In Vitro pH-Dependent Drug Release from N^4-(4-Carboxybutyryl)-1-β-D-arabinofuranosylcytosine and Its Conjugate with Poly-L-lysine or Decylenediamine-dextran T70

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N^4-(4-Carboxybutyryl)-1-β-D-arabinofuranosylcytosine (glu-ara-C), the conjugate of glu-ara-C and poly-L-lysine (PLL), (PLL–glu-ara-C), and the conjugate of glu-ara-C and decylenediamine-dextran T70 (T70-C_{10}), (T70-C_{10}–glu-ara-C), were prepared. Drug regeneration from glu-ara-C and the conjugates was investigated in buffered solutions of pH 4, 5, 7, 7.4 and 8. The character of the drug release from the conjugates was different from that from glu-ara-C. Namely, the release of 1-β-D-arabinofuranosylcytosine (ara-C) from glu-ara-C was accelerated under both weakly acidic and weakly basic conditions, while it was accelerated only under weakly basic conditions from the conjugates. Overall, the drug release profiles from the conjugates showed similar patterns. However, under neutral and weakly basic conditions, ara-C was regenerated more rapidly from PLL–glu-ara-C than from T70-C_{10}–glu-ara-C. During the incubation of glu-ara-C and the conjugates under weakly acidic conditions, 1-β-D-arabinofuranosyluracil (ara-U) was detected and its amount increased with time to similar extents.

Keywords ara-C; glu-ara-C; PLL–glu-ara-C; T70-C_{10}–glu-ara-C; drug release; pH

Introduction

1-β-D-Arabinofuranosylcytosine (ara-C), being a cell-phase specific antitumor agent,[1] must remain in the body fluid for a prolonged period of time in order to exhibit high chemotherapeutic activity. However, ara-C must be administered by continuous infusion or on a complex schedule because of its rapid inactivation to 1-β-D-arabinofuranosyluracil (ara-U) and quick excretion.[2,3] Therefore, attempts have been made to modify the structure of ara-C to overcome these problems. Derivation of the N-4 amino group introduces resistance to deamination by cytidine deaminase,[4] and the introduction of a lipophilic residue is useful for improving the tissue affinity and prolonging residence in the body.[4,5] N^4-Stearoyl-ara-C and N^4-behenoyl-ara-C are effective derivatives.[4,5]

Besides those small-molecular prodrugs, it was demonstrated in a chemotherapeutic study of the conjugate between poly-L-glutamic acid (PLGA) and ara-C (PLGA–ara-C) by Kato *et al.* that the N-4 acylation of ara-C with a macromolecule was useful for overcoming the above defects of ara-C.[6] In the synthesis of PLGA–ara-C, the direct coupling of ara-C with the acid anhydride of PLGA was done in an organic solvent. In the present work, in order to obtain a macromolecular prodrug of ara-C, N^4-(4-carboxybutyryl)-1-β-D-arabinofuranosylcytosine (glu-ara-C) has been synthesized initially as a derivative having an N^4-acyl chain with the ω-carboxyl group as a reactive group. Subsequently, conjugation between glu-ara-C and a macromolecule, poly-L-lysine (PLL) or decylenediamine-dextran T70 (T70-C_{10}), has been carried out by condensation using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). Further, the pH-dependent drug release was investigated from glu-ara-C, the conjugate of glu-ara-C and PLL (PLL–glu-ara-C), and the conjugate of glu-ara-C and T70-C_{10} (T70-C_{10}–glu-ara-C).

Experimental

General Procedure Ultraviolet (UV) absorption spectra were recorded on a Hitachi model 200-20 spectrometer. Nuclear magnetic resonance (NMR) spectra were obtained on a JEOL JNM-GX 400 spectrometer. Mass spectra (MS) were obtained using a Hitachi M-80 B mass spectrometer. Thin layer chromatography (TLC) was carried out on TLC plates (Silica gel 60 F-254, from E. Merck Co., Ltd.), using a solvent system of chloroform–methanol–acetic acid (16:4:1, v/v).

Chemicals Ara-C, ara-U, 1-(5′-*o*-trityl-β-D-arabinofuranosyl)cytosine (Tr-ara-C), glutaric anhydride and poly-L-lysine hydrobromide (PLL·HBr) with an average molecular weight of 109000 were purchased from Sigma Chemical Co. Dextran T70 (T70) with an average molecular weight of 70000 was obtained from Tokyo Kasei Kogyo Co., Ltd. 1,10-Diaminodecane (C_{10}) was purchased from Wako Pure Chemical Industries, Ltd. EDC was obtained from Nakarai Chemicals, Ltd. Silica gel (70—230 mesh ASTM, from E. Merck Co., Ltd.) was purified before use by following the method of Resnik *et al.*[7] T70-C_{10} was prepared by Schiff's base formation between C_{10} and periodate-oxidized T70, followed by borohydride reduction, as described in the previous paper.[8] All other chemicals were of reagent grade.

Synthesis of Glu-ara-C Tr-ara-C (1 g, 2.06 mmol) and glutaric anhydride (352 mg, 3.09 mmol) were mixed in 48 ml of dioxane. After one week of stirring at room temperature, the solvent was evaporated off *in vacuo*. The residue was added to 30 ml of 50% (v/v) aqueous acetic acid, and then the mixture was heated at 90 °C for 10 min. The precipitate was filtered off, and the filtrate was concentrated *in vacuo*. The residue was dissolved in a small amount of aqueous ethanol, and chromatographed on a silica gel column using the same solvent system as in TLC. After the fractions were checked by TLC, the fractions showing a single spot with *Rf* 0.20 were collected and concentrated to dryness *in vacuo*. The resulting solid was crystallized from ethanol; 48% yield. ^{1}H-NMR (DMSO-d_6): 1.72—1.80 (2H, m, $COCH_2\underline{CH_2}CH_2CO$), 2.22—2.45 (4H, m, $COC\underline{H_2}CH_2C\underline{H_2}CO$), 3.60—4.07 (5H, m, C_2·H, C_3·H, C_4·H, C_5·H_2), 5.06—5.48 (3H, m, C_2·OH, C_3·OH, C_5·OH), 6.05 (1H, d, J=3.7 Hz, C_1·H), 7.20 (1H, d, J=7.5 Hz, C_5H), 8.05 (1H, d, J=7.5 Hz, C_6H), 10.81 (1H, br s, $N\underline{H}COCH_2$), 12.07 (1H, br s, COOH). SIMS m/z: 358 (M+1). UV $\lambda_{max}^{1/15\ M\ phosphate\ buffer\ (pH\ 7.4)}$ nm (ε): 214 (15100), 248 (13900), 299 (8200).

Preparation of PLL–glu-ara-C Conjugate and T70-C_{10}–glu-ara-C Conjugate Glu-ara-C (15 mg), PLL·HBr (500 mg) and EDC (500 mg) were dissolved in 20 ml of purified water, and then stirred for 24 h at pH 7 (adjusted with NaOH or HCl). After that, the conjugate of PLL and glu-ara-C was separated from the unbound glu-ara-C by applying the reaction mixture to a Sephadex G-50 column (4.1 × 26 cm; each fraction, 15 ml; elution solvent, 50 mM NaCl). Each fraction was checked spectrophotometrically at 300 nm. Macromolecular fractions were combined and dialyzed sufficiently against water. The final product was obtained by lyophilization of the dialyzed sample. In the case of T70-C_{10}–glu-ara-C, glu-ara-C (10 mg), T70-C_{10} (1 g) and EDC (700 mg) were mixed in 20 ml of purified water. The reaction and the purification were carried out under the same conditions as those of PLL–glu-ara-C. Finally, T70-C_{10}–glu-ara-C was obtained by lyophilization.

Analysis Ara-C, ara-U and glu-ara-C were determined by high-performance liquid chromatography (HPLC) with a Shimadzu LC-3A apparatus equipped with a Nucleosil 10C_{18} column (4.6 × 150 mm; particle size, 10 μm) and an UV detector set at 272 nm. The mobile phase used was

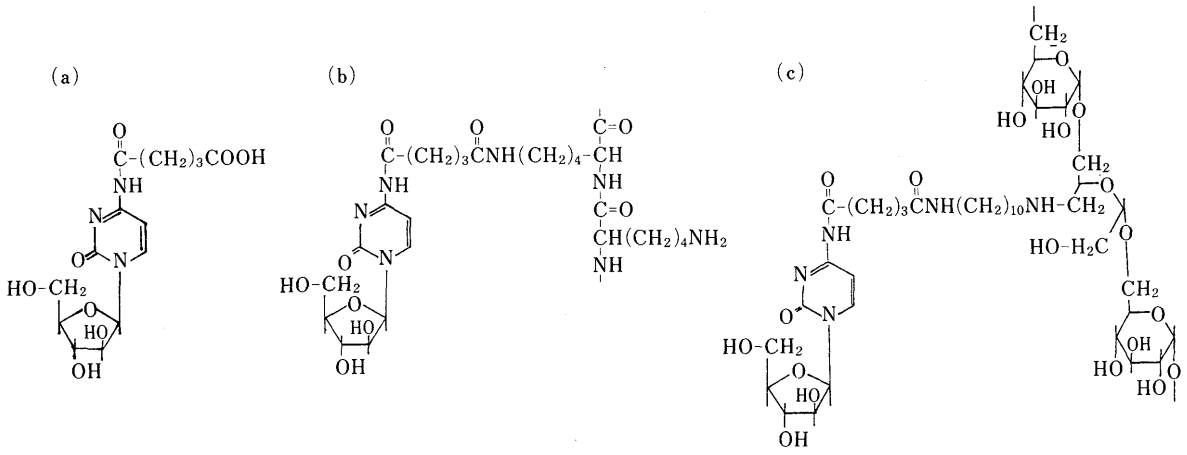

Fig. 1. Structures of Glu-ara-C (a), PLL–glu-ara-C (b) and T70-C_{10}–glu-ara-C (c)

a mixture of 2.5 mM phosphate buffer (pH 7.0) and methanol (31 : 1, v/v).

Drug Contents of the Conjugates The drug content of each conjugate was estimated from that of glu-ara-C, which was calculated from the difference between the UV absorption at 315 nm of the conjugate and that of the polymer support at the same concentration (w/v) in 1/10 M acetate buffer, pH 4.0, based on the molecular extinction coefficient at 315 nm (ε, 4000) for glu-ara-C in the same buffer. When a small amount of ara-C or ara-U was detected, it was determined by HPLC. The total amount of ara-C contained in the conjugates was estimated as the sum of ara-C from the UV method and that from HPLC.

***In Vitro* Drug Release** As incubation media, 1/15 M phosphate buffers of pH 5, 7, 7.4 and 8, and 1/10 M acetate buffer of pH 4 were used. All incubations were carried out at 37 °C.

Glu-ara-C was incubated for 120 h, at the concentration of 28 μg/ml. Aliquots were withdrawn after 0, 40, 80 and 120 h. PLL–glu-ara-C and T70-C_{10}–glu-ara-C were incubated for 7 d at the concentrations of 6 and 7 mg/ml, respectively. Namely, T70-C_{10}–glu-ara-C was used at a slightly higher concentration based on its lower drug content compared with that of PLL–glu-ara-C, as found in the drug content study described above. Aliquots were taken at appropriate times. The pH values of those aliquots were checked by using pH-test paper from Toyo Roshi Co., Ltd. Analysis of every aliquot by HPLC was carried out.

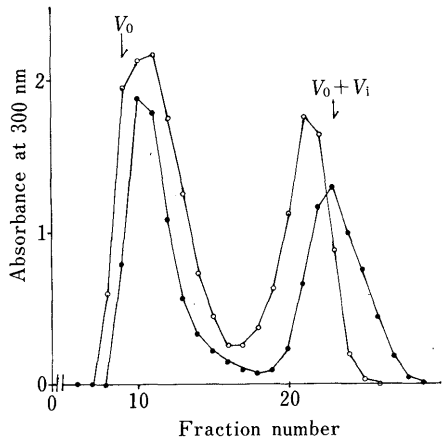

Fig. 2. Gel-filtration Patterns after Conjugation Reactions

(○), PLL·HBr, glu-ara-C and EDC; (●), T70-C_{10}, glu-ara-C and EDC. V_0, void volume; V_0+V_i, total volume.

Results and Discussion

Glu-ara-C The derivative synthesized by the reaction of Tr-ara-C and glutaric anhydride was identified as N^4-(4-carboxybutyryl)ara-C (glu-ara-C) on the basis of UV, NMR and MS spectra. The structure is shown in Fig. 1 (a). The change of UV maximum caused by the derivation suggested the acylation of the N-4 amino group of ara-C. In the NMR spectrum, signals of all the protons of glu-ara-C could be seen. Namely, the signals and integrated intensities indicated that one 4-carboxybutyryl group had been introduced into ara-C *via* the N-4 amide bond. Neither imide nor ester was observed. MS also supported the assigned structure.

Conjugates Since glu-ara-C was considered to bind through its ω-carboxyl group to amino groups of the polymer support as a result of condensation by EDC, the structures of the conjugates are proposed to be as shown in Fig. 1 (b) and (c). Figure 2 shows the gel-chromatographs after each conjugation reaction.

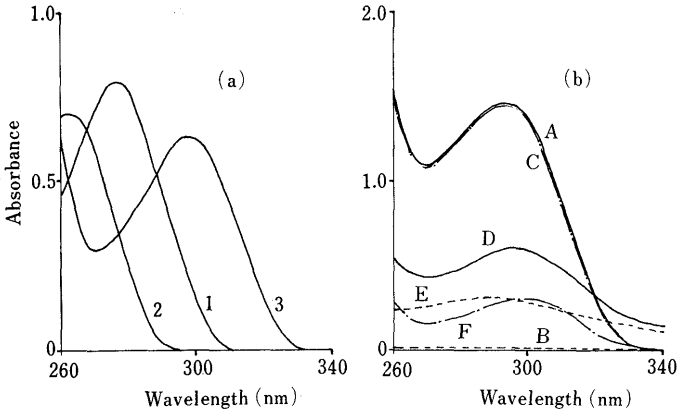

Fig. 3. UV Absorption Spectra Used for the Drug Content Study

(a) 1, ara-C (68 μM); 2, ara-U (55 μM); 3, glu-ara-C (78 μM). (b) A, PLL–glu-ara-C; B, PLL·HBr; C, difference spectrum estimated from A and B; D, T70-C_{10}–glu-ara-C; E, T70-C_{10}; F, difference spectrum estimated from D and E. A and B, 3.7 mg/ml; D and E, 4.1 mg/ml. The difference spectra are shown on the conjugates with the above concentrations. The solvent used was 1/10 M acetate buffer, pH 4.

In order to determine the drug contents of the conjugates, the UV spectra of ara-C, ara-U and glu-ara-C were measured in 1/10 M acetate buffer, pH 4, as standard UV profiles. The spectra are shown in Fig. 3 (a). Since only glu-ara-C showed UV absorption at 315 nm, the molecular extinction coefficient at 315 nm (ε, 4000) could be used for estimation of the amount of the glu-ara-C moiety even in the presence of ara-C or ara-U. Ara-C, ara-U and glu-ara-C were detected at 2.5, 3.5 and 8.1 min, respectively, at the flow rate of the elution solvent of 2 ml/min in HPLC. The coexistence of these compounds in each conjugate product

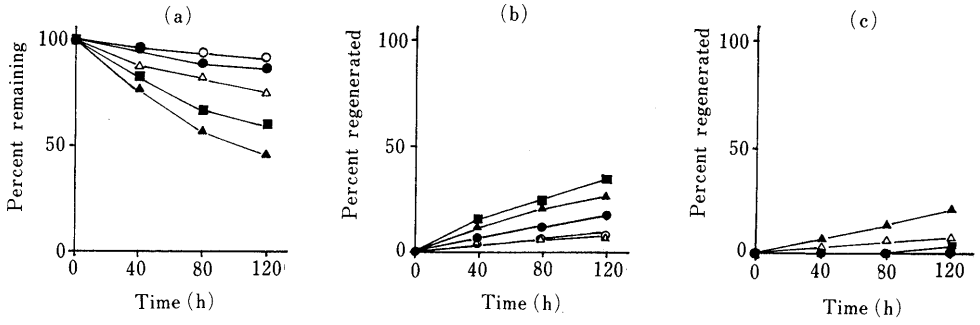

Fig. 4. Conversion of Glu-ara-C into Ara-C and Ara-U in Various pHs

(a), glu-ara-C; (b), ara-C; (c), ara-U. ▲, pH 4; △, pH 5; ○, pH 7; ●, pH 7.4; ■, pH 8. The percentages remaining or regenerated mean the molar ratio to the used amount of glu-ara-C.

could be determined by HPLC.

The obtained conjugate products showed the UV spectra illustrated in Fig. 3 (b). In the case of PLL–glu-ara-C, the difference spectrum between the conjugate and the polymer support indicated some breakdown of the N-4 amide bond, that is, coexistence of ara-C or ara-U. The UV absorption at 315 nm corresponded to 43.3 μM ara-C/g, and HPLC showed the coexistence of 8.7 μM ara-C/g, which was consistent with the difference of the conjugate spectrum from the standard glu-ara-C spectrum. As a whole, the drug content of PLL–glu-ara-C was 52.0 μM ara-C/g. In the case of T70-C_{10}–glu-ara-C, the difference spectrum between the conjugate and T70-C_{10} showed almost the same UV absorption profile as glu-ara-C. The UV absorption at 315 nm corresponded to 10.5 μM ara-C/g, and only 0.1 μM ara-C/g was detected by HPLC. Therefore, the ara-C content of T70-C_{10}–glu-ara-C was 10.6 μM/g. Ara-U and glu-ara-C were not detected as coexisting compounds in each conjugate by HPLC. From these results, it was considered that the N-4 amide bond was more unstable in PLL–glu-ara-C than in T70-C_{10}–glu-ara-C in the purification process or during storage at room temperature.

***In Vitro* Drug Release** Regeneration of ara-C was found in glu-ara-C, PLL–glu-ara-C and T70-C_{10}–glu-ara-C from HPLC analysis. Further, since ara-C is subject to deamination even in the absence of enzyme,[9] ara-U would be expected to be formed in the incubation studies, and this was confirmed by HPLC analysis. In every incubation, the pH values of samples were maintained at the pHs of the buffers used.

On glu-ara-C, its degradation and the drug release are shown in Fig. 4. Although the degradation of glu-ara-C was more rapid at pH 4 than at pH 8, the amount of ara-C in the media was lower at pH 4. The reason was that the decomposition to ara-U was significant at pH 4 but very little at pH 8. Namely, ara-U appearing after incubation for 120 h corresponded to about 40% of the degraded glu-ara-C at pH 4, but only 3% at pH 8.

The drug release profiles of the conjugates are shown in Figs. 5 and 6. Over 7 d of incubation, glu-ara-C was not detected or was found in a negligible amount. Therefore, it was considered that the release of ara-C occurred through the direct hydrolysis of the N-4 amide bond of the bound glu-ara-C, that is, the amide bonds between the ω-carboxyl group of glu-ara-C and the amino groups of the polymer supports, formed by EDC, were relatively stable.

The pH-dependency of the drug release from the conjugate was different from that from glu-ara-C itself. Namely, the release rate of ara-C increased with increase in pH, and was not accelerated under weakly acidic conditions. Concerning the accelerated drug release from glu-ara-C under weakly acidic conditions, the free carboxyl group might act as an intramolecular catalytic group under such conditions. In the conjugates, ara-C was found to be released following almost pseudo-first-order kinetics under neutral and weakly basic conditions. At pH 7.4, the time of

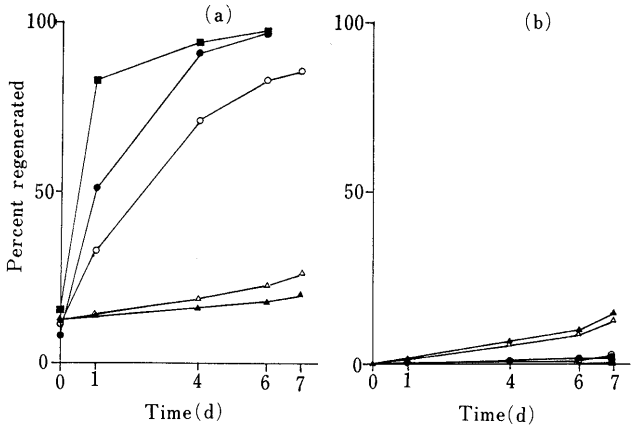

Fig. 5. Drug Release from PLL-glu-ara-C in Various pHs

(a), ara-C; (b), ara-U. ▲, pH 4; △, pH 5; ○, pH 7; ●, pH 7.4; ■, pH 8. The percentages regenerated mean the molar ratio to the whole amount of the drug loaded in PLL–glu-ara-C.

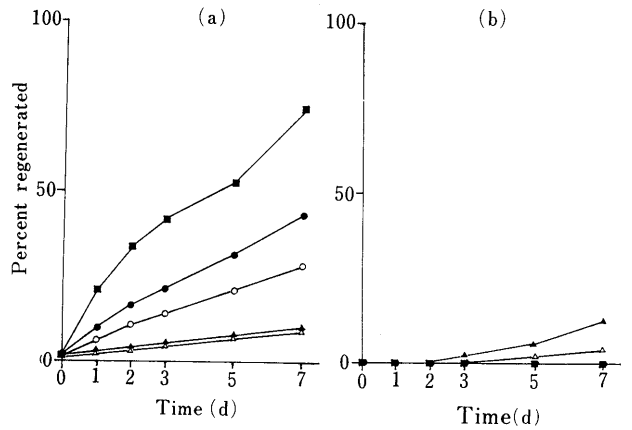

Fig. 6. Drug Release from T70-C_{10}-glu-ara-C in Various pHs

(a), ara-C; (b), ara-U. ▲, pH 4; △, pH 5; ○, pH 7; ●, pH 7.4; ■, pH 8. The percentages regenerated mean molar ratio to the whole amount of the drug loaded in T70-C_{10}–glu-ara-C.

50% release of ara-C was 2 d for PLL–glu-ara-C and 7 d for T70-C_{10}–glu-ara-C. On the other hand, under weakly acidic conditions for each conjugate, ara-C in the media was less than 25% of the loaded amount of the drug after 7 d and the decomposition to ara-U, being less than 15%, was detected at that time. The results suggest that the macromolecular conjugates of glu-ara-C should be effective under physiological conditions because the appropriate regeneration of ara-C was obtained at pH 7.4, while the drug release rate was slow at acidic lysosomal pHs of 4 and 5. Since macromolecules are known to be taken up through endocytosis by tumor cells,[10] the behavior of the macromolecules on endocytosis by tumor cells, *i.e.*, lysosomotropism, will be an important factor affecting the utility of the conjugate.

Comparing the drug regeneration from PLL–glu-ara-C with that from T70-C_{10}–glu-ara-C, the former was faster under weakly basic and neutral pH conditions. At weakly acidic pHs, the drug release profiles were similar. Therefore, the release of ara-C was considered to be affected by the kind of polymer support. Namely, since PLL–glu-ara-C has more amino groups than T70-C_{10}–glu-ara-C, the difference in the chemical circumstance around the N-4 amide of the bound glu-ara-C could be suggested between each conjugate, and this difference might affect the difference in the drug regeneration rate between each conjugate.

Our results suggest that conjugates between glu-ara-C and macromolecules having amino groups will be useful as macromolecular prodrugs, which would release ara-C effectively under physiological conditions. *In vivo*, the loaded amount of the drug, and the toxicity and the degradation properties of the macromolecule should affect the chemotherapeutic activity of the conjugates. These points need to be studied next.

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