investigated.

The anthracycline antibiotics doxorubicin (DXR) and daunorubicin are excellent antitumor agents, especially DXR which is widely used in the treatment of a number of solid tumors and leukemias in humans.[1,2] However these drugs have dose limiting toxicities such as cardiac damage and bone marrow inhibition.[3]

In recent years a variety of drug delivery systems for the anthracyclines have been reported. In most cases, the drugs were linked to high molecular compounds such as dextran,[4,5] DNA,[6] maleinic acid-divinyl ether co-polymer[7] and the like, so that the conjugates had very intricate structures. Our attention has been focused on the synthesis of a new conjugate of DXR as a prodrug. The approach used was based on (i) use of a water soluble carrier bearing a linker which is cleavable by an enzyme or mild hydrolysis, (ii) regio-selective preparation of a conjugate, and (iii) change in drug delivery compared to the free drug, and (iv) reduced toxicity.

The present paper describes the synthesis and characterization of DXR-γ-cyclodextrin conjugates, and the relationship between sustained release of DXR and *in vitro* antitumor activities.

Chemistry

Cyclodextrins are extensively used in foods, pharmaceuticals and in various other fields.[8] We chose γ-cyclodextrin (γ-CD) as a carrier in this program because of its low toxicity and high water solubility. In addition we used several dicarboxylic acid compounds as linkers.

When γ-CD in dimethylformamide (DMF) was reacted with phthalic anhydride (0.5 equiv.) in the presence of triethylamine (0.5 equiv.) at room temperature for several hours, the esterification gave a mixture containing mono 2-O-, mono 3-O- and mono 6-O-phthaloyl half esters of γ-CD as main products.

While the reaction in pyridine or pyridine-DMF afforded selectively mono half-ester compounds attached to a 6-hydroxyl group of γ-CD. Table 1 shows the reaction conditions and the results when one 6-hydroxyl group was selectively esterified with dicarboxylic anhydrides and the products ratio was determined by HPLC analysis. The resulting half esters were purified by reverse phase column chromatography.

In the ^{1}H NMR spectrum (DMF-d_7) of **1**, the chemical shifts of the esterified glucose moiety were observed down field compared with those of other uncharged glucose moieties, paticularly the signals of the esterified C-6 methylene group revealed at 4.14 ppm (dd, $J_{5,6a}=6.6$ and $J_{6a,6b}=11.5$ Hz) and 4.55 ppm

(d, $J_{6b,6a}=11.5$ Hz). These values shifted down field by 0.5~0.8 ppm. The MS spectrum of **1** showed a molecular ion peak $[M+H]^+$ at *m/z* 1397. Similarly other compounds gave suitable ^1H NMR and MS spectra. These results supported that compounds **1**~**4** had the mono half-ester structure linked to a 6-hydroxyl group of γ-CD.

DXR-γ-CD conjugates was prepared by a convenient manner, namely 14-bromodaunorubicin hydrochloride[1] was coupled with one equivalent of the half-esters (**1**~**4**) in the presence of 2 equivalents of triethylamine in DMF, yielding DXR-γ-CD conjugates (**5**~**8**). The conjugates were chromatographed on a reverse phase column.

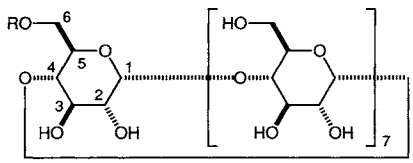
In ^1H NMR analysis, the methylene signals at the 14-position of DXR moiety in **5** (DMF-d_7) and in **6** (DMSO-d_6) were observed at 5.34 and 5.41 ppm ($J_{14a,14b}=18$ Hz), and at 5.45 ppm (2H, broad), respectively. These down field shifts resulted from the esterification of the 14 position of DXR.[9] Structures of the conjugates (**5**~**8**) were also

Table 1.

Compound	Reaction conditions: Acid anhydride/γ-CD[a]	Reaction conditions: Pyr/DMF/γ-CD[b]	Products 6-O-R/2- & 3-O-R[c]
1	SA 1/1	5/1/1	4.6/1
2	PA 1/3	5/0/1	13.6/1
3	NA 1/2	5/1/1	14.0/1
4	CA 1/2	5/1/1	14.7/1

[a] Molar ratio.
[b] vol./vol./weight.
[c] Formation ratio by HPLC, SA: succinic anhydride, PA: phthalic anhydride, NA: naphthalene dicarboxylic anhydride, CA: cyclohexane dicarboxylic anhydride.

Fig. 1.



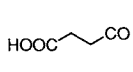
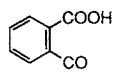
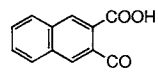
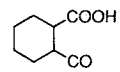
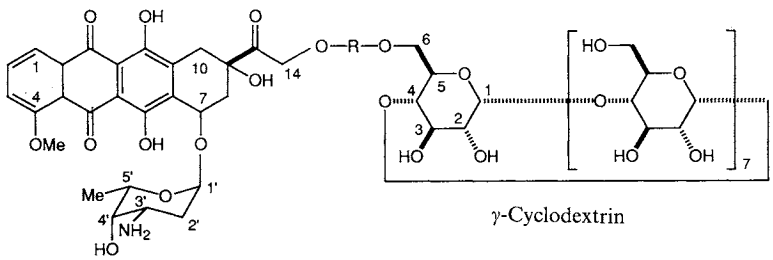
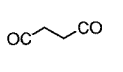
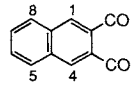
Compound	1	2	3	4
R				

Fig. 2.



Doxorubicin

γ-Cyclodextrin

Compound	5	6	7	8
R				

supported by their MS, IR and UV-Vis spectra. In addition, UV-Vis spectra of the conjugates in aqueous solution showed no bathochromic shift, therefore suggesting that the adriamycin chromophore was not in the lipophilic cavity of γ-CD.[8]

Thus, the new DXR-γ-CD conjugates were constructed *via* regioselective preparation of mono half-ester derivatives of γ-CD.

Table 2.

Compound	50% Release of DXR (hours)	Antitumor activity IC_{50} (μM)
DXR		0.10
5	3	0.11
6	45	0.25
7	65	0.39
8	>70	1.05

Antitumor test: L1210 leukemia cells (5×10^4 cells/ml) were cultured in RPMI 1640 medium containing 10% horse serum with test compounds (0.001 ~ 10 μg/ml) at 37°C under 5% CO_2 - 95% air for 2 days. The cell growth was determined using a hemocytometer by counting viable cells after staining with trypan blue. Antitumor activity was expressed as 50% inhibitory concentration (IC_{50}) of the control growth.

Results

One mg each of the conjugates (**5**~**8**) was dissolved in 1 ml of 0.0067 M saline phosphate buffer solution (initial pH: 7.36) and kept at 37°C, release of DXR was then monitored by HPLC. The liberated DXR was calculated by the amount of residual conjugate because the DXR free base was unstable at this pH. As shown in Table 2, the release rate depends on the structure of the linker (dicarboxylate moiety), that is, hydrolysis of conjugate **5** was over 20-times faster than that of **8** which has a bulky cyclohexane structure.

The *in vitro* antitumor activity of **5**~**8** was assayed against cultured L1210 leukemia cells in comparison with DXR (Table 2). The antitumor activity of **5** was similar to that of parent DXR because of the rapid release of DXR from the carrier, whereas the compounds **6**, **7** and **8** showed activities corresponding to their liberated DXR concentrations. As a result, it is believed that the activity of the intact conjugates is very weak or absent. The activity of the conjugates on mice bearing L1210 leukemia was tested by daily intraperitoneal administration on days 1 to 10. The compounds **5** and **6** exhibited the excellent T/C values at their optimal doses (**5**: T/C (%) = 552 at a dose of 5 mg/Kg/day as DXR, **6**: T/C (%) = 468 at 10 mg/Kg/day as DXR, and DXR: T/C (%) = 255 at 1.25 mg/Kg/day). However the antitumor efficacy of **5** and **6** against Sarcoma-180 and Ehrlich in mice was similar to that of DXR. A detailed evalution of their *in vivo* antitumor activity will be reported elsewhere.

Experimental

General

NMR spectra were recorded on a JEOL JNM-GSX400 spectrometer using TMS as an internal standard and are expressed as ppm (δ). Splitting patterns are abbreviated as: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, b = broad. Molecular weight was determined by MS spectrometry (FAB technique) on a JEOL JMS-SX102A instrument. UV-Vis and IR spectra were taken on a Hitachi U-3210 and a JASCO FT/IR-5300 spectrophotometers, respectively. HPLC monitoring of reactions and chromatography was performed with a Shimadzu liquid chromatograph LC-5A and LC-6AD equiped with a refractive index (RI) detector (Shimadzu RID-6A) or a UV detector (Shimadzu SPD-2A). An octadesyl silica gel column (Yamamura Inst. Co., ODS A-312) was used, eluting at 1.5 ml/minute with 0.1% AcOH - MeOH (9 : 1 ~ 3 : 1, in compounds **1**~**4**; RI detector) and with 0.05 M ammonium formate buffer (pH 4) - MeCN (**5** = 7 : 3; **7** = 7 : 4; **8** = 6 : 4; UV detector 254 nm), and with 0.05 M ammonium formate buffer (pH 4) - MeOH (2 : 3 in compound **6**; UV detector 254 nm).

DXR-succinyl-γ-CD Conjugate **5** (general procedure)

(i) 6-O-Succinyl-γ-CD mono half ester **1**: γ-CD (1.02 g, 1.53 mmol) was suspended in DMF (1 ml),

and pyridine (5 ml) was added and dissolved. Succinic anhydride (94.4 mg, 0.943 mmol, 1.2 equiv.) was added under stirring and the reaction was run at room temp. for 18 hours. The mixture was poured into chloroform (100 ml) to obtain a precipitate. The precipitate was filtered and washed with chloroform (20 ml) and methanol (20 ml), and then dried at 60°C to afford 496 mg of crude **1** (containing about 60 mol% of **1**). The crude **1** was purified on an ODS column (Yamamura Inst. Co., ODS A-120-350/250; eluent water-methanol 7:1~3:1) to yield 220 mg of **1**.

1: FAB-MS (glycerin): positive m/z 1397=$[M+H]^+$, negative m/z 1395=$[M-H]^-$; ^{1}H NMR (in DMF-d_7 ,40°C): Ester moiety δ 2.44~2.63 (4H, m, succinyl methylene), *Esterified glucose moiety* δ 4.14 (1H, dd, J=6.6, 11.5 Hz, H-6a), 4.55 (1H, d, J=11.5 Hz, H-6b).

(ii) DXR-succinyl-γ-CD conjugate **5**: Purified **1** (220 mg, 0.157 mmol) was dissolved in DMF (4.4 ml) and triethylamine (44 μl, 0.315 mmol) was added. 14-Bromodaunorubicin hydrochloride (100 mg, 0.157 mmol) was then added to the solution and stirred at room temp. for 2 hours. The mixture was poured into a mixture of chloroform/methanol (10 ml/5 ml). The resulting precipitate was filtered and dried to obtain 219 mg of a reddish powder. The powder was purified on the above mentioned ODS column (eluent 0.1% acetic acid-acetonitrile 13:4) to provide 119 mg of **5**.

5: UV-Vis (λmax in water): 234, 254, 289, 482 nm; IR (KBr; ν C=O) 1730, 1622 cm^{-1}; FAB-MS (glycerin/thioglycerin=1/1): positive m/z 1922=$[M+H]^+$, negative m/z 1920=$[M-H]^-$; ^{1}H NMR (in DMF-d_7): *DXR moiety* δ 1.28 (3H, d, J=6.6 Hz, 5'-CH_3), 1.86 (1H, b, J=12 Hz, H-2'a), 2.02 (1H, b, J=12 Hz, H-2'b), 2.23 (1H, dd, J=5.1, 14 Hz, H-8a), 2.50 (1H, d, J=14 Hz, H-8b), 3.02 (1H, d, J=18 Hz, H-10a), 3.21 (1H, d, J=18 Hz, H-10b), 4.10 (3H, s, 4-OCH_3), 4.35 (1H, q, J=6.6 Hz, H-5'), 5.14 (1H, b, H-7), 5.34 (1H, d, J=18 Hz, H-14a), 5.41 (1H, d, J=18 Hz, H-14b), 5.43 (1H, d, J=3.7 Hz, H-1'), 7.73 (1H, m, H-3), 7.97 (2H, m, H-1, -2); *Linker moiety* δ 2.78 (4H, m, 2×CH_2); *Esterified glucose moiety* δ 3.98 (1H, m, H-5), 4.30 (1H, dd, J=5.1, 11 Hz, H-6a), 4.44 (1H, d, J=11 Hz, H-6b).

DXR-phthaloyl-γ-CD Conjugate **6**

2: FAB-MS: positive (glycerin) m/z 1455=$[M+H]^+$, negative (glycerin/thioglycerin=1/1) m/z 1443=$[M-H]^-$; ^{1}H NMR (in DMSO-d_6 ,60°C): *Estrified glucose moiety* δ 3.90 (1H, m, H-5'), 4.23 (1H, dd, J=6.2, 11 Hz, H-6a), 4.58 (1H, d, J=11 Hz, H-6b), *Ester moiety* δ 7.40~7.62 (4H, m, phthaloyl-H).

6: UV-Vis (λmax in water): 233, 252, 285, 482 nm; IR (KBr; ν C=O) 1726, 1620 cm^{-1} ; FAB-MS (glycerin/thioglycerin=1/1): positive m/z 1970=$[M+H]^+$, negative m/z 1968=$[M-H]^-$; ^{1}H NMR (in DMSO-d_6): *DXR moiety* δ 1.19 (3H, d, J=6.6 Hz, 5'-CH_3), 1.67 (1H, bd, J=12 Hz, H-2'a), 1.86 (1H, m, H-2'b), 2.17 (1H, m, H-8a), 2.32 (1H, d, J=13 Hz, H-8b), 2.94 (1H, d, J=18 Hz, H-10a), 3.11 (1H, d, J=18 Hz, H-10b), 3.99 (3H, s, 4-OCH_3), 4.22 (1H, q, J=6.6 Hz, H-5'), 5.00 (1H, b, H-7), 5.32 (1H, b, H-1'), 5.45 (2H, b, H-14), 7.66 (1H, m, H-3), 7.92 (2H, m, H-1, -2); *Linker moiety* δ 7.72 (2H, m, H-4, -5), 7.80, 7.86 (each 1H, m, H-3, -6); *Esterified glucose moiety* δ 3.95 (1H, m, H-5), 4.36 (1H, m, H-6a), 4.53 (1H, d, J=11 Hz, H-6b).

DXR-naphthalene Dicarbonyl-γ-CD Conjugate **7**

3: FAB-MS (glycerin): positive m/z 1505=$[M+H]^+$.

7: UV-Vis (λmax in water): 236, 285, 338, 486 nm; IR (KBr, ν C=O) 1724, 1620 cm^{-1} ; FAB-MS (glycerin/thioglycerin=1/1): positive m/z 2020=$[M+H]^+$; ^{1}H NMR (in DMSO-d_6): *DXR moiety* δ 1.21 (3H, d, J=6.6 Hz, 5'-CH_3), 1.69 (1H, bd, J=12 Hz, H-2'a), 1.90 (1H, m, H-2'b), 2.20 (1H, m, H-8a), 2.34 (1H, d, J=13 Hz, H-8b), 2.98 (1H, d, J=18 Hz, H-10a), 3.13 (1H, d, J=18 Hz, H-10b), 4.00 (3H, s, 4-OCH_3), 4.23 (1H, q, J=6.6 Hz, H-5'), 5.02(1H, b, H-7), 5.33 (1H, b, H-1'), 5.49 (2H, b, H-14), 7.69 (1H, m, H-3), 7.92 (2H, m, H-1, -2); *Linker moiety* δ 7.75 (2H, m, H-6, -7), 8.18 (2H, dd, J=3.3, 5.9 Hz, H-5, -8), 8.43, 8.49 (each 1H, s, H-1, -4): *Esterified glucose moiety* δ 4.01 (1H, m, H-5), 4.39 (1H, m, H-6a), 4.59 (1H, bd, J=11 Hz, H-6b).

DXR-cyclohexane Dicarbonyl-γ-CD Conjugate **8**

4: FAB-MS (glycerin): positive m/z 1451=$[M+H]^+$, negative m/z 1499=$[M-H]^-$; ^{1}H NMR (in DMSO-d_6 ,60°C): *Ester moiety* δ 1.36 (4H, m, H-4, -5), 1.67, 1.90 (each 2H, m, H-3, -6), 2.72 (2H, m, H-1, -2), *Esterified glucose moiety* δ 3.77 (1H, dd, J=5.1, 10.3 Hz, H-5), 4.00 (1H, dd, J=5.1, 11.5 Hz, H-6a), 4.43 (1H, d, J=11.5 Hz, H-6b).

8: UV-Vis (λmax in water): 234, 254, 291, 481 nm; IR (KBr, ν C=O) 1730, 1620 cm^{-1} ; FAB-MS (glycerin/thioglycerin=1/1): positive m/z 1976=$[M+H]^+$, negative m/z 1974=$[M-H]^-$; ^1H NMR (in DMSO-d_6): *DXR moiety* δ 1.16 (3H, d, J=6.6 Hz, 5'-CH_3), 1.66 (1H, b, J=11 Hz, H-2'a), 1.86 (1H, m, H-2'b), 2.12 (1H, m, H-8a), 2.24 (1H, bd, J=14 Hz, H-8b), 2.87 (1H, d, J=18.5 Hz, H-10a), 3.06(1H, d, J=18.5 Hz, H-10b), 3.99 (3H, s, 4-OCH_3), 4.18 (1H, q, J=6.6 Hz, H-5'), 4.97 (1H, b, H-7), 5.10 (1H, d, J=17 Hz, H-14a), 5.29 (1H, d, J=17 Hz, H-14b), 5.30 (1H, b, H-1'), 7.66 (1H, m, H-3), 7.92 (2H, m, H-1, -2); *Linker moiety* δ 1.30~1.50 (4H, m, H-4, -5), 1.69~1.82 (2H, m, H-3a, -6a), 1.86, 1.98 (each 1H, m, H-3b, -6b), 2.85, 3.02 (each 1H, m, H-1, -2): *Esterified glucose moiety* δ 3.78 (1H, m, H-5), 4.03 (1H, m, H-6a), 4.41 (1H, bd, J=11 Hz, H-6b).

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