Pharmaceutical Research, Vol. 22, No. 3, March 2005 (© 2005)
DOI: 10.1007/s11095-004-1875-x

Research Paper

A New Class of 5-Fluoro-2′-deoxyuridine Prodrugs Conjugated with a Tumor-Homing Cyclic Peptide CNGRC by Ester Linkers: Synthesis, Reactivity, and Tumor-Cell–Selective Cytotoxicity

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Received May 27, 2004; accepted November 29, 2004

Purpose. Tumor-targeting prodrugs of 5-fluoro-2′-deoxyuridine (5-FdUrd), which are chemical conjugations of 5-FdUrd with a tumor-homing cyclic peptide CNGRC by succinate and glutarate linkers, were synthesized to investigate the structural effects of linkers on the hydrolytic release of 5-FdUrd and the tumor-cell–selective cytotoxicity.
Methods. A solid phase synthesis method was used to produce 5-FdUrd prodrugs. The kinetics and efficiency of hydrolytic 5-FdUrd release from the prodrugs were investigated in phosphate buffer (PB), fetal bovine serum (FBS), HT-1080 cell lysate, MDA-MB-231 cell lysate, and MEM containing 10% FBS. The tumor-cell–selective cytotoxicity of prodrugs was evaluated by an MTT method.
Results. Two tumor-targeting prodrugs **CNF1** and **CNF2** bearing 5-FdUrd conjugated with a common cyclic peptide CNGRC by succinate and glutarate linkers, respectively, and their control compounds **CN1** and **CN2** without 5-FdUrd moiety were synthesized and identified. **CNF1** underwent hydrolysis to release 5-FdUrd more rapidly and efficiently than **CNF2**. Both prodrugs were of lower cytotoxicity compared to 5-FdUrd, showing more selective cytotoxicity toward APN/CD13 positive cells (HT-1080) than toward APN/CD13 negative cells (HT-29, MDA-MB-231).
Conclusions. A new class of tumor-targeting 5-FdUrd prodrugs **CNF1** and **CNF2** were successfully synthesized. These prodrugs targeted a tumor marker APN/CD13 to cause tumor-cell–selective cytotoxicity due to 5-FdUrd release, the rate of which could be controlled by the structure of ester linker.

KEY WORDS: anticancer prodrug; cytotoxicity; 5-fluoro-2′-deoxyuridine; hydrolysis; tumor-homing peptide.

INTRODUCTION

Among the cytotoxic anticancer drugs, 5-fluoro-2′-deoxyuridine (5-FdUrd) plays a considerable function in the treatment of metastatic cancers (1). While exerting an appropriate antitumor ability via blocking thymidylate synthase to inhibit DNA synthesis and/or incorporation of its metabolites into DNA or RNA (2,3), 5-FdUrd produces various side effects including nonspecific toxicity toward rapidly proliferating normal tissues (1). Because of such pharmacological properties, efforts have been made to develop a variety of prodrugs undergoing hydrolysis to release 5-FdUrd: for example, ester derivatives (4,5) and phosphoramidate derivatives of 5-FdUrd (6). There is also a different family of prodrugs that are activated to release 5-FdUrd by ionizing radiation (7,8) or UV light (9). For more sophisticated current 5-FdUrd prodrugs, targeting strategies are used to improve the efficiency of drug delivery into tumor tissues: for example, packaging of 5-FdUrd or its prodrugs into the nanoparticles (10,11), and conjugation of 5-FdUrd with tumor-cell–targeting antibodies (12). These drug delivery systems consist of nanoparticles that can readily be transported to the well vascularized region, but not to the ischemic region with less vascularization, of tumor tissues (13–15). In a different drug delivery system using a tumor-cell–targeting antibody of foreign origin, immune response from humans/animals will cause rapid elimination of the antibody. Furthermore, because the gene of tumor cell is quickly mutated without interruption to lose the characteristic epitope, the tumor-cell–targeting antibody will become practically inactive (16,17).

Currently, particular attention has been given to the tumor vascular targeting strategy originated from the specialization of tumor vasculature (18,19). The tumor vasculature is morphologically abnormal and carries various types of tumor molecular markers that can be used to discriminate tumor vessels from normal vasculature: for example, integrin $\alpha v\beta 3$, $\alpha v\beta 5$, and aminopeptidase N (APN/CD13) (18,20). As demonstrated by the *in vivo* phage display technology, some of these markers could be recognized by certain peptides and antibody fragments (20–23). Although these findings would stimulate development of a new class of tumor-targeting anticancer prodrugs (24,25), there are still few studies on the application of such a strategy. In this view, an attempt was herein made to develop tumor-targeting prodrugs of 5-FdUrd, employing chemical conjugation with a tumor-

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homing cyclic peptide CNGRC that should target to a specific APN/CD13 isoform expressing within tumor tissues but to none of the other isoforms in normal epothelia and myeloid cells (26–27).

Previous studies demonstrated that ester derivatives of 5-FdUrd undergo hydrolysis to effectively release 5-FdUrd under physiologic conditions and thereby produce a considerable antitumor effect. It was noted that 3′-*O*-esters of 5-FdUrd showed higher antitumor effect than 3′-*O*-ester analogs *in vivo*, due in part to improved pharmacokinetic characteristics with sufficiently slow hydrolysis rate for arriving at tumor tissues (4–6). However, a quantitative structure-activity relationship of the prodrugs releasing 5-FdUrd by hydrolysis, especially the influence of linker structures on the hydrolytic activation rate, has not yet been clarified and is still a subject of further investigations. We have therefore designed and synthesized two prototypes of tumor targeting 5-FdUrd prodrugs, in which 3′-oxygen of 5-FdUrd was conjugated with a common cyclic peptide CNGRC by known linkers based on succinate (**CNF1**) and glutarate (**CNF2**) esters, respectively (28,29). As the control compounds without 5-FdUrd moiety, amide-linked derivatives of cyclic peptide CNGRC with succinic acid (**CN1**) and glutaric acid (**CN2**) were also synthesized. These peptide conjugates were investigated to elucidate the structural effect of ester linkers on the hydrolytic 5-FdUrd release from 5-FdUrd–CNGRC conjugates, the hydrolysis reaction pathway, and the cytotoxicity toward both APN/CD13 positive and negative cell lines by reference to 5-FdUrd.

MATERIALS AND METHODS

Materials

5-Fluoro-2′-deoxyuridine (5-FdUrd) and 4,4′-dimethoxytriphenylmethyl chloride were obtained from Tokyo Kasei (Tokyo, Japan). Fmoc-Cys(Acm)-PEG-PS-resin and Fmoc-amino acids such as Arg(Pbf), Asn(Trt), Cys(Acm), and Gly were purchased from Applied Biosystems (Warrington, UK). *N,N*-Diisopropylethylamine (DIPEA) and *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HATU) were received from Applied Biosystems. Succinic anhydride, glutaric anhydride, trifluoroacetic acid (TFA), trimethylsilyl chloride (TMSCl), 2-propanol, culture media PRMI-1640, and Eagle's minimal essential medium (MEM) were purchased from Nacalai Tesque (Kyoto, Japan). Fetal bovine serum (FBS) was obtained from Thermo Trace (Melbourne, Australia). 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (Steinheim, Germany). Acetonitrile, dimethylsulfoxide (DMSO), and other chemicals were purchased from Wako (Osaka, Japan). RIPA lysis buffer was received from Upstate Biotechnology (Lake Placid, NY, USA). All the chemicals were of either analytic or high-performance liquid chromatography (HPLC) grade. Cell line of HT-1080 human fibrosarcoma was supplied by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Cell lines of MDA-MB-231 human adenocarcinoma and HT-29 human colorectal adenocarcinoma were obtained from the American Type Culture Collection (www.attc.org).

General Methods

Proton nuclear magnetic resonance spectra (^{1}H NMR) and carbon nuclear magnetic resonance spectra (^{13}C NMR) were recorded on a JEOL JNM-EX-400 (400 MHz) spectrometer (JEOL, Tokyo, Japan) or a JNM-AL300 (300 MHz) spectrometer. Fast atom bombardment mass spectrometry (FAB-MS) was performed on a JEOL JMS-SX102A mass spectrometer, using glycerol or 3-nitrobenzyl alcohol (NBA) matrix. High-performance liquid chromatography was performed on a Hitachi D-7000 HPLC system (Hitachi, Tokyo, Japan), using aqueous CH_3CN solution containing 0.05% TFA as a mobile phase. An Interstil ODS-3 column (4.6ϕ × 250 mm, GL Science Inc., Tokyo, Japan) was used for analytical HPLC. Sample solution was eluted with a linear gradient of aqueous CH_3CN at a flow rate of 0.6 ml/min and detected by UV absorbance at wavelength 210 nm (for compounds **CN1**, **CN2,** and **CN3**) or 260 nm (for compounds 5-FdUrd, **CNF1,** and **CNF2**). A preparative Interstil ODS-3 column (10ϕ × 250 mm, GL Science Inc.) was used for preparative HPLC. Sample solution was eluted with a linear gradient of aqueous CH_3CN (10–26%, 20 min) at a flow rate of 3.0 ml/min and detected by UV absorbance at 210 nm.

Synthesis of 2′-Deoxy-5′-*O*-[bis(4-methoxyphenyl) phenylmethyl]-5-fluoro-3′-*O*-(3-carboxypropanoyl)uridine (Compound 2)

Intermediate compound **2** leading to the prodrug **CNF1** was synthesized as outlined in Fig. 1a. The 5′-OH of 5-FdUrd was selectively protected using 4,4′-dimethoxytriphenylmethyl chloride to give 2′-deoxy-5′-*O*-[bis(4-methoxyphenyl)phenylmethyl]-5-fluorouridine (**1**) (12). Succinic anhydride was allowed to react with **1** (0.3 mmol) in dichloromethane (DCM; 16 ml) in the presence of 4-dimethylaminopyridine (0.45 mmol). The reaction mixture was stirred for 16 h at room temperature, poured into ice-cooled aqueous solution of 5% $NaHCO_3$ (30 ml), acidified with 1 M HCl to pH 4, and then extracted with DCM (50 ml). The extract was washed by water and dried with Na_2SO_4. Evaporation of the solvent and purification by column chromatography ($CHCl_3$/MeOH, 12:1) gave **2** (169 mg, 87%): R_f = 0.25 ($CHCl_3$/MeOH, 12:1). ^{1}H NMR (300 MHz, [D_6] acetone): δ = 2.50 (m, 2 H, 2′-CH_2), 2.63 (t, J = 2.7 Hz, 4 H, Succ-CH_2), 3.36 (dd, J = 10.4, 3.2 Hz, 1 H, 5′-H_a), 3.53 (dd, J = 10.5, 4.3 Hz, 1 H, 5′-H_b), 3.78 (s, 6 H, OCH_3), 4.20 (dt, J = 3.8, 3.1 Hz, 1 H, 3′-H), 5.47 (dt, J = 5.9, 2.7 Hz, 1 H, 4′-H), 6.27 (td, J = 7.0, 1.7 Hz, 1 H, 1′-H), 6.89, 7.23–7.50 (2 m, 13 H, arom H), 7.87 (d, J = 6.7 Hz, 1H, 6-H). ^{13}C NMR (100 MHz, [D_6] acetone): δ = 29.4, 30.2 (Succ-CH_2), 38.2(C-2′), 55.4 (2 signals, OCH_3), 64.5 (C-5′), 75.4 (C-3′), 84.6(C-4′), 85.7 (C-1′), 87.6 (C-Ph_3), 113.9 (2 signals, *m*-C in MeOPh), 124.6 (d, J = 34.0 Hz, C-6), 127.6 (*p*-C in Ph), 128.6 (*o*-C in Ph), 128.7 (*m*-C in Ph), 130.8 (*o*-C in MeOPh), 136.1, 136.3 (*i*-C in MeOPh), 141.3 (d, J = 233.1 Hz, C-5), 145.6 (*i*-C in Ph), 150.3 (C-2), 157.3 (d, J = 27.3 Hz, C-4), 159.5 (C-OMe in MeOPh), 172.4, 173.4 (Succ-C=O). FAB-MS (positive mode, NBA): m/z = 648 $[M]^+$, HRMS calcd. for $C_{34}H_{33}FN_2O_{10}$ $[M]^+$ 648.2119, found 648.2142.

Synthesis of 2′-Deoxy-5′-*O*-[bis(4-methoxyphenyl) phenylmethyl]-5-fluoro-3′-*O*-(4-carboxybutanoyl)uridine (Compound 3)

Intermediate compound **3** leading to the prodrug **CNF2** was synthesized in the same way as intermediate **2** (Fig.

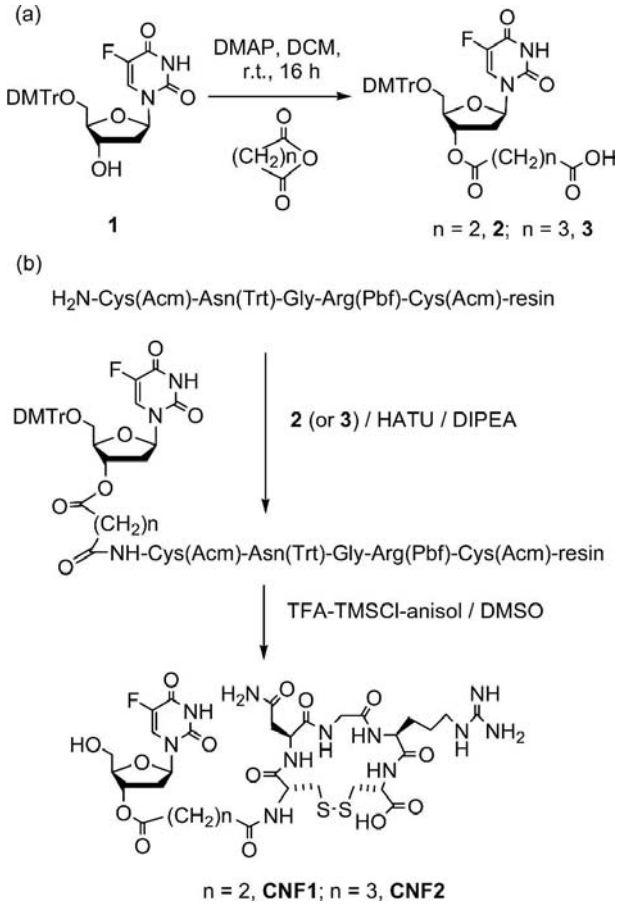

Fig. 1. (a) Syntheses of intermediate compounds **2** and **3**. (b) Syntheses of 5-FdUrd–CNGRC conjugates **CNF1** and **CNF2** with succinate and glutarate linkers, respectively.

1a). Reaction of glutaric anhydride (1.8 mmol) with compound **1** (0.3 mmol) and purification by column chromatography ($CHCl_3$/MeOH, 12:1) gave **3** (170 mg, 85%): R_f = 0.27 ($CHCl_3$/MeOH, 12:1). ^{1}H NMR (300 MHz, [D_6] acetone): δ = 1.88, (m, 2 H, Glut-CH_2), 2.35–2.52 (m, 6 H, Glut-$COCH_2$, 2′-H), 3.36 (dd, J = 10.4, 3.2 Hz, 1 H, 5′-H_a), 3.54 (dd, J = 10.4, 4.2 Hz, 1 H, 5′-H_b), 3.78 (s, 6 H, OCH_3), 4.12, 4.18 (dd*2, J = 3.3, 3.2 Hz, total 1 H, 3′-H), 5.38, 5.48 (dt*2, J = 5.9, 2.7 Hz, total 1 H, 4′-H), 6.27 (td, J = 7.9, 1.7 Hz, 1 H, 1′-H), 6.87, 7.20–7.50 (2 m, 13 H, Ph), 7.87 (d, J = 6.7 Hz, 1H, 6-H). ^{13}C NMR (75 MHz, [D_6] acetone): δ = 20.8 (Glut-CH_2CH_2), 33.1, 33.6 (Glut-CH_2C=O), 38.2(C-2′), 55.4 (2 signals, OCH_3), 64.5 (C-5′), 75.3 (C-3′), 84.8 (C-4′), 85.82 (C-1′), 87.7 (C-Ph_3), 114.0 (2 signals, *m*-C in MeOPh), 124.7 (d, J = 34.2 Hz, C-6), 127.7 (*p*-C in Ph), 128.7 (*o*-C in Ph), 128.9 (*m*-C in Ph), 130.9 (*o*-C in MeOPh), 136.3, 136.5 (*i*-C in MeOPh), 141.5 (d, J = 233.2 Hz, C-5), 145.8 (*i*-C in Ph), 149.7 (C-2), 157.4 (d, J = 26.8 Hz, C-4), 159.7 (C-OMe in MeOPh), 172.9, 174.1 (Glut-C=O). FAB-MS (positive mode, NBA): m/z = 662 $[M]^+$, HRMS calcd. for $C_{35}H_{35}FN_2O_{10}$ $[M]^+$ 662.2276, found 662.2255.

Synthesis of 5-FdUrd-3′-*O*-Succ-CNGRC Conjugate (CNF1)

Prodrug **CNF1**, 5-FdUrd-3′-*O*-Succ-CNGRC conjugate, was synthesized as shown in Fig. 1b. Protected peptide CNGRC-resin was prepared on a Pioneer peptide synthesis system (PerSeptive Biosystems Inc., Framingham, MA, USA), using Fmoc-Cys(Acm)-PEG-PS-resin (0.18 meq/g, 0.2 mmol scale) and Fmoc-amino acids Arg(Pbf), Asn(Trt), Cys(Acm), and Gly, along with HATU and DIPEA as activators. Intermediate compound **2** (330 μmol) was manually condensed with the protected peptide CNGRC-resin [equal to 66 μmol Fmoc-Cys(Acm)-PEG-PS-resin], activated by HATU (330 μmol) and DIPEA (660 μmol) in dimethylformamide (DMF; 3 ml) for 0.5 h at room temperature. After filtration and washing with DMF (5 × 5 ml) and DCM (4 × 4 ml), the resulting protected 5-FdUrd-3′-*O*-Succ-CNGRC–resin was treated with a mixture of TFA (86 ml)-TMSCl (0.72 ml)-anisole (0.99 ml) at room temperature (30). After 1 h, DMSO (12.5 ml) was added to the reaction mixture and kept for another 1 h at 4°C with stirring. After removal of the resin by filtration, ice-cooled dry diethyl ether (600 ml) was added to the filtrate. The precipitate was collected by centrifugation and then washed with ice-cooled dry diethyl ether (4 × 20 ml). The crude product was purified by preparative HPLC and freeze-dried to give fluffy white powder of prodrug **CNF1** [11.6 mg, 20% calculated from the Fmoc-Cys(Acm)-PEG-PS-resin]: Analytical HPLC: t_r = 30.1 min (1–30% CH_3CN, 40 min). ^{1}H NMR (400 MHz, [D_6] DMSO): δ = 1.42–1.54 (m, 2 H, Arg-γ-CH_2), 1.85 (m, 2 H, Arg-β-CH_2), 2.26, 2.31 (2 m, 2 H, 5-FdUrd-2′-CH_2), 2.48–2.53 (2 m, 4 H, Succ-CH_2), 2.72 (m, 2 H, Arg-δ-CH_2), 2.89 (m, 2 H, Asn-β-CH_2), 3.05–3.25 (2 m, 4 H, Cys_1-β-CH_2, Cys_2-β-CH_2), 3.63 (br. s, 1 H, 5-FdUrd-5′-CH_2), 4.00 (br. s, 1 H, 5-FdUrd-3′-CH), 4.12 (d, J = 7.6 Hz, 1 H, Gly-α-CH_a), 4.17 (d, J = 7.6 Hz, 1 H, Gly-α-CH_b), 4.27–4.35 (2 m, 2 H, Arg-α-CH, Asn-α-CH), 4.44–4.49 (m, 2 H, Cys_1-α-CH, Cys_2-α-CH), 5.20 (br. s, 1 H, 5-FdUrd-4′-CH), 6.15 (t, J = 6.8 Hz, 1 H, 5-FdUrd-1′-CH), 6.80 (br. s, 3 H, Arg-δ-NH, N=CNH_2), 7.29 (s, 2 H, Asn-$CONH_2$), 7.46 (t, J = 4.0 Hz, 1 H, Gly-α-NH), 7.72–7.74 (2 m, 2 H, CONH, C=NH), 8.17–8.21 (m, 2 H, CONH, 5-FdUrd-6-H), 8.38 (d, J = 7.6 Hz, 1 H, CONH), 8.62 (d, J = 7.2 Hz, 1 H, CONH), 11.85 (d, J = 4.8 Hz, 1 H, COOH). ^{13}C NMR(75 MHz, [D_6] DMSO): δ = 24.9 (Arg-γ-C), 28.4 (Arg-β-C), 29.0 (Succ-C=O), 29.7 (Succ-C=O), 35.8 (Asn-β-C), 36.9 (5-FdUrd-C-2′), 40.1 (Cys-β-C), 40.3 (Cys-β-C), 41.8 (Arg-δ-C), 42.7 (Gly-α-C), 49.7 (Asn-α-C), 51.2 (Arg-α-C), 52.0 (Cys-α-C), 52.5 (Cys-α-C), 61.2 (5-FdUrd-C-5′), 74.8 (5-FdUrd-C-3′), 84.5, 84.9 (5-FdUrd-C-4′, 5-FdUrd-C-1′), 124.2/124.7 (5-FdUrd-C-6), 138.6/141.6 (5-FdUrd-C-5), 149.0 (5-FdUrd-C-2), 156.7 (C=NH), 156.8/157.2 (5-FdUrd-C-4), 169.4, 170.5, 170.6, 171.5, 171.6, 172.0 (8C, C=O). FAB-MS (positive mode, glycerol): m/z = 878 $[M+H]^+$, HRMS calcd. for $C_{31}H_{45}FN_{11}O_{14}S_2$ $[M+H]^+$ 878.2573, found 878.2573.

Synthesis of 5-FdUrd-3′-*O*-Glut-CNGRC Conjugate (CNF2)

Prodrug **CNF2**, 5-FdUrd-3′-*O*-Glut-CNGRC conjugate, was synthesized in the same way as prodrug **CNF1** (Fig. 1b). Condensation of protected CNGRC-resin [equal to 66 μmol Fmoc-Cys(Acm)-PEG-PS-resin] with intermediate compound **3** and purification by preparative HPLC gave fluffy white powder of prodrug **CNF2** [10.6 mg, 18% calculated from the Fmoc-Cys(Acm)-PEG-PS-resin]: Analytical HPLC: t_r = 30.8 min (1–30% CH_3CN, 40 min). ^{1}H NMR (400 MHz, [D_6] DMSO): δ = 1.42–1.56 (m, 2 H, Arg-γ-CH_2), 1.75 (m, 2 H, Glut-β-CH_2), 1.83 (m, 2 H, Arg-β-CH_2), 2.12–2.37 (3

m, 6 H, Glut-α-CH_2, Glut-γ-CH_2, 5-FdUrd-2′-CH_2), 2.70 (m, 2 H, Arg-δ-CH_2), 2.86 (m, 2 H, Asn-β-CH_2), 3.00–3.2 (2 m, 4 H, Cys_1-β-CH_2, Cys_2-β-CH_2), 3.62 (br. s, 2 H, 5-FdUrd-5′-CH_2), 3.99 (m, 1 H, 5-FdUrd-3′-CH), 4.12 (d, J = 7.6 Hz, 1 H, Gly-α-CH_a), 4.17 (d, J = 7.6 Hz, 1 H, Gly-α-CH_b), 4.27 (m, 1 H, Arg-α-CH), 4.36 (m, 1 H, Asn-α-CH), 4.42–4.49 (m, 2 H, Cys_1-α-CH, Cys_2-α-CH), 5.21 (m, 1 H, 5-FdUrd-4′-CH), 6.14 (t, J = 7.6 Hz, 1 H, 5-FdUrd-1′-CH), 6.79 (br. s, 3 H, Arg-δ-NH, N=CNH_2), 7.32 (s, 2 H, Asn-$CONH_2$), 7.55 (dd, J = 7.6, 7.6 Hz, 1 H, Gly-α-NH), 7.74 (d, J = 8.4 Hz, 1 H, CONH), 7.80 (m, 1 H, C=NH), 8.18–8.22 (m, 2 H, CONH, 5-FdUrd-6-CH), 8.27 (d, J = 8.0 Hz, 1 H, CONH), 8.64 (d, J = 8.0 Hz, 1 H, CONH), 11.86 (d, J = 4.8 Hz, 1 H, COOH). ^{13}C NMR(100 MHz, [D_6] DMSO): δ = 20.4 (Glut-β-C), 25.0 (Arg-γ-C), 28.5 (Arg-β-C), 32.8, 34.1 (Glut-α-C=O, Glut-γ-C=O), 35.8 (Asn-β-C), 36.9 (5-FdUrd-C-2′), 40.1 (Cys-β-C), 40.4 (Cys-β-C), 42.0 (Arg-δ-C),42.7 (Gly-α-C), 49.6 (Asn-α-C), 51.1 (Arg-α-C), 52.0 (Cys-α-C), 52.3 (Cys-α-C), 61.2 (5-FdUrd-C-5′), 74.6 (5-FdUrd-C-3′), 84.4, 84.9 (5-FdUrd-C-4′, 5-FdUrd-C-1′), 124.2/124.6 (5-FdUrd-C-6), 138.8/141.6 (5-FdUrd-C-5), 148.9 (5-FdUrd-C-2), 156.6 (C=NH), 156.7/156.9 (5-FdUrd-C-4), 169.2 (C=O), 170.4 (C=O), 170.5 (C=O), 171.3 (C=O), 171.4 (C=O), 171.5 (C=O), 172.1 (2 signal, 2 C, C=O). FAB-MS (positive mode, NBA): m/z = 892 $[M+H]^+$, HRMS calcd. for $C_{32}H_{47}FN_{11}O_{14}S_2$ $[M+H]^+$ 892.2729, found 892.2744.

Synthesis of N^{α}-Hemisuccinimide-CNGRC (CN1)

Protected CNGRC-resin [equal to 20 μmol Fmoc-Cys(Acm)-PEG-PS-resin] was condensed with succinic anhydride (80 μmol) in pyridine (3 ml) for 1 h at room temperature. After filtration and washing with DMF (5 × 5 ml) and DCM (4 × 5 ml), the resulting protected N^{α}-hemisuccinimide-CNGRC-resin was treated with a mixture of TFA (26 ml)-TMSCl (0.22 ml)-anisole (0.3 ml)-DMSO (3.9 ml) in the same manner as in synthesis of **CNF1**. Precipitation and preparative HPLC gave fluffy white powder of **CN1** [5.6 mg, 43% calculated from the Fmoc-Cys(Acm)-PEG-PS-resin]: Analytical HPLC: t_r = 25.0 min (1–30% CH_3CN, 40 min). ^{1}H NMR (400 MHz, [D_6] DMSO): δ = 1.46–1.52 (m, 2 H, Arg-γ-CH_2), 1.86 (m, 2 H, Arg-β-CH_2), 2.28–2.30 (m, 4 H, Succ-α-CH_2, Succ-β-CH_2), 2.72 (m, 2 H, Arg-δ-CH_2), 2.90 (m, 2 H, Asn-β-CH_2), 3.06–3.20 (2 m, 4 H, Cys_1-β-CH_2, Cys_2-β-CH_2), 4.13 (d, J = 8.0 Hz, 1 H, Gly-α-CH_a), 4.16 (d, J = 8.0 Hz, 1 H, Gly-α-CH_b), 4.26–4.34 (m, 2 H, Arg-α-CH, Asn-α-CH), 4.42 (dt, J = 6.8, 6.8 Hz, 1 H, Cys_1-α-CH), 4.50 (dt, J = 9.2, 3.2 Hz, 1 H, Cys_2-α-CH), 6.80 (br. s, 3 H, Arg-δ-NH, N=CNH_2), 7.29 (s, 2 H, Asn-$CONH_2$), 7.48 (t, J = 5.4 Hz, 1 H, Gly-α-NH), 7.70 (d, J = 8.4 Hz, 1 H, CONH), 7.75 (m, 1 H, C=NH), 8.17 (d, J = 8.0, 1 H, CONH), 8.38 (d, J = 8.0 Hz, 1 H, CONH), 8.60 (d, J = 7.2 Hz, 1 H, CONH). ^{13}C NMR(100 MHz, [D_6] DMSO): δ = 25.0 (Arg-γ-C), 28.4 (Arg-β-C), 29.0, 30.0 (Succ-α-C=O, Succ-β-C=O), 35.7 (Asn-β-C), 40.1 (Cys-β-C), 40.4 (Cys-β-C), 42.7 (Arg-δ-C), 45.7 (Gly-α-C), 49.8 (Asn-α-C), 51.2 (Arg-α-C), 52.0 (Cys-α-C), 52.6 (Cys-α-C), 156.5 (C=NH), 169.2, 170.4, 170.5, 171.2, 171.4, 171.5, 171.8, 173.7 (8 C, C=O). FAB-MS (positive mode, glycerol): m/z = 650 $[M+H]^+$, HRMS calcd. for $C_{22}H_{36}N_9O_{10}S_2$ $[M+H]^+$ 650.2027, found 650.2022.

Synthesis of N^{α}-Hemiglutarimide-CNGRC (CN2)

CN2 was synthesized in the same way as **CN1**. Condensation of protected CNGRC-resin [equal to 20 μmol Fmoc-Cys(Acm)-PEG-PS-resin] with glutaric anhydride (80 μmol) and purification by preparative HPLC gave fluffy white powder of **CN2** [6.0 mg, 45% calculated from the Fmoc-Cys(Acm)-PEG-PS-resin]: Analytical HPLC: t_r = 24.5 min (1–30% CH_3CN, 40 min). ^{1}H NMR (400 MHz, [D_6] DMSO): δ = 1.43–1.56 (m, 2 H, Arg-γ-CH_2), 1.70 (m, 2 H, Glut-β-CH_2), 1.85 (m, 2 H, Arg-β-CH_2), 2.12–2.33 (2 m, 4 H, Glut-α-CH_2, Glut-γ-CH_2), 2.69 (m, 2 H, Arg-δ-CH_2), 2.87 (m, 2 H, Asn-β-CH_2), 3.00–3.23 (2 m, 4 H, Cys_1-β-CH_2, Cys_2-β-CH_2), 4.14 (d, J = 8.0 Hz, 1 H, Gly-α-CH_a), 4.18 (d, J = 8.0 Hz, 1 H, Gly-α-CH_b), 4.29 (td, J = 8.4, 4.8 Hz, 1 H, Arg-α-CH), 4.36 (m, 1 H, Asn-α-CH), 4.41–4.49 (m, 2 H, Cys_1-α-CH, Cys_2-α-CH), 6.80 (br. s, 3 H, Arg-δ-NH, N=CNH_2), 7.31 (s, 2 H, Asn-$CONH_2$), 7.49 (t, J = 5.6 Hz, 1 H, Gly-α-NH), 7.72 (d, J = 8.4 Hz, 1 H, CONH), 7.80 (m, 1 H, C=NH), 8.18 (d, J = 8.4, 1 H, CONH), 8.26 (d, J = 8.0 Hz, 1 H, CONH), 8.64 (d, J = 8.0 Hz, 1 H, CONH). ^{13}C NMR (100 MHz, D_2O): δ = 21.0 (Glut-β-C), 25.0 (Arg-γ-C), 28.4 (Arg-β-C), 33.6, 35.1 (Glut-α-C=O, Glut-γ-C=O), 36.3 (Asn-β-C), 41.1 (Cys-β-C), 41.2 (Cys-β-C), 42.0 (Arg-δ-C), 43.4 (Gly-α-C), 51.3 (Asn-α-C), 53.5 (Arg-α-C), 54.1 (Cys-α-C), 54.3 (Cys-α-C), 157.3 (C=NH), 171.7 (C=O), 172.6 (C=O), 173.9 (C=O), 174.5 (C=O), 175.3 (C=O), 176.6 (C=O), 178.4 (C=O). FAB-MS (positive mode, glycerol): m/z = 664 $[M+H]^+$, HRMS calcd. for $C_{23}H_{38}N_9O_{10}S_2$ $[M+H]^+$ 664.2183, found 664.2186.

Hydrolysis of 5-FdUrd–CNGRC Conjugates CNF1 and CNF2

CNF1 or **CNF2** (0.05 μmol) was dissolved in 10 mmol/L phosphate buffer (PB, 0.5 ml, pH 7.4), fetal bovine serum (FBS) / H_2O (v/v = 1:1, 0.5 ml), MEM containing 10% FBS (0.5 ml), HT-1080 cell lysate (0.5 ml) or MDA-MB-231 cell lysate (0.5 ml) and then incubated at 37°C. At various time intervals, an aliquot (10 μl) was sampled for analytical HPLC. The samples in PB and FBS were eluted with a linear gradient of aqueous CH_3CN (1–30%, 40 min), while those in MEM-10%FBS and HT-1080 cell lysate were eluted with another linear gradient of aqueous CH_3CN (5.9–30.4%, 30 min). The HPLC elution peaks of starting prodrug and hydrolysates were fractionated and identified by FAB-MS.

The concentrations of prodrugs and hydrolysates during hydrolysis were quantified by analytical HPLC, using the respective calibration curves prepared by reference to the authentic samples except for less stable intermediate **CN3** produced in the hydrolysis of **CNF1** (Fig. 3). For convenience, the concentration of **CN3** was estimated by HPLC analysis 1 h after the hydrolysis of **CNF1**, assuming that the theoretical material balance of C_t(**CN3**) = $C_{t=0}$(**CNF1**) − C_t(**CNF1**) − C_t(**CN1**) is satisfied in the initial stage of hydrolysis, where $C_{t=0}$ and C_t are the concentrations of each product at the initial time t = 0 and a given time t, respectively. Evidently, this assumption became invalid as the hydrolysis proceeded, probably due to the side reactions of **CNF1** that occurred without 5-FdUrd release.

Because the hydrolytic decompositions of **CNF1** and **CNF2** in all the media (PB, FBS, MEM-10% FBS, and HT-1080 and MDA-MB-231 cell lysates) were well represented in

terms of the first-order kinetics (for **CNF1** shown in Fig. 2d), the hydrolysis rate constants (k_1) were evaluated from Eq. (1):

$$C_t = C_{t=0} \cdot \exp(-k_1 t) \quad (1)$$

Accordingly, the half-life periods ($t_{(1/2)}$) of **CNF1** and **CNF2** were also evaluated as the measures of their stability from Eq. (2):

$$t_{(1/2)} = \ln 2/k_1 \quad (2)$$

In Vitro Cytotoxicity Assays

HT-1080 and MDA-MB-231 cells were cultured in MEM containing 1% nonessential amino acids and 10% FBS, while HT-29 cells were in RPMI-1640 medium containing 10% FBS. All the cells were grown in an atmosphere of 5% CO_2 and 90% relative humidity at 37°C.

Inhibition of the cellular growth was evaluated using a method of MTT assay described by Mosmann (31). Suspensions of 5000 cells (50 μl) were seeded in each well of a 96-well cell culture microplate and treated with either 50 μl fresh drug–medium solution at various concentrations from 10^{-10} to 10^{-3} mol/L or 50 μl control medium solution. These plates were incubated for 72 h at 37°C under an atmosphere of humidified 5% CO_2, and then 10 μl of 5 mg/ml MTT in PBS was added to each well. After MTT cleavage for 4 h at 37°C, 100 μl of 0.04 N HCl in 2-propanol was added to each well to dissolve the dark blue crystals. The absorbance at a test wavelength of 570 nm was measured on a Model-550 Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA). The concentration of drug to reduce the cell survival to half of the control value (IC_{50}) was calculated from a regression analysis of cell survival vs drug concentration.

Preparation of Cell Lysates

HT-1080 or MDA-MB-231 cells (about 4×10^7 cells) were collected from 8 dishes (ϕ100 mm dish/75 cm^2), washed twice with ice-cold PBS, followed by centrifugation at 1200 rpm in a table-top centrifuge for 5 min to pelletize the cells. The resulting cell pellet was added to ice-cold RIPA lysis buffer (4 ml, pH 7.4) and shaken for 15 min to lyse cells. The lysate was centrifuged at 14,000 rpm for 15 min at 4°C, and then the supernatant was transferred to seven fresh tubes for hydrolysis studies of peptide conjugates **CNF1** and **CNF2**.

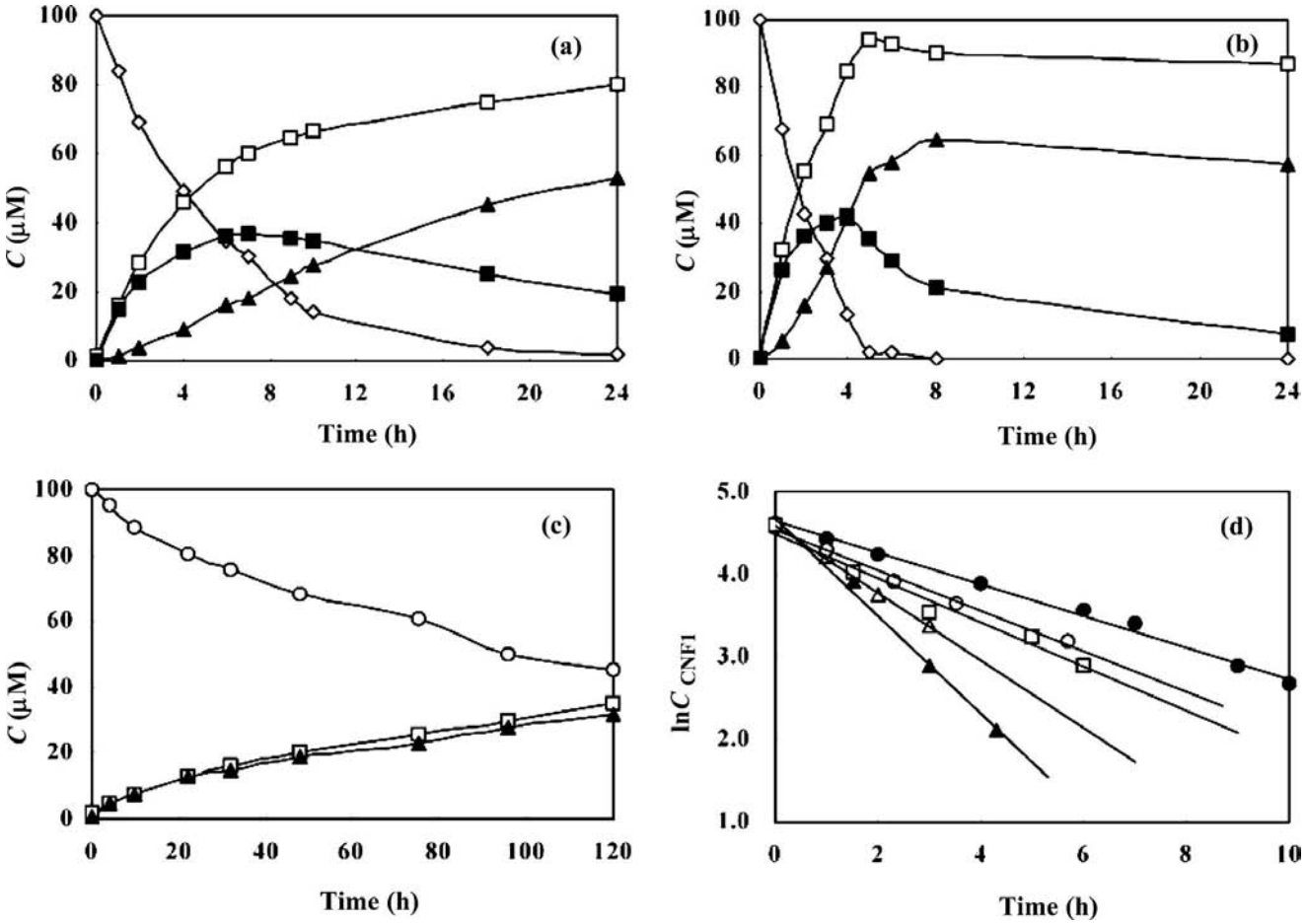

Fig. 2. (a) Concentration *vs.* time plots for (◇) **CNF1**, (□) 5-FdUrd, (■) **CN3**, and (▲) **CN1** in the hydrolysis of 0.1 mM **CNF1** in 10 mM phosphate buffer (PB, pH 7.4) at 37°C. (b) Concentration *vs.* time plots for (◇) **CNF1**, (□) 5-FdUrd, (■) **CN3**, and (▲) **CN1** in the hydrolysis of 0.1 mM **CNF1** in fetal bovine serum (FBS) at 37°C. (c) Concentration *vs.* time plots for (○) **CNF2**, (□) 5-FdUrd, and (■) **CN2** in the hydrolysis of 0.1 mM **CNF2** in 10 mM PB (pH 7.4) at 37°C. (d) First-order kinetic plots for the hydrolysis of **CNF1** in (●) PB (pH 7.4), (○) HT-1080 cell lysate, (□) MDA-MB-231 cell lysate, (Δ)FBS, and (▲) MEM + 10% FBS.

Extracellular and Intracellular Concentrations of 5-FdUrd and Its Prodrugs CNF1 and CNF2

For analyzing the cellular concentrations of 5-FdUrd and its prodrugs (**CNF1** and **CNF2**), we used a similar method as previously reported in the analysis of 5-FdUrd within EMT6 cells (32). 5-FdUrd, **CNF1** or **CNF2** (14 μmol) was added to 70 ml of HT-1080 (or MDA-MB-231) cell suspension (10^7 cells/ml in MEM containing 10% FBS medium) in a spinner flask rotated at 100 rpm at room temperature. At appropriated time intervals, aliquots of the cell suspensions (6 ml) were centrifuged at 1200 rpm for 5 min. The supernatant (0.1 ml) was transferred to a tube and 0.1 ml of 1% aqueous CH_3CN containing HCl (pH 0.5) was added to stop the hydrolysis. The resulting solution was sampled for analyzing extracellular concentration of 5-FdUrd or prodrug by analytical HPLC with a linear gradient of aqueous CH_3CN (5.9–30.4%, 30 min). After washing by PBS, the cell pellet was treated by 0.1 ml of 1% aqueous CH_3CN containing HCl (pH 0.5) and sonicated for 5 min to dissolve 5-FdUrd or prodrug. The suspension was transferred to a centrifugal filter Microcon YM-3 (Millipore Co., Bedford, MA, USA) and centrifugated at 14,000 rpm for 20 min at 4°C. The filtrate thus obtained was sampled for analyzing intracellular concentration of 5-FdUrd or prodrug by analytical HPLC with a linear gradient of aqueous CH_3CN (5.9–30.4%, 30 min).

RESULTS AND DISCUSSION

Synthesis of 5-FdUrd–CNGRC Conjugates with Ester Linkers

The 5′-OH–protected 5-FdUrd derivative **1**, which was synthesized according to the procedure of Goerlach (12), was condensed with succinic anhydride or glutaric anhydride to obtain two types of intermediate compounds **2** and **3** leading to the corresponding 5-FdUrd prodrugs **CNF1** and **CNF2**, respectively (Fig. 1a). Protected peptide CNGRC-resin prepared by the Fmoc solid phase synthesis method was condensed with the intermediate compounds **2** and **3**, followed by elimination of protecting groups and disulfide cyclization with the TFA-TMSCl-anisole-DMSO system in one-pot procedure to produce **CNF1** and **CNF2**, respectively, as shown in Fig. 1b (30). The overall yields of prodrugs **CNF1** and **CNF2** were 20% and 18%, respectively. The lower yields than those of **CN1** and **CN2** (see below), are attributable to partial decompositions of 5-FdUrd-3′-*O*-Succ-CNGRC-resin and 5-FdUrd-3′-*O*-Glut-CNGRC-resin at their 3′-*O*-ester linkages during the treatment with TFA-TMSCl-anisole-DMSO.

In order to identify the molecular mechanism of **CNF1** and **CNF2** as the potential antitumor prodrugs, the corresponding control compounds N^{α}-hemisuccinimide-CNGRC (**CN1**) and N^{α}-hemiglutarimide-CNGRC (**CN2**) were also synthesized. The protected peptide CNGRC-resin was similarly condensed with succinic anhydride or glutaric anhydride, followed by elimination of protecting groups and disulfide cyclization with the TFA-TMSCl-anisole-DMSO system to yield **CN1** (43%) or **CN2** (45%). The purity of each peptide conjugate was more than 97%, as confirmed by analytical HPLC.

Hydrolysis of 5-FdUrd–CNGRC Conjugates Releasing 5-FdUrd

Ester compounds have been well-known to undergo both nonenzymatic and enzymatic hydrolysis in physiologic environment (28,29). We therefore studied the hydrolysis of 5-FdUrd–peptide conjugates, **CNF1** with succinate linker, and **CNF2** with glutarate linker in phosphate buffer (PB) at pH 7.4, fetal bovine serum (FBS), MEM containing 10% FBS, and HT-1080 and MDA-MB-231 cell lysates.

Figure 2a illustrates a time-course profile observed for the hydrolysis of **CNF1** to release 5-FdUrd in phosphate buffer at pH 7.4. The 5-FdUrd release accompanied the formation of N^{α}-succinimide-CNGRC (**CN3**: positive FAB-MS (glycerol) m/z = 632 $[M+H]^+$; HRMS found 632.1872, calcd. for $C_{22}H_{34}N_9O_9S_2$ $[M+H]^+$ 632.1921; analytical HPLC retention time t_r = 29.5 min) as an intermediate. The formations of similar intermediates were previously reported in the hydrolysis of 3′-azido-3′-deoxythymidine-5′-*O*-Succ-peptide conjugates (28). The intermediate peptide **CN3** was subsequently hydrolyzed and converted to the final product **CN1**. The main hydrolysis mechanism of **CNF1** is outlined in Fig. 3, while the side reactions leading to unknown by-products occurred simultaneously with the main hydrolysis, as confirmed by the analytical HPLC. Similar characteristics involving the intermediate **CN3** were also observed in the hydrolysis of **CNF1** in FBS (Fig. 2b), MEM-10% FBS and cell lysates (data not shown). As plotted in Fig. 2d, the hydrolysis of **CNF1** in each medium was of a first-order reaction kinetics with respective rate constant (Table I). Obviously, the hydrolysis rates of **CNF1** in FBS-containing media and cell lysates are greater than that in PB. This acceleration effect suggests that some

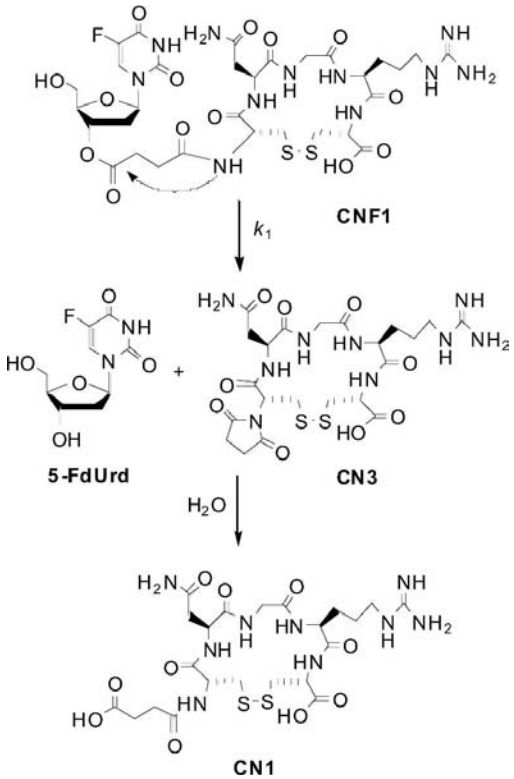

Fig. 3. Reaction pathway for the hydrolysis to release 5-FdUrd of 5-FdUrd–CNGRC conjugate **CNF1** with succinate linker.

Table I. Hydrolyses of 5-FdUrd-CNGRC Conjugates in Various Media at 37°C

Prodrug	Medium	k_1 (h^{-1}) (mean ± SD, n = 3)	$t_{1/2}$ (h) (mean ± SD, n = 3)	Yield of 5-FdUrd (%) after hydrolysis for a given time (h) shown in the parentheses	Selectivity of 5-FdUrd release (%)
CNF1	PB (pH 7.4)	0.185 ± 0.004	3.74 ± 0.08	80 (24)	82
CNF1	FBS	0.410 ± 0.003	1.69 ± 0.01	90 (6)	93
CNF1	MEM + 10% FBS	0.580 ± 0.010	1.19 ± 0.04	83 (6)	85
CNF1	HT-1080 cell lysate	0.255 ± 0.011	2.72 ± 0.11	70 (6)	92
CNF1	MDA-MB-231 cell lysate	0.263 ± 0.01	2.63 ± 0.08	71 (6)	87
CNF2	PB (pH 7.4)	0.0066 ± 0.0001	105.0 ± 1.6	13 (24), 35 (120)	63
CNF2	FBS	0.0098 ± 0.0007	70.9 ± 5.3	13 (24), 41 (120)	66
CNF2	MEM + 10% FBS	0.0414 ± 0.0019	16.7 ± 0.8	40 (24), 57 (48)	66
CNF2	HT-1080 cell lysate	0.043 ± 0.004	16.2 ± 1.4	32 (24)	48
CNF2	MDA-MB-231 cell lysate	0.041 ± 0.001	16.9 ± 0.4	31 (24)	49

enzymatic catalysis may be involved in the hydrolysis of **CNF1**.

In contrast to the behavior of **CNF1**, hydrolysis of **CNF2** in all media (PB, FBS, cell culture medium, or cell lysates) led directly to final products 5-FdUrd and **CN2** in almost equimolar amounts (see Fig. 2c for a representative time-course in PB) without formation of intermediate peptide like **CN3**. The hydrolysis of **CNF2** in each medium was also represented by a first-order reaction kinetics with its own rate constant (Table I). The hydrolysis rate constants also show the presence of enzymatic catalysis accelerating the hydrolysis of **CNF2** in FBS-containing media and cell lysates.

Structural Effect of Ester Linkers on the Stability and 5-FdUrd Release Efficiency of 5-FdUrd–CNGRC Conjugates

The 5-FdUrd–CNGRC conjugates, **CNF1** with succinate and **CNF2** with glutarate linkers, showed their stability and 5-FdUrd releasing efficiency, depending on their linker structures (Table I). The hydrolysis rate constants of **CNF1** are about 6~42 times greater than the corresponding values of **CNF2**. The hydrolysis of **CNF1** in each medium also gave higher 5-FdUrd yield than that of **CNF2**. For example, the hydrolysis of **CNF1** in MEM-10% FBS for 6 h gave 83% yield of 5-FdUrd, while the hydrolysis of **CNF2** even for up to 48 h only 57% yield. **CNF1** showed much higher selectivity of hydrolysis to release 5-FdUrd than **CNF2:** the hydrolysis of **CNF1** and **CNF2** resulted in 82~93% and 48~66% selectivity, respectively (Table I). The higher hydrolysis reactivity of **CNF1** relative to **CNF2** for releasing antitumor agent 5-FdUrd in both nonenzymatic and enzymatic hydroyses, can be interpreted in terms of intramolecular catalysis effect of succinate linker, as is evident from the formation of intermediate hydrolysate **CN3** bearing succinimide moiety (see Figs. 2 and 3).

In another aspect, by reference to similar conjugates consisting of ester linkers derived from primary alcohols such as 3′-azido-3′-deoxythymidine-5′-*O*-Succ-peptide conjugates (unstable in FBS) (28), doxorubicin-14-*O*-Glut-CNGRC conjugate ($t_{(1/2)}$ = 53 min in human serum; $t_{(1/2)}$ = 76 min in cell culture medium DMEM containing 10% FCS) (33), and another doxorubicin-14-*O*-Glut-peptide conjugate AN-152 ($t_{(1/2)}$ = 19.5 min in mouse serum) (29); the greater stability of **CNF1** and **CNF2** should be attributed to their ester linkers derived from the secondary alcohol 3′-OH of 5-FdUrd. Thus, the ester linker structure is one of the significant factors that control the pharmacokinetic properties of CNGRC-conjugated anticancer prodrugs.

Tumor-Cell–Selective Cytotoxicity

In order to get insight into the anticancer effect and the specificity to tumor marker APN/CD13 of 5-FdUrd–CNGRC conjugates as the prodrugs, *in vitro* cytotoxicity was evaluated by an MTT method. Three kinds of cells were used for the *in vitro* cytotoxicity experiments, including APN/CD13 positive cell line of HT-1080 as well as APN/CD13 negative cell lines of HT-29 and MAD-MB-231 (33–35). As summarized in Table II, neither **CN1** nor **CN2** affected the cell survival fraction in the concentration range from 10 nM to 1 mM. While **CNF1** and **CNF2** had lower cytotoxicity in comparison with the typical anticancer agent of 5-FdUrd, their apparent cytotoxicity was related to their hydrolysis rate and 5-FdUrd yield: **CNF1** with higher hydrolysis rate and yield of toxic 5-FdUrd (Table I) showed about 6–200 times higher cytotoxicity than **CNF2**. It should be more striking that **CNF1** showed almost the same level (only 1.6 times lower) of cytotoxicity as

Table II. Cytotoxicity of 5-FdUrd and Its Prodrug 5-FdUrd-CNGRC Conjugates **CNF1** and **CNF2** Toward Various Tumor Cells

Cell lines	IC_{50} (M, mean ± SD, n = 5)				
	CNF1	**CNF2**	5-FdUrd	**CN1**	**CN2**
HT-1080	$(4.0 \pm 1.6) \times 10^{-8}$	$(9.0 \pm 3.6) \times 10^{-6}$	$(2.5 \pm 0.2) \times 10^{-8}$	Nontoxic	Nontoxic
HT-29	$(5.0 \pm 0.4) \times 10^{-6}$	$(1.0 \pm 0.2) \times 10^{-4}$	$(1.0 \pm 0.2) \times 10^{-8}$	Nontoxic	Nontoxic
MDA-MB-231	$(8.0 \pm 0.7) \times 10^{-6}$	$(5.0 \pm 0.8) \times 10^{-5}$	$(7.0 \pm 2.0) \times 10^{-9}$	—	—

5-FdUrd toward APN/CD13 positive cell lines of HT-1080, while being 500 to almost 1000 times lower cytotoxicity than 5-FdUrd toward APN/CD13 negative cell lines HT-29 and MAD-MB-231, respectively. Similar tumor-selectivity was also observed for **CNF2**, though its selectivity was obviously lower than that of **CNF1**. **CNF2** caused about 360 times lower cytotoxicity toward HT-1080, while about 7100 to 10,000 times lower cytotoxicity toward HT-29 and MDA-MB-231 cells, relative to 5-FdUrd.

In order to clarify the tumor-selective cytotoxicity, we further investigated the cellular concentrations of 5-FdUrd and prodrugs (**CNF1** and **CNF2**) after incubation of 0.2 mM 5-FdUrd or prodrugs, respectively, in HT-1080 (or MDA-MB-231) cell suspension at room temperature. As shown in Fig. 4, the intracellular concentration of 5-FdUrd within HT-1080 cells exposed to 0.2 mM **CNF1** quickly reached the same level as in the exposure to 0.2 mM 5-FdUrd, while the concentration within MDA-MB-231 cells exposed to 0.2 mM **CNF1** was considerably lower level. It is also noted that the intracellular concentration of **CNF1** within HT-1080 cells was obviously higher than that within MDA-MB-231 cells, and even higher than the corresponding extracellular concentration. Similar results were also observed for **CNF2**. After exposure to 0.2 mM **CNF2**, the intracellular concentration of **CNF2** within HT-1080 cells was up to about 0.1 mM within 1 h and maintaining on this level for more than 12 h, while being only on a level of 0.04~0.05 mM within MDA-MB-231 cells during 12 h. After exposure for 12 h, the intracellular concentration of 5-FdUrd within HT-1080 and MDA-MB-231 cells arrived at 0.055 mM and 0.035 mM, respectively. These are obviously lower than those observed for **CNF1**, in accord with the lower hydrolysis reactivity and cytotoxicity of **CNF2** comparing to **CNF1**. The above results indicate that 5-FdUrd-CNGRC conjugates would target APN/CD13 positive cells and enhance the intracellular concentration of their hydrolysate 5-FdUrd, thereby showing the tumor-selective cytotoxicity. Such a tumor-selective cytotoxicity of **CNF1** and **CNF2** is attributable to the conjugation of 5-FdUrd with a tumor homing peptide CNGRC that has an ability of binding selectively to a specific protein APN/CD13 overexpressed on the tumor tissue. In a separate experiment, we have also confirmed that a fluorescent probe molecule bearing the tumor homing peptide CNGRC could penetrate into APN/CD13 positive HT-1080 cells, but not into APN/CD13 negative MDA-MB-231 cells (data reported elsewhere). In these contexts, the previous study demonstrated that CNGRC enhances the selective accumulation of its conjugate with TNF (tumor necrosis factor alpha) in tumor tissues, thereby resulting in higher immunotherapeutic effect *in vivo* (27,36). On the other hand, in view of lower cytotoxicity toward APN/CD13 negative cells, the 5-FdUrd–CNGRC conjugates **CNF1** and **CNF2** may be tumor vascular targeting anticancer prodrugs that show lower side effect on normal cells. A similar result was previously observed where the doxorubicin–CNGRC conjugate exhibited lower side effect than free doxorubicin (24). The strategy of tumor vascular targeting prodrug should be further promoted by the investigation on the mapping of human vasculature (37), and the tumor-homing peptide could also be a useful device for modifying nanoparticles that can deliver packaged agents to tumor tissues (38).

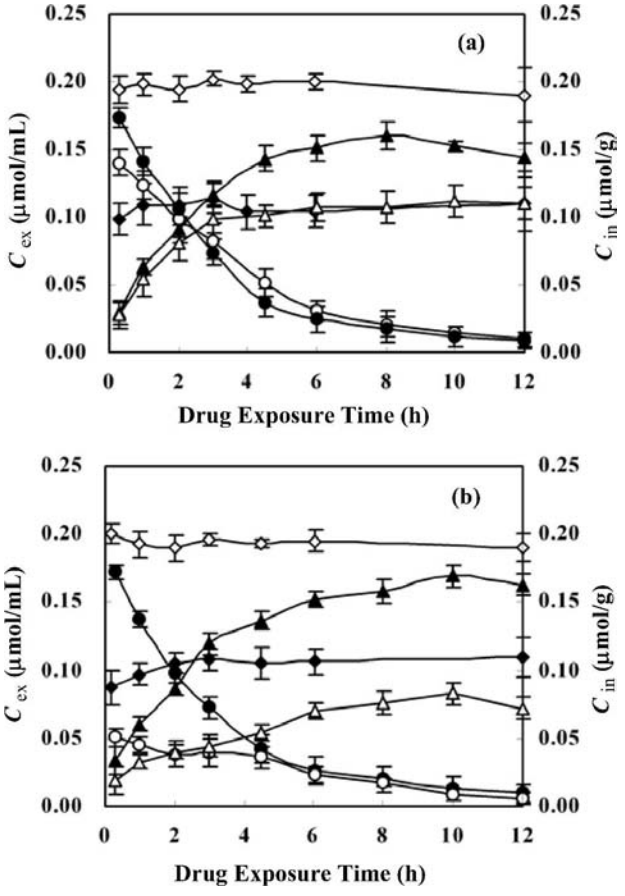

Fig. 4. (a) Extracellular concentration (◊) and intracellular concentration (♦) of 5-FdUrd after incubation of 0.2 mM 5-FdUrd in HT-1080 cell suspension; extracellular concentrations of (●) **CNF1** and (▲) 5-FdUrd and intracellular concentrations of (○) **CNF1** and (Δ) 5-FdUrd after incubation of 0.2 mM **CNF1** in HT-1080 cell suspension. (b) Extracellular concentration (◊) and intracellular concentration (♦) of 5-FdUrd after incubation of 0.2 mM 5-FdUrd in MDA-MB-231 cells suspension; extracellular concentrations of (●) **CNF1** and (▲) 5-FdUrd and intracellular concentrations of (○) **CNF1** and (Δ) 5-FdUrd after incubation of 0.2 mM **CNF1** in MDA-MB-231 cell suspension.

CONCLUSIONS

A new class of 5-FdUrd prodrugs **CNF1** and **CNF2** conjugated with a tumor-homing cyclic peptide CNGRC by succinate and glutarate linkers have been designed and synthesized. The linker structure affected the hydrolysis mechanism and efficiency of 5-FdUrd–CNGRC conjugate to release 5-FdUrd, thus being a useful tool to control the pharmacokinetic property. Although both **CNF1** and **CNF2** had higher selectivity of cytotoxicity toward APN/CD13 positive tumor cell line of HT-1080 than toward APN/CD13 negative cell lines of HT-29 and MDA-MB-231, **CNF1** is evidently more promising as tumor-targeting prodrug of 5-FdUrd than **CNF2**.

ACKNOWLEDGMENTS

The authors thank Prof. Isao Morishima for kindly supplying the Peptide Synthesis System. The authors are grateful to Prof. Side Yao, Prof. Yuta Shibamoto, Dr. Satoshi Takahashi, and Dr. Tetsunari Kimura for their valuable discussions.

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