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NOTES

Synthesis, Hydrolysis, and Antitumor Activity of Conjugates of 5-Fluorouracil with Poly(L-lysine)

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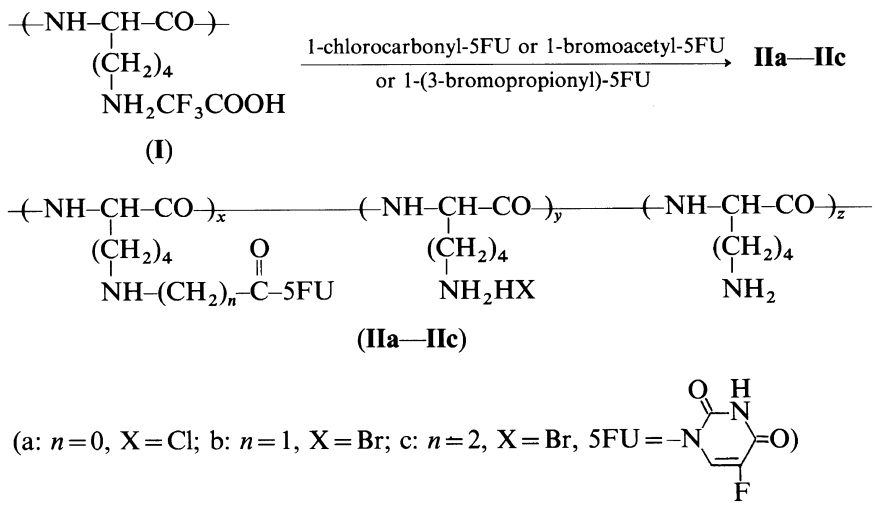
The preparation of polymer-drug conjugates, in which active substances are linked to polymeric matrices by means of covalent bonds that can be enzymatically degraded or hydrolyzed in body fluids, is presently recognized as an effective way to prolong pharmacological activity by the gradual release of free drugs, which react with the receptor, from the macromolecular matrix. Unfavorable side effects can also be minimized.

Very recently, a great deal of interest has centered on utilizing biocompatible polymers to regulate the delivery of pharmaceuticals and veterinary drugs. So far, a number of drug delivery systems by using polymer as a carrier has been successfully developed to control the rate of drug administration and prolong the duration of therapeutic action as well as target the delivery of drugs. Poly(amino acid)s have been used as polymer carriers as they are biocompatible, biodegradable and lack of immunogenicity. It has been reported that basic poly(amino acid)s labeled with fluorescein are readily taken up by tumor cells in culture.[1] Therefore, poly(amino acid)s may be effective carriers for antitumor agents.

5-Fluorouracil (5FU) is known to have remarkable antitumor activity, but it has strong toxic side effects. In our previous papers, it was reported that 5FU was linked covalently to poly(L-lysine),[2] poly(L-glutamic acid),[3] poly(L-tyrosine),[4] poly(L-cysteine),[5] and a,β-poly(L-aspartic acid) derivatives[6] as a mean of achieving increased drug uptake by tumor cells and reducing its toxic side effects. In consideration that poly(L-lysine) can act as powerful "transport vector" and the conjugation of methotrexate or human serum albumin or horseradish peroxidase to poly(L-lysine) markedly increases the cellular uptake of the drug or the proteins,[7-9] we prepared the oligomers of poly[N^{ε}-(5-fluorouracil-1-alkylacyl)-L-lysine] by the polymerization of NCAs of L-lysine containing 5FU.[2] In this paper, we prepared the conjugates of 5FU and poly(L-lysine) with different linkages from those reported by reactions of poly(L-lysine) with 5FU derivatives according to the followng schemes. Hydrolysis *in vitro* and antitumor activity *in vitro* and *in vivo* of the conjugates were also investigated.



EXPERIMENTAL

Materials

Poly(L-lysine)[10] (*I*, $\bar{M}_{vpo,H_2O}=8{,}100$), 1-chlorocarbonyl-5FU,[11] 1-bromoacetyl-5FU[12] and 1-(3-bromopropionyl)-5FU[12] were prepared according to the literatures. L-Lysine was a biochemical reagent. DMSO was dried over a molecular sieve (Ca-Y) and distilled under reduced pressure. All other chemicals used were of analytical reagent grade.

Preparation of the Conjugates

Poly(L-lysine)-*5FU* (**IIa**). 1-Chlorocarbonyl-5FU in pyridine (25 ml, 0.011 mol) was added dropwise to a solution of poly(L-lysine) (1.28 g, 0.010 mol) and Et_3N (4 ml) in 20 ml of DMSO. It was stirred for 4 h and filtered. Pyridine was distilled off from the filtrate under reduced pressure and the residue was poured slowly into 400 ml of dry acetone. The precipitate was reprecipitated from DMSO–acetone, washed with acetone, extracted from absolute ethanol in a continuous extractor and dried thoroughly *in vacuo* to give the conjugate (**IIa**), 1.3 g.

Poly(L-*lysine*)-*5FU* (**IIb** *and* **IIc**). Sodium bicarbonate (0.84 g, 0.010 mol) and 1-bromoacetyl-5FU (2.5 g, 0.010 mol) in 5 ml of DMSO was added to a solution of poly(L-lysine) (1.28 g, 0.010 mol) in 20 ml of DMSO. It was stirred at room temperature for 2 days and filtered. The filtrate was poured slowly into 500 ml of dry acetone with vigorously stirring. The precipitate was purified with the same procedure as for **IIa**; yield **IIb** 1.5 g.

Conjugates (**IIc**) was prepared in a similar way described for conjugate (**IIb**) according to the reaction scheme. Characterization data of all conjugates (**IIa—IIc**) are listed in Table I.

Determination of the Release Rate of 5FU from the Conjugates (**II**)

Hydrolysis of the conjugates (**II**) was carried out by placing the sample inside a dialysis membrane under shaking at 37°C in 0.1 *N* NaOH, 0.1 *N* HCl or 0.1 M phosphate buffer, solution (pH 7.4). By measuring with HPLC (silica gel column, 10×0.5, eluent: MeOH/H_2O; detector: UV_{271}), the conjugates (**II**) were found to be hydrolyzed *in vitro* to give free 5FU, but not to afford any 5FU derivatives. Consequently, the amount of 5FU released was determined using the UV spectrum method. (Shimadzu double beam spectrophotometer UV-240 type).

Measurement of Antitumor Activity

The growth-inhibitory effects of the con-

Table I. Characterization data of conjugates (**IIa—IIc**)

No.	X	5FU%[a] wt%	x	y	z[b]	X% Found	N% Found	N% Calcd
IIa	Cl	32.35	0.67	0.16	0.17	2.60	19.13	19.59
IIb	Br	36.53	0.53	0.31	0.16	5.53	18.06	18.54
IIc	Br	23.74	0.48	0.42	0.10	3.56	18.17	18.46

[a] Determined by the UV spectrum method after complete hydrolysis in 5 *N* NaOH aq. soln.
[b] x, y, and z were calculated from 5FU% and X%.

jugates were tested against Ehrlich ascites carcinoma (EAC) *in vitro* and the survival effect of the conjugates was measured against EAC in mice (i.p./i.p.) according to a typical NCI procedure. The inhibition ratio and the ratio of prolongation of life, T/C (%), *i.e.*, the ratio of mean survival of treated mice (T) to that of control (C), were evaluated.

RESULTS AND DISCUSSION

Preparation and Determination of the Composition of the Conjugates

Poly(L-lysine) can be chemically modified *via* its free amino groups using the activated ester[13,14] or carbodiimide[7,8] methods. In this paper, 5FU was conjugated to poly(L-lysine) through typical acylation or alkylation, as shown in the scheme. Reactions of 5FU derivatives with poly(L-lysine) were carried out in DMSO solution in the presence of triethylamine or sodium bicarbonate at room temperature. The conjugates obtained had different infrared absorption spectra from those of the starting compounds, and had absorptions assigned to 5FU.

Ouchi[15] reported that carbamoyl bonds in the conjugates of 5FU and vinyl polymeric carriers could be perfectly hydrolyzed by refluxing in 5 *N* NaOH aq. soln. for 2 days to give free 5FU. In our experiment, we found the bonds linked 5FU, carbamoyl or carbonyl, in the conjugates (**II**), which were more hydrophilic than vinyl polymeric carriers, could be perfectly hydrolyzed by refluxing in

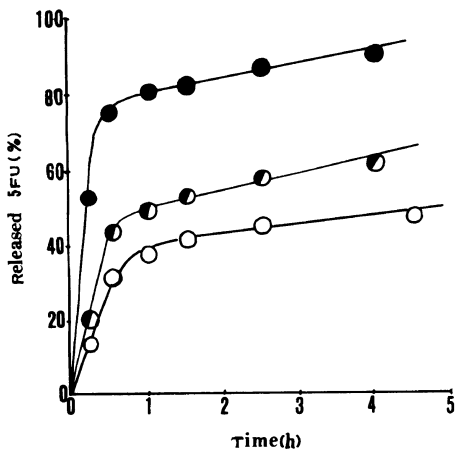


Figure 1. Release rate of 5FU from conjugate (**IIc**) in 0.1 *N* NaOH aq. soln. (●), 0.1 M phosphate buffer soln. (pH 7.4) (◐), 0.1 *N* HCl aq. soln. (○).

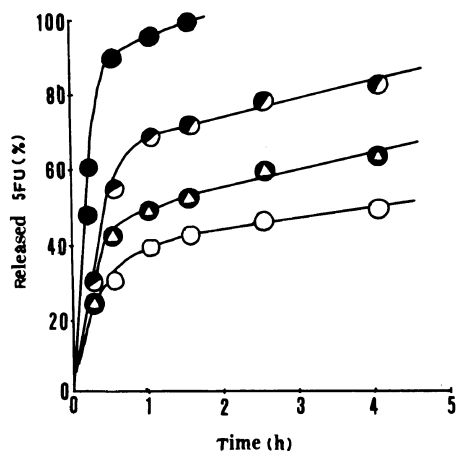


Figure 2. Release rates of 5FU from 1-(3-bromopropionyl)-5FU (●), conjugate (**IIa**) (◐), conjugate (**IIc**) (△) in 0.1 M phosphate buffer soln. (pH 7.4) and from conjugate (**IIc**) in 0.1 M phosphate buffer soln. (pH 7.4) containing 0.05% Tween 80 (○).

5 *N* NaOH aq. soln. for 10 h to give free 5FU. The content of 5FU in the conjugates (**II**) was determined by UV measurement of the amount of 5FU released by full hydrolysis of the conjugates and that the composition could be calculated from the contents of 5FU and halogen (see Table I). The content of nitrogen calculated from these compositions agreed with the experimental values. The bonds linking 5FU in these conjugates (**IIa**—**IIc**) can thus be perfectly hydrolyzed by refluxing in 5 *N* NaOH aq. soln. for 10 h.

Release Behavior of 5FU from Conjugates in Vitro

In order to evaluate the release behavior of 5FU *in vivo*, the hydrolysis of the conjuates (**II**) was carried out using the method described above. The results are shown in Figures 1 and 2. The order of release rates of 5FU was as follows: 0.1 *N* NaOH aq. soln. > 0.1 M phosphate buffer soln. (pH 7.4) > 0.1 *N* HCl aq. soln. (Figure 1); in 0.1 M phosphate buffer, 1-(3-bromopropionyl)-5FU > **IIa** > **IIc** (Figure 2). The release rate of 5FU from **IIc** was depressed by the addition of emulsifying agent, Tween 80.

Table II. Growth-inhibitory effects of conjugates against EAC *in vitro*[a]

No.	Inhibition ratio/%		
	1000 Dose $\mu g\,ml^{-1}$	100 Dose $\mu g\,ml^{-1}$	10 Dose $\mu g\,ml^{-1}$
IIa	87.5	76.9	30.7
IIb	36.8	17.6	26.1
IIc	32.3	34.8	4.8
5FU	61.8	88.8	35.6

[a] Tumor cell concentration $(1—4) \times 10^4$ cells ml^{-1}; Blank test: 1% NaCl aq. soln., 4 h.
Growth-inhibition ratio (%) =

$$\left(1 - \frac{\text{Survival cell number treated}}{\text{Survival cell number of control}}\right) \times 100 .$$

Antitumor Activity of Conjugates

The antitumor activity of the conjugates (**IIa**—**IIc**) was evaluated by two methods. The growth-inhibitory effects of the conjugates against EAC *in vitro* and survival effects against EAC in mice (i.p./i.p.) are shown in Tables II and III, respectively. As shown in Table II, the inhibition ratio of **IIa** (87.5%) at a dose of 1000 $\mu g\,ml^{-1}$ was higher than that of 5FU (61.8%) and the other two conjugates (**IIb** and **IIc**) had lower inhibition ratios compared with that of 5FU in the dose range tested. Table II shows that the conjugates (**IIa**—**IIc**) exhibit significant survival effects and have higher T/C values and ratios of longterm survival than 5FU at some dosages. Moreover, these samples did not show acute toxicity at the dosages tested, which suggests that the conjugates (**IIa**—**IIc**) are incorporated into tumor cells by an endocytosis mechanism.

Table III. Survival effects of conjugates against EAC in mice[a]

No.	Dose		Survival days		T/C	Longterm survival/% (>60 days)
	$mg\,kg^{-1}$	mmol 5FU kg^{-1}	C	T	%	
IIa	75	0.19	23.6	39.0	165	60
	105	0.25	23.6	29.7	126	30
IIb	50	0.10	19.1	29.2	153	20
	100	0.20	19.1	30.9	162	30
IIc	75	0.14	23.6	37.5	159	30
	125	0.23	23.6	39.6	168	33
5HU	20	0.15	23.6	34.0	144	40
	20	0.15	19.1	30.9	162	20

[a] i.p. × 8 (i.p. × 4 for 5FU), p values < 0.01.

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