SYNTHESIS AND ANTITUMOR ACTIVITY OF 6-O-CARBOXYMETHYL CHITIN FIXING 5-FLUOROURACILS THROUGH PENTAMETHYLENE, MONOMETHYLENE SPACER GROUPS *VIA* AMIDE, ESTER BONDS

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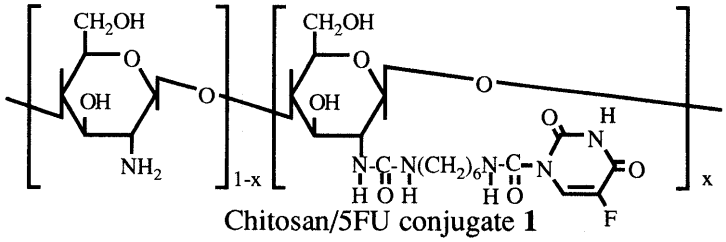
In order to provide the water-soluble and biodegradable macromolecular prodrug of 5-fluorouracil (5FU), the fixation of 5FUs to 6-O-carboxymethyl chitin(CM-chitin) through pentamethylene, monomethylene spacer groups *via* amide, ester bonds was carried out. The obtained CM-chitin/5FU conjugate showed the slow release of 5FU and exhibited remarkable antitumor activity against P388 *lymphocytic leukemia* in mice by intraperitoneal(*i.p.*) implantation / *i.p.* injection.

KEYWORDS 6-O-carboxymethyl chitin; 5-fluorouracil; drug delivery system; macromolecular prodrug; antitumor activity; P388 *lymphocytic leukemia*

5-Fluorouracil(5FU) has been used for a long time as an antineoplastic agent.[1,2] However, its undesirable side-effects have also been cited.[3,4] In comparison with low molecular weight prodrugs, polymer-drug conjugates are expected to show reduced side-effects and prolonged duration of activity. Chitin and chitosan are noteworthy as low or non-toxic, non-immunogenetic, compatible and biodegradable polymers. In particular, partially N-acetylated chitosan was reported to be selectively accumulated in certain tumor cells and to inhibit growth of the tumor cells.[5] Recently, water-soluble N-acetylated chitohexaose(NACOS6; hexamer of chitin) was found to show the immunoenhanced growth-inhibitory effect against tumor cells.[6] Therefore, chitin and chitosan are expected to be applied as drug carrier of a polymer-drug conjugate. In previous studies[7-9], we had found that chitosan fixing 5FUs through hexamethylene spacer groups *via* urea, urea bonds, chitosan/5FU conjugate **1**, exhibited higher survival effect against P388 *lymphocytic leukemia* in CDF1 mice by intraperitoneal (*i.p.*) implantation / *i.p.* injection and showed higher growth-inhibitory effect against Meth-A *fibrosarcoma* in mice by subcutaneous(*s.c.*) implantation / intravenous(*i.v.*) injection than free 5FU. However, the conjugates obtained were water-insoluble, and the way of injection and their dose capacity were restricted. Moreover, the hydrolysis rate of urea bonds of conjugate **1** was relatively fast. The appropriate hydrolysis rate of the covalent bonds between drugs and carrier polymer was desired for slow release of drugs from the carrier polymer.



Chitosan/5FU conjugate **1**

In order to provide a biodegradable and water-soluble macromolecular prodrug of 5FU showing slow release of 5FU, reducing side-effects, having an affinity for tumor cells and exhibiting strong antitumor activity, we employed 6-O-carboxymethyl chitin(CM-chitin), which is water-soluble chitin derivative as a drug carrier, and

carried out fixation of 5FUs to CM-chitin at 6-positions through pentamethylene, monomethylene spacer groups *via* amide, ester bonds. The release behavior of 5FU from the conjugate obtained was studied *in vitro* at 37°C in physiological saline media. Moreover, the survival effect of the conjugate against P388 *lymphocytic leukemia* in female CDF1 mice *i.p./i.p.* was investigated.

EXPERIMENTAL

CM-chitin sodium salt (degree of carboxymethylation: DCM=70%) obtained from Katokichi Co. Ltd. was treated with 3N-HCl aq. to give desalted CM-chitin (Mw=10,000). 1-[(amino-*n*-pentyl)-ester]-methylene-5FU hydrochloride **2** was prepared by the method reported previously.[10] The fixation of **2** to CM-chitin *via* amide bonds was carried out as shown in Chart 1. 1.04g (5.06mmol) of N,N'-dicyclohexylcarbodiimide(DCC) was added to 15ml of DMF containing 1.49g (4.22mmol of carboxyl unit) of CM-chitin and 1.31g (4.22mmol) of **2** and 0.59ml (4.22mmol) of triethylamine. The solution obtained was stirred at 0°C for 3h and at room temperature for 20h. After the N,N'-dicyclohexylurea(DCU) formed was removed by filtration, the condensed supernatant was purified by gel filtration chromatography (Sephadex LH-20, eluent: DMF) to give CM-chitin fixing 5FUs through pentamethylene, monomethylene spacer groups *via* amide, ester bonds, CM-chitin/5FU conjugate **3**. The degree of substitution of 5FU of the conjugates in mol% per glucosamine unit(D5FU) was determined by means of GPC measurement of the amount of 5FU released after enough hydrolysis with 6N-NaOH aq.(column: Shodex OHpak KB-803; eluent: H_2O; detector: UV 265nm). The release behavior of 5FU from the conjugates was investigated *in vitro* in physiological saline(0.9 wt.%, pH=6.80) media at 37°C. The amount of 5FU released from the conjugates was estimated by the GPC method described above. The survival effect of the conjugate was tested against P388 *lymphocytic leukemia* in female CDF1 mice (30 untreated mice/group and 6 mice/group) by *i.p./i.p.* according to the typical protocol of the Japanese Foundation for Cancer Research (JFCR). $1x10^6$ *leukemia* cells were injected *i.p.*on day 0. The conjugates were dissolved in a sterile normal saline solution and administered *i.p.* The mice twice received doses of 200-800mg/kg of the conjugates, at 1 and 5 days. The ratio of prolongation of life of the tested 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 was generally obtained to be 10 days.

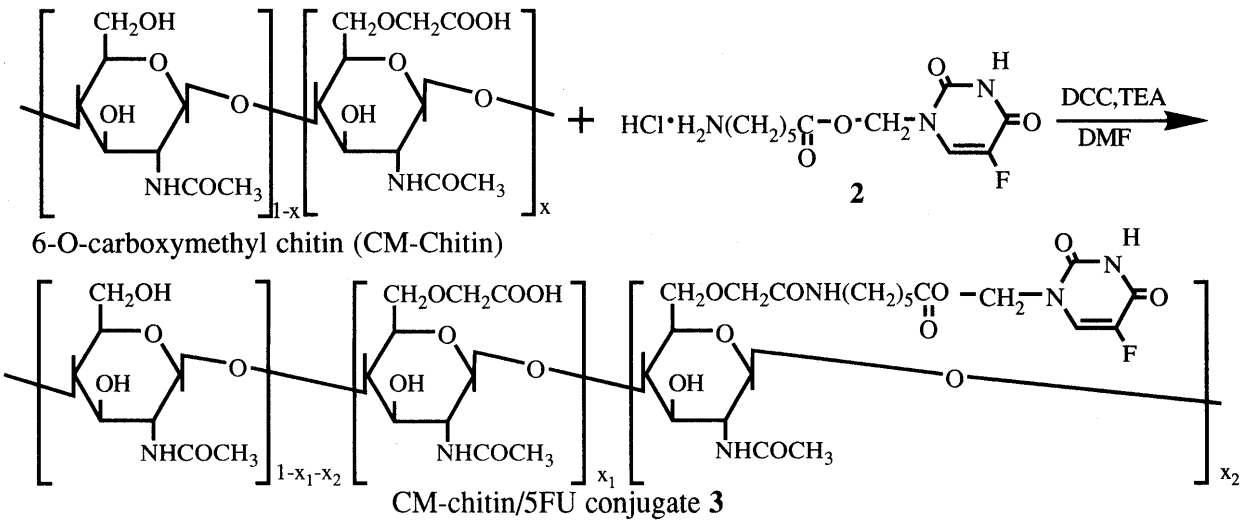


Chart 1. Synthesis of CM-Chitin/5FU Conjugate **3**

RESULTS AND DISCUSSION

The fixation of 5FU to CM-chitin through pentamethylene, monomethylene spacer groups *via* amide, ester bonds was accomplished by the coupling reaction of CM-chitin with 5FU derivative **2** to give water-soluble CM-chitin/5FU conjugate **3**(D5FU=27.6 or 35.2 mol%). The water-solubility of conjugate **3** results from the high water-solubility of CM-chitin as a backbone polymer; the good water-solubility is due to the existence of residual carboxyl groups in this conjugate.

In order to evaluate the release behavior of 5FU from CM-chitin/5FU conjugate, the hydrolyses of **1** and **3** were studied *in vitro* at 37°C in physiological saline media. The results are shown in Fig.1. In the hydrolyses of these conjugates, only generation of free 5FU itself was recognized, while no 5FU derivative was detected. The release rate of 5FU from the CM-chitin/5FU conjugate **3** was slower than that from chitosan/5FU conjugate **1**. Therefore, the slow release of 5FU from the conjugate was achieved by the fixation of 5FU to CM-chitin through hexamethylene, monomethylene spacer groups *via* amide, ester bonds.

The results of the survival effect for conjugate **3**, 5FU derivative **2** and 5FU against P388 *lymphocytic leukemia* in female CDF1 mice *i.p./i.p.* are shown in Fig. 2. The prolongation of life for conjugate **3** tended to increase with an increase in dose. Although T/C value for 5FU derivative **2** was lower than that for 5FU, T/C value for conjugate **3** was higher than that for 5FU. Moreover, the conjugate **3** obtained did not cause a rapid decrease of body weight in the treated mice even in the high dose ranges shown in Fig. 2; they did not display an acute toxicity in such high dose ranges. Therefore, CM-chitin/5FU conjugate technique can be concluded to derive the depression of the side-effects of 5FU. The conjugate **1** using chitosan as a drug carrier was water-insoluble, while the conjugate **3** using CM-chitin was water-soluble. Therefore, the conjugate **3** can be applied easily for the test of antitumor activity by intravenous injection.

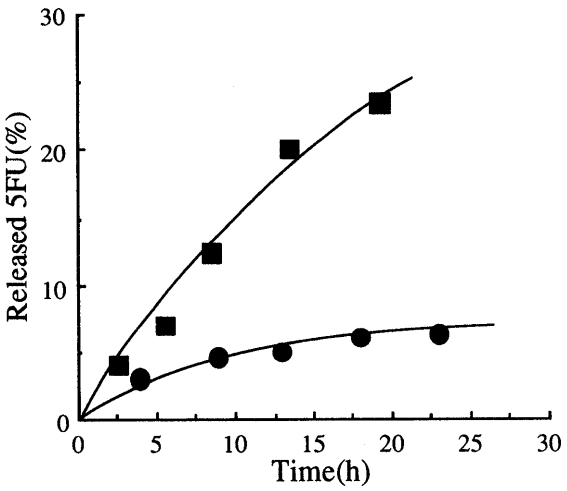


Fig. 1. Release Behavior of 5FU from CM-Chitin/5FU Conjugate **3** and Chitosan/5FU Conjugate **1** in Physiological Saline at 37°C *in Vitro*. ■: conjugate **1**(D5FU=18.0mol%); ●: conjugate **3**(D5FU=27.6mol%).

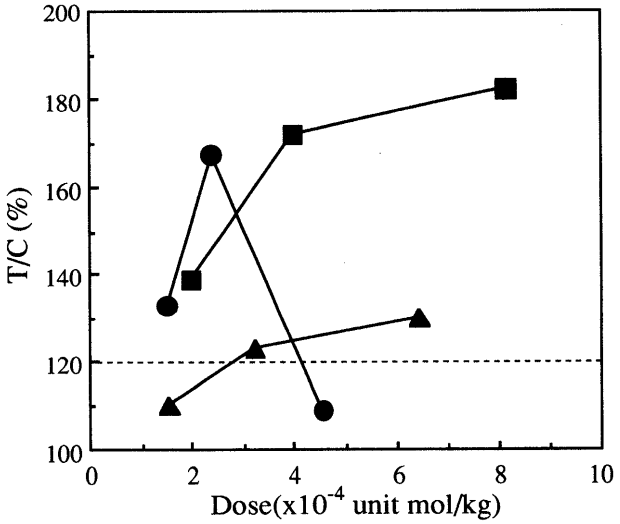


Fig. 2. Survival Effect per Unit mol of 5FU for **2**, CM-Chitin/5FU Conjugate **3** and 5FU against p388 *Lymphocytic leukemia* in mice *i.p./i.p.*. ●: 5FU; ▲: **2**; ■: conjugate **3**(D5FU=35.2mol%).

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