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Syntheses and Anti-AIDS Activities of Polyrotaxane-3′-azido-3′-deoxythymidine Conjugates

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Introduction

Many attempts have been made to develop chemotherapeutic agents that may be effective against human immunodeficiency virus (HIV). It has been reported that several antiviral nucleoside analogues such as 3′-azido-3′-deoxythymidine (AZT), 2′,3′-dideoxycytidine (DDC), 2′,3′-dideoxyinosine (DDI), and 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T) inhibit the viral reverse transcriptase (RT) of HIV as well as terminate DNA chain synthesis from viral RNA inside the infected cells, and also various oligo- and polysaccharides have potent anti-HIV activities.[1-5] However, since it has been known that long-term use of dideoxy nucleoside analogues induce serous side effects such as bone marrow suppression and the appearance of drug-resistant viruses, it is necessary to search for a new anti-AIDS agent.[6,7] In spite of the many efforts for the development of new drugs, there have been no report on the drugs without side effects to cure AIDS. Thus new molecular designs and the syntheses of more potent drugs are required to develop compounds that are less toxic against normal cells and more selective against target cells without drug resistance. It is well known that one of the methods for the reduction of side effects is to synthesize polymers containing drug moiety for drug delivery, because polymeric drugs can be expected to have some advantages such as higher specificity of actions, longer duration of actions and lower toxic side effects compared with low molecular weight drugs.[8-15]

Cyclodextrins (CDs) are a class of cyclic oligosaccharides that have a common chemical structure of six to eight D-glucose units, and have been used as non-toxic drug delivery carriers.[16-18]

Therefore, the long term aim of this work is to synthesize new anti-AIDS active agents containing AZT in order to reduce their side effects mentioned above. In this study, first the new nucleoside derivative containing AZT moiety, 3′-azido-3′-deoxy-5′-*O*-succinyl-thymidine (suc-AZT) was prepared from AZT with succinic anhydride. Polyrotaxane-AZT conjugates were synthesized by the reaction of suc-AZT and polyrotaxane, in which many α-cyclodextrins (α-CDs) were threaded onto a poly(ethylene glycol) encapped with 2,4-dinitrofluorobenzene (DNFB). Fourier transform infrared (FTIR), ^{1}H NMR, ^{13}C NMR, and elemental analysis were used to confirm the syntheses of the various conjugates. The *in vitro* anti-AIDS activities of the conjugates were evaluated with MT-4 cell lines infected or uninfected with the AIDS virus.

Experimental

Materials. α-Cyclodextrin (α-CD; Tokyo Kasei Kogyo Co.), succinic anhydride (Aldrich Co.), poly(ethylene glycol) bis(3-aminopropyl) terminated (M_n = 1,500) (PEG-BA; Aldrich Co.), 2,4-dinitrofluorobenzene (DNFB; Aldrich Co.), 4-dimethylaminopyridine (DMAP; Aldrich Co.), and *N*,*N*-dicyclohexylcarbodiimide (DCC; Aldrich Co.) were used without further purification. 3′-Azido-3′-deoxythymidine (AZT) was kindly supplied by Samchully Pharm. Co. (Seoul, Korea). All other chemicals were reagent grade and were used without further purification.

Measurements. Nuclear magnetic resonance spectra (^{1}H and ^{13}C NMR) were recorded on a FT-300 MHz Varian Gemini 2,000 spectrophotometer; chemical shifts are expressed as δ units (part per million) downfield from TMS (tetramethyl silane). IR spectra were obtained with a Jasco FT/IR-5300 spectrophotometer by using KBr pellet for analysis. Elemental analyses were performed on a Carlo Erba Model EA1180 elemental analyzer.

Synthesis of Polyrotaxane. The nucleoside derivative, 3′-azido-3′-deoxy-5′-*O*-succinyl-thymidine (suc-AZT), was synthesized and characterized from succinic anhydride and AZT according to our previous paper.[16] A polyrotaxane was prepared according to the method previously reported by Yui[17,18] and Harada *et al.*[19,20] with a minor modification.

Briefly, DNFB was allowed to react with the terminal amino groups in the inclusion complex of α-CD and PEG-BA in DMF. The mixture was heated to 80 °C for 2 hrs. After cooled to room temperature, the product was precipi-

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tated by dropwise addition of diethyl ether to the reaction mixture. The precipitate was filtered, washed with diethyl ether. The residue was dissolved in DMSO and then precipitated from methanol to remove unreacted DNFB, PEG, and the dinitrophenyl derivatives of PEG, and from water to remove free α-CD. The product was collected, washed with diethyl ether, and dried under vacuum to obtain pure polyrotaxane.

Suc-AZT: ^{1}H NMR (DMSO-d_6): δ (ppm)=7.5 (s, 1H, =C<u>H</u>-N-), 6.2 (t, 1H, -O-C<u>H</u>-N-), 4.5 (m, 1H, -C<u>H</u>-N_3), 4.3 (m, 2H, -C<u>H</u>$_2$-O-CO-), 4.0 (m, 1H, -O-C<u>H</u>-C<u>H</u>$_2$-), 3.0 (s, 4H, -C<u>H</u>$_2$-C<u>H</u>$_2$-), 2.5~2.3 (m, 2H, -C<u>H</u>$_2$-C-N_3), 1.8 (s, 3H, C<u>H</u>$_3$); ^{13}C NMR (DMSO-d_6) [Figure 3(a)]: δ (ppm)=174.5 (<u>C</u>OOH), 172.5 (<u>C</u>OO), 164.0 (coupled C=O of thymine), 151.0 (C=O of thymine), 136.2 (=CH of thymine), 110.5 (=<u>C</u>-CH_3), 84.0 (-O-<u>C</u>H-N-), 81.2 (-O-<u>C</u>H-CH_2-), 63.8 (-<u>C</u>H$_2$-OCO-), 60.2 (-<u>C</u>H-N_3), 36.0 (-<u>C</u>H$_2$-C-N_3), 31.0, 29.0 (-<u>C</u>H$_2$-<u>C</u>H$_2$-), 12.3 (-<u>C</u>H$_3$); IR (KBr) [Figure 2(a)] : 3400~2800 cm^{-1} (OH stretch of acid moiety), 2121 cm^{-1} (azide group, N_3).

Inclusion Complex of PEG with α-CD: ^{1}H NMR (DMSO-d_6 + D_2O) : δ (ppm)=4.81 (d, 6H, C(1)H of α-CD), 3.89 (t, 6H, C(3)H of α-CD), 3.67 (m, 12H, C6(H) of α-CD), 3.60 (m, 6H, C(5)H of α-CD), 3.51 (s, 8H, CH_2 of PEG), 3.43 (t, 6H, C(2)H of α-CD), 3.27 (t, 6H, C(4)H of α-CD); ^{13}C NMR (DMSO-d_6+D_2O): δ (ppm)=102.6 (C1 of α-CD), 82.5 (C4 of α-CD), 73.9 (C2 of α-CD), 72.5 (C3 and C5 of α-CD), 70.2 (CH_2 of PEG), 60.5 (C6 of α-CD).

Synthesis of Polyrotaxane-AZT Conjugates. The solutions of obtained polyrotaxane (0.3 g), DMAP (0.33 g, 2.77 mmol), and suc-AZT (0.68 g, 1.85 mmol) in dimethyl formamide (DMF; 20 mL) was stirred for 1.5 hrs at room temperature. DCC (0.76 g, 3.7 mmol) in DMF (5 mL) was added dropwise to the solution for 1 hr. The mixture was allowed to stir overnight at room temperature. After the reaction was completed, the *N,N′*-dicyclohexylurea (DCU) that formed was filtered off and the filtrate was concentrated on a rotary evaporator; this was followed by the addition of acetone. The yellowish precipitate was collected by filtration, washed several times with acetone and diethyl ether, then dried to a constant weight under vacuum to obtain pure polyrotaxane-AZT conjugate (Yield : 80%). The schematic diagram for the conjugate syntheses is shown in Figure 1.

Polyrotaxane-AZT Conjugate: ^{13}C NMR (DMSO-d_6) (Figure 3(b)): δ=172.2 (C=O of succinyl moiety), 164.1 (coupled C=O of thymine), 150.7 (C=O of thymine), 136.2 (=CH of thymine), 110.1 (=<u>C</u>-CH_3), 102.1 (C1 of α-CD), 83.9 (-O-<u>C</u>H-N-), 82.3 (C4 of α-CD), 80.6 (-O-<u>C</u>H-CH_2-), 73.7 (C2 of α-CD), 72.1 (C3 of α-CD), 72.0 (C5 of α-CD), 69.7 (CH_2 of PEG), 65.0 (-<u>C</u>H$_2$-OCO-), 63.9 (esterified C6 of α-CD), 60.2 (C6 of α-CD), 35.8 (-<u>C</u>H$_2$-C-N_3), 28.5 (-<u>C</u>H$_2$-<u>C</u>H$_2$-), 12.3 (-<u>C</u>H$_3$); IR (KBr) [Figure 2(b)]: 3,700~2,800 cm^{-1} (OH stretch), 2,120 cm^{-1} (azide group, N_3), 1,723 (C=O stretch).

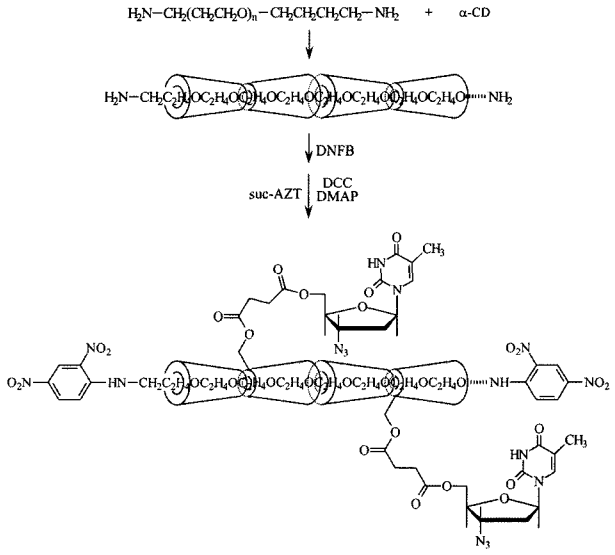

Figure 1. Synthesis of polyrotaxane-AZT conjugates.

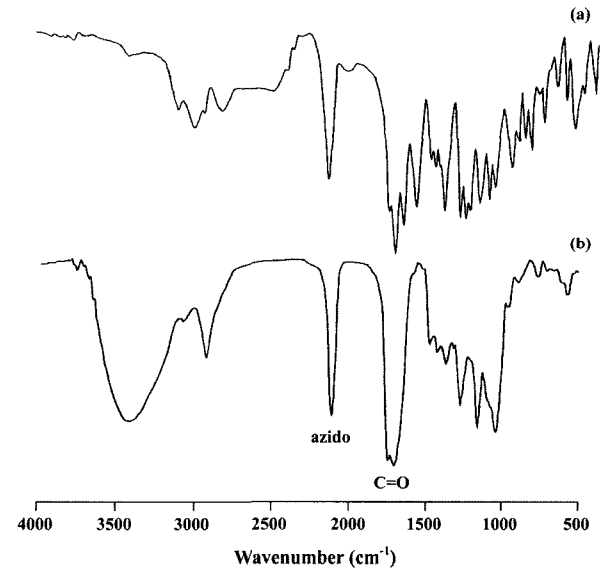

Figure 2. FTIR spectra of (a) suc-AZT and (b) its conjugates with polyrotaxane.

In Vitro Anti-AIDS Activity

Cells and Medium. HIV-infected and uninfected MT-4 cells, a human T4-positive cell line carrying HTLV-1 (human T-lymphotropic virus type-1), were obtained through the Pharmaceutical Screening Center of Korea Research Institute of Chemical Technology (KRICT) in Korea. The cell line having RPMI-1640 medium supplemented with 10% (w./v.) fetal bovine serum (FBS), gentamycin (40 μg/mL), and 2 mM L-glutamine (growth medium) was incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The viability of cells was investigated by trypan blue dye exclu-

sion method. For an assay, the cells were seeded at a density of 1.0×10^5 cells/well.

Virus. HIV-$1_{HTLV\text{-}IIIB}$ and HIV-2_{ROD} were used in the anti-HIV assay. Human immunodeficiency virus type-1 (HIV-1) was obtained from the culture supernatant of H9 cells persistently infected with HTLV-IIIB[21] (derived from a pool of American patients with AIDS). To harvest HIV-1, the cells were pelleted by centrifugation and the supernatant containing infectious HIV-1 was aliquoted, and stored at -70 °C by a refrigerator (Deep Freezer; REVOC, ULT-1685).

Antiviral Assay on MT-4 Cell Line. The MT-4 cells[22] (1.0×10^5 cells/mL) were exposed to cell-free HIV-$1_{HTLV\text{-}IIIB}$ or HIV-2_{ROD} at a dose of 100 $TCID_{50}$/mL (50% tissue culture infectious dose) and cultured at 37 °C for 1.5 hrs. Compounds were tested and compared to AZT from the Samchully Pharm. Co. for cytotoxicity and for their ability to inhibit HIV replication. Compounds were first dissolved in 100% of DMSO and then diluted with RPMI 1640/10% FBS just before use. The maximum final concentration of DMSO added to the cell cultures was 0.5% at the highest concentration of compound. We have determined the concentration that DMSO does not interfere with cell growth. The HIV-1 induced cytopathic effects (CPE) were monitored by the MTT viability assay. In the microplate tests (96-well), 50 μL of each compound dilution or PBS alone was distributed in triplicate. The cells were adjusted to 1.0×10^5 cells/mL, then were plated in each well at the rate of 200 L per well. Virus suspension (200 μL) was added to cells with or without drugs and cultured for 6 day.

Cytotoxicity by MTT Assay.[23,24] Infected cultures were carried out in a parallel to determine the cytotoxicity of the compound. Briefly, 100 μL of cell suspension were collected and mixed with 10 μL of a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 7.5 mg/mL in phosphate buffered 0.85% (w./v.) sodium chloride (PBS). After 1.5 hrs incubation at 37 °C, most of the supernatant was removed and the formazan precipitate dissolved in 100 μL of 0.04 N HCl in 2-propanol. The absorbance at 540 and 690 nm was measured on a multiwell scanning spectrophotometer (ELISA plate reader; SLT, ERA-400). The percentage of toxicity was defined with uninfected and untreated control cells. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration required to reduce the viability of uninfected cells at 5 day of incubation in the presence of the compounds. The concentration achieving 50% protection or concentration required to inhibit HIV-induced destruction of MT-4 cells by 50% was defined as 50% effective concentration (EC_{50}). HIV-infected or uninfected MT-4 cells (1.0×10^5 cells/mL) as target cells were suspended with the various concentrations of samples and cultured for 5 day in a CO_2 incubator at 37 °C. Anti-AIDS activities of the conjugates were evaluated after 5 day of HIV infection.

Results and Discussion

Identification of the Polyrotaxane-AZT Conjugates. In ^{13}C NMR spectrum of the conjugates (Figure 3(b)), the absorption peaks of carbonyl carbon, which was esterified from carboxylic acid group, and an adjacent methylene of the succinyl ester moiety shifted upfield to 172.2 and 28.5 ppm. A part of the C6 absorption of α-CD in polyrotaxane shifted downfield from 60.2 to 63.9 ppm because of the esterification of 6-hydroxyl group in α-CD. Because there was no change in the C2 and C3 carbon absorptions of α-CD after the reaction, it is clear that AZT was introduced into only the C6 position of α-CD in polyrotaxane. This result agrees with the earlier report by D'Souza *et al.*[25] that the 6-modified CD was obtained by the esterification of CD with a carboxylic acid functional group with pyridine and a noncomplexing electrophile.

The molecular weight of the polyrotaxane was also confirmed by the end group analysis using the UV absorption spectrum, which showed the wavelength of the absorption maximum at 362.0 nm.

$$M_w = 2c/c' = 2c\varepsilon L/A$$

where c (g/L) is the concentration of the product (the weight of the polyrotaxane dissolved in solvent), c' (mol/L) is the concentration of end groups determined by the UV spectrum, $c' = A/\varepsilon L$, ε is molar extinction coefficient of dinitrophenyl group (17,950 $Lmol^{-1}cm^{-1}$), L is cell length (1 cm), and A = absorbance. Thus, the molecular weight of the polyrotaxane obtained was 15,600. The number of CDs in the polyrotaxane was also calculated from the molecular weight of the polyrotaxane (Mw). The molecular weights of PEG and α-CD are 1,500 and 973, respectively. Thus, the number of CDs in polyrotaxane is

$$N_{CD} = (M_w - 1{,}500)/973$$

The number of CDs calculated is ca. 15, and this result is excellent agreement with that determined from the ^1H NMR spectra by comparing the integration of the peak of α-CD (1H) and that of the methylene group of PEG.

Contents of the AZT Unit. As shown by the results in Table I, the degree of AZT substitution per one cyclodextrin molecule in polyrotaxane increased with increasing equivalent of suc-AZT, and ranged from 1.4 to 3.2, which was calculated from the elemental analysis.

Anti-HIV Activities of the Polyrotaxane-AZT Conjugates. The anti-AIDS virus activity *in vitro* of the conjugates were measured on MT-4 cell line infected or uninfected with HIV-1 (III_B) and HIV-2 (ROD) cell supernatants, and summarized in Table I together with those of AZT. The estimations of toxicity *in vitro* anti-AIDS activity were obtained

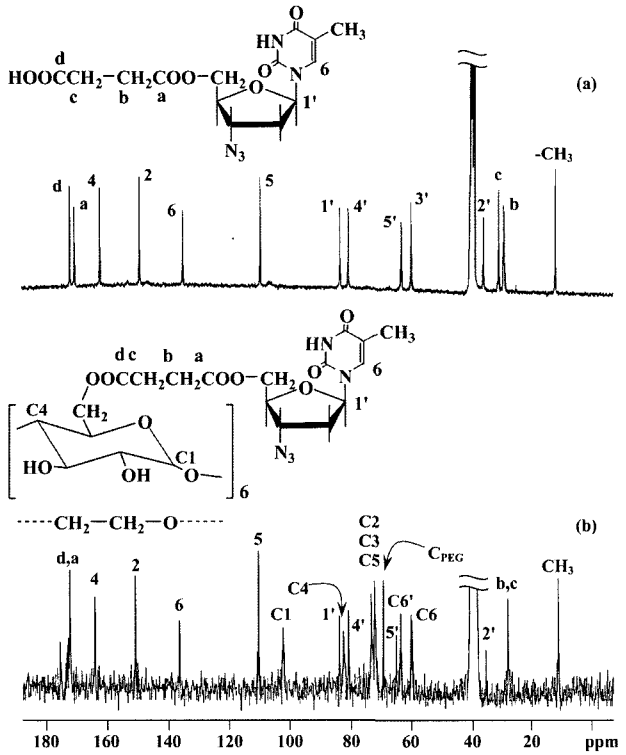

Figure 3. ^{13}C NMR spectra of (a) suc-AZT and (b) polyrotaxane-AZT conjugates.

by determining the EC_{50} values for infected cells with AIDS virus and CC_{50} values for uninfected cells using the MTT cell viability assay in culture supernatants. The drug concentration effective for 50% inhibition of virus infection in 5 days HIV-infected MT-4 cell was defined as 50% effective concentration (EC_{50}), and cytotoxic concentration (CC_{50}) means the 50% cytotoxic concentration required to reduce the viability of uninfected MT-4 cells. As shown in Table I, the CC_{50} and EC_{50} values for HIV-1 (III_B) of the polyrotaxane-AZT conjugates prepared from various molar ratios of suc-AZT in the feed were determined as follows : CC_{50} = 35.29, EC_{50}=0.031 for 3 : 1, CC_{50}=24.40, EC_{50}=0.014 for 6 : 1, and CC_{50}=49.39, EC_{50}=0.042 for 9 : 1. For HIV-1 (III_B), the selectivity index (SI) values in the series of the conjugates increased in the following order : 6 : 1 ≥ AZT > 9 : 1 ≥ 3 : 1, and had the highest value of 1,715 when degree of AZT substitution was 2.2. And the conjugates have much more reduced toxicities than free AZT maintaining SI values as effective as free AZT. We have also synthesized AZT-CD conjugates directly for comparison,[16] that have more potent SI values than those of polyrotaxane-AZT conjugates. This results may be attributed to the fact that polyrotaxane has been encapped with DNFB by non-biodegradable linkage, which made it impossible that CD-AZT conjugates in polyrotaxane could be released from them. Therefore, future studies should focus on syntheses of polyrotaxanes with various biodegradable linkages on both ends, such as ester or anhydride linkages. Studies on the release of AZT from the carrier by an enzymatic hydrolysis and the anti-HIV activity of the released AZT will also be done.

Conclusions

The new anti-AIDS agents, polyrotaxane-AZT conjugates, were synthesized from 3′-azido-3′-deoxythymidine (AZT) and succinic anhydride, followed by condensation reactions to be conjugated with polyrotaxane, which was prepared from PEG and α-CD. The number of α-CDs in polyrotaxane was determined to be ca. 15 from ^1H NMR and UV spectra. The degree of AZT substitution per one conjugate molecule ranged from 1.4 to 3.2 based on elemental analysis. *In vitro* anti-AIDS activities, the CC_{50} values of the synthesized conjugates were much greater than that of AZT, and their SI values were as effective as that of AZT. This research describes novel structures that may have potential applications in drug delivery system for anti-AIDS drugs.

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Table I. Yields, AZT Contents, and Anti-HIV Activities of Polyrotaxane-AZT Conjugates

[AZT]/[Polyrotaxane] in the Feed Ratio	Yield (%)	DS_{AZT}[a] (AZT-bound)	CC_{50}[b]	EC_{50} (μg/mL)[c]		SI[f]	
			(μg/mL)	III_B[d]	ROD[e]	III_B	ROD
3 : 1	64	1.4	35.29	0.031	2.51	1,130	14
6 : 1	80	2.2	24.40	0.014	1.95	1,715	13
9 : 1	84	3.2	49.39	0.042	7.82	1,180	6
AZT	-	-	0.9	0.0005	0.0126	1,701	71

[a]Degree of AZT substitution to α-CD was determined by elemental analysis. [b]Cytotoxic effect (μg/mL): Drug concentration 50% cytotoxicity in 5 days MT-4 cell culture. [c]Anti-HIV activity (μg/mL): Drug concentration effective for 50% inhibition of virus infection in 5 days HIV-infected MT-4 cell culture. [d]HIV-1 strain. [e]HIV-2 strain. [f]Selectivity index : Ratio of CC_{50} to EC_{50}.

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