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Report

Nonenzymatic and Enzymatic Hydrolysis of 5-Fluoro-2′-deoxyuridine (FUdR) Esters

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The chemical and enzymatic reactivity of 5-fluoro-2′-deoxyuridine prodrugs esterified at the 3′ and 5′ positions with several acyl groups has been investigated. The enzymatic reactivity was affected by the acyl structure, the site of esterification, and the number of esters in the prodrug molecule.

KEY WORDS: 5-fluoro-2′-deoxyuridine ester; ester prodrug; esterase; hydrolysis; enzyme specificity.

INTRODUCTION

5-Fluoro-2′-deoxyuridine (FUdR) diesters (Fig. 1), a series of FUdR prodrugs, are highly active against mouse leukemia (1,2) and rabbit hepatoma (3). Since generation of FUdR from the prodrugs requires enzymatic hydrolysis under physiological conditions (2,4), specificity of esterases for the structure and the site of esterification is an important determinant of activity. We have previously studied the structure–reactivity relationships for several esterases (4). Casida *et al.* (5) also reported on the structure–reactivity relationship in various enzyme systems including human tissue homogenates. Those results suggest that a controlled release of the parent compound in the body can be achieved by selecting an appropriate ester structure and/or site(s) of esterification.

The present report describes the hydrolytic reactivity of the 3′- and 5′-ester groups of FUdR diesters and of two series of the monoesters in several enzymatic and nonenzymatic systems.

EXPERIMENTAL

Materials. 3′, 5′-Di-acyl-FUdR was synthesized according to the method of Nishizawa *et al.* (6). 3′-Mono-acyl-FUdR was synthesized by acylation of 5′-triphenylmethyl-FUdR and subsequent detriphenylmethylation. 5′-Mono-acyl-FUdR was obtained by reacting FUdR with an equimolar acid anhydride below 0°C and was purified by removing a small amount of 3′-mono-acyl-FUdR and 3′, 5′-di-acyl-FUdR with silica gel column chromatography. All the esters were more than 98% pure as shown by one major peak in high-performance liquid chromatography (HPLC). Stock solutions of all the esters were prepared in ethanol to give a concentration of 4×10^{-3} *M* and were stored at 4°C.

3′-Acetyl-FUdR: mp 199-201°C; NMR ($CDCl_3$) δ: 1.91 (3H,s,$COCH_3$), 2.25–2.40 (2H,m,C2′), 3.98–3.99 (2H,m,C5′), 4.10–4.11 (1H,m,C4′), 5.33–5.37 (1H,m,C3′), 6.33 (1H,t,C1′), 8.04 (1H,d,J=6.5Hz,CHCF); mass spectrum, *m/e* 288 (M^+).

5′-Acetyl-FUdR: mp 145–147°C; NMR ($CDCl_3$) δ: 1.88 (3H,s,$COCH_3$), 2.31–2.41 (2H,m,C2′), 4.14–4.50 (4H,m, C3′,C4′,C5′), 6.24 (1H,t,C1′), 7.71 (1H,d,J=6.5Hz, CHCF); mass spectrum, *m/e* 288 (M^+).

3′-Pentanoyl-FUdR: mp 83–84°C; NMR ($CDCl_3$) δ: 0.89 (3H,t,CH_3), 1.25–1.37 (4H,m,CH_2), 1.60–1.67 (2H,m,$COCH_2$), 2.24–2.42 (2H,m,C2′), 3.96–3.99 (2H,m,C5′), 4.11-4.12 (1H,m,C4′), 5.34–5.37 (1H,m,C3′), 6.43 (1H,t,C1′), 8.00 (1H,d,J=6.5Hz,CHCF); mass spectrum, *m/e* 330 (M^+).

5′-Pentanoyl-FUdR: oil; NMR δ: 0.88 (3H,t,CH_3), 1.24–1.36 (4H,m,CH_2) 1.63–1.68 (2H,m,$COCH_2$), 2.29–2.42 (2H,m,C2′), 4.12–4.49 (4H,m,C3′, C4′, C5′), 6.22 (1H,t,C1′), 7.98 (1H,d,J=6.5Hz,CHCF); mass spectrum, *m/e* 330 (M^+).

3′-Octanoyl-FUdR: mp 133–134°C; NMR ($CDCl_3$) δ: 0.90 (3H,t,CH_3), 1.22–1.34 (1OH,m,CH_2), 1.61–1.67 (2H,m,$COCH_2$), 2.22–2.42 (2H,m,C2′),3.96–3.97 (2H,m,C5′), 4.10-4.14 (1H,m,C4′), 5.34–5.37 (1H,m,C3′), 6.43 (1H,t,C1′), 8.02 (1H,d,J=6.5Hz,CHCF); mass spectrum, *m/e* 372 (M^+).

5′-Octanoyl-FUdR: mp 114°C; NMR δ: 0.89

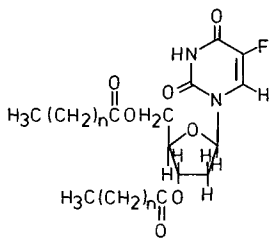

Fig. 1. Structure of 3′,5′-di-acyl-5-fluoro-2′-deoxyuridine (n = 0–10).

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(3H,t,CH_3), 1.24–1.36 (1OH,m,CH_2), 1.63–1.68 (2H,m,$COCH_2$), 2.29–2.42 (2H,m,C2′), 4.12–4.49 (4H,m, C3′, C4′, C5′), 6.22 (1H,t,C1′), 7.98 (1H,d,J=6.5Hz, CHCF); mass spectrum, *m/e* 372 (M^+).

3′-Decanoyl-FUdR: mp 130–131°C; NMR ($CDCl_3$) δ: 0.90 (3H,t,CH_3), 1.21–1.38 (14H,m,CH_2), 1.60–1.69 (2H,m,$COCH_2$), 2.22–2.41 (2H,m,C2′), 3.94–3.97 (2H,m,C5′), 4.11–4.16 (1H,m,C4′), 5.34–5.37 (1H,m,C3′), 6.43 (1H,t,C1′), 8.02 (1H,d,J=6.5Hz,CHCF); mass spectrum, *m/e* 400 (M^+).

5′-Decanoyl-FUdR: mp 99–101°C; NMR δ: 0.89 (3H,t,CH_3), 1.20–1.36 (14H,m,CH_2), 1.63–1.68 (2H,m,$COCH_2$), 2.27–2.40 (2H,m,C2′), 4.13–4.49 (4H,m,C3′, C4′,C5′), 6.22 (1H,t,C1′), 8.00 (1H,d,J=6.5Hz, CHCF); mass spectrum, *m/e* 400 (M^+).

3′-Dodecanoyl-FUdR: mp 134°C; NMR ($CDCl_3$) δ: 0.88 (3H,t,CH_3), 1.17–1.38 (18H,m,CH_2), 1.63–1.69 (2H,m,$COCH_2$), 2.25–2.41 (2H,m,C2′), 3.95-3.97 (2H,m,C5′), 4.11–4.16 (1H,m,C4′), 5.33–5.37 (1H,m,C3′), 6.43 (1H,t,C1′), 8.04 (1H,d,J=6.5Hz,CHCF); mass spectrum, *m/e* 428 (M^+).

5′-Dodecanoyl-FUdR: mp 125–126°C; NMR δ: 0.89 (3H,t,CH_3), 1.20–1.36 (18H,m,CH_2), 1.63–1.68 (2H,m,$COCH_2$), 2.27–2.40 (2H,m,C2′), 4.13–4.49 (4H,m,C3′, C4′, C5′), 6.22 (1H,t,C1′), 8.00 (1H,d,J=6.5Hz, CHCF); mass spectrum, *m/e* 428 (M^+).

Porcine liver esterase was purchased from Sigma Chemical Co. (St. Louis, Mo.). The esterase suspension (1500 U/ml) in 3.2 *M* $(NH_4)_2SO_4$ solution was diluted with isotonic 0.1 *M* phosphate buffer, pH 7.0, to give a final concentration of 200 U/ml, then the resultant solution was filtered through a membrane filter (Gelman Scientific, 0.45 μm). The esterase preparation was stored at 4°C for no more than 50 hr before use.

Human blood was collected from four volunteers with a heparinized syringe. The blood was centrifuged at 1000*g* for 15 min; the resulting plasma was transferred to 1-ml glass tubes and stored at −80°C until use.

Analytical Methods. The nonenzymatic hydrolysis rates of the FUdR esters were measured in 1 *N* HCl and 0.01 *N* NaOH solutions at 40°C. Reaction was initiated by adding a 10-μl standard solution to a 2-ml preheated solution in a glass tube. A 10-μl portion of the reacting mixture was periodically injected into a reversed-phase HPLC column (Nucleosil RP-18); and concentrations of FUdR, each of two monoesters, and a diester in each sample were determined.

The enzymatic hydrolysis rates were determined in the presence of the porcine liver esterase preparation and the human plasma diluted with an isotonic phosphate buffer, pH 7.0, containing 0.19 *M* sucrose. The experiments were performed at 37°C. The hydrolysis was initiated by adding the stock ester solution to the test solution to give an initial concentration of 4×10^{-5} *M*, the lowest concentration suitable for quantitative analysis of the ester, and 8×10^{-5} *M*. The changes in concentrations of the diester, the monoesters, and the FUdR were followed by HPLC analysis of samples taken periodically from the reaction mixture. The enzymatic reaction was not saturated at the higher substrate concentration (8×10^{-5} *M*).

Kinetic Methods. Since FUdR is expected to be regenerated from the diesters via four separate hydrolytic cleavage reactions; diester to 5′-monoester (k_1), diester to 3′-monoester (k_2), 3′-monoester to FUdR (k_3), and 5′-monoester to FUdR (k_4), the overall hydrolytic reaction can be described by Fig. 2.

In this scheme, k_{1-4} are pseudo-first-order rate constants for each hydrolytic process. The rate equation corresponding to this model can be integrated by standard integrating factor methods to give the following equations:

$$C(\mathrm{D}) = C_0(\mathrm{D})\, e^{-(k_1+k_2)t} \quad (1)$$

$$C(5\mathrm{M}) = C_0(\mathrm{D})\, k_1 [e^{-(k_1+k_2)t} - e^{-k_4 t}]/(k_4 - k_1 - k_2) \quad (2)$$

$$C(3\mathrm{M}) = C_0(\mathrm{D})\, k_2 [e^{-(k_2+k_1)t} - e^{-k_3 t}]/(k_3 - k_2 - k_1) \quad (3)$$

$$C(\mathrm{F}) = C_0(\mathrm{D}) \{1 - k_1[e^{-(k_1+k_2)t} - e^{-k_4 t}]/(k_4 - k_1 - k_2) - k_2 [e^{-(k_1+k_2)t} - e^{-k_3 t}]/(k_3 - k_2 - k_1) - e^{-(k_1+k_2)t}\} \quad (4)$$

where $C_0(\mathrm{D})$ represents the initial concentration of diester, and $C(\mathrm{D})$, $C(5\mathrm{M})$, $C(3\mathrm{M})$, and $C(\mathrm{F})$ are the concentration of diester, 5′-monoester, 3′-monoester, and FUdR at time *t*, respectively.

Based on the above equations, curve fitting and parameter estimation were carried out using a nonlinear least-squares program (7). Independent experiments and calculation using the monoester standards gave k_3 and k_4 values within 10% variation from those obtained by the simultaneous line fitting.

RESULTS AND DISCUSSION

Nonenzymatic Hydrolysis. Hydrolytic rate constants (k_1–k_4) for five leaving groups (C2-, C5-, C8-, C10-, and C12-acyl) in 1 *N* HCl at 40°C are shown in Table I. No significant difference in the hydrolytic rates was observed among the acyl groups, except for the acetates (C2-acyl). The higher reactivity of the acetates may be attributed to hyperconjugation of a methyl group in the esters. The 5′-esters, primary alcohol esters, are more reactive than the 3′-esters, secondary alcohol esters. Since the values of k_1 and k_2 are essentially the same as those of k_3 and k_4, respectively, chemical interaction between 3′- and 5′-ester groups is expected to be little.

Rate constants in 0.01 *N* NaOH at 40°C are shown in Table II. The effect of the acyl structure on alkali lability was not significant. The reactivity of the 3′- and 5′-ester was essentially the same, k_1/k_2 and k_3/k_4 were 0.8~1.1, and little

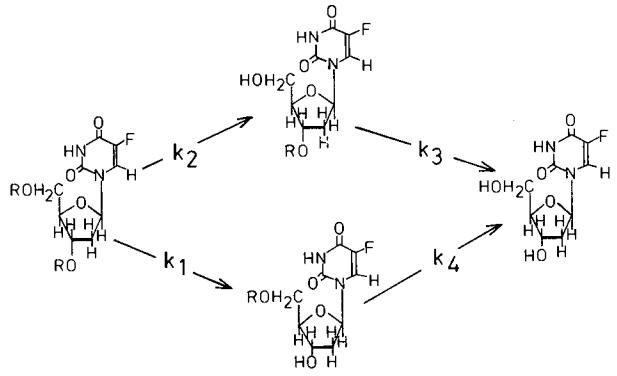

Fig. 2. Letters k_1–k_4 represent the pseudo-first-order rate constants for each hydrolysis step.

Table I. Hydrolytic Rate Constants of FUdR Esters in 1 *N* HCl at 40°C[a]

Acyl group	Rate constant (hr^{-1})				Site specificity (3′-ester/5′-ester)	
	k_1	k_2	k_3	k_4	k_1/k_2	k_3/k_4
Acetyl	0.608 ± 0.042	1.051 ± 0.061	0.550 ± 0.038	1.074 ± 0.120	0.578	0.512
Pentanoyl	0.298 ± 0.011	0.571 ± 0.020	0.322 ± 0.045	0.612 ± 0.006	0.522	0.526
Octanoyl	0.251 ± 0.036	0.492 ± 0.042	0.289 ± 0.034	0.560 ± 0.013	0.510	0.516
Decanoyl	0.200 ± 0.021	0.501 ± 0.041	0.198 ± 0.007	0.504 ± 0.022	0.399	0.393
Dodecanoyl	0.225 ± 0.015	0.456 ± 0.031	0.205 ± 0.019	0.406 ± 0.095	0.493	0.505

[a] Mean ± SD; $N = 3$. k_1, k_2, k_3, and k_4 are the pseudo-first-order rate constants of the following reactions: diester → 5′-monoester (k_1); diester → 3′-monoester (k_2); 3′-monoester → FUdR (k_3); 5′-monoester → FUdR (k_4).

Table II. Hydrolytic Rate Constants of FUdR Esters in 0.01 *N* NaOH at 40°C[a]

Acyl group	Rate constant (hr^{-1})				Site specificity (3′-ester/5′-ester)	
	k_1	k_2	k_3	k_4	k_1/k_2	k_3/k_4
Acetyl	0.336 ± 0.028	0.378 ± 0.019	0.305 ± 0.034	0.353 ± 0.047	0.889	0.864
Pentanoyl	0.299 ± 0.061	0.277 ± 0.050	0.284 ± 0.019	0.257 ± 0.019	1.080	1.105
Octanoyl	0.298 ± 0.070	0.302 ± 0.052	0.325 ± 0.001	0.339 ± 0.049	0.987	0.959
Decanoyl	0.335 ± 0.033	0.411 ± 0.055	0.315 ± 0.015	0.389 ± 0.027	0.815	0.810
Dodecanoyl	0.399 ± 0.096	0.467 ± 0.076	0.458 ± 0.071	0.511 ± 0.022	0.854	0.896

[a] Mean ± SD; $N = 3$. k_1, k_2, k_3, and k_4 are the pseudo-first-order rate constants of the following reactions: diester → 5′-monoester (k_1); diester → 3′-monoester (k_2); 3′-monoester → FUdR (k_3); 5′-monoester → FUdR (k_4).

Table III. Hydrolytic Rate Constants of FUdR Esters in the Presence of Porcine Liver Esterase (1.0 U/ml)[a]

Acyl group	Rate constant (hr^{-1})				Site specificity (3′-ester/5′-ester)	
	k_1	k_2	k_3	k_4	k_1/k_2	k_3/k_4
Acetyl	0.0024 ± 0.0003	0.049 ± 0.008	0.0019 ± 0.0002	0.0033 ± 0.0003	0.049	0.576
Pentanoyl	97.1 ± 6.2	12.6 ± 1.5	10.5 ± 0.71	13.3 ± 0.82	7.706	0.789
Octanoyl	60.1 ± 4.5	7.25 ± 0.9	39.8 ± 2.9	72.2 ± 5.9	8.290	0.551
Decanoyl	$k_1 + k_2 = 0.0056 \pm 0.0013$		25.2 ± 4.1	32.8 ± 1.0	—	0.768
Dodecanoyl	<0.0001	<0.0001	2.82 ± 0.09	14.4 ± 1.9	—	0.196

[a] Mean ± SD; $N = 3$. k_1, k_2, k_3, and k_4 are the pseudo-first-order rate constants of the following reactions: diester → 5′-monoester (k_1); diester → 3′-monoester (k_2); 3′-monoester → FUdR (k_3); 5′-monoester → FUdR (k_4).

chemical interaction between 3′- and 5′-ester groups was observed ($k_1 \fallingdotseq k_3$, $k_2 \fallingdotseq k_4$).

The above results show limited effects of the acyl structure and the site of esterification on the chemical reactivity of the FUdR esters.

Enzymatic Hydrolysis. Hydrolytic rate constants of the FUdR esters in the presence of porcine liver esterase (1.0 U/ml) at pH 7.0 are shown in Table III. The reactivity of the esterase depends remarkably on the acyl structure; the difference in the reactivity of esters was beyond 20,000-fold. Although the relative site specificity of the diesters (k_1/k_2) depends highly on the acyl group, that of the monoesters (k_3/k_4) to the acyl structure was not significant (k_3/k_4 = 0.2~0.7). In terms of an interaction between 3′- and 5′-esters, reactivities of 3′-pentanoate, 5′-acetate, and 5′-octanoate are affected by the coexistence of an ester group at the corresponding site; k_1/k_3 for the pentanoate was 9.3, and k_2/k_4 ratios were 14.9 and 0.1 for the acetate and the octanoate, respectively. Although 5′-pentanoate, 3′-acetate, and 3′-octanoate affect the reactivity of 3′-pentanoate, 5′-acetate, and 5′-octanoate, respectively, only a small effect was observed on the reactivity of the former esters by the coexistence of the latter. Since hydrolysis of the monodecanoates was much faster than that of the didecanoate, k_1 and k_2 for the decanoyl-FUdR could not be estimated separately. k_1 and k_2 of the dodecanoyl-FUdR could not be obtained because of the low reactivity of the didodecanoate to the esterase.

Hydrolytic rate constants in human plasma (20%, v/v, in the isotonic phosphate buffer, pH 7.0) are shown in Table IV. Again, significant dependence of the reactivity on the acyl structure was observed: the higher reactivity of pentanoate and octanoate in the diesters and lower reactivity of acetates in the monoesters are similar to the specificity ob-

Table IV. Hydrolytic Rate Constants of FUdR Esters in Human Plasma (20%)[a]

Acyl group	Rate constant (hr^{-1})				Site specificity (3′-ester/5′-ester)	
	k_1	k_2	k_3	k_4	k_1/k_2	k_3/k_4
Acetyl	<0.01	0.15 ± 0.028	<0.01	0.081 ± 0.018	<0.067	<0.123
Pentanoyl	0.21 ± 0.05	25.1 ± 10.5	0.18 ± 0.043	3.37 ± 0.54	0.008	0.053
Octanoyl	0.18 ± 0.04	0.75 ± 0.06	0.12 ± 0.042	8.49 ± 5.0	0.24	0.014
Decanoyl	<0.01	0.022 ± 0.01	0.43 ± 0.01	88.5 ± 13.1	<0.455	0.005
Dodecanoyl	<0.01	<0.01	0.066 ± 0.02	1.39 ± 0.51	—	0.047

[a] Mean ± SE; $N = 4$. k_1, k_2, k_3, and k_4 are the pseudo-first-order rate constants of the following reactions: diester → 5′-monoester (k_1); diester → 3′-monoester (k_2); 3′-monoester → FUdR (k_3); 5′-monoester → FUdR (k_4).

tained in the porcine liver esterase. Higher reactivity of the monodecanoates and monododecanoates over the corresponding diesters is also common to the human and porcine enzyme systems. Site specificity of the human plasma to the 3′ and 5′ positions was quite different from that of the porcine liver esterase; much higher reactivity of the 5′-esters (primary alcohol esters) was observed irrespective of the acyl structure and the presence of an acyl group at the 3′ position. Since the k_3/k_4 ratios of the octanoate, decanoate, and dodecanoate in rabbit plasma (preliminary data, 0.62, 1.12, and 0.86, respectively) were comparable to those in the porcine liver esterase, the specificity to the 5′-esters may be characteristic of human plasma.

The effect of the acyl structure on the enzymatic reactivity of FUdR esters has been evident in spite of the similar chemical reactivity of those esters. The structure–reactivity relationship in the enzymatic hydrolysis of the FUdR esters can be used to develop controlled-release preparations of FUdR *in vivo* by selecting an appropriate acyl group and/or site(s) of esterification. Although Casida *et al.* (5) concluded that the rate of acid liberation from the FUdR diesters approximated the summation of the rates with the two corresponding monoesters, the present study indicates that enzymatic reactivity of some esters is affected by an acyl group in the other position. This observation suggests the presence of an interaction between 3′- and 5′-ester groups, which affects the affinity of the FUdR ester molecules to the esterases. Different reactivity of the 3′- and 5′-esters has been observed with human plasma and with porcine liver esterase. Casida *et al.* (5) also referred to site specificity of butyryl and acetyl esters in some enzyme systems. In the present study, however, the site specificity depends on the chemical structure of the acyls and the number of ester(s) as well as the enzyme system responsible for the hydrolysis. These findings suggest that reactivity of ester prodrugs to human enzymes cannot be predicted simply from animal systems.

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