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Characterization of alkylcarbamoyl derivatives of 5-fluorouracil and their application to liposome

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Summary

Four alkylcarbamoyl derivatives of 5-fluorouracil (I): butyl- (II), hexyl- (III), octyl- (IV), and octadecylcarbamoyl 5-fluorouracil (V), were prepared and their physicochemical and biological properties were investigated. In addition, the utility of their liposomal formulations was evaluated. The compounds showed an increase in lipophilicity according to the natures of the functional groups introduced. The derivatives except for V showed antimicrobial activities equal to I because of their rapid conversion to I in pH 7.4 buffer. They were also converted to I at various rates in rat plasma and liver homogenate. Although V was stable in the buffer, it showed conversion in biological media. Test compounds, I, II, IV and V, showed antitumor activities against murine L1210 leukemia in i.p.-i.p. system. Degree of incorporation into liposomes of the derivatives was proportional to their lipophilicities. Compound I was not efficiently entrapped into liposomes. V showed the highest incorporation and the slowest release among them, and thus, was chosen as the best prodrug for applying to liposomes. Liposomal V showed prolonged retention in the injection site and enhanced delivery into the lymphatics after intramuscular injection in rats, suggesting its usefulness in cancer chemotherapy. Furthermore, V maintained the higher antitumor activity when administered in the form of liposome.

Introduction

In cancer chemotherapy, it is necessary to control the pharmacokinetic behavior of a cytotoxic drug for effective treatment, and many attempts have been made to deliver such drugs to the tumor site by means of drug delivery systems (Gregoriadis, 1977; Juliano, 1980). Among different approaches, the use of a physical device or the chemical transformation of a drug molecule into latent form (prodrug design) appears to be promising for improving the delivery of anticancer agents. For the past decade, we have been engaged in studies on cancer drug delivery systems by using physical devices (Hashida et al., 1977, 1980; Sezaki et al., 1982; Yoshioka et al., 1981) or chemical modification of drug molecules to prodrugs (Hashida et al., 1978; Kojima et al., 1980; Sasaki et al., 1983a, b and c; Takakura et al., 1984).

Although these delivery systems demonstrated their utilities in their own right, further optimal drug delivery system seems to be developed

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through rational systematic combination of dosage form design, chemical modification, and alteration of dose regimen. Recently, we reported the development of lipoidal delivery systems of mitomycin C (MMC), where its lipophilic prodrug entrapped in the formulations showed topical sustained release and lymphotropic delivery of MMC (Sasaki et al., 1984, 1985a and b). This combined approach seemed to have some benefits from a pharmaceutical viewpoint such as large scale preparation of sterile and pyrogen-free liposomes (Rao, 1983), compared with the ordinary method.

In the present study, we prepared four alkylcarbamoyl derivatives of 5-fluorouracil (I) and examined their physicochemical and biological properties. They were also incorporated into liposomes and the utility of these formulations was investigated.

Materials and Methods

Chemicals

5-Fluorouracil (5-FU, I) was supplied from Kyowa Hakko Kogyo Co. Octylcarbamoyl 5-FU (IV) and hexylcarbamoyl 5-FU (III) were supplied from Mitsui Pharmaceuticals, Inc. Butylcarbamoyl 5-FU (II) and octadecylcarbamoyl 5-FU (V) were synthesized as reported by Ozaki et al. (1977) and purified by using a silica gel column. 6-Carboxyfluorescein was obtained from Eastman Kodak Co. All other chemicals were of reagent grade. Egg phosphatidylcholine was prepared from egg yolks by a method described by Tanaka et al. (1975).

Melting points were determined in capillary tubes using a Yanagimoto serial no. 17 micromelting point apparatus and were uncorrected. Ultraviolet (UV) absorption spectra were recorded on a Hitachi model 220 UV-VIS spectrophotometer. Elemental analyses were performed by the Center for Organic Elemental Microanalysis, Kyoto University. The analytical results of 5-FU derivatives obtained were within $\pm 0.4\%$ of the theoretical values.

Lipophilicity and lipid solubility studies

Apparent partition coefficients of compounds were determined in a 1-octanol and pH 4.0 citrate buffer solution at 37 °C according to the method of Kakemi et al. (1967). 1-Octanol and the buffer solution were saturated with the relevant aqueous or organic phase before use. The initial concentration of compounds used was 1 mM dissolved in 1-octanol.

The relative lipophilic indices (log k') were determined by high-performance liquid chromatography (HPLC) employing the following equation (Yamana et al., 1977):

$$\log k' = \log[(t_r - t_0)/t_0]$$

where t_r is the retention time and t_0 is the elution time of a solvent. The lipophilic indices (log k'_0) were determined by extrapolation of k' to 0% methanol in water elution. The solubilities in pH 4.0 buffer solution, sesame oil, isopropyl myristate, and *n*-hexane were determined by suspending an excess amount of the compound in the solvent, followed by filtration and analysis by HPLC.

Stability measurement in aqueous solution and biological media

Stability experiments were carried out in an isotonic pH 7.4 phosphate buffer (ionic strength = 0.3) or 10% methanol/pH 10 carbonate buffer at 37 ± 0.2 °C. Degradation was initiated by the addition of the stock solution to a preheated buffered solution to give a concentration of 1×10^{-4} M. Aliquots of the solution were withdrawn at suitable time intervals for assay.

Male Wistar rats weighing 200–220 g were used to obtain plasma and liver homogenates. The liver was homogenized at 0–5 °C in a glass–Teflon homogenizer, centrifuged at 4 °C and $600 \times g$ for 10 min, and the supernatant was used for the experiments. An isotonic phosphate buffer (pH 7.4) containing 0.25 M sucrose was used to prepare and dilute tissue homogenate and plasma samples.

Bioactivation experiments were performed at 37 ± 0.2 °C and initiated by adding the stock solution to give a final concentration of 1×10^{-4} M. At appropriate time intervals aliquots of the solution were withdrawn, added to acetonitrile to precipitate the protein, and the supernatant after centrifugation was subjected to analysis.

Antimicrobial activity studies

Antimicrobial activities were determined by the ordinary paper disc method using *Staphylococcus aureus* 209P as a test organism. The test compounds were dissolved in 50% methanol solution for analysis because of their various aqueous solubilities. The antimicrobial activity was determined by measuring a diameter of growth-inhibitory zone after 24 h incubation at 37 °C.

Antitumor activity studies

L1210 leukemia cells were maintained by weekly transplantation of tumor cells into the peritoneal cavity of male DBA/2 mice. Animals used for test were male hybrid BDF_1 mice (C57Bl/6 × DBA/2). Six mice for each group weighing 20–25 g were inoculated intraperitoneally with a suspension of 1×10^5 leukemia cells and the chemotherapy was given intraperitoneally at 24 h after inoculation. All drugs used were administered as a saline suspension or liposomes. Activities were calculated at T/C %, the ratio of the mean survival time of the treated group (T) divided by that of the control group (C). The observation period of survival time was 60 days.

Preparation of liposomes

Liposomes were prepared from egg phosphatidylcholine. A chloroform solution of phosphatidylcholine 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 by Vortex shaking, and the resulting suspension was sonically disrupted at 0 °C for 3 min under nitrogen atmosphere. In the gel filtration experiment, compound I and 6-carboxyfluorescein were dissolved in a saline solution and added to the dry lipid film containing 0, 20, or 40% (mol/mol %) cholesterol for preparing liposome.

Entrapment and release measurement

Incorporation of compound I and 6-carboxyfluorescein into liposomes with or without 20% or 40% cholesterol contents (mol/mol %) was determined by gel filtration. The liposomes incorporating drug and dye, as inner aqueous space indicator, were chromatographed on Sephadex G75 column and the entrapment percent was determined from the elution patterns. Liposomal fraction was monitored by determining the optical absorbance at 600 nm. Compound I and 6-carboxyfluorescein were determined by HPLC and fluorophotometer (EX : 490 nm and EM : 520 nm, Shimadzu RF-540 spectrofluorophotometer, Japan), respectively.

The extent of entrapment of drugs by liposomal lipid was determined by ultrafiltration. Liposome suspension (containing 0.3 mM drug and 1.2 mM phosphatidylcholine) was ultrafiltrated with Micro-partition System MPS-1 (Amicon) and the filtrate was subjected to analysis for drug. The entrapment was calculated by subtracting filtrate concentration from initial concentration of drug.

The release of the test compounds from liposomes was examined by a modified method of a dynamic dialysis system. A visking dialysis tube (20/32) containing 5 ml of liposome suspension was immersed in 70 ml of pH 4.0 citrate buffer maintained at 37 °C. The inner fluid was stirred continuously and 0.1 ml of sample was withdrawn from the outer medium at fixed time intervals for analysis.

Intramuscular injection experiment of liposomal drug

Male Wistar albino rats weighing between 200–220 g were used in the experiment. The animals were anesthetized by ether and injected with 100 μl of the formulations at a dose of 1.0 mg/kg (as I) into the right thigh muscle. At various times after injection, rats were sacrificed, and blood was removed via the aorta. The muscle and the regional lymph node were excised rapidly. The excised samples were homogenized with a Teflon homogenizer and adequately diluted with citrate buffer (pH 4.0). To an aliquot of the blood and the homogenate samples, ethylacetate was added for extraction of compound V. The organic layer was evaporated and the residue was dissolved in a little DMSO for assay of the prodrug using HPLC. Another aliquot was washed by chloroform and the aqueous phase was supplied for bioassay of I.

Assay

Compound I and its derivatives were determined by HPLC system (TRIROTAR, Jasco,

Japan) equipped with a variable wavelength UV-detector (UVIDEC 100-II, Jasco, Japan). The stationary phase used was Cosmosil $5C_{18}$ packed column (4.6 × 150 mm, Nakarai Chemicals) and the mixtures of methanol and water were used as the mobile phase with a flow rate of 0.8 ml/min. As the mobile phase for I, 0.1% acetate was used. The standard solutions were chromatographed and calibration lines were constructed on the basis of peak-area measurements.

Concentration of compound I in the rat muscle and lymph node homogenates was determined by bioassay using *Staphylococcus aureus* 209P.

Results

Chemistry

The prodrugs studied in the present paper were synthesized by ordinary alkylcarbamoylation methods (Ozaki et al., 1980). The structures and physical data are listed in Table 1. Butyl-, hexyl-, octyl- and octadecylcarbamoyl groups were introduced to 1-position of compound I, respectively. All derivatives had a UV maximum at approximately 260 nm, which was used in detection of each compound by HPLC assay.

Table 2 shows physicochemical constants of test compounds such as apparent partition coefficients (P_{oct}), lipophilic indices (log k'_0) in HPLC, and solubilities in various solvents. All derivatives showed an increase in lipophilicity due to the introduction of the alkylcarbamoyl group. The partitioning properties of these compounds covered a range of almost four orders of magnitude. They also exhibited decreased aqueous solubilities and increased organic solubilities to various degrees.

Biological activity

The antimicrobial activities of the compounds against *Staphylococcus aureus* 209P are shown in Fig. 1. Each activity is expressed as the corresponding concentration of I (ordinate) which shows growth inhibition activity equal to that of the test compound. All derivatives except for V exhibited approximately equal antimicrobial activities to I. Compound V showed a remarkable decrease in activity.

The effects of the prodrugs on the survival time of mice given intraperitoneal inoculation of L1210 leukemia are shown in Fig. 2. Compounds II, III and IV showed lower efficacy on the leukemia. Compound V showed higher activity than I following intraperitoneal treatment.

Stability and bioactivation

The stability of derivatives were investigated in an isotonic pH 7.4 phosphate buffer at 37 °C, and the results are shown in Fig. 3. Prodrugs II, III and IV degraded to I with a half-life of 10 min in a buffer solution. Under this condition, compound

TABLE 1

Structures and physical properties of 5-fluorouracil and its alkylcarbamoyl derivatives

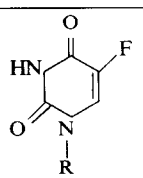
Compound	 R	mol.wt.	m.p. (°C)	UV_{max} ($\epsilon \times 10^{-3}$) (nm), EtOH
I 5-Fluorouracil (5-FU)	–H	130	> 270	267 (8.2)
II Butylcarbamoyl 5-FU	$-CONH(CH_2)_3CH_3$	229	130–133	257 (9.2)
III Hexylcarbamoyl 5-FU	$-CONH(CH_2)_5CH_3$	257	112–113	257 (9.5)
IV Octylcarbamoyl 5-FU	$-CONH(CH_2)_7CH_3$	285	92– 94	257 (10.6)
V Octadecylcarbamoyl 5-FU	$-CONH(CH_2)_{17}CH_3$	425	98–100	257 (11.2)

TABLE 2

Lipophilicity and solubility of 5-fluorouracil and its alkylcarbamoyl derivatives

Compound	Log P_{oct} [a]	Log k'_0 [b]	Solubility at 37 °C (mM)			
			pH 4.0 [c] buffer	Sesame oil	Isopropyl myristate	Hexane
I	−1.08	0.20	> 11.7	0.06	0.08	0.0006
II	1.48	2.40	1.1	15.85	38.05	0.368
III	2.63	2.60	0.093	50.15	44.88	0.582
IV	3.90	3.61	0.009	54.24	48.41	1.072
V	3.71	11.83	0.002	4.26	5.66	0.027

[a] Logarithmic value of apparent partition coefficient (P_{oct}) between 1-octanol and pH 4.0 buffer solution at 37 °C.
[b] Lipophilic index in HPLC (see text).
[c] Solubility in pH 4.0 buffer solution at 37 °C (mg/ml).

V remained stable in a form of suspension. In the 10% methanol/pH 10 carbonate buffer, compound V also degraded rapidly the same as II, III and IV (Fig. 4).

In contrast with the stability in buffer, prodrugs II, III, IV and V disappeared at various rates in biological media such as rat plasma and liver homogenate (Fig. 5).

Entrapment and release of test compounds in liposomes

Incorporation of parent drug I and 6-carboxyfluorescein as inner space indicator into liposome was determined by gel filtration as shown in Table 3. Both drug and dye showed low entrapment efficacy with or without cholesterol.

Table 4 shows entrapment into and release from liposomes of test compounds determined by ultrafiltration method. Although I was hardly incorporated into the lipid carrier, its prodrugs showed higher incorporation according to their lipophilicity. Especially, compound V having fairly high lipophilicity was completely incorporated into liposomes. Also, their release rates decreased with an increase in their lipophilicities.

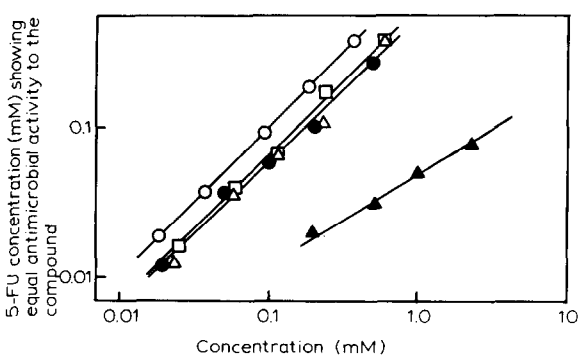


Fig. 1. Comparison of antimicrobial activities of 5-fluorouracil and its alkylcarbamoyl derivatives. ○, I; △, II; □, III; ●, IV; ▲, V.

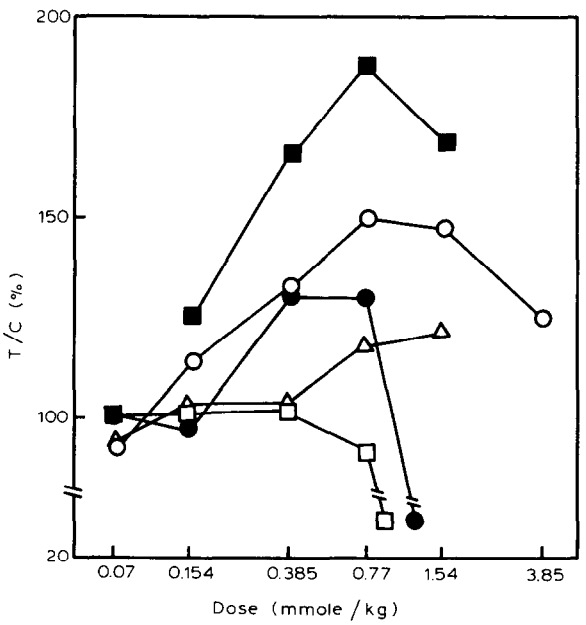


Fig. 2. Effect of 5-fluorouracil and its alkylcarbamoyl derivatives on survival time of mice bearing L1210 leukemia. ○, I; △, II; □, III; ●, IV; ■, V.

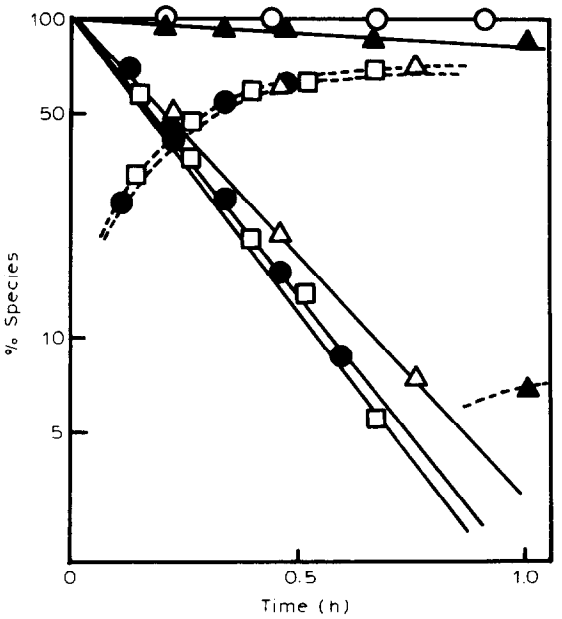


Fig. 3. Stability of 5-fluorouracil and its alkylcarbamoyl derivatives in a pH 7.4 buffer solution at 37°C. The solid line represents time course of degradation of prodrug and the dashed line is for regeneration of I from prodrug, respectively. ○, I; △, II; □, III; ●, IV; ▲, V.

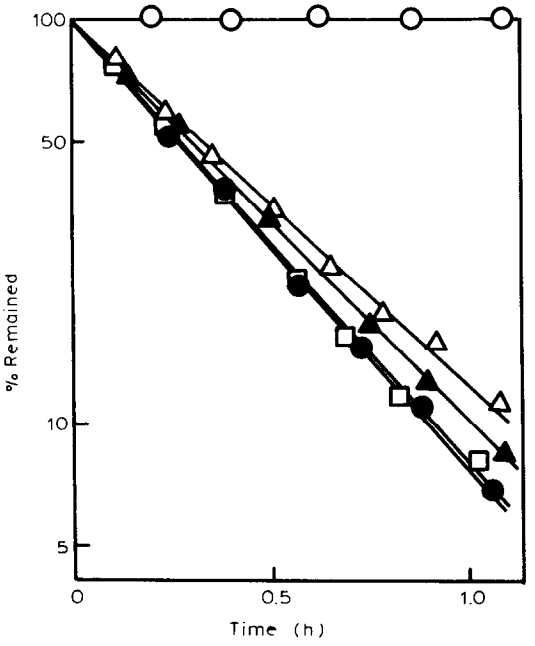


Fig. 4. Disappearance of 5-fluorouracil and its alkylcarbamoyl derivatives in 10% methanol/pH 10 buffer solution at 37°C. ○, I; △, II; □, III; ●, IV; ▲, V.

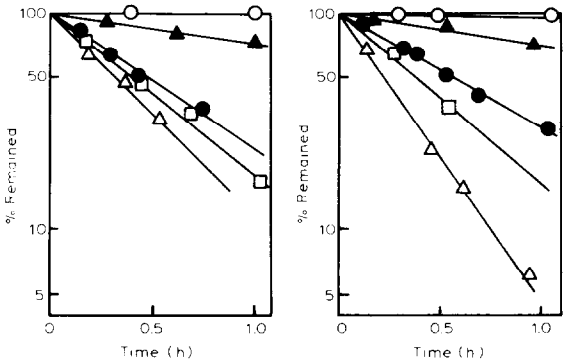


Fig. 5. Disappearance of 5-fluorouracil and its alkylcarbamoyl derivatives in 10% rat plasma (left) and 10% rat liver homogenate (right). ○, I; △, II; □, III; ●, IV; ▲, V.

Intramuscular injection experiment

The disappearance of I and V from the thigh muscle after intramuscular administration with liposomes is shown in Fig. 6. Whereas I was rapidly absorbed from the injection site even when administered in the form of liposome, V was retained for a considerably longer period. In the

TABLE 3

Entrap percent of 5-fluorouracil and 6-carboxyfluorescein (6-CF) in liposome determined by gel filtration

Components of liposome (%)		Entrap (%)	
cholesterol	PC [a]	5-FU	6-CF
0	100	0.16	0.20
20	80	0.10	0.11
40	60	0.11	0.15

[a] Egg phosphatidylcholine.

TABLE 4

Entrapment and release of 5-fluorouracil and its alkylcarbamoyl derivatives in liposome

Compound	Entrap (%)	Release % in 30 min
I	0.03	97.0
II	19.91	73.5
III	48.99	42.0
IV	94.10	12.0
V	99.96	0.1

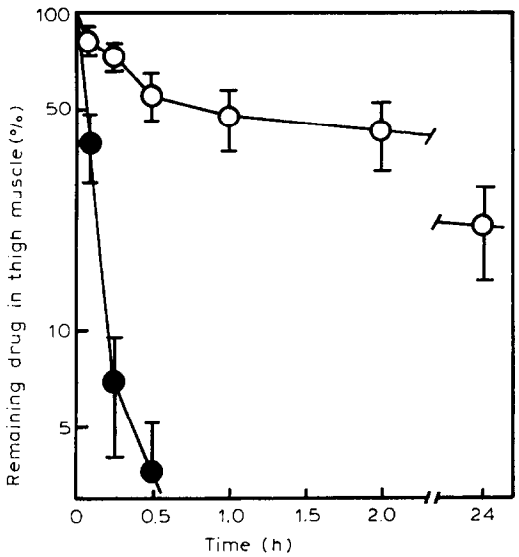


Fig. 6. Disappearance from the thigh muscle of liposomally entrapped 5-fluorouracil or octadecylcarbamoyl 5-fluorouracil after intramuscular administration. ●, I; ○, V. Each point represents the mean ± S.D. of at least 3 experiments.

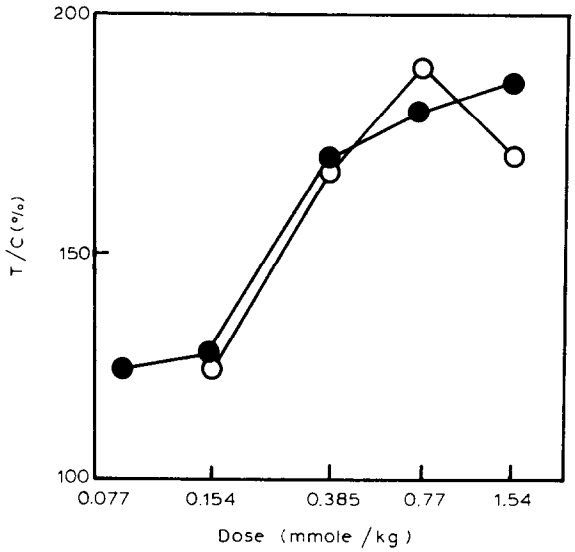


Fig. 8. Effect of octadecylcarbamoyl 5-fluorouracil administered with saline suspension and liposomes on survival time of mice bearing L1210 leukemia. ○, saline suspension; ●, liposomes.

same experiment, drug concentration in blood and iliac lymph node were determined and results are shown in Fig. 7. After administration of compound V, V and I were not detected in blood although compound I showed high blood level in the initial period. On the other hand, compound I arrived at the regional lymph nodes immediately after injection, but then disappeared rapidly. In contrast with this result, compound V administered with liposomes appeared in the lymph nodes at relative high concentration without detectable regeneration of I at the early stage after injection. Thereafter, its concentration decreased gradually but a considerable amount of V still remained even after 2 h and became undetectable after 24 h.

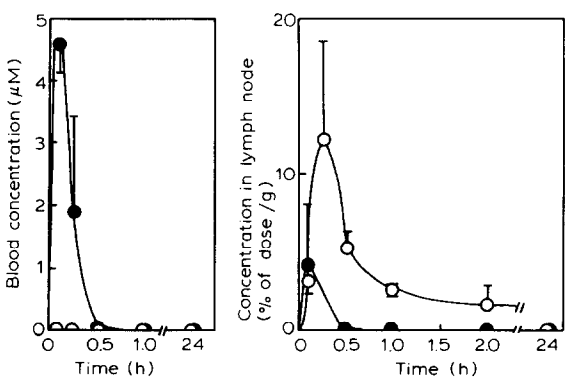


Fig. 7. Concentration in the blood (left) and the iliac lymph node (right) of liposomally entrapped 5-fluorouracil or octadecylcarbamoyl 5-fluorouracil after intramuscular administration. ●, I; ○, V. Each point represents the mean ± S.D. of at least 3 experiments except for some points (two experiments; 1.0, 2.0 and 24 h of I).

Antitumor activity of compound V entrapped in liposome against L1210 leukemia

Fundamental antitumor activities of compound V administered as the forms of liposomes are compared with its saline suspension in Fig. 8. Liposomal V showed equally high activity as the saline suspension.

Discussion

Recently, lipidic drug carriers such as liposomes have attracted much interest in cancer chemotherapy (Gregoriadis, 1980; Juliano and Stamp, 1978). However, there is a drawback in the appli-

cation of these lipid dispersion systems to certain drugs for example, hydrophilic compounds are poorly entrapped and amphiphilic compounds cannot be stably retained in liposomes. In these cases, the encapsulated drug must be separated from unencapsulated (free) drug and be concentrated before use. These procedures are disadvantageous to the large scale sterile production and preservation of a liposome formulation (Rao, 1983). Although numerous attempts have been reported to improve the entrapment efficiency by pharmaceutical approaches, no definitive improvement has been accomplished.

One promising approach seems to be application of chemical transformation of drug molecules to prodrugs having adequate physicochemical properties for incorporation in lipidic carriers. For example, the drug derivatives with appropriate hydrophobicity should spontaneously distribute to the lipid phase of the liposomes, and thus these compounds are easily incorporated into liposomes with the simple procedure. These properties would further encourage the application of novel preparation methods of liposome such as freeze-thawing and freeze-drying methods (Ohsawa et al., 1985). Based on these considerations, lipophilic prodrugs derivatized from compound I were characterized and applied to liposome in the present investigation.

Compound I has been widely used for the treatment of carcinoma of breast and gastrointestinal tract (Heidelberger et al., 1957; Cline, 1971). In this investigation, 4 alkylcarbamoyl derivatives were tested as prodrugs of I (Table 1). The compound prepared showed various lipophilicities according to their promoieties (Table 2). The chemical modification of I with introduction of a carbamoyl group was first reported by Ozaki et al. (1980). Among these derivatives, III showed higher therapeutic efficacy than I by oral administration (Hoshi et al., 1976; Iigo et al., 1978), and is supplied for clinical use (Okabayashi et al., 1979). On the other hand, V has not been reported in previous papers.

It is considered that compound III is chemically converted to I in the body (Kobari et al., 1981; Buur and Bundgaard, 1985). In fact, test compounds were rapidly converted to the parent drug in the buffer solution (Fig. 3). Although compound V was stable in a buffer because of its low solubility, it is rapidly converted to I in 10% methanol/buffer solution (Fig. 4). The regeneration behavior of I from prodrugs in aqueous medium was reflected in their antimicrobial activities (Fig. 1). Recently, it was reported that binding of prodrug of I to plasma protein markedly decreased its decomposition rate (Buur and Bundgaard, 1985). Similar observations were obtained in the present results (Fig. 5).

The antitumor activities of prodrugs are expected to reflect their regeneration and pharmacokinetic characteristics (Fig. 2). The activities of compounds II, III, and IV were lower than that of parent drug I because they, especially III, exhibited unexplained side effects to the host animals in i.p. administration. However, it was reported that compound III shows high activity by oral administration (Hoshi et al., 1976). This contradictory phenomenon might be explained in part by absorption behavior in the digestive tract: we have reported that the absorption rates of these prodrugs depended on their dissolution and III showed considerably slow absorption (Sasaki et al., 1986). On the other hand, compound V showed superior activity to I probably due to the slower regeneration of I from a suspension form of V. The continual contact with cancer cells is a necessary requirement for an antimetabolite like I to exhibit its cytocidal activity but its definitive contribution to the present results remains unclear.

Table 3 shows incorporation of compound I and hydrophilic dye (6-carboxyfluorescein) into liposome determined by gel filtration. The inner aqueous space of the present liposomes was determined to be only 0.1–0.2% of total aqueous volume from the entrapping efficiency of 6-carboxyfluorescein. Compound I also showed poor entrapment, suggesting that it distributes in the intravesicular space marked by the hydrophilic dye, as reported by Tsukada et al. (1984). Addition of cholesterol could not increase the entrapment of I. Consequently, some additional pharmaceutical techniques seemed to be necessary for preparing effective liposomal formulation of I. On the other hand, distribution of the lipophilic drug into the liposomal membrane lipids, not the in-

travesicular space, is generally considered to correspond with its organic solvent/water partition coefficient (Arrowsmith et al., 1983; Knight, 1981) and this was confirmed in the present study (Table 4). Compound V with the highest lipophilicity was almost completely entrapped into liposome using the simple procedure by its high attachment to the liposomal membrane lipids.

The utilities of liposomal preparation for prolonging retention in the injection site and increasing lymphotropicity have been demonstrated in topical administration (Juliano, 1980; Knight, 1981). A sufficient supply of anticancer agents to the lymphatic system seems to offer a promising means of preventing lymph node metastasis in cancer chemotherapy (Hashida et al., 1977; Khato et al., 1983; Tsuruo et al., 1980). Therefore, the usefulness of the liposomal dosage form containing prodrug V was evaluated using the thigh muscle of rat as a model injection site (Figs. 6 and 7). As a result, the liposomal prodrug V remained at the injection site and in the regional lymph node for a considerably longer period compared with the formulation of I. Furthermore no drug was detected in the blood after administration of liposomal V, suggesting a decrease in side-effect. These behaviors of drug will be well adapted for locally injectable dosage form with sustained release and lymphotropic delivery.

The prodrug V incorporated in liposome exhibited significant antitumor activities against L1210 leukemia (Fig. 8). Retention in the cavity and sustained release from liposome of V may cause to the high activity as same as saline suspension. These results seemed to guarantee further application of this formulation to special therapeutic use such as lymphatic metastasis prevention.

Thus the approach of integrated modification, i.e. combined use of physical and chemical modifications, seems to offer a promising future in optimization of drug delivery. The prodrug manipulation can be used as another variable in the design of carriers with controlled drug release and may expand the applicability of existing dosage forms.

Acknowledgement

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