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# Activity of Esterase in the Hydrolysis of 3′,5′-Diesters of 5-Fluoro-2′-deoxyuridine in Relation to the Structure of the Diester Prodrugs[1)]

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The activity of porcine liver esterase towards diesters of 5-fluoro-2′-deoxyuridine with saturated aliphatic acids including acetic, propionic, butyric, hexanoic, octanoic, decanoic and dodecanoic acids was investigated. The susceptibility of the 3′,5′-diesters increased as the acyl chain was lengthened up to octanoyl, but further increase in the acyl chain length resulted in a sharp decrease in the susceptibility. The susceptibility of 3′- and 5′-monoesters increased as the chain was lengthened to decanoyl and slightly decreased on going to dodecanoyl.

These results suggest that the higher antitumor activity of longer alkyl chain diesters of 5-fluoro-2′-deoxyuridine is partly due to their slow rates of hydrolysis by non-specific esterase.

**Keywords**——5-fluoro-2′-deoxyuridine; ester prodrug; partition coefficient; enzymatic hydrolysis; alkyl chain length; antitumor activity; hydrophobicity; regeneration

5-Fluorouracil (FU) and its derivatives are used clinically against various solid tumors. Although their cytotoxicity depends markedly upon the duration of the exposure of tumor cells to the drug, many of those antimetabolites have rather short half-lives *in vivo*.[2,3)]

In *in vitro* studies, 5-fluoro-2′-deoxyuridine (FUdR), one of the FU metabolites, showed up to 100 times higher activity than FU against several tumor lines,[4-7)] and it is believed to be a better precursor of 5-fluoro-2′-deoxyuridine-5′-monophosphate, an active form of FU. In *in vivo* studies, however, FUdR has been reported to be less effective than FU, partly because of the rapid degradation of FUdR to FU.[8-10)] Recently, Kanzawa *et al.* reported that several 3′,5′-diesters of FUdR with long-chain aliphatic acids show marked activity against L1210 cells *in vivo* and give higher therapeutic indices than FUdR, but 3′,5′-diesters having shorter-chain aliphatic acid appeared to be less active than FUdR.[11)] They also reported that long-alkyl-chain esters acted as FUdR precursors, affording slow release of FUdR and higher and more persistent levels of FUdR than those obtained by direct administration of FUdR.

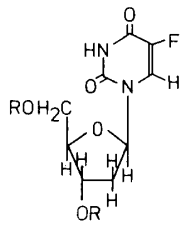
Since the ester prodrugs release FUdR *in vivo*, presumably as a result of metabolism by non-specific esterase, the rate of FUdR release from the prodrugs might be controlled by modifying the ester groupings.[12)] In the present study, the susceptibility of seven 3′,5′-diesters of FUdR with saturated aliphatic acids to porcine liver esterase was investigated.

## Experimental

**Chemicals**——FUdR was purchased from Heinrich Mack Nachf. The 3′,5′-diesters of FUdR shown in Table I were prepared according to the method of Nishizawa *et al.*[6)] The purity of each ester was determined by high performance liquid chromatography (HPLC) analysis. All other chemicals were of reagent grade quality and obtained commercially.

Stock solutions of all compounds were prepared in methanol to give a concentration of $4 \times 10^{-3}$ M, and 10 $\mu$l of

TABLE I. Structures and Physicochemical Properties of 3′,5′-Diesters of 5-Fluoro-2′-deoxyuridine

| General structure | Compound No. | –R | log $P_{oct}$ | $T_{1/2}$ (h) (pH = 7.0, 37 °C) | HPLC condition (Buffer[a]: methanol) |
|---|---|---|---|---|---|
|  | E-1 | $-CO-CH_3$ | −0.3365 | 5000 | 80:20 |
| | E-2 | $-CO-CH_2CH_3$ | 0.7760 | 5000 | 65:35 |
| | E-3 | $-CO-(CH_2)_2CH_3$ | 1.8999 | 5000 | 50:50 |
| | E-4 | $-CO-(CH_2)_4CH_3$ | 4.2500 | 1030 | 35:65 |
| | E-5 | $-CO-(CH_2)_6CH_3$ | — | 300 | 25:75 |
| | E-6 | $-CO-(CH_2)_8CH_3$ | — | 240 | 11:89 |
| | E-7 | $-CO-(CH_2)_{10}CH_3$ | — | 300 | 8:92 |
| | FUdR | –H | | | 95: 5 |

a) 0.02 M, pH 4.00 acetate buffer.

the stock solution was mixed with 1 ml of aqueous buffer or esterase preparation for kinetic studies.

**Stability Measurement in Aqueous Solutions**——The chemical hydrolysis rates were determined in 0.1 M phosphate buffer solution (pH = 7.00, $\mu = 0.22$) at 37 °C. Degradation was initiated by the addition of the stock solution to a preheated buffered solution to give a concentration of $4 \times 10^{-5}$ M. Aliquots of the solution were withdrawn at appropriate time intervals.

**Hydrophobicity Measurement**——Partition coefficients of compounds E-1—4 (see Table I) were determined in an *n*-octanol–phosphate buffer system at 25 °C according to the method of Kakemi *et al.*[13]

**Stability Measurements with Esterase System**——A partially purified porcine liver esterase (Sigma # E-9627) was used. A 1 ml aliquot of the esterase suspension (in 3.2 M $(NH_4)_2SO_4$ solution, 1500 units/ml) was diluted with 4.0 ml of 0.1 M pH 7.00 phosphate buffer, then this solution was filtered through a membrane filter (Gelman Science, 0.45 μm), and used for the experiments. The preparation was stored at 0—5 °C for no more than 50 h before use.

The hydrolytic rates of the esters were determined in the presence of the esterase preparation diluted appropriately with an isotonic phosphate buffer (pH = 7.00) containing 0.19 M sucrose. The experiments were performed at 37 °C and initiated by adding the stock solution to give a final concentration of $4 \times 10^{-5}$ and $8 \times 10^{-5}$ M. Both the decrease in concentration of diester and the increase in concentration of released FUdR were followed by HPLC analysis of samples taken periodically from the reaction mixture.

**Analysis**——Degradation of the esters and regeneration of FUdR were monitored by HPLC (LC-3A apparatus, Shimadzu) equipped with a variable-wavelength ultraviolet (UV) detector (SPD-2A, Shimadzu). Mixtures of methanol and 0.02 M pH 4.00 acetate buffer in the ratios shown in Table I were used as the mobile phase at a flow rate of 1.0 ml/min. A 10 μl sample was injected directly into a μ-Bondapack C-18 column fitted with a guard pre-column (Lichrosorb RP-18, Brownlee). Detection was achieved by UV absorption measurement at 270 nm. The detector signal was processed and recorded using a reporting integrator (C-R1B, Shimadzu).

## Results

### Hydrophobicity

Partition coefficients ($P_{oct}$) of compounds E-1—4 were determined (Table I). Plots of log $P_{oct}$ *versus* alkyl chain length of the esters showed a reasonably good linear relationship. The relationship between the number of $CH_2$ moieties ($n$) in the esters and log $P_{oct}$ is expressed by the following equation:

$$\log P_{oct}n = \log P_{oct}0 + n \times 0.573$$

$$N = 4 \quad r = 0.999$$

where $N$ and $r$ are the number of experiments and the correlation coefficient, respectively. The Partition coefficients of compounds E-5—7 could not be determined experimentally because they were very high.

### Enzymatic Hydrolysis of Diesters

The relative susceptibility of the diester prodrugs to enzymatic hydrolysis was studied *in*

*vitro* with partially purified porcine liver esterase at 37 °C. The time courses of disappearance of diesters at various esterase concentrations are shown in Fig. 1. The slopes of semi-logarithmic plots of diester concentration against time gave pseudo-first-order rate constants. Table II summarizes the effect of esterase concentration and substrate concentration on the

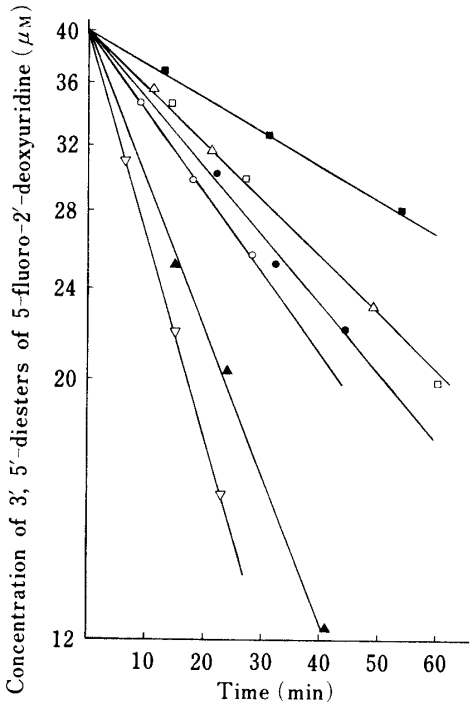



Fig. 1. Porcine Liver Esterase-Catalyzed Hydrolysis of 3',5'-Diesters of 5-Fluoro-2'-deoxyuridine

Acetyl (●, 4 units), propionyl (○, 0.1 units), butyryl (△, 0.02 units), hexanoyl (▲, 0.03 units), octanoyl (▽, 0.03 units), decanoyl (■, 0.3 units), dodecanoyl (□, 300 units).

TABLE II. Effects of Esterase Concentration and Substrate Concentration on Enzymatic Hydrolysis Rates of 3',5'-Diesters of 5-Fluoro-2'-deoxyuridine

| Compound | Enzyme concn. (unit/ml) | $K_1$ obs ($min^{-1}$) ($4 \times 10^{-5}$ M). | $K_1$ obs ($min^{-1}$) ($8 \times 10^{-5}$ M) |
|---|---|---|---|
| E-1 | 3.0 | 0.0099 | 0.0095 |
| | 12.0 | 0.036 | — |
| | 24.0 | 0.058 | — |
| E-2 | 0.15 | 0.027 | 0.028 |
| | 0.3 | 0.055 | — |
| | 0.45 | 0.082 | — |
| E-3 | 0.015 | 0.0087 | 0.0085 |
| | 0.03 | 0.021 | — |
| | 0.05 | 0.039 | — |
| E-4 | 0.015 | 0.019 | 0.021 |
| | 0.03 | 0.033 | — |
| | 0.045 | 0.055 | — |
| E-5 | 0.0075 | 0.012 | — |
| | 0.015 | 0.023 | 0.021 |
| | 0.02 | 0.033 | — |
| E-6 | 0.3 | 0.0065 | 0.0060 |
| | 0.6 | 0.014 | — |
| | 1.0 | 0.022 | — |
| E-7 | 75.0 | 0.0017 | — |
| | 150.0 | 0.0041 | 0.0048 |
| | 300.0 | 0.0092 | — |

hydrolysis rates of the diesters. Their degradation rate constants showed a significant dependence on the enzyme concentration, while the rate constants were not decreased at higher substrate concentrations. The half-lives of the diesters of FUdR in 0.1 M pH 7.00 phosphate buffer solution at 37 °C (see Table I) show that the esters are relatively stable, with half-lives of over 200 h. Since the enzymatic hydrolysis experiments were carried out within 2 h, the influence of chemical reaction on the evaluated enzymatic hydrolysis rate constants is considered to be negligible.

**Stability of FUdR in Esterase Solution**

Stability of FUdR in the presence of esterase was examined. Even at concentration of 300 units/ml, enzymatic degradation was not observed.

**Enzymatic Hydrolysis of Monoesters**

To evaluate the rate constants of enzymatic hydrolysis of the monoesters, the time courses of appearance of FUdR from each diester prodrug were measured at various esterase concentrations. Since the regeneration of FUdR by esterase should occur *via* two separate hydrolytic cleavages, diester to monoester and monoester to FUdR, the overall decomposition reaction may be described roughly by the following scheme for all diester prodrugs.

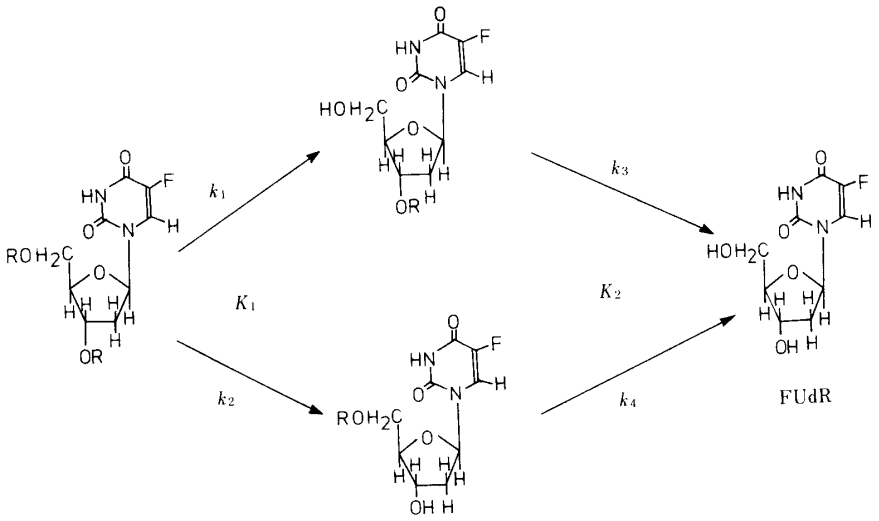



Chart 1

In this scheme, $k_1$—$k_4$ are pseudo-first-order rate constants for enzyme-mediated reactions (where $k_1+k_2=K_1$, $k_3+k_4=K_2$). The rate equation corresponding to this model can be integrated by usual integrating factor methods to give the following equations:

$$C(\mathrm{A})=C_0(\mathrm{A})e^{-K_1t} \tag{1}$$

$$C(\mathrm{C})=C_0(\mathrm{A})\,[1+1/(K_1-K_2)\times(K_2e^{-K_1t}-K_1e^{-K_2t})] \tag{2}$$

where $C_0(\mathrm{A})$ represents the initial concentration of diester prodrug, and $C(\mathrm{A})$ and $C(\mathrm{C})$ are the concentrations of diester and FUdR at time $t$.

Based on these equations, curve-fitting and parameter estimation were done by using a non-linear least-squares program.[14] Concentrations of diesters and FUdR were fitted simultaneously. Figures 2 and 3 show the time courses of disappearance of E-5 and E-2, and the appearance of FUdR in a solution of 1 unit of esterase. The full lines represent the theoretical curve based on the calculated $K_1$ and $K_2$ values. The theoretical curves were also in fair agreement with the experimental values for other prodrugs. The calculated rate constants are

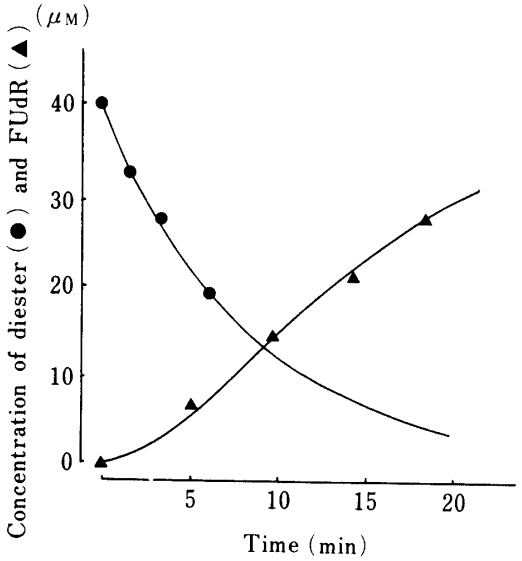



Fig. 2. Time Courses of Disappearance of 3′,5′-Dioctanoyl-5-fluoro-2′-deoxyuridine and Appearance of 5-Fluoro-2′-deoxyuridine

The solid lines represent the theoretical curves based on the calculated $K_1$ and $K_2$ values.

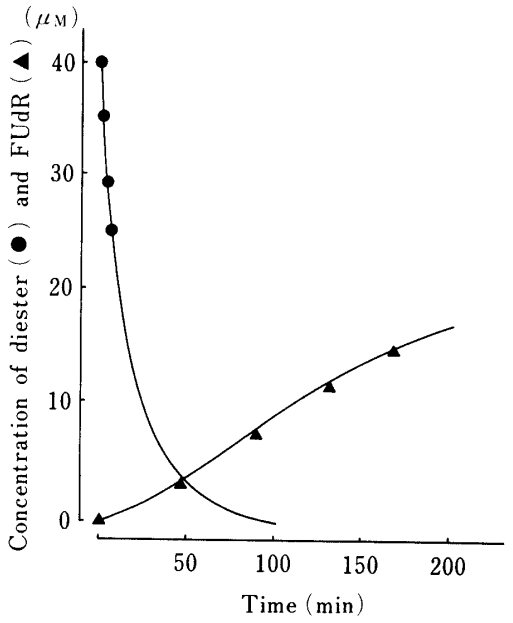



Fig. 3. Time Courses of Disappearance of 3′,5′-Dipropionyl-5-fluoro-2′-deoxyuridine and Appearance of 5-Fluoro-2′-deoxyuridine

The solid lines represent the theoretical curves based on the calculated $K_1$ and $K_2$ values.

TABLE III. Enzymatic Hydrolysis Rate Constants of Ester Prodrugs

| Compound | $K_1$ (unit$^{-1}$ min$^{-1}$) | $K_2$ (unit$^{-1}$ min$^{-1}$) | $K_1/K_2$ |
|---|---|---|---|
| E-1 | $3.14 \times 10^{-3}$ | $3.74 \times 10^{-4}$ | 8.40 |
| E-2 | $8.22 \times 10^{-2}$ | $6.62 \times 10^{-3}$ | 12.3 |
| E-3 | $6.08 \times 10^{-1}$ | $3.91 \times 10^{-2}$ | 15.6 |
| E-4 | 1.30 | $6.40 \times 10^{-1}$ | 2.03 |
| E-5 | 1.73 | 1.17 | 1.48 |
| E-6 | $3.01 \times 10^{-2}$ | 1.55 | 0.0194 |
| E-7 | $3.83 \times 10^{-5}$ | $2.70 \times 10^{-1}$ | 0.000142 |

summarized in Table III. Independent calculation from the slopes of semilogarithmic plots of diester concentration against time gave $K_1 s$ within 10% variation from those obtained by the simultaneous two-line fitting.

## Discussion

Since the cytotoxicity of FUdR depends markedly upon the duration of exposure of tumor cells to the drug, it seems possible that the cytotoxicity of FUdR would be enhanced if the FUdR level could be maintained by administering a depot form of FUdR. It was reported recently that several 3′,5′-diesters of FUdR with aliphatic acids of longer chain length are markedly active against L1210 cells *in vivo*.[9] These ester prodrugs are presumably able to suppress tumor growth by being slowly metabolized *in vivo* to FUdR.

Although many bioactivation pathways may take part in the regeneration process, non-specific esterase might be mainly responsible for this reaction.[15] Therefore, studies on the relationship between the chemical structure of the ester pro-moieties and the reactivity with

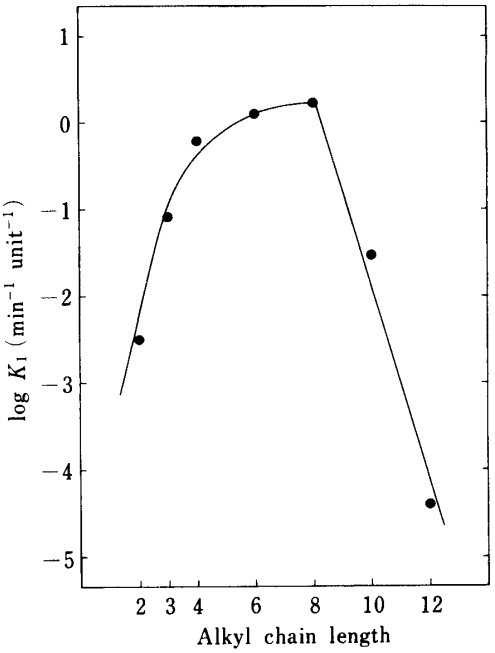



Fig. 4. Relationship between $K_1$ Values and Alkyl Chain Length

The numbers on the abscissa indicate the number of carbon atoms on the acyl moiety

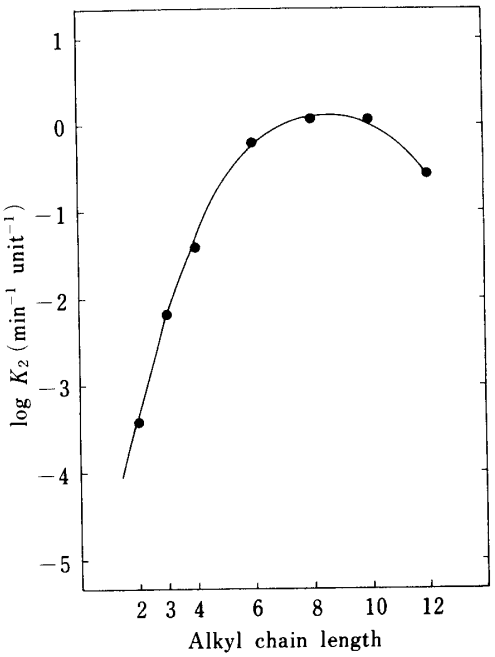



Fig. 5. Relationship between $K_2$ Values and Alkyl Chain Length

The numbers on the abscissa indicate the number of carbon atoms of the acyl moiety

non-specific esterase would be useful to provide basic data for the rational design of desirable pro-moieties for nucleoside-related compounds.

Figure 4 shows the relationship between alkyl chain length of 3′,5′-diesters of FUdR and susceptibility to porcine liver esterase. The reactivity increases as the chain is lengthened up to octanoyl. Further increase in the alkyl chain length results in a sharp decrease in the reactivity. Similarly, a plot of log $K_2$, the rate constant for enzymatic hydrolysis of 3′- and 5′-monoesters of FUdR, *versus* alkyl chain length is shown in Fig. 5. The rate ($K_2$) increases as the chain is lengthened up to octanoyl, as in the case of $K_1$, but the rate does not decrease sharply beyond decanoyl, where the rate constant is nearly equal to that of octanoate. Even for dodecanoate the decrease of $K_2$ is small. As shown in Table III, the $K_1/K_2$ ratios change to a considerable degree depending on the pro-moiety. In the cases of acetyl, propionyl and butyryl, $K_1$*s* are larger than $K_2$*s* by a factor of 10 to 20 but in the cases of hexanoyl and octanoyl the factor decreases to around 2. In compounds with longer chains, the $K_1/K_2$ ratios are reversed, being 0.0194 and 0.00014 for decanoyl and dodecanoyl, respectively. Non-specific esterase, which may be responsible for the hydrolysis of the ester prodrugs, is believed to carry a hydrophobic pocket on its active site.[16,17] The small $K_1/K_2$ ratios of E-6 and E-7 suggest that the hydrophobic pocket might be too small for the diesters with those pro-moieties, but could be reasonable size for the monoesters. On the other hand, the relatively large $K_1/K_2$ ratios for E-1 and E-2 suggest that these moieties are too short to give rise to a sufficient interaction with the hydrophobic pocket when they are introduced as monoesters. On the basis of the above results, we estimate the hydrophobic pocket to be around the size of a decyl moiety. These observations suggest that considerable specificity could perhaps be built into other nucleoside-related prodrugs as well.

The higher antitumor activity of longer alkyl chain diesters of FUdR is presumably related to the slow reactivity of E-6 and E-7 with esterase, though E-1, which is not as active as FUdR, was also observed to release FUdR slowly through enzymatic hydrolysis. One possible explanation for the conflicting observation is that the short-alkyl-chain ester may be

eliminated from the body before it is subjected to enzymatic regeneration. The low hydrophobicity, as shown in Table I, may affect the kinetic behavior of the acetyl diester of FUdR *in vivo*.

Since the present study was carried out only with porcine liver esterase, further study with other enzyme systems is necessary. For example, in the presence of pyrimidine nucleoside phosphorylases, which are responsible for the degradation of FUdR, but might also be active towards some of those FUdR prodrugs, the kinetic behavior of these prodrugs might be affected. To check this point, the kinetic behavior of these ester prodrugs has been examined with rat tissue homogenates and plasma. These results will be reported in a subsequent paper.

**References and Notes**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