[Chem. Pharm. Bull. 36( 8 )3186—3189(1988)]

# Characterization of a Lipophilic Prodrug of 5-Fluorouracil with a Cholesterol Promoiety and Its Application to Liposomes

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(Received January 29, 1988)

A lipophilic prodrug of 5-fluorouracil (FU) with a cholesterol promoiety, cholesteryl-5-(5-fluorouracilcarbamoyl)capronate (ChFU), was synthesized and its physicochemical and biological properties were studied in comparison with those of octadecylcarbamoyl FU (C18FU) having the same linkage structure. ChFU and C18FU showed enhanced lipophilicity and almost complete incorporation into liposomes while incorporation of FU was very small. In neutral and alkaline media, FU was rapidly regenerated from ChFU with a half-life of about 14 min and C18FU showed a similar rapid conversion. The conversion of ChFU to FU was suppressed by the incorporation into liposomes and the regenerated FU was released from liposomes with a half-life of about 1 h. The release of FU from the liposomal formulation of C18FU was much slower than that of ChFU. The liposomal formulation of ChFU showed almost the same *in vivo* antitumor activity against P388 leukemia as that of FU solution with less side effects. The liposomal formulation of C18FU exhibited superior *in vivo* antitumor effect to those of ChFU and free FU. The mode of incorporation of these lipophilic FU prodrugs into liposomes seemed to affect the conversion kinetics of the prodrugs and their subsequent pharmacological efficacy.

**Keywords**——5-fluorouracil; lipophilic prodrug; cholesterol; regeneration property; liposome; antitumor activity

In cancer chemotherapy, it is important to control the pharmacokinetic behavior of antitumor drugs for effective treatment and various approaches have been used to deliver drugs to tumor tissues.[1] Among them, the use of liposomes as carriers of antitumor agents seems to be of great interest.[2] However, most antitumor drugs are poorly entrapped in liposomes owing to their low lipophilicity.

One promising approach seems to be application of chemical modification of drug molecules to prodrugs having adequate physicochemical properties for incorporation in lipidic carriers.[3] We have synthesized various kinds of lipophilic prodrugs of mitomycin C[4] and demonstrated the usefulness of their liposomal formulations as either a lymphotropic delivery system or a local sustained-release system. We have also investigated the applicability of lipophilic prodrugs of 5-fluorouracil (FU) with alkyl promoieties and a carbamoyl linkage to the liposomal formulation.[5]

In the present study, a new lipophilic prodrug of FU having a cholesteryl group was synthesized, and its physicochemical and biological properties and the applicability to liposomal formulation were studied. These characteristics were compared with those of octadecylcarbamoyl FU (C18FU),[5] which has the same carbamoyl linkage but a straight alkyl side chain.

## Experimental

**Chemicals**——FU was supplied by Kyowa Hakko Kogyo Co., Japan. C18FU was synthesized as reported

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previously[5,6] and purified by using a silica gel column. 1-(5-Carboxypentylcarbamoyl)-5-fluorouracil (CPEFU) was a gift from Sankyo Co., Japan. Cholesteryl-5-(5-fluorouracilcarbamoyl)-capronate (ChFU) was synthesized by coupling CPEFU with cholesterol (Nakarai Chemicals, Japan) in a mixture of dimethylformamide and chloroform using *N,N'*-dicyclohexylcarbodiimide. The reaction mixture was transferred to a silica gel column and eluted with chloroform–ethyl acetate (9 : 1). The ChFU fraction was further purified by a liquid chromatography system (Waters, Prep LC/system 500A) using hexane–ethyl acetate (56 : 44) as the solvent. Egg phosphatidylcholine was prepared from egg yolks.[7] All other chemicals were commercial reagent-grade products.

**Partition Coefficient Measurement**——Apparent partition coefficients of prodrugs were determined in a chloroform/0.1 N HCl system at 37 °C.

**Stability Study**——Buffer solution (pH 1.6, 7.4) or 0.1 N NaOH solution was added to 10 volumes of methanol solution of each prodrug and the mixture was maintained at 37 °C. Aliquots of the solution were withdrawn at suitable time intervals for determining both prodrug and regenerated FU.

**Preparation of Liposomes**——Liposomes were prepared from egg phosphatidylcholine. A chloroform solution of phosphatidylcholine (70 mg) and the drug at a molar ratio of 4 : 1 was evaporated under vacuum to a thin lipid film. The dry lipid film was then suspended in a saline solution (4 ml) containing small amount of HCl (0.001 N), and the resulting suspension was sonicated at 0 °C for 3 min under a nitrogen atmosphere. The obtained liposomes showed multilamellar structure on electron microscopic observation. Liposomal drug entrapment was calculated by determining the aqueous phase concentration of the drug after centrifugation. For the animal experiment, a liposome formulation containing 10 times more lipid and drug was prepared.

**Release from Liposome Formulation**——Liposomes containing prodrugs were diluted 100 times with pH 7.4 isotonic buffer solution and maintained at 37 °C. At fixed time intervals, an aliquot was withdrawn and ultrafiltered with a Micro-partition System MPS-1 (Amicon, U.S.A.), and the filtrate was subjected to assay.

**Assay**——FU and prodrug was determined by the use of an high performence liquid chromatography (HPLC) system (LC-5A, Shimadzu, Japan) equipped with a variable-wavelength detector (SPD-2A, Shimadzu, Japan). The stationary phase used was a Cosmosil $5C_{18}$ packed column (46 × 150 mm, Nakarai Chemicals, Japan). The mobile phases used were water for FU, water–methanol (40 : 60) for CPEFU, and methanol–ethanol (50 : 50) for ChFU and C18FU, with a flow rate of 0.8 ml/min.

***In Vivo* Antitumor Activity**——Six male B6D2$F_1$ mice (weighing 20—23 g) for each group were inoculated intraperitoneally with $1 \times 10^6$ P388 leukemia cells and the chemotherapy was given intraperitoneally 24 h after inoculation. The antitumor activities were indicated as T/C (%), the ratio of the mean survival time of the treated group (T) to that of the control group (C). To check for side effects, the body weight of each mouse was measured every day.

## Results and Discussion

### Physicochemical Properties and Liposomal Entrapment of Prodrugs

ChFU showed an exceedingly high partition coefficient (PC = 12368) compared with FU (PC = $6.9 \times 10^{-4}$) and CPEFU (PC = 215.2), which suggested that the cholesterol moiety enhanced the lipophilicity of FU to a great extent. Reflecting the increased lipophilicity, more than 99% of ChFU was incorporated into liposomes, while the entrapment percentages were low in the case of FU (0.03%) and CPEFU (27.8%). C18FU also exhibited high partition coefficient (PC = 6100) and incorporation percentage (99.2%). These lipophilic prodrugs are considered to distribute to the membrane lipid, not the intravesicular aqueous space, in liposomes.[8]

### Stability of Prodrugs in Solution

Under acidic conditions, ChFU was stable and regeneration of FU was not observed for at least 90 min. On the other hand, ChFU was rapidly converted to FU with half-lives of 13.0 min and 13.9 min in pH 7.4 phosphate buffer and 0.1 N NaOH solution, respectively, according to first-order kinetics. The amount of regenerated FU corresponded quantitatively with that of degraded ChFU, and no intermediate compound, including CPEFU, was detected in the solution. The regeneration kinetics both of CPEFU and C18FU were similar to that of ChFU. These results suggested that all prodrugs were chemically converted to FU at almost the same rate by hydrolytic cleavage of the cabamoyl linkage in spite of the difference in the structure of the promoiety.

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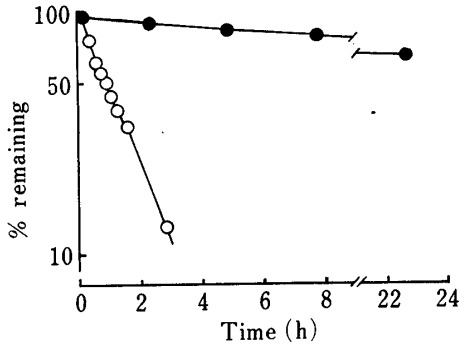


Fig. 1. Release of FU from Liposomes Containing Lipophilic FU Prodrugs in pH 7.4 Isotonic Buffer Solution at 37 °C

○, FU released from liposomes containing ChFU; ●, FU released from liposomes containing C18FU. Liposomes containing each prodrug were incubated in pH 7.4 isotonic buffer solution at 37 °C and released FU was determined by the ultrafiltration method. In each case, the prodrug itself was not detected.

TABLE I. Effects of FU, ChFU, and C18FU on the Survival Time and Body Weight of Mice Bearing P388 Leukemia

| Compound | Dose (mg equivalent FU/kg) | T/C[a] (%) | Change in body weight (%)[b] Day 4 | Day 7 |
|---|---|---|---|---|
| Saline | | 100 | +12.3 | +22.9 |
| FU | 20 | 109.4 | +14.9 | +28.1 |
| (Solution) | 50 | 121.9 | +15.6 | +28.1 |
| | 100 | 120.3 | +2.7 | +21.3 |
| | 200 | 134.4 | −13.3 | +16.8 |
| | 500 | 146.9 | −11.8 | +6.1 |
| ChFU | 50 | 118.8 | +15.6 | +25.5 |
| (Liposomes) | 100 | 134.4 | +11.3 | +26.3 |
| | 200 | 135.9 | +5.4 | +26.0 |
| | 400 | 75.0 | −9.8 | +3.0 |
| C18FU | 50 | 156.3 | +11.2 | +21.0 |
| (Liposomes) | 100 | 151.6 | +6.6 | +18.0 |
| | 200 | 157.8 | −3.8 | +6.7 |
| | 400 | 170.3 | −15.1 | −18.9 |

*a*) The ratio of the mean survival time of the treated group to that of the control group. *b*) The increase or decrease in the ratio of the mean body weight at day 4 and day 7 to that at day 0.

**Release from the Liposomal Formulation**

Figure 1 shows the release of FU from the liposomal formulation incorporating ChFU or C18FU. Prodrugs were not detected in the aqueous medium in either case. The release half-life of FU was about 1 h in the case of ChFU, which was about four times longer than the regeneration rate of FU in the outer aqueous medium. It was suggested that liposomal entrapment protected the prodrug from hydrolysis to FU. In the case of C18FU, incorporation into liposomes resulted in extreme suppression of conversion and the release half-life of FU was about 20 h.

The difference in mode of incorporation of these prodrugs into liposomes might affect the regeneration reaction of FU from prodrugs in liposomes. In the ChFU molecule, the carbamoyl linkage, which is subject to hydrolysis, is separated from the cholesterol moiety by a 6-carbon spacer and may be exposed to the aqueous phase of liposomes when the cholesteryl group is anchored to the membrane lipid. On the other hand, the carbamoyl linkage in C18FU should be close to the lipid bilayer since the carbamoyl linkage is adjacent to the C18 alkyl chain. Although another mechanism might operate, the accessibility of water seems to be a predominant factor which determines the rates of chemical hydrolysis of these prodrugs.

***In Vivo* Antitumor Activity**

The effects of FU and its prodrugs on the survival of mice bearing P388 leukemia are summarized in Table I, together with the change in body weight of the mice. Saline solution of

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FU gave the maximal effect at the dose of 500 mg/kg (T/C = 147%) but a significant decrease in body weight was observed at a dose of more than 200 mg/kg. The liposome formulation of ChFU showed almost the same antitumor effect as FU solution, with no obvious side effects at the dose of 200 mg equivalent FU/kg (T/C = 134%). C18FU in liposomes exhibited a superior antitumor effect to other compounds at any dose tested; the T/C value was 170% at the dose of 400 mg equivalent FU/kg. However, severe side effects were observed at doses of more than 200 mg equivalent FU/kg. Sustained release of FU from this formulation would result in enhancement in the efficacy of FU, a time-dependent antimetabolite. On the other hand, side effects might also be enhanced due to the prolonged supply of FU in the body. Liposomal formulation of ChFU and C18FU showed almost equal *in vitro* antitumor activities to FU against L1210 leukemia, while simple suspensions of these compounds had negligible effects (data not shown).

In conclusion, the combination of prodrug modification and lipidic carrier devices seems to offer a promising means to control the *in vivo* fate and increase the therapeutic efficacy of anticancer agents. In order to accomplish this purpose, the prodrug should be designed to be efficiently retained in carriers and to supply the parent drug at an adequate rate. In the case of FU prodrugs with a carbamate linkage, the chemical structure and the resulting mode of interaction with phosphatidylcholine membrane of the drug are considered to determine the regeneration kinetics of the parent drug and consequently the pharmacological efficacy.

**Acknowledgment** This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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