Synthesis of Poly(vinyl alcohol)–Doxorubicin Conjugates Containing *cis*-Aconityl Acid-Cleavable Bond and Its Isomer Dependent Doxorubicin Release

Atsufumi KAKINOKI,[a] Yoshiharu KANEO,*[,a] Yuka IKEDA,[a] Tetsuro TANAKA,[a] and Kahee FUJITA[b]

[a] *Laboratory of Biopharmaceutics, Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University; Fukuyama, Hiroshima 729–0292, Japan: and* [b] *Department of Molecular Medicinal Sciences, Graduate School of Biomedical Sciences, Nagasaki University; Nagasaki 852–8521, Japan.* Received August 20, 2007; accepted October 11, 2007

Aconityl-doxorubicin (ADOX) was synthesized by the modified method of Shen and Ryser. Two isomers of *cis*-ADOX (*cis*-configuration) and *trans*-ADOX (*trans*-configuration) were generated in the reaction of DOX and *cis*-aconitic anhydride. These products were separated completely by using HPLC and analyzed by TOF-MS spectroscopy and ^{1}H- and ^{13}C-NMR experiments. The yields of *cis*-ADOX and *trans*-ADOX were 36.3 and 44.8%, respectively. The free γ-carboxylic group of ADOX molecule was coupled to poly(vinyl alcohol) (PVA) *via* ethylenediamine spacer, resulting the macromolecular conjugates of PVA–*cis*-ADOX and PVA–*trans*-ADOX, respectively. The DOX content of the conjugates estimated by the hydrolysis method detected the aglycone of DOX which can be estimated as the PVA-bound DOX selectively was 4.4 w/w% which was similar to 4.6 w/w% by the ordinary UV method. Both PVA–*cis*-ADOX and PVA–*trans*-ADOX were very stable at neutral pH, but the release of DOX was increased markedly under acidic conditions. Half-life of the release of DOX from PVA–*cis*-ADOX at pH 5.0 was 3 h which was 4.7-fold shorter than that from PVA–*trans*-ADOX (14 h). The cytotoxicities of PVA–*cis*-ADOX and PVA–*trans*-ADOX were evaluated by using J774.1 cells employing a [^{3}H]uridine incorporation assay as a measure of RNA synthesis. A significant difference in antitumor activity between PVA–*cis*-ADOX and PVA–*trans*-ADOX was observed where the former was much active than the later. It was suggested that the conjugate enters the cells and reaches the lysosomal/endosomal compartment, and that the aconityl spacer releases DOX from the conjugate in the acidic compartment of lysosomes/endosomes due to the participation of a free carboxylic group.

Key words poly(vinyl alcohol); doxorubicin; acid-sensitive spacer; macromolecular prodrug; cytotoxicity; cellular uptake

Doxorubicin (DOX) is effective antineoplastic agent and widely used in the therapy of cancer.[1—3] It is used clinically to treat a variety of tumors, but this anthracycline drug is limited by its toxic dose-related side effect, such as cumulative cardiotoxicity, myelosuppression, nephrotoxicity, and extravasation.[4]

The consequence of attachment of low molecular weight drugs to macromolecular carriers alters their rate of excretion from the body, changes their toxicity and immunogenicity, and limits their uptake by cells *via* endocytosis, thus providing the opportunity to direct the drug to the particular cell type where its activity is needed.[5] In addition, these macromolecular conjugates can accumulate in solid tumors due to the enhanced microvasculature of tumor tissue.[6,7] This phenomenon has been termed enhanced permeability and retention in relation to tumor targeting (EPR-phenomenon).[8]

In recent years the use of soluble synthetic polymers as drug delivery systems has been received increasing attention. *N*-(2-Hydroxypropyl)methacrylamide (HPMA) copolymers containing a tetrapeptide side chain (Gly-Phe-Leu-Gly) terminated in DOX and, optionally, galactosamine, as a targeting moiety,[9] were recently approved in the United Kingdom for phase I/phase II clinical trials to treat primary hepatoma. HPMA copolymer prodrugs produced increased life spans and an increased number of long-term survivors[10—13] depending on the structure of the conjugate, timing of administration, and number of doses. Similarly, HPMA copolymers containing the anthracycline antibiotics, Daunorubicin (DNR)[12,13] and DOX[11] were modified with fucosylamine, and it was shown that they interact with the receptor on L1210 cells *in vitro* and *in vivo*. Subsequent experiments were performed to test the pharmacologic activity of these conjugates *in vivo* against L1210 leukemia in DBA_2 mice.

Poly(vinyl alcohol) (PVA) is a polymer which is synthesized by polymerizing not a vinyl alcohol monomer but a vinyl acetate monomer. This monomer is polymerized in to poly(vinyl acetate) and then hydrolyzed to produce PVA. PVA's biocompatibility makes it an excellent material for use in medical applications such as soft contact lenses. Recently, PVA has been used for long-term implants, including a bioartificial pancreas, artificial cartilage, nonadhesive film, and esophagus or scleral buckling material.[14] PVA can be available various molecular weight according to the usage. PVA also provide a potential targetable drug delivery system.[15]

Drug–polymer conjugates are potential candidates for the selective delivery of antineoplastic agents to tumor tissue. Incorporating acid-sensitive bonds between the drug and the polymer is an attractive approach because it ensures effective release of the polymer-bound drug at the tumor site. This release is either extracellular, resulting from the slightly acidic condition in tumor tissue, or intracellular, in acidic lysosomes/endosomes after cellular uptake of drug-polymer conjugate.[16]

Such delivery systems can be usefully distinguished into those formulations where the anthracyclines is bound to a carrier molecule using a covalent linkage that is stable in the bloodstream, being cleaved only after internalization into cells. In this paper we report on the synthesis of the poly(vinyl alcohol)–doxorubicin conjugates, their release experiment, cellular uptake, and cytotoxicity.

* To whom correspondence should be addressed. e-mail: kaneo@fupharm.fukuyama-u.ac.jp

MATERIALS AND METHODS

Materials Doxorubicin hydrochloride and fluorescein isothiocyanate isomer I (FITC) were obtained from Wako Pure Chemical, Osaka, Japan. *cis*-Aconitic anhydride was obtained from Aldrich, Milwaukee, WI, U.S.A. PVA (80 K, MW=80520) was kindly supplied by Japan Vam & Poval Co., Ltd., Osaka, Japan. All other chemicals and reagents were of the highest grade commercially available.

Synthesis of Acontityl-doxorubicin Acontityl-doxorubicin (ADOX) was synthesized by the modified method of Shen and Ryser.[17] Briefly, doxorubicin hydrochloride (10 mg) was dissolved in distilled water (10 ml) and cooled on ice. *cis*-Aconitic anhydride (10 mg) was dissolved in *p*-dioxane (6 ml) and added slowly to the solution with stirring, and the pH was immediately adjusted to 9.0 by careful addition of 0.5 M NaOH. After 15 min, the pH was adjusted to 7.0 and continued for another 1 h. The reaction mixture was analyzed and purified by using HPLC. Analysis of ADOX was carried out using a Shimadzu liquid chromatographic system (LC-6A, Kyoto, Japan) with a variable-wavelength UV detector (SPD-6A) operated at 200 nm. A 4.6×150 mm, 5-μm particle size, C_{18} reversed-phase column (Cosmosil 5C_{18}, Nacalai, Kyoto, Japan) was used at ambient temperature. The mobile phase was $(NH_4)_2CO_3$ (3 w/w%) : methanol : acetonitrile=50 : 45 : 5, v/v/v. The injection volume was 20 μl, and the flow rate was 1.0 ml/min.

Purification of ADOX was achieved by HPLC where a 250×20 mm, 5-μm particle size, C_{18} reversed-phase column (YMC-PackPro C18RS, YMC, Kyoto, Japan) was used at ambient temperature. The mobile phase was $(NH_4)_2CO_3$ (3 w/w%) : methanol=48 : 52, v/v. The injection volume was 1 ml, and the flow rate was 4.0 ml/min. Fractions containing ADOX-isomers were collected and lyophilized.

The TOF-MS spectrum of ADOX displayed the expected pseudomolecular ions at *m/z* 722 (M+Na) and 738 (M+K). Its structure was confirmed by one- and two-dimensional NMR (^{1}H, ^{13}C, ^{1}H, ^{1}H COSY, ^{1}H, ^{13}C HMQC). DOX moieties of *cis*-ADOX and *trans*-ADOX: ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 7.91 (1H, d, H-1), 7.91 (1H, t, H-2), 7.64 (1H, t, H-3), 3.99 (3H, s, 4-OMe), 4.94 (1H, t, H-7), 2.12 and 2.18 (1+1H, dd, H_2-8), 4.85 (1H, t, 9-OH), 4.59 (2H, d, 9-$COCH_2$), 2.89 and 2.99 (1+1H, dd, H_2-10), 5.29 (1H, d, H-1′), 1.70 and 1.90 (1+1H, dd and dt, H_2-2′), 3.95 (*cis*-ADOX: 1H, m, H-3′)/4.00 (*trans*-ADOX: 1H, m, H-3′), 3.60 (1H, d, H-4′), 4.85 (1H, d, 4′-OH), 4.18 (1H, q, H-5′), 1.16 (3H, d, H_3-6′). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 118.9 (C-1), 136.1 (C-2), 119.6 (C-3), 160.7 (C-4), 56.5 (4-OMe), 120.0 (C-4a), 186.4 (C-5), 110.7 (C-5a), 156.0 (C-6), 133.9 (C-6a), 69.8 (C-7), 36.6 (C-8), 74.9 (C-9), 213.7 (9-CO), 32.0 (C-10), 134.6 (C-10a), 154.4 (C-11), 110.6 (C-11a), 186.4 (C-12), 135.4 (C-12a), 100.2 (C-1′), 29.4 (C-2′), 45.1 (*cis*-ADOX: C-3′)/45.7 (*trans*-ADOX: C-3′), 67.7 (C-4′), 66.6 (C-5′), 16.9 (C-6′). Acontityl moiety of *cis*-ADOX: ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 5.70 (1H, s, =CH), 3.03 and 3.01 (1+1H, d, CH_2), 8.85 (1H, d, NH). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 164.4 (CONH and COOH), 127.2 (=CH), 138.8 (=C), 43.9 (CH_2), 171.1 ($CH_2\underline{C}OOH$). *trans*-ADOX: ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 6.42 (1H, s, =CH), 3.18 and 3.19 (1+1H, CH_2), 7.57 (1H, d, NH). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 166.8 (CONH and COOH), 132.0 (=CH), 135.4 (=C), 37.8 (CH_2), 171.9 ($CH_2\underline{C}OOH$).

Preparation of PVA–ADOX Conjugates Ethylenediamine residues were introduced to the hydroxyl groups of PVA molecules by the 1,1′-carbonyldiimidazole (CDI) activation method.[18—20] Four milliliters of dimethyl sulfoxide (DMSO) containing CDI (74 mg) was added to 400 mg of PVA dissolved in 60 ml of DMSO, followed by stirring for 1 h at room temperature. After several precipitations in butanol to remove unreacted reagents, the fraction of CDI-activated PVA was dried *in vacuo*. Then, ethylenediamine (2 g) was added to the CDI-activated PVA (200 mg) dissolved in 50 ml of DMSO and stirred for 48 h at 50 °C. After several precipitations in butanol to remove unreacted reagents, the fraction of PVA-ethylenediamine was dried *in vacuo*. The free amino groups of ethylenediamine spacers introduced into the PVA molecule were measured by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method.[21—23]

One hundred microliters of DMSO containing 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) (0.27 mg) and *N*-hydroxysuccinimide (NHS) (0.16 mg) were added to 1 mg of the ADOX-isomers dissolved in 0.1 ml of DMSO, respectively, followed by stirring for 2 h at room temperature. Then, PVA-ethylenediamine (10 mg) dissolved in 0.2 ml DMSO was added to the activated-ADOX-isomers and stirred for 4 h at room temperature. The reaction mixture was dialyzed against distilled water for 48 h at 4 °C. Excess reagents and low molecular by-products of the reaction were removed by this process. The PVA–ADOX conjugates were obtained from lyophilization of the final solution. Characterizations of the conjugates were carried out using a high-performance size exclusion chromatography (HPSEC) described later.

DOX Content of the Conjugates The conjugates were dissolved in distilled water, and the absorbance at 476 nm was measured. The DOX content of the conjugates was estimated using the calibration curve of doxorubicin standard (UV method).

The DOX content of the conjugates was estimated by the modified method of Seymour.[24] The DOX of the conjugates were hydrolyzed with 5 M HCl for 10 min at 85 °C. Then, 5 M NaOH was added to neutralize the solution. The hydrolysis products were measured by HPLC described later. The amount of DOX was calculated from the quantity of aglycone, hydrolyzed product of DOX (hydrolysis method).

***In Vitro* Release Experiment** The release of DOX from the conjugates was determined in a 0.1 M citrate buffer solution (pH 5.0, 6.0, 7.0, μ=0.3) at 37 °C. The experiment was initiated by the addition of the stock solution to a preheated buffer solution to give a concentration of 1.0 mg/ml of PVA–ADOX conjugates, respectively. At fixed time intervals, the amounts of DOX released were determined by using a HPLC method described later.

Preparation of FITC-Labeled PVA FITC-labeled PVA (F-PVA) was prepared by the modified method of deBelder and Granath.[25] PVA (300 mg) was dissolved in DMSO (8 ml) containing 1 drops of pyridine. FITC-I (50 mg) was added, followed by dibutyltin dilaurate (20 mg), and the mixture was heated for 2 h at 95 °C. After several precipitations in butanol to remove free dye, the fraction of F-PVA was dried *in vacuo* at 80 °C. The F-PVA was further purified by size-exclusion chromatography on Sephadex G-25, and then freeze-dried.

The F-PVA sample was dissolved in 25 mM borate buffer (pH 9.0), and the absorbance at 495 nm was measured. The fluorescein content of F-PVA was estimated using the calibration curve of fluorescein sodium (uranine).

Preparation of [^{125}I]-Labeled PVA ([^{125}I]-PVA) PVA-ethylenediamine (200 μg) was labeled with 0.25 mCi of [^{125}I]iodine by using Bolton and Hunter reagent for protein iodination (GE Healthcare Bio-Sciences, Tokyo, Japan). Unreacted [^{125}I] was removed by chromatography on a PD-10 column (Amersham Pharmacia Biotech).

Cells Mouse macrophage-like tumor J774.1, mouse leukemia P388, mouse hepatoma MH134-TC and human histiocytic lymphoma U937 were kindly provided by Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). Cells were typically kept in continuous logarithmic growth at 37 °C in a humidified atmosphere in 5% CO_2–95% air in RPMI 1640 medium (Nacalai, Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Tokyo, Japan) and 50 U/ml penicillin and 50 μg/ml streptomycin (Dainippon Sumitomo Pharma Co., Ltd., Tokyo, Japan). The number of viable cells was determined by the trypan blue exclusion method by using a Burker-Turk hematocytometer 3 d after incubation.

Fluorescence Microscopic Examination The cells (8×10^5) were placed in a 35 mm culture dish (Iwaki, Funabashi, Japan) and incubated in humidified air with 5% CO_2 at 37 °C for 24 h. F-PVA was added to the dishes, which were incubated at 37 °C for 5 h. The concentration of F-PVA in the medium was adjusted to be 500 μg/ml. After incubation, the dishes were washed three times with phosphate-buffered saline (PBS) (pH 7.4). Specimens were examined with a Nikon transmitted light fluorescence microscope.

Uptake of [^{125}I]-PVA For uptake experiments, [^{125}I]-PVA was used as the substrate against J774.1 and P388 cells. The cells (5×10^3) were placed in a 96-well culture plate (Nunc, Denmark) and cultured in humidified air with 5% CO_2 at 37 °C for 24 h. The medium containing [^{125}I]-PVA was added to the wells, which were incubated at 37 °C. The concentration of [^{125}I]-PVA in the medium was adjusted to be 10 μg/ml. After incubation, the wells were washed with PBS (pH 7.4) then washed with ice-cold 10% trichloroacetic acid three times. To the washed wells, 0.1 M NaOH (150 μl) was added and allowed to stand at room temperature for 30 min. The radioactivity of a solubilized aliquot of 100 μl was determined with a gamma counter (ARC-380CL, Aloka, Tokyo, Japan). The amount of protein in the aliquot was determined by the method of Lowry *et al.*[26]

Uptake of PVA–ADOX Conjugates The cells (4×10^5) were placed in a 35 mm culture dish (Iwaki, Funabashi, Japan) and cultured in humidified air with 5% CO_2 at 37 °C for 24 h. PVA–ADOX conjugates and free DOX were added to the dishes, which were incubated at 37 °C for 6 h. The drug concentration for the microscopic examination and the intracellular release experiment were 20 μg/ml and 125 μg/ml in DOX equivalent, respectively. After incubation, the dishes were washed with PBS (pH 7.4) three times. Specimens were observed with a Nikon transmitted light fluorescence microscope. Subsequently, the amount of intracellular release of DOX from the conjugates was determined by the modified method of Seymour.[24] The cells were homogenized in 0.5 ml of PBS (pH 7.4). Daunorubicin (0.5 μg) was added to each tube as an internal standard, vortex-mixed, and then 33% silver nitrate (0.1 ml) was added. Six milliliters of acetonitrile was added as an extract medium, samples were vortex-mixed three times during a 30 min period. The upper layer was carefully removed from the silver chloride precipitate. The samples were evaporated to dryness and redissolved in mobile phase (0.1 ml). The amount of DOX was determined by HPLC described later. The amount of protein in the aliquot was determined by the method of Lowry *et al.*[26]

***In Vitro* Cytotoxicity** J774.1 cells (5×10^3) were placed in a 96-well culture plate and cultured in humidified air with 5% CO_2 at 37 °C for 24 h. Subsequently, the cells were incubated with various drug concentrations for 5 h. Control wells were identical, except that the test compound was absent. Then, the wells were washed with fresh medium and incubated for 48 h. After incubation, [^{3}H]uridine incorporation assay was carried out. Two microCi of [^{3}H]uridine was added to the wells, which were incubated for 2 h. After incubation, the wells were washed with PBS (pH7.4) then washed with ice-cold 10% trichloroacetic acid three times. To the washed wells, 0.5 M NaOH (150 μl) was added and allowed to stand at room temperature for 30 min. A solubilized aliquot of 0.1 ml was neutralized with HCl and added 2 ml of liquid scintillator (Clear-sol I, Nacalai, Kyoto, Japan). The radioactivity was determined with a liquid scintillation counter (LSC-1100, Aloka, Tokyo, Japan).

Analytical Methods The amount of DOX and its hydrolyzed product was determined by HPLC. Chromatography was carried out using a Shimadzu liquid chromatographic system (LC-6A, Kyoto, Japan) with a variable-wavelength fluorescent detector (RF-10AXL, Shimadzu). The excitation and emission wavelength were set at 470 nm and 560 nm, respectively. A 4.6×150 mm, 5-μm particle size, C_{18} reversed-phase column (Cosmosil 5C_{18}, Nacalai, Kyoto, Japan) was used at ambient temperature. The mobile phase was 34% acetonitrile in 1% triethylamine adjusted to pH 4.0 with formic acid. The injection volume was 20 μl, and the flow rate was 1.0 ml/min. For every experimental sample the content of DOX and its hydrolyzed product was calculated by measuring the relevant peak area and calibrating against the corresponding peak area derived from the daunorubicin internal standard.

High-performance size-exclusion chromatography (HPSEC) was carried out using a Shimadzu liquid chromatographic system (LC-9A, Kyoto, Japan) equipped with a variable-wavelength UV detector (MCPD-3600, Otsuka, Osaka, Japan) and a differential refractometer (RI-8000, Tosho). A 7.8×300 mm, TSKge1 G4000PWXL column (Tosoh) was used at 40 °C. The mobile phase was 0.2 M NaCl in 0.05 M phosphate buffer, pH 7.0. The injection volume was 100 μl, and the flow rate was 1.0 ml/min.

RESULTS

Synthesis of Aconityl-doxorubicin Aconityl-doxorubicin (ADOX) was synthesized by the modified method of Shen and Ryser.[17] The amino group of the sugar ring of doxorubicin (DOX) was reacted with *cis*-aconitic anhydride to form α-unsaturated amide bond. Two isomers of *trans*-ADOX (*trans*-configuration) and *cis*-ADOX (*cis*-configuration) were generated (Fig. 1).

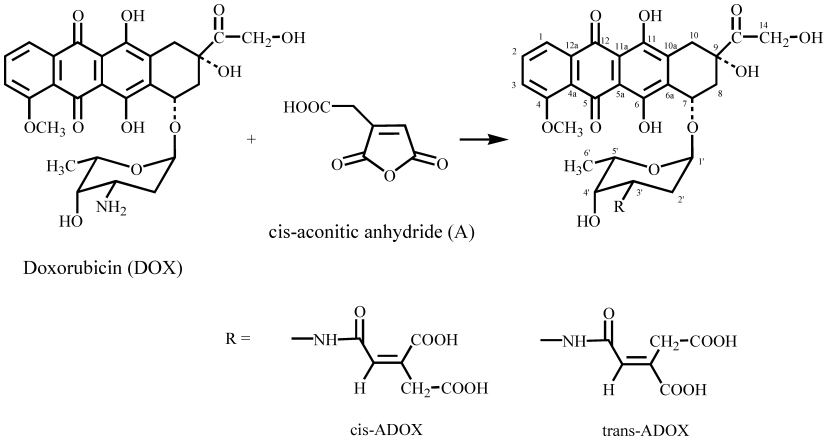

Fig. 1. Synthetic Pathway of Acontyl-doxorubicin (ADOX)

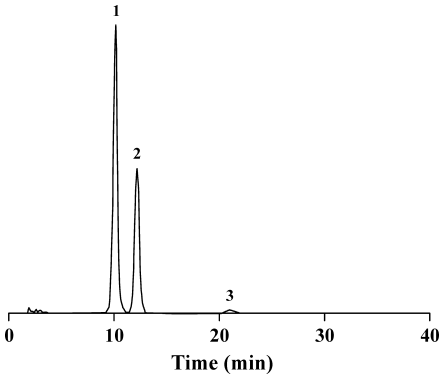

Fig. 2. HPLC Chromatogram of ADOX

Chromatography was carried out using a HPLC system equipped with a variable-wavelength fluorescent detector. The excitation and emission wavelength were set at 470 nm and 560 nm, respectively. A 4.6×150 mm, 5-μm particle size, C18 reversed-phase column was used at ambient temperature. The mobile phase was $(NH_4)_2CO_3$ (3 w/w%) : methanol : acetonitrile = 50 : 45 : 5, v/v/v. The injection volume was 20 μl, and the flow rate was 1.0 ml/min. Peaks: 1, *trans*-ADOX; 2, *cis*-ADOX; 3, DOX.

A representative HPLC chromatogram demonstrated the separation of *trans*-ADOX, *cis*-ADOX and unmodified DOX as shown in Fig. 2. The retention times were 10.2, 12.2 and 21.0 min, respectively, and were highly reproducible. Fractions corresponding to the isomers were collected and analyzed by TOF-MS spectrum. Their structure were confirmed by one- and two-dimensional NMR (^{1}H, ^{13}C, ^{1}H, ^{1}H COSY, ^{1}H, ^{13}C HMQC). The yields of *trans*-ADOX and *cis*-ADOX were 44.8 and 36.3%, respectively.

Preparation of PVA–ADOX Conjugates ADOX was bound to PVA according to the synthetic scheme shown in Fig. 3. The overall synthetic pathway involved two steps. The first was the preparation of PVA-ethylenediamine and the second was the binding of the ADOX and PVA-ethylenediamine.

Hydroxyl group of PVA was activated by CDI. Then, a 10-fold weight excess of ethylenediamine was reacted with CDI-activated PVA. An excess of the ethylenediamine was used in order to prevent cross-linking and cyclisation of the PVA chains. The number of free amino groups of the PVA-ethylenediamine was estimated by the TNBS method.[21—23] One mole of PVA showed the color intensity of 25 mol of free amino group. Free γ-carboxylic group of ADOX molecule was activated with an equimolar amount of EDC and NHS, and then reacted with PVA-ethylenediamine. The DOX content of the conjugates estimated by the UV method and the hydrolysis method were 4.6 and 4.4 w/w%, respectively, corresponding to 6.1 and 6.4 mol of DOX/mol of PVA.

HPSEC Analysis The size exclusion chromatography of the conjugates was also performed by HPLC on a TSK gel G4000PWXL column. PVA–*cis*-ADOX and PVA–*trans*-ADOX were spectrophotometrically detected at 476 nm, whereas PVA was analyzed by a differential refractometer. Elution peaks of the conjugates and PVA were detected similarly, suggesting that the ADOX-substitution has no effect on the molecular weight of PVA (Fig. 4).

Release of DOX from PVA–ADOX Conjugates The release of DOX from the conjugates was determined in a 0.1 M citrate buffer solution (pH 5.0, 6.0, 7.0, μ=0.3) at 37 °C (Fig. 5). Both PVA–*cis*-ADOX and PVA–*trans*-ADOX were very stable at neutral pH, but the release of DOX was increased markedly under acidic conditions. Half-life of the release of DOX from PVA–*cis*-ADOX at pH 5.0 was 3 h which was 4.7-fold shorter than that from PVA–*trans*-ADOX (14 h). Furthermore, the amount of DOX released from PVA–*cis*-ADOX at pH 6.0 was approximately 3-fold higher than that released from PVA–*trans*-ADOX.

Uptake of PVA Fluorescence microscopic examination of the cellular uptake of F-PVA was carried out using various kinds of cells (Fig. 6). It was found that MH134-TC and U937 cells internalized F-PVA marginally. In contrast, J774.1 and P388 cells accumulated F-PVA very efficiently.

In addition, Fig. 7 shows that [^{125}I]-PVA were gradually accumulated in J774.1 and P388 cells at 37 °C. The level of [^{125}I]-PVA accumulated in the J774.1 cells was three times higher than that in the P388 cells. The uptake of [^{125}I]-PVA by these cells was saturated in 5—6 h.

Uptake of PVA–ADOX Conjugates To compare the cellular uptake and intracellular distribution of DOX with those of PVA–*cis*-ADOX, J774.1 cells were incubated in the medium which contained the drug preparations at 37 °C for 6 h. The fluorescence of DOX was observed with a conventional fluorescence microscopy. As shown in Fig. 8, DOX was internalized in the cells very efficiently. PVA–*cis*-ADOX was also internalized and the fluorescence of DOX was observed in the nuclear region.

Subsequently, the amount of intracellular release of DOX from the conjugates was determined by the modified method

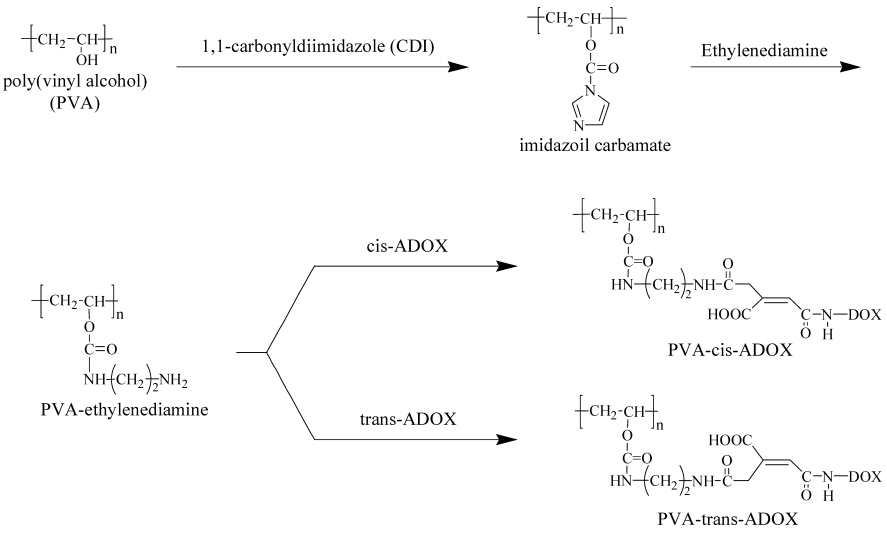

Fig. 3. Synthetic Pathway of PVA–ADOX Conjugates

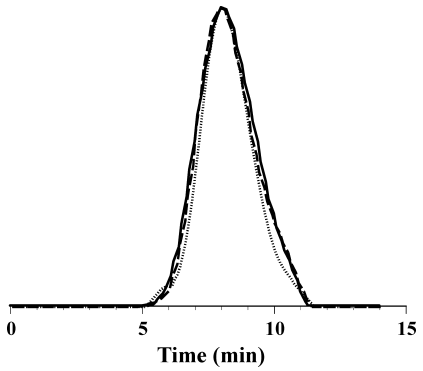

Fig. 4. HPSEC Chromatograms of PVA (……), PVA–*cis*-ADOX (– – –) and PVA–*trans*-ADOX (——)

HPSEC was carried out on a TSKge1 G4000PWXL column (7.8×300 mm) with 0.2 M NaCl in 0.05 M phosphate buffer, pH 7.0. PVA–*cis*-ADOX and PVA–*trans*-ADOX were spectrophotometrically detected at 476 nm, whereas PVA was analyzed by a differential refractometer.

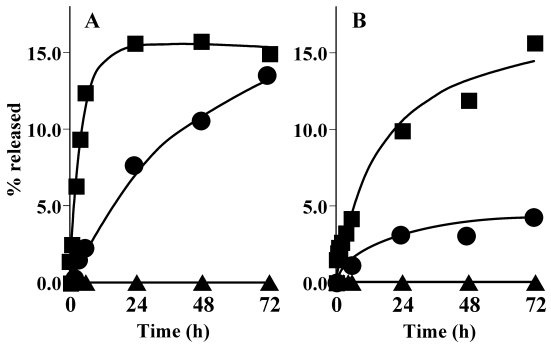

Fig. 5. Effect of pH on the Release of DOX from PVA–*cis*-ADOX (A) and PVA–*trans*-ADOX (B)

The release of DOX from the conjugates was determined in 0.1 M citrate buffer solution (μ=0.3) of pH 5.0 (■), 6.0 (●), and 7.0 (▲) at 37 °C.

of Seymour.[24] J774.1 cells were incubated in the medium which contained PVA–*cis*-ADOX and PVA–*trans*-ADOX at 37 °C for 6 h. Figure 9 shows that the amount of DOX released from PVA–*cis*-ADOX was approximately 2-fold higher than that from PVA–*trans*-ADOX.

***In Vitro* Cytotoxicity** The cytotoxicity of PVA–*cis*-ADOX and PVA–*trans*-ADOX were evaluated in comparison with that of DOX by using J774.1 cells employing a [^{3}H]uridine incorporation assay as a measure of RNA synthesis (Fig. 10). As is evident, the cytotoxicity effects of these compounds were concentration dependent. PVA–*cis*-ADOX is more active than PVA–*trans*-ADOX, but less active than DOX where the IC_{50} values for PVA–*cis*-ADOX and DOX were 18 μg eq DOX/ml and 0.48 μg/ml, respectively. PVA–*trans*-ADOX are only marginally active, the IC_{50} value was not evaluated at the concentrations tested. Significant difference in cytotoxicity was observed between the two conjugates.

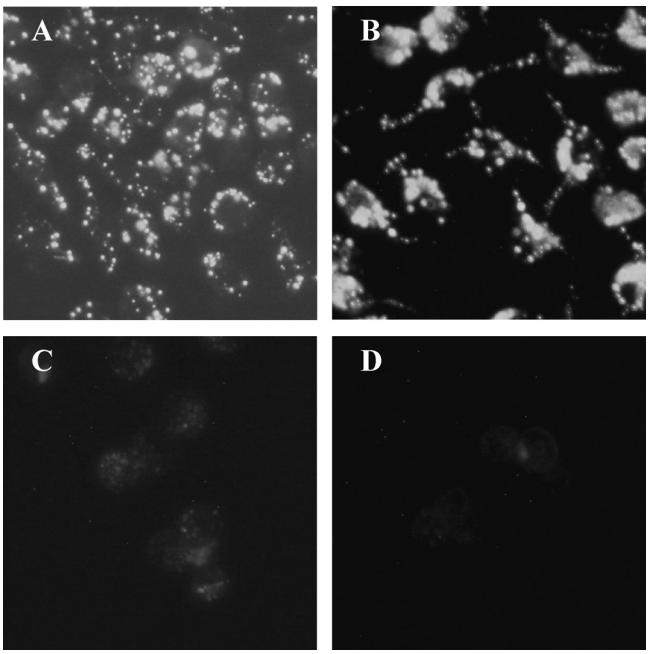

Fig. 6. Fluorescence Microscopic Examination of the Cellular Distribution of FITC-Labeled PVA in J774.1 (A), P388 (B), MH134-TC (C) and U937 (D)

Cells (8×10^5) were incubated with F-PVA (500 μg/ml) at 37 °C for 5 h. After incubation, the cells were washed three times with PBS (pH 7.4) and then examined with a Nikon transmitted light fluorescence microscope of 400 magnifications.

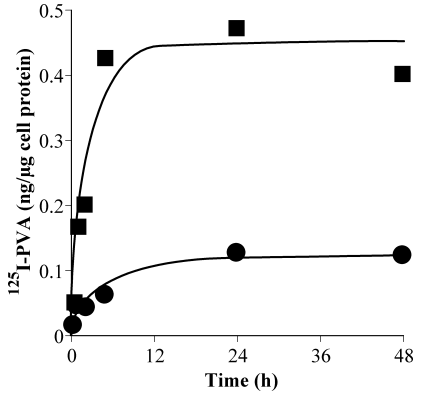

Fig. 7. Cellular Uptake of [^{125}I]-PVA in J774.1 (■) and P388 (●)

Cells were incubated with [^{125}I]-PVA (10 μg/ml) at 37 °C. After incubation, the cells were washed and solubilized to measure the cell-accumulated radioactivity.

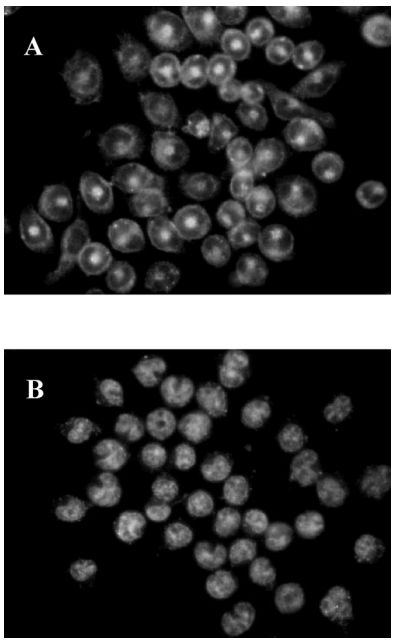

Fig. 8. Fluorescence Microscopic Examination of the Cellular Distribution of PVA–*cis*-ADOX (A) and DOX (B) in J774.1

Cells (4×10^5) were incubated with PVA–*cis*-ADOX (20 μg/ml in DOX equivalent) at 37 °C for 6 h. After incubation, the cells were washed three times with PBS (pH 7.4) and then examined with a Nikon transmitted light fluorescence microscope of 400 magnifications.

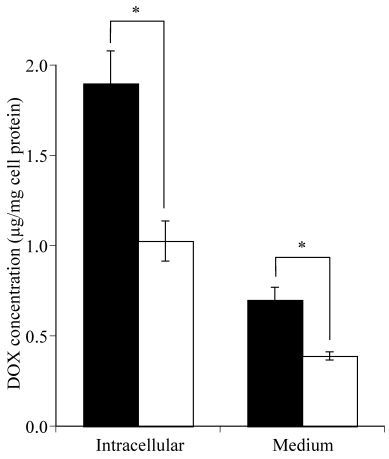

Fig. 9. Intracellular Release of DOX from PVA–*cis*-ADOX (Close Column) and PVA–*trans*-ADOX (Open Column) in J774.1

Cells (4×10^5) were incubated with PVA–ADOX conjugates (125 μg/ml in DOX equivalent) at 37 °C for 6 h. After incubation, the cells were washed and homogenized. The amount of DOX was estimated by using a HPLC equipped with a variable-wavelength fluorescent detector. The excitation and emission wavelength were set at 470 nm and 560 nm, respectively. Values are given as means±S.E. ($n=3$). The statistical significance was determined based on Student's *t*-test ($*p<0.05$).

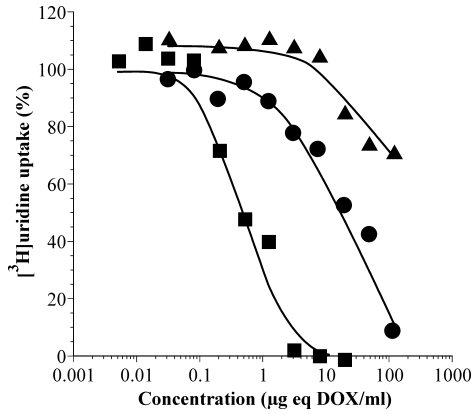

Fig. 10. Effects of PVA–*cis*-ADOX (●), PVA–*trans*-ADOX (▲) and DOX (■) on the Proliferation of J774.1 Cells

Cells (5×10^3) were incubated with various drug concentrations for 5 h and then washed with fresh medium and incubated for another 48 h. After incubation, the cells were incubated with [^{3}H]uridine for 2 h and harvested. Results are shown as percent of the cellular uptake of [^{3}H]uridine uptake.

DISCUSSION

The first acid-sensitive drug–polymer conjugate was synthesized by Shen and Ryser.[17] These authors coupled daunorubicin to aminoethyl polyacrylamide polymer beads or poly-D-lysine using the aconityl spacer. Thereafter, a number of daunorubicin conjugates with monoclonal antibodies and synthetic polymers have been developed with this method.[16,27] Apart from daunorubicin, anthracycline doxorubicin has been also bound to monoclonal antibodies using the *cis*-aconityl spacer.[28]

On the route of acylation of DOX with *cis*-aconitic anhydride four isomeric amide products should be expected, considering the spontaneous inter-conversion of *cis*-aconitic anhydride to propene-1,2,3-tricarboxylic acid-1,3-anhydride.[29—31] Acylated products derived from the primer aconitic anhydride consist of *Z*-substituted double bond (in respect of N–C=O and COOH substituents), whereas the products of the secondary propene-1,2,3-tricarboxylic acid-1,3-anhydride could serve either as an isomer where the COOH and amide substituents are *E* arranged or an isomer with *E* located carboxylic groups.

We found that two isomers of *cis*-ADOX (*cis*-configuration) and *trans*-ADOX (*trans*-configuration) were generated in the reaction of DOX and *cis*-aconitic anhydride as shown in Fig. 1. These products were separated completely by using HPLC (Fig. 2) and were analyzed by TOF-MS spectroscopy and one- and two-dimensional NMR (^{1}H, ^{13}C, ^{1}H, ^{1}H COSY, ^{1}H, ^{13}C HMQC). Two isomers of *cis*-ADOX and *trans*-ADOX, 36.3% and 44.8% yields, were chief products in this reaction. Similar findings were reported by Remenyi *et al.* in the case of DNR. They demonstrated that two isomers of aconityl-daunorubicin (ADNR) belong to the *cis*- and *trans*-isomers of the a-monoamide of ADNR, respectively, by MS-

spectroscopy and ^{1}H- and ^{13}C-NMR experiments.[32]

ADOX was bound to PVA according to the synthetic scheme shown in Fig. 3. It has been suggested that most of the free γ-carboxylic group of the aconityl spacer molecule is coupled to macromolecular moiety.[17] In this case the free γ-carboxylic group may also participate to the coupling to PVA *via* ethylenediamine spacer, resulting the macromolecular conjugates of PVA–*cis*-ADOX and PVA–*trans*-ADOX, respectively. The conjugates were generated from the combination of PVA and DOX for the first time. The contents of DOX of the conjugates were estimated by the separated procedures with the ordinary UV method and the hydrolysis method.[24] The glycosidic bond between the daunosamine ring and the aglycone moiety is hydrolysed relatively easily, releasing the free aglycone.[33] The hydrolysis method detected the aglycone of DOX which can be estimated as the PVA-bound DOX selectively. The DOX content estimated by this method was 4.4 w/w% which was similar to 4.6 w/w% by the UV method. These results indicated that the DOX was linked approximately 24% of the ethylenediamine spacer which was introduced to PVA, corresponding 6.1—6.4 mol of DOX/26 mol of ethylenediamine/1 mol of PVA.

In the release experiments, both PVA–*cis*-ADOX and PVA–*trans*-ADOX were very stable at the neutral pH, whereas acidic conditions accelerated the release of DOX from the conjugates. Furthermore, it was found that half-life of the release of DOX from PVA–*cis*-ADOX was significantly shorter than that observed with PVA–*trans*-ADOX under the same conditions as shown in Fig. 5. Release mechanism was considered to be due to the participation of a free carboxylic group in the acidic condition and the *cis*-transformation was much advantageous to catalyze the intramolecular hydrolysis of the amide bond.

We found that the cellular uptake of F-PVA varied between cell lines, with mouse macrophage-like tumor J774.1 and mouse leukemia P388 showing the highest levels of uptake (Fig. 6). Fluorescence microscopic examination revealed that F-PVA was distributed mainly in the lysosomal/endosomal fraction. Furthermore, the level of [^{125}I]-PVA accumulated in the J774.1 cells was significant higher than that in the P388 cells (Fig. 7). Half-lives of uptake and elimination in the J774.1 cells were 6 h and 14 h, respectively (data are not shown). Mean residence time (MRT) of [^{125}I]-PVA in the J774.1 cells was estimated to be 20 h. These data indicated that the tumor cells remained to incorporate PVA enough to release the DOX.

Intracellular distribution of DOX was also observed by fluorescence microscopy (Fig. 8). The fluorescence of DOX in the cells treated with PVA–*cis*-ADOX was detected mainly in the cytoplasm and nuclear region, whereas the fluorescence treated with DOX was widely distributed in the cells. The intracellular release of DOX from PVA–ADOX conjugates was estimated by the HPLC method of Seymour.[24] The level of DOX released from PVA–*cis*-ADOX was significant higher than that from PVA–*trans*-ADOX (Fig. 9). Furthermore, the intracellular concentration of DOX was significantly higher than that in the medium in both cell lines. Therefore, it was confirmed that the tumor cells took the macromolecular conjugates which released the free DOX in the lysosomal/endosomal acidic conditions.

The cytotoxicities of PVA–*cis*-ADOX and PVA–*trans*-ADOX were evaluated by using J774.1 cells employing a [^{3}H]uridine incorporation assay as a measure of RNA synthesis. A significant difference in antitumor activity between PVA–*cis*-ADOX and PVA–*trans*-ADOX was observed where the former was much active than the later (Fig. 10). It was suggested that the conjugate enters the cells and reaches the lysosomal/endosomal compartment, and that the aconityl spacer releases DOX from the conjugate in the acidic compartment of lysosomes/endosomes due to the participation of a free carboxylic group.

In conclusion, it is possible to exploit the pH difference between the lysosome/endosome and the extracellular environment, as Shen and Ryser[17] first demonstrated, in the development of the acid sensitive macromolecular prodrug, PVA–*cis*-ADOX. Due to a different configuration between PVA–*cis*-ADOX and PVA–*trans*-ADOX, release rates of DOX from the conjugates were very different. A higher effect can be expected by using PVA–*cis*-ADOX in the treatment of cancer.

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