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DESIGN OF CHITOSAN-5FU CONJUGATE EXHIBITING ANTITUMOR ACTIVITY

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ABSTRACT

In order to provide a macromolecular prodrug of 5-fluorouracil (5FU) reducing the side effects, having an affinity for tumor cells, and exhibiting strong antitumor activity, the covalent attachments of 5FUs to chitosan and chitosamino-oligosaccharide (COS) through hexamethylene spacer groups via urea, urea bonds were carried out. The effect of prolongation of life was tested *in vivo* against p388 *lymphocytic leukemia* in female CDF_1 mice by intraperitoneal (i.p.) transplantation/i.p. injection and the growth-inhibitory effect on Meth-A *fibrosarcoma* or MH-134Y *hepatoma* was evaluated *in vivo* in SPF-C3H/He scl male or Balb/c male mice by subcutaneous (s.c.) implantation/intravenous (i.v.) injection. The effects of the degree of polymerization of chitosan and

the degree of substitution of 5FU on the conjugate based on the number of glucosamine groups (D5FU) on the prolongation of life were investigated. Moreover, the effect of balance of hydrophobicity and hydrophilicity of carrier chitosan on the prolongation of life was studied. The chitosan-5FU conjugate exhibited a strong survival effect against p388 *lymphocytic leukemia* mice. Furthermore, the chitosan-5FU and COS-5FU conjugates showed remarkable growth-inhibitory effects on Meth-A *fibrosarcoma* and MH-134Y *hepatoma*. These chitosan-5FU and COS-5FU conjugates did not display an acute toxicity even in higher dose ranges.

INTRODUCTION

5-Fluorouracil (5FU) has remarkable antitumor activity [1-3] which is, however, accompanied by undesirable side effects [4, 5]. Chitosan and chitin are noteworthy as low or nontoxic, nonimmunogenetic, compatible, and biodegradable polymers. Partially *N*-acetylated chitosan was reported to be selectively internalized into the tumor cells and to inhibit the growth of tumor cells [6]. Moreover, *O*-benzoyl chitosan was reported to demonstrate a 10-fold increase in aggregating potency into L1210 *leukemia* when compared to chitosan [7]. Recently, water-soluble chitohexaose (COS6; hexamer of chitin) was found to show an immunoenhanced growth-inhibitory effect of tumor cells [8].

In comparison with a low molecular weight prodrug of 5FU, a macromolecular prodrug can be generally expected to overcome the problem of side effects and to have a prolonged duration of activities. In addition, the polymer may show a possible affinity for tumor cells. Therefore, polymer-5FU and the oligomer-5FU conjugates can be expected to exhibit strong antitumor activities.

In order to provide a macromolecular prodrug of 5FU with reduced side-effects, having an affinity for tumor cells, and exhibiting strong antitumor activity, we previously investigated the design of chitosan-5FU and chitin-5FU conjugates [9, 10]. The antitumor activities of four kinds of conjugates of 5FU attached to chitosan and chitin derivatives through some kinds of spacer groups via ether, amide, ester, or urea bonds were tested. The conjugate of 5FU attached to chitosan at the 2-positions and the conjugate of 5FU attached to chitin at the 6-positions through hexamethylene spacer groups via urea, urea bonds were found to exhibit remarkable effects on the prolongation of life against p388

lymphocytic leukemia in female CDF_1 mice by intraperitoneal (i.p.) transplantation/i.p. injection.

In the present paper the design of the conjugate of 5FU attached to chitosan at the 2-positions through hexamethylene spacer groups via urea, urea bonds is discussed in detail. The effect of the degree of substitution of 5FU on the conjugate based on the number of glucosamine groups (D5FU) and the effect of balance of hydrophobicity and hydrophilicity of carrier chitosan on the survival effect against p388 *lymphocytic leukemia* in female CDF_1 mice i.p./i.p. are investigated. Moreover, by using COS6, chitosaminotriose (COS3; trimer of chitosan) and chitosamino-oligosaccharides mixture (COS(mix)-H) having an end sugar alcohol group, instead of chitosan, the effect on the prolongation of life for the conjugates of 5FU attached to chitosamino-oligosaccharides at the 2-positions through the examethylene spacer groups via urea, urea bonds against p388 *lymphocytic leukemia* in female CDF_1 mice i.p./i.p. and the growth-inhibitory effect against MH-134Y *hepatoma* or Meth-A *fibrosarcoma* in SPF-C3H/He or Balb/c mice by subcutaneous (s.c.) plantation/intravenous (i.v.) injection are further studied. The release behavior of 5FU from the conjugates was studied *in vitro* in various aqueous media at 37°C.

EXPERIMENTAL

Materials

Chitosan (η of 0.5 wt% in 0.5% acetic acid at 25°C = 6.5 cPs) supplied by Kimitsu Chemical Co. was treated with 12 *N* HCl to give lower molecular weight chitosans ($\bar{P}_n$ = 30, 75). COS6 and COS3, provided by Ihara Chemical Industry Co., were confirmed to be chromatographically homogeneous when they were separately examined on a column of Biogel P-4 (2.5 × 90 cm) connected with an RI monitor and using water as the eluent. COS(mix)-H (n = 2–7), chitosamino-oligosaccharides mixture, whose reducing sugar end residue was derived from a sugar alcohol group, was also supplied by Ihara Chemical Industry Co. α-Carboxylmethyl-ω-methoxy-poly(oxy-1,2-ethanediyl) (methoxy PEG acid; MeO-PEG acid; 9, MW = 400, 1000) was provided by Kawaken Fine Chemical Co. Hexamethylene diisocyanate (HMDI), dimethylsulfoxide (DMSO), and pyridine were purified by distillation. 5FU and triethylamine (TEA) were of commercial grade and were used without further purification.

Attachment of 5FUs to Chitosan through Hexamethylene Spacer Groups via Urea, Urea Bonds (3a)

The conjugate of 5FU attached to chitosan through hexamethylene spacers via urea, urea bonds (**3a**) was prepared. 5FU (1.2–4.7 g, 9.6–36 mmol) and HMDI (1.3–5.0 g, 8.0–30 mmol) were stirred in 30–80 mL anhydrous pyridine at 90–100°C for 10 h to give 6-(5-fluorouracil-1-yl)hexamethylene isocyanate **1**. Without isolation of 5FU isocyanate intermediate **1**, the pyridine solution of **1** was added to a solution of chitosan hydrochloride ($\overline{P}_n$ = 30 or 75; 1.0 g) swollen in 100 mL DMSO and then stirred at 60°C for 12 h. The reaction mixture was poured into acetone to precipitate the chitosan–5FU conjugate **3a**. The conjugate **3a** was washed well with pyridine and acetone. The IR spectrum (KBr disk) showed absorptions at 2940–2840 (CH), 1740 (C=O/5FU), 1620, 1575 (NHCONH), and 1080 cm^{-1} (C—O—C). The chitosan–5FU conjugate **3a** was insoluble in water and organic solvents.

Since all urea bonds of chitosan–5FU conjugate **3a** were confirmed to be completely hydrolyzed to give only free 5FU by refluxing in 3 *N* NaOH aqueous solution for 2 days [9–11], the degree of substitution of 5FU on the chitosan in mol% 5FU based on the number of glucosamine groups (D5FU) of chitosan–5FU conjugate **3a** was determined by means of UV measurement of the amount of 5FU released in the hydrolyzed solution (standard: 6500 of ϵ_{max}^{285} for 5FU in 3 *N* NaOH aqueous solution).

Attachment of 5FUs to COS through Hexamethylene Spacer Groups via Urea, Urea Bonds (3b)

The conjugate of 5FU attached to COS through hexamethylene spacer groups via urea, urea bonds (**3b**) was prepared. 5FU (3.9 g, 30 mmol) and HMDI (4.2 g, 25 mmol) were stirred in 80 mL dry pyridine at 90–100°C for 10 h to give 6-(5-fluorouracil-1-yl) hexamethylene isocyanate **1**. Without isolation of **1**, the pyridine solution of **1** was added to a solution of COS hydrochloride (**2b**; 1.0 g) in 100 mL DMSO and then stirred at room temperature for 12 h. The reaction mixture was poured into a large amount of acetone to precipitate **3b**. The **3b** obtained was washed well with pyridine and acetone. The IR spectrum showed absorptions at 2940–2840 (CH), 1740 (C=O/5FU), 1620, 1575 (NHCONH), and 1080 cm^{-1} (C—O—C). The **3b** obtained was insoluble in water and organic solvents other than DMSO.

Since only free 5FU was confirmed after hydrolysis of **3b** by refluxing in 3 *N* NaOH aqueous solution for 2 days, the D5FU values of COS6-5FU (**3b**; $n = 6$) and COS3-5FU (**3b**; $n = 3$) conjugates were estimated to be 44 and 34 mol%, respectively, by UV difference spectral measurement of the amount of 5FU released in the hydrolyzed solutions.

Attachment of 5FUs to COS(mix)–H through Hexamethylene Spacer Groups via Urea, Urea Bonds (3c)

The conjugate of 5FU attached to COS(mix)–H through hexamethylene spacer groups via urea, urea bonds (**3c**) was synthesized by a coupling reaction of COS(mix)–H (**2c**; $n = 2$–7) with **1**.

5FU (3.1 g, 24 mmol) and HMDI (3.3 g, 20 mmol) were stirred in 50 mL dry pyridine at 90–100°C for 10 h to give **1**. Without isolation of **1**, the pyridine solution of **1** was added into a solution of COS(mix)–H hydrochloride (**2c**; $n = 2$–7; 1.0 g) in 100 mL DMSO and then stirred at room temperature for 12 h. The reaction mixture was poured into a large amount of acetone to precipitate **3c**. After washing well with pyridine and acetone, the **3c** dissolved in DMSO was fractionated to separate methanol-soluble (**3c-S**) and methanol-insoluble (**3c-I**) parts. The values of D5FU for **3c-S** and **3c-I** were estimated by the GPC method described above.

Synthesis of the Conjugate of 5FU Attached to Hydrophobic Chain-Grafted Chitosan through Hexamethylene Spacer Groups via Urea, Urea Bonds (7)

First, hydrophobic chain-grafted chitosan was prepared by the methods of Hirano et al. [12–14] and Tokura et al. [15].

O-Benzoylchitosan (**6a**), *O*-myristoylchitosan (**6b**), and *O*-caproylchitosan (**6c**) were prepared via their Schiff-base intermediates. The 4-methoxyphenyl Schiff base of chitosan (**4**) was prepared through the reaction of chitosan with 4-methoxybenzaldehyde by the method of Hirano et al. [13, 14]. The Schiff base of **4** obtained in gel form was isolated as a dried product. The product has IR adsorptions at 1640 (C=N), 830, and 777 cm^{-1} (phenylene), and the degree of substitution for the Schiff base was 90 mol% on the basis of the C/N ratio. The *O*-acylations of the Schiff base of chitosan were performed with benzoyl, myristoyl, and caproyl chlorides to give the Schiff bases of *O*-substituted chitosans **5a**, **5b**, and **5c**, respectively. The presence of benzoyl, myristoyl, and caproyl

groups in the products was detected by strong IR adsorptions at 710 (phenyl), 2910, 2850 (CH), and 1720 cm^{-1} (C=O). The values of the degree of *O*-substitution of hydrophobic chain on the chitosan on the number of glucosamine groups (DR) for **5a**, **5b**, and **5c** were estimated to be 145, 90–120, and 97 mol%, respectively. The Schiff-base groups were selectively removed by treatment with 0.5 *N* HCl aqueous solution in ethanol at room temperature, and, essentially, the release of *O*-substituted groups was not found in the treatment described above to give the hydrochloride salts of **6a**, **6b**, and **6c**.

Second, conjugates of 5FU attached to *O*-acylchitosans through hexamethylene spacer groups via urea, urea bonds (**7**) were prepared by the reaction of **6** with 6-(5-fluorouracil-1-yl)hexamethylene isocyanate **1**.

The pyridine solution of **1** obtained by the same method as described above was added to the solution of *O*-benzoylchitosan hydrochloride (**6a**; $\overline{P}_n$ = 30; 1.5 g) swollen in a mixture of 100 mL DMSO and 150 mL pyridine, the solution of *O*-myristoylchitosan hydrochloride (**6b**; $\overline{P}_n$ = 30: 1.5 g) swollen in 150 mL pyridine and the solution of *O*-caproylchitosan hydrochloride (**6c**; $\overline{P}_n$ = 30; 1.5 g) swelled in 80 mL DMSO, and then stirred at 50–70°C for 10–60 h. The reaction mixtures were poured into acetone to precipitate *O*-acylchitosan-5FU conjugated **7a**–**7c**. The products were washed well with pyridine and acetone. IR spectra (KBr disk) showed adsorptions at 1740 (C=O/5FU), 1620, 1575 (NHCONH), and 1080 cm^{-1} (C—O—C). The *O*-acylchitosan-5FU conjugates obtained were isoluble in organic solvents.

It was confirmed that only free 5FU was obtained after hydrolyses of **7a**–**7c** by refluxing in 3 *N* NaOH aqueous solution for 2 days. The values of D5FU for **7a**, **7b**, and **7c** were determined to be 45–49, 13–33, and 11–19 mol%, respectively. The value of D5FU for **7a** was obtained by GPC measurement of the amount of 5FU released in the hydrolyzed solution (column: Shodex OHpak B-805, eluent; 1/75 *M* KH_2PO_4–Na_2HPO_4 buffer solution of pH 7.0; detector: UV_{270}). The values of D5FU for **7b** and **7c** were obtained by UV spectral measurement of the amount of 5FU released in the hydrolyzed solutions.

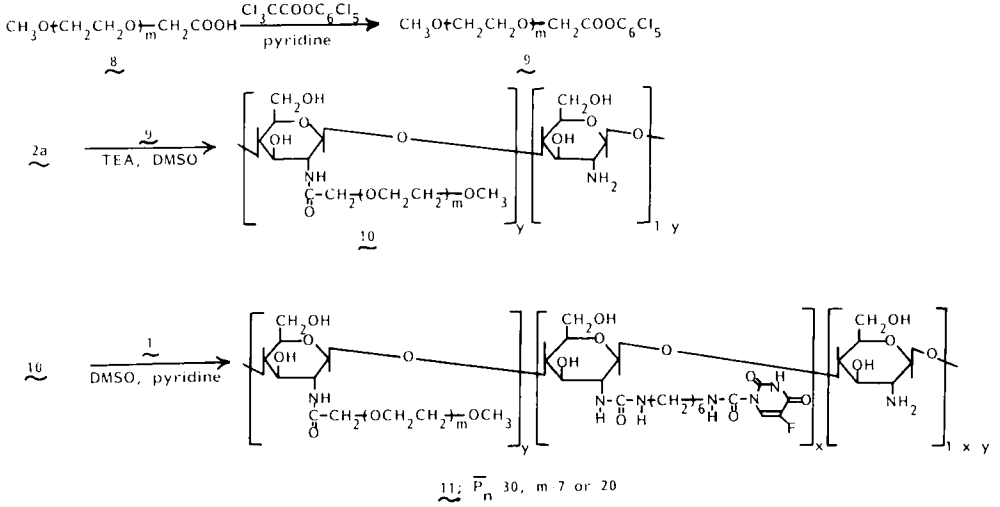
Synthesis of the Conjugate of 5FU Attached to Hydrophilic PEG Grafted Chitosan through Hexamethylene Spacer Groups via Urea, Urea Bonds (11)

First, MeO-PEG grafted chitosan **10** was prepared by the coupling reaction of chitosan with MeO-PEG acid by applying the active-ester

method. Pentachlorophenyl trichloroacetate (4.2 g; 10.2 mmol) was added to a solution of MeO-PEG acid **8** (m = 7; 3 g, 7.5 mmol) in pyridine (50 mL) and then stirred at room temperature for 12 h. The insoluble materials were filtered off and the solvent was evaporated under reduced pressure. Triethylamine (1.2 mL; 8.5 mmol) was added to a solution of chitosan hydrochloride ($\bar{P}_n$ = 30; 1.9 g) dissolved in 150 mL DMSO and the solution of the active ester of MeO-PEG acid obtained in DMSO (10 mL) was further added and then stirred at room temperature for 12 h. The reaction mixture was poured into acetone to precipitate the crude PEG-grafted chitosan **10**. The **10** obtained was washed well with acetone. IR spectrum (KBr disk) showed adsorptions at 1670, 1550 (NHCO), and 1150–1050 cm^{-1} (C—O—C). ^1H NMR(D_2O) showed the signals of δ3.6 ppm ($C\underline{H}_2\underline{H}_2O$).

MeO-PEG (m = 20) grafted chitosan was also obtained by the method described above. Values of the degree of N-substitution of the PEG chain on the chitosan on the number of saccharide groups (DPEG) for 10 (m = 7) and 10 (m = 20) were estimated to be 26 and 18 mol%, respectively, by determination of the unreacted amino group through colloidal titration by N/400-potassium polyvinyl sulfate solution.

Second, the conjugate of 5FU attached to MeO-PEG grafted chitosan through haxamethylene spacer groups via urea, urea bonds (**11**) was



SCHEME 1.

prepared by the reaction of **10** with 6-(5-fluorouracil-1-yl)haxamethylene isocyanate **1** shown in Scheme 1.

The pyrydine solution of **1** obtained by the same method as described above was added to the solution of **10** (1.0–1.5 g) dissolved in 100 mL DMSO and then stirred at room temperature for 12 h. The reaction mixture was poured into acetone to precipitate *N*-PEG-grafted chitosan–5FU conjugated **11**. The product was washed well with pyridine and acetone.

The values of D5FU for **11** ($m = 7$) and **11** ($m = 20$) were estimated to be 18 and 24 mol%, respectively, by the method described above.

Determination of the Extent of Release of 5FU

The release behavior of 5FU from the chitosan-5FU conjugates obtained was investigated *in vitro* at 37°C under shaking in physiological saline, 0.01 *N* NaOH or 0.1 *N* HCl aqueous solution. The amounts of 5FU released from the conjugates were estimated by the GPC method reported previously [16].

Measurement of the Antitumor Activities

The survival effect was tested against p388 *lymphocytic leukemia* in female CDF_1 mice (30 untreated mice/group and 6 treated mice/group) *in vivo* i.p./i.p. according to a typical protocol of the Japanese Foundation for Cancer Research (JFCR). 1×10^6 *leukemia* cells were injected i.p. on day 0. The samples were sonicated in 0.05% sorbate 80 in a sterile normal saline solution and administered i.p. The mice received doses of 200–800 mg/kg twice for the conjugate at 1 and 5 days. The ratio of prolongation of life of the test mice, T/C (%), which means the ratio of the median survival of treated mice (T) to that of the control (C), was evaluated as a survival effect. The average C value obtained was generally 10 days. These survival effect data are the results of the screening performed at the Cancer Chemotherapy Center of JFCR.

The growth-inhibitory effect was tested against Meth-A *fibrosarcoma* or MH-134Y *hepatoma* in male mice (6 untreated mice/group and 6 treated mice/group) *in vivo* s.c./i.v. The water-insoluble chitosan–5FU, COS6-5FU, and COS3-5FU conjugates, dissolved in a small amount of DMSO, were added to a physiological saline or buffer solution, and then the suspension obtained was administered i.v. Meth-A *fibrosarcoma* or 5×10^5 MH-134H *hepatoma* cells were implanted s.c. on day 0. The

mice received doses of 10 mg/kg of COS–5FU conjugate on 14 days (one time) or on 7, 12, and 17 days (three times). The mice were sacrificed after 30 days and then the solid tumor weights were measured. The growth-inhibitory effects of chitosan–5FU, COS6–5FU, and COS3–5FU conjugates against such solid tumor cells were evaluated by the following equation:

$$\text{Growth-inhibitory effect (\%)} = \frac{\text{Average tumor weight in controlled mice (g)} - \text{average tumor weight in treated mice (g)}}{\text{Average tumor weight in controlled mice (g)}} \times 100.$$

RESULTS AND DISCUSSION

Synthesis of Chitosan–5FU (3a), COS–5FU (3b), and COS(mix)–H–5FU (3c) Conjugates

The attachment of 5FUs to chitosan at 2-positions through hexamethylene spacer groups via urea, urea bonds was accomplished by reaction of the hydrochloride salt (**2a**) of chitosan with 6-(5-fluorouracil-1-yl)hexamethylene isocyanate (**1**) in a heterogeneous system without isolation of the 5FU isocyanate intermediate **1**. The values of D5FU for the chitosan-5FU (**3a**) obtained were calculated to be 7–27 mol%. We could not obtain a chitosan–5FU conjugate with a high 5FU content by the polymer reaction technique; the polymer reaction of chitosan with 5FU did not proceed easily because of the low solubility of chitosan.

The COS6–5FU and COS3–5FU conjugates (**3b**) were synthesized individually through coupling reactions of the corresponding chitosamino-oligosaccharide hydrochlorides **2** with 6-(5-fluorouracil-1-yl)hexamethylene isocyanate **1**. The D5FU values for the COS6–5FU and COS3–5FU conjugates obtained were estimated to be 44 and 34 mol%, respectively. In spite of the oligomeric nature of the reactant, we could not obtain the conjugates of COS6 and COS3 with a high content of 5FU.

Moreover, the COS(mix)–H-5FU conjugate (**3c**) was also synthesized through the coupling reaction of COS(mix)–H (n = 2–7) with **1**. The D5FU values for the methanol-soluble part (**3c-S**) and the methanol-insoluble part (**3c-I**) of the COS(mix)–H-5FU conjugate obtained were estimated to be 30 and 33 mol%, respectively.

Synthesis of Hydrophobic Chain Grafted Chitosan–5FU Conjugate (7a–7c)

Conjugates of 5FU attached to *O*-benzoylchitosan, *O*-myristoylchitosan, and *O*-caproylchitosan through hexamethylene spacer groups via urea, urea bonds, **7a**, **7b**, and **7c**, were prepared by the heterogeneous reactions of 6-(5-fluorouracil-1-yl)hexamethylene isocyanate **1** with the corresponding O-acylchitosans.

The measured values of DR/D5FU for **7a**, **7b**, and **7c** were 145/45–49, 90–120/13–33, and 97 mol%/11–19 mol%, respectively.

Synthesis of MeO–PEG Grafted Chitosan–5FU Conjugate (11)

Conjugates of 5FU attached to MeO-PEG grafted chitosan through hexamethylene spacer groups via urea, urea bonds, **11** (m = 7) and **11** (m = 20), were prepared by the reaction of 6-(5-fluorouracil-1-yl)hexamethylene isocyanate **1** with MeO-PEG grafted chitosans, **10** (m = 7) and **10** (m = 20), respectively (Scheme 1).

The values of DPEG/D5FU for **11** (m = 7) and **11** (m = 20) were estimated to be 26/18 and 18 mol%/24 mol%, respectively.

Release Behavior of 5FU from Chitosan–5FU Conjugates

In order to evaluate the release behavior of 5FU from chitosan–5FU conjugates, the hydrolyses of **3**, **7**, and **11** were studied *in vitro* at 37°C in various aqueous media. The urea bonds of these chitosan–5FU conjugates were found to be cleaved to give only free 5FU but not to afford and 5FU derivative. One example of the results is shown in Fig. 1. The hydrolytic rate of the urea bonds *in vitro* was obtained in the following order: 0.01 *N* NaOH aqueous solution > physiological saline > 0.1 *N* HCl aqueous solution.

Antitumor Activity of Chitosan–5FU Conjugate and COS-5FU Conjugate

The results of the survival effect for chitosan–5FU conjugate (**3a**) against p388 *lymphocytic leukemia* in female CDF_1 mice i.p/i.p. are shown in Fig. 2. The prolongation of life for conjugate **3a** tended to increase with an increase in the value of D5FU.

The results of the effect of the degree of polymerization ($\bar{P}_n$) of carrier

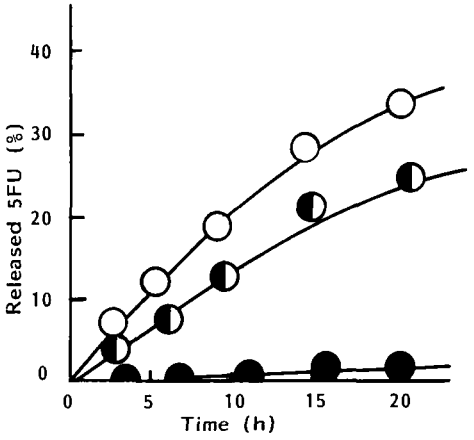


FIG. 1. Release rate of 5FU from **3a** in various aqueous media at 37°C. **3a** ($\overline{P}_n$ = 30, D5FU = 18 mol%); 5.0 × 10^{-2} g in 3 mL aq soln, (○) in 0.01 *N* NaOH; (●) in 0.1 *N* HCl; (◐) in physiological saline.

chitosan on the prolongation of life for the chitosan-5FU conjugate **3a** are plotted in Fig. 3. The prolongation of life for conjugate **3a** by using chitosan of $\overline{P}_n$ = 30 was almost the same as that by using chitosan of $\overline{P}_n$ = 75.

Moreover, the results of the survival effect for hydrophobic chain-grafted chitosan-5FU conjugates (**7a–7c**) against p388 *lymphocytic leukemia* in mice i.p/i.p. are summarized in Fig. 4. Although the smooth incorporation of the conjugates into the tumor cells by the introduction of grafted hydrophobic chains was expected, the survival effects for hydrophobic chain-grafted chitosan-5FU conjugates **7a–7c** were almost the same as for nongrafted chitosan-5FU conjugate **3a**.

On the other hand, the results of the survival effect for MeO-PEG-grafted chitosan-5FU conjugate **11** against p388 *lymphocytic leukemia* in mice i.p./i.p. are shown in Fig. 5. The survival effect for hydrophilic MeO-PEG-grafted chitosan-5FU conjugate **11** was lower than that for the nongrafted chitosan-5FU conjugate **3a**. Such a lowering of activity may be due to the inadequacy of the association state of MeO-PEG-grafted chitosan-5FU conjugate in aqueous solution; the chitosan chain of conjugate **11** in aqueous solution may not be located on the surface of the associates.

The results of the survival effect for the methanol-soluble COS(mix)-

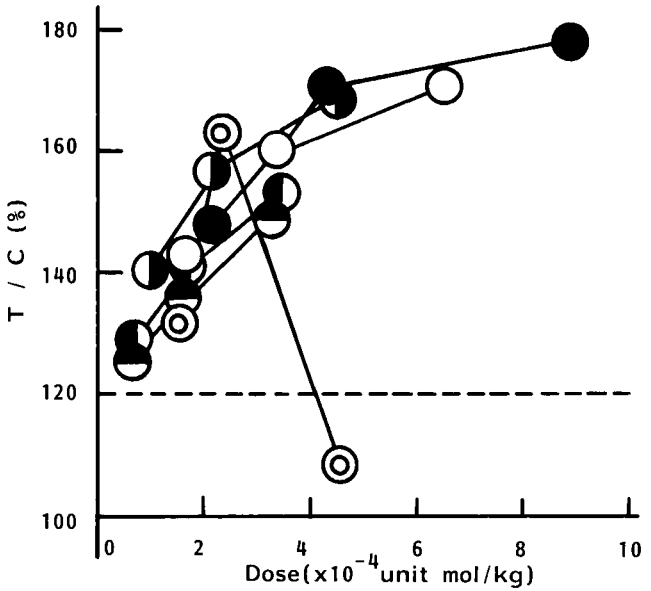


FIG. 2. Effect of D5FU on the prolongation of life for chitosan-5FU conjugate **3a** [degree of polymerization ($\bar{P}_n$) of chitosan = 30] against p388 *lymphocytic leukemia* in mice i.p./i.p. D5FU = 27 mol% (●); 18 mol% (○); 11 mol% (◑); 8.3 mol% (◐); 7.0 mol% (◓); 5FU (◎).

H-5FU conjugate (**3c-S**) and the methanol-insoluble COS(mix)-H-5FU conjugate (**3c-I**) and 5FU against *leukemia* in female CDF_1 mice i.p./i.p. are shown in Fig. 6. In spite of their solubility in methanol, the prolongation of life for these COS(mix)-H-5FU conjugates was lower than that for the chitosan(n = 30)-5FU conjugate.

Moreover, the results of the growth-inhibitory effect by chitosan-5FU conjugate **3a** against Meth-A *fibrosarcoma* in SPF-C3H/He mice, COS6-5FU conjugate (**3b**; n = 6) against MH-134Y *hepatoma* in SPF-C3H/He scl male mice, and COS3-5FU conjugate (**3b**; n = 3) against Meth-A *fibrosarcoma* in Balb/c male mice s.c./i.v. are shown in Fig. 7. The chitosan-5FU, COS6-5FU, and COS3-5FU conjugates were known to exhibit significant growth-inhibitory effects against Meth-A *fibrosarcoma* or MH-134Y *hepatoma*. From the results shown in Fig. 7, these chitosan-5FU, COS6-5FU, and COS3-5FU conjugates were found to exhibit stronger growth-inhibitory effects against solid tumor cells than do free 5FU, chitosan, COS6, COS3, and their blend s.c./i.v.

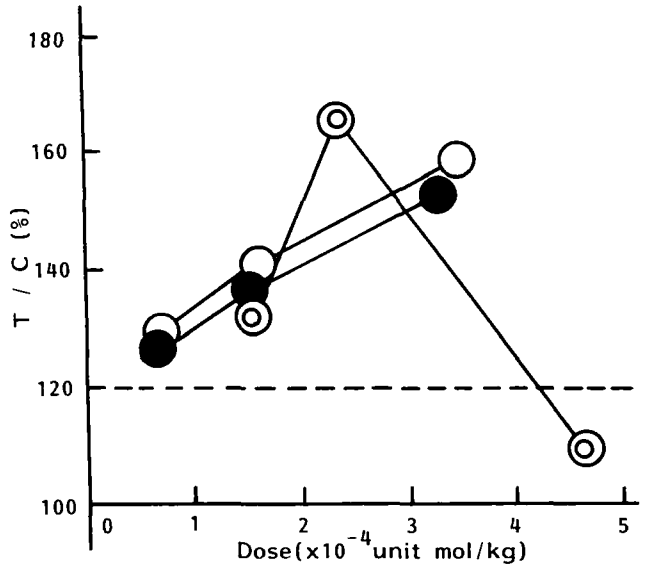


FIG. 3. Effect of $\overline{P}_n$ of chitosan on the prolongation of life for chitosan-5FU conjugate **3a** ($\overline{P}_n$ of chitosan = 30) against p388 *lymphocytic leukemia* in mice i.p./i.p. (○) $\overline{P}_n$ of chitosan = 30, D5FU = 8.3 mol%; (●) $\overline{P}_n$ of chitosan = 75, D5FU = 8.0 mol%; (◎) 5FU.

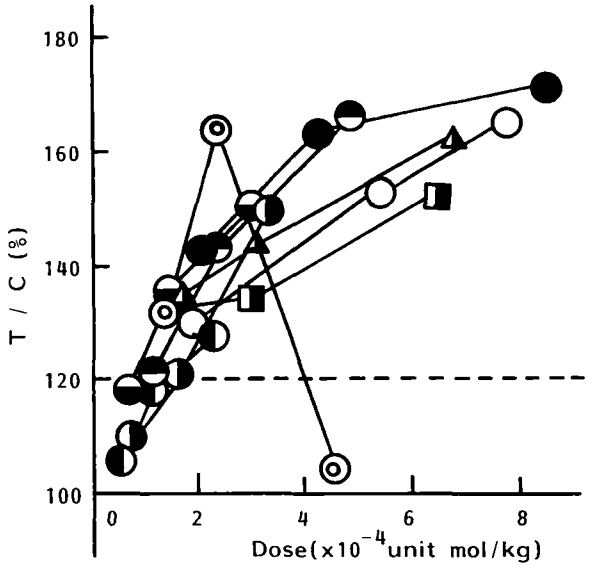


FIG. 4. The prolongation of life for hydrophobic chain-grafted chitosan-5FU conjugates **7a**–**7c** ($\overline{P}_n$ of chitosan = 30) against p388 *lymphocytic leukemia* in mice i.p./i.p. (●) **7a**: DR = 145 mol%, D5FU = 49 mol%; (○) **7a**: DR = 145 mol%, D5FU = 45 mol%; (◨) **7b**: DR = 120 mol%, D5FU = 33 mol%; (◑) **7b**: DR = 120 mol%; D5FU = 20 mol%; (◐) **7b**: DR = 120 mol%, D5FU = 13 mol%; (▲) **7b**: DR = 90 mol%, D5FU = 28 mol%; (◓) **7c**: DR = 97 mol%, D5FU = 19 mol%; (◒) **7c**: DR = 97 mol%, D5FU = 11 mol%; (◎) 5FU.

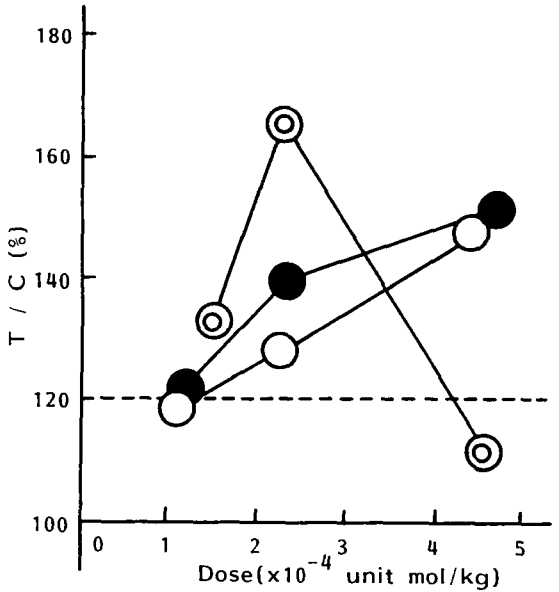


FIG. 5. The prolongation of life for hydrophilic MeO-PEG-grafted chitosan-5FU conjugate **11** ($\bar{P}_n$ of chitosan = 30) against p388 *lymphocytic leukemia* in mice i.p./i.p. (●) m = 7, DPEG = 26 mol%, D5FU = 18 mol%; (○) m = 20, DPEG = 18 mol%, D5FU = 24 mol%; (◎) 5FU.

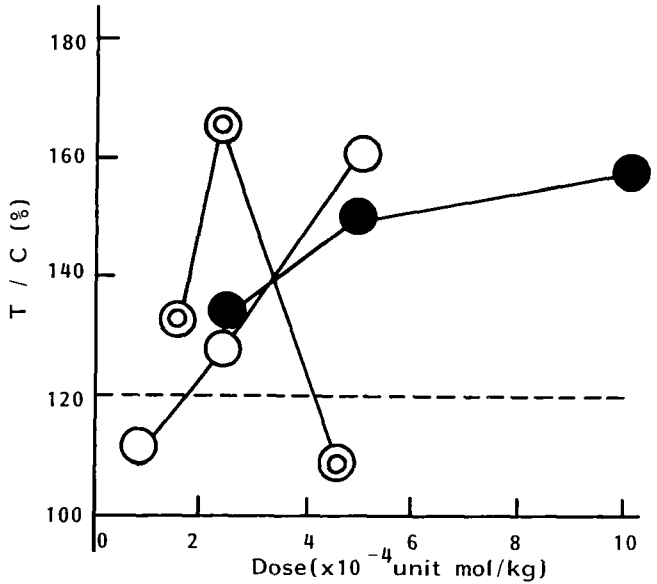


FIG. 6. The prolongation of life for COS(mix)-H-5FU conjugate **3c** and 5FU against p388 *lymphocytic leukemia* in mice i.p./i.p. (●) Methanol-soluble COS(mix)-H-5FU (**3c-S**) ($\bar{P}_n$ = 2-7, D5FU = 30 mol%), (○) methanol-insoluble COS(mix)-H-5FU[b] (**3c-I**) ($\bar{P}_n$ = 2-7, D5FU = 33 mol%); (◎) 5FU.

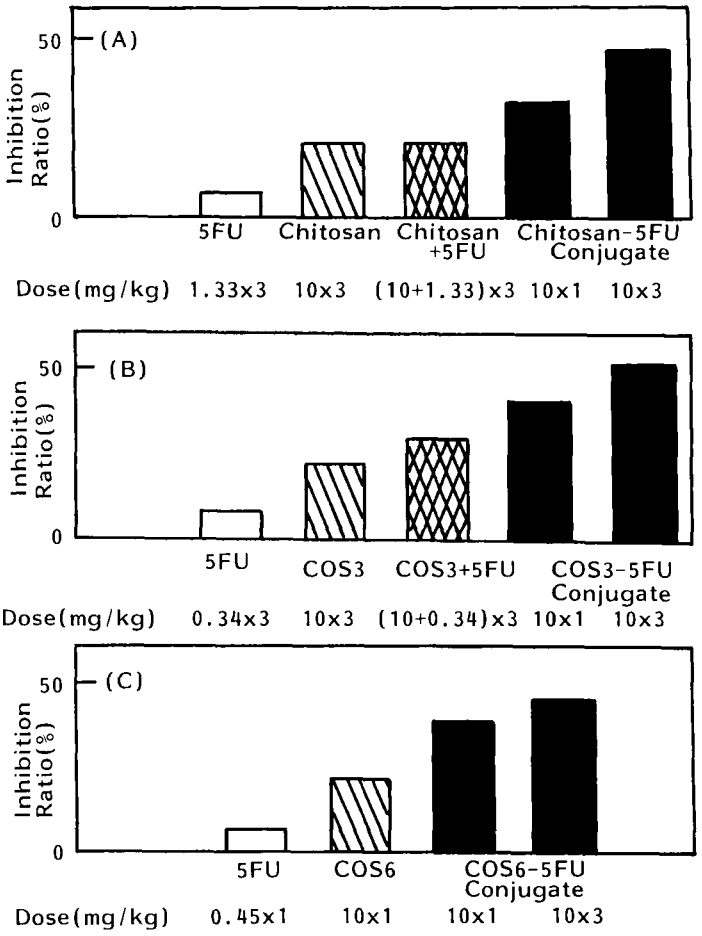


FIG. 7. Growth-inhibitory effects by chitosan-5FU conjugate **3a** [(A): $\overline{P}_n$ of chitosan = 30, D5FU = 27 mol%], COS3-5FU conjugate **3b** [(B): $\overline{P}_n$ of D5FU = 34 mol%] against Meth-A *fibrosarcoma* and growth-inhibitory effect by COS6-5FU conjugate **3b** [(C): $\overline{P}_n$ of D5FU = 44 mol%] against MH-134Y *hepatoma* in mice s.c./i.v.

The chitosan-5FU and chitosamino-oligosaccharide-5FU conjugates obtained did not exhibit a rapid decrease of body weight of the treated mice even in the high dose range shown in Figs. 2–7; they did not display an acute toxicity in such high does ranges.

Thus, it is expected that the chitosan-5FU and chitosamino-oligosaccharide-5FU conjugates obtained will act clinically as macromolecular and oligomolecular prodrugs of 5FU.

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