Bioorganic & Medicinal Chemistry 20 (2012) 5164–5168

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and one-electron reduction characteristics of radiation-activated prodrugs possessing two 5-fluorodeoxyuridine units

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ARTICLE INFO

Article history:
Received 13 June 2012
Revised 2 July 2012
Accepted 3 July 2012
Available online 14 July 2012

Keywords:
Prodrug
X-ray irradiation
One-electron reduction
5-Flurodeoxyuridine

ABSTRACT

Two molecules of an antitumor agent, 5-fluorodeoxyuridine (5-FdUrd), were connected by a 2-oxoalkyl linker (Oxo-linker) at the N(3) position to obtain radiation-activated prodrugs, $FdUrd_2$ A and $FdUrd_2$ B. The prodrugs in this study released 5-FdUrd via one-electron reduction initiated by hypoxic X-irradiation. The release of 5-FdUrd from $FdUrd_2$ A and $FdUrd_2$ B proceeded more efficiently than that of previous prodrug, Oxo-FdUrd, which possessed one molecule of 5-FdUrd. $FdUrd_2$ A exhibited increased cytotoxicity against A549 cells when the $FdUrd_2$ A solution had been irradiated with a large dose of X-rays before administration to the cells. However, we observed no effect on cytotoxicity when the cells were X-irradiated under hypoxic conditions in the presence of $FdUrd_2$ A because the amount of 5-FdUrd released in the cells seemed to be too low to induce cytotoxic activity.

1. Introduction

A number of stimuli-responsive prodrugs have been developed and investigated extensively with the aim of maximizing their potential and minimizing side effects. Several stimuli such as pH change,[1,2] enzymatic reaction,[3–5] and photoirradiation[6–9] have been used to activate prodrugs to express their original activities. The use of stimuli-responsive prodrugs offers an interesting opportunity for the efficient and precise onset of drug potency.[10,11]

One new strategy is the use of X-irradiation as an external trigger to activate a prodrug.[12–17] Since the chemical reactions triggered by this stimulation can be controlled spatially and temporally, the prodrug can be converted to its active form with exact control over the area, time, and dosage. The prodrug does not exhibit cytotoxicity without irradiation, but a prodrug can be activated irreversibly by X-irradiation to produce the original cytotoxicity by removing labile substituents to form parent drug. We have identified a series of 2-oxoalkyl groups as reactive substituents that are removable by hypoxic X-irradiation in aqueous solution.[18–21] An activation mechanism has been proposed by which the 2-oxoalkyl group undergoes one-electron reduction by hydrated electrons (e_{aq}^-) generated via radiolysis of water to form the corresponding π^* anion radical, followed by thermal activation into the σ^* anion radical, which is readily hydrolyzed to release the 2-oxoalkyl group. We have applied these characteristics of the 2-oxoalkyl group to develop the following prodrugs: 1-(2′-oxopropyl)-5-fluorouracil,[18] 2′-deoxy-5-fluoro-3-(2′-oxoalkyl)uridine[20] and 4-(2-oxopropylamino)-1-(β-arabinofuranosyl)pyrimidine-2-one.[21] These were activated to release 5-fluorouracil (5FU), 5-fluoro-2′-deoxyuridine (5-FdUrd) and cytarabine upon hypoxic X-irradiation. Because of the highly cytotoxic effect, these drugs have been widely used as an antineoplastic and antiviral agent. However, high dose of drug causes serious side effects, and thereby there has been a demand for giving a target specific feature to drugs that could discriminate between tumor and normal cells for cytotoxicity.

Herein, we describe our design of the radiation-activated prodrug of 5-FdUrd, $FdUrd_2$ A and $FdUrd_2$ B (Fig. 1), to establish guides for the molecular design of 5-FdUrd prodrugs. For an emergence of highly potent cytotoxicity, two 5-FdUrd units were connected with a 2-oxoalkyl linker (Oxo-linker) to produce the prodrugs, which undergo one-electron reduction by X-irradiation to liberate the two parent drugs of 5-FdUrd. The release of 5-FdUrd upon X-irradiation proceeded predominantly under hypoxic conditions, which has been identified as one of the most important features of solid tumors.[22] We report on the synthesis of the prodrugs and characterization of their radiolytic reduction and cytotoxic effect in living cells.

2. Results and discussion

The syntheses of the 5-FdUrd derivatives, $FdUrd_2$ A and $FdUrd_2$ B, are outlined in Scheme 1. Two molecules of acetylated 5-FdUrd 1[23] were coupled with 1,3-dibromo-2-propanone, and the acetyl groups were then removed under basic conditions to give $FdUrd_2$ A.[24] $FdUrd_2$ B and the control compound $FdUrd_2$ C were obtained

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http://dx.doi.org/10.1016/j.bmc.2012.07.008

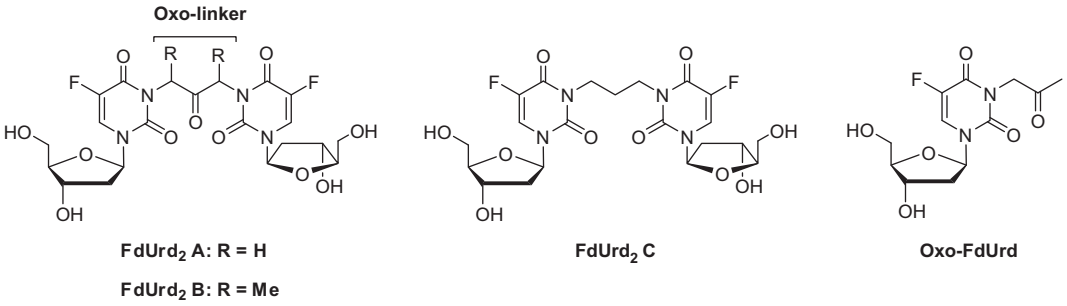
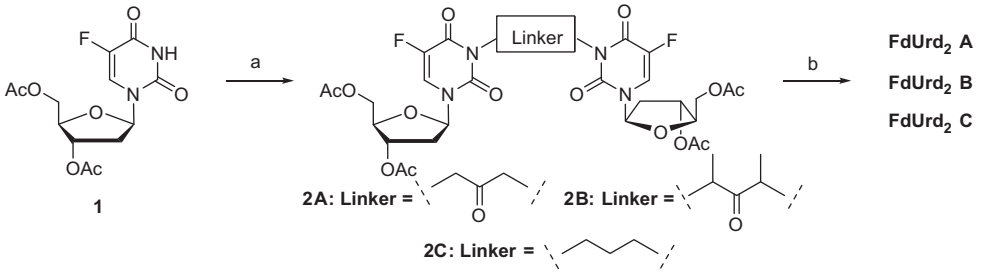


Figure 1. Chemical structure of prodrugs and drug ($FdUrd_2$ A, $FdUrd_2$ B, $FdUrd_2$ C and Oxo-FdUrd) used in this study.



Scheme 1. Reagents and conditions: (a) 1,3-dibromo-2-propanone (for 2A), 2,4-dibromo-3-pentanone (for 2B), 1,3-dibromopropane (for 2C), NaH, DMF, 71% (for 2A), 20% (for 2B), 63% (for 2C); (b) NaOH, THF, MeOH, quant. (for $FdUrd_2$ A), 87% (for $FdUrd_2$ B), quant. (for $FdUrd_2$ C).

similarly using 2,4-dibromo-3-pentanone and 1,3-dibromopropane, respectively.[24]

We performed the one-electron reduction of $FdUrd_2$ A using X-radiolysis in argon-purged aqueous solution containing an excess amount of 2-methyl-2-propanol, which scavenges hydroxyl radicals to suppress any possible side reactions.[25] Under these conditions, hydrated electrons (e_{aq}^-) are generated as one of the active species by the radiolytic degradation of water molecules. The representative reaction profile of $FdUrd_2$ A is shown in Figure 2. Hypoxic X-irradiation of $FdUrd_2$ A induced the formation of a single new product, which was assigned as 5-FdUrd according to the overlapped injection of authentic sample. As shown in Table 1, the G values[26] for the decomposition of $FdUrd_2$ A and the formation of 5-FdUrd were estimated to be 319.1 and 125.9 nmol/J, respectively. In contrast to the efficient reaction upon hypoxic X-irradiation, the release of 5-FdUrd from $FdUrd_2$ A was less efficient upon X-radiolysis under aerobic conditions, under which a significant amount of the starting material, $FdUrd_2$ A, was recovered. In

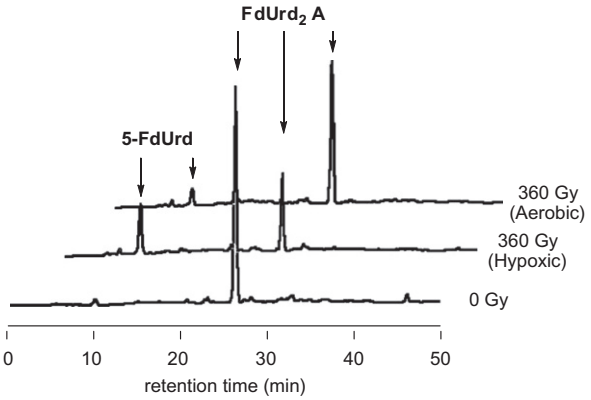


Figure 2. HPLC profiles for the one-electron reduction of $FdUrd_2$ A (100 μM) in the X-radiolyses (0 and 360 Gy) of aqueous solution containing 2-methyl-2-propanol (5 mM) under hypoxic or aerobic conditions.

view of the well-documented evidence that molecular oxygen captures reducing species e_{aq}^- to inhibit the reduction, the hypoxic radiolysis of $FdUrd_2$ A is most likely to occur via one-electron reduction by e_{aq}^- in a hypoxia selective manner. We also confirmed that a similar reaction for $FdUrd_2$ B proceeded to release 5-FdUrd upon hypoxic X-irradiation; however, the formation efficiency of 5-FdUrd from $FdUrd_2$ B was slightly lower than that from $FdUrd_2$ A. In a separate experiment, radiolysis of the control compound $FdUrd_2$ C failed to release 5-FdUrd, indicating that a carbonyl group in the Oxo-linker is indispensable for the radiolytic release of 5-FdUrd.

We compared the radiolytic release of 5-FdUrd from $FdUrd_2$ A with that from a previous prodrug, Oxo-FdUrd, which has one 5-FdUrd unit intramolecularly.[20] As shown in Table 1, the two compounds showed similar G values for the decomposition, whereas the G value for the formation of 5-FdUrd derived from the radiolysis of $FdUrd_2$ A was larger than that of Oxo-FdUrd. These results strongly indicate that $FdUrd_2$ A has greater ability to release 5-FdUrd than does Oxo-FdUrd because $FdUrd_2$ A possesses two units of 5-FdUrd.

To understand the function of $FdUrd_2$ A and $FdUrd_2$ B in living cells, we next assessed their cytotoxic properties toward the human cell line of lung adenocarcinoma A549 (Fig. 3). The cells were cultured for 72 h in the presence of various concentrations of $FdUrd_2$ A, $FdUrd_2$ B, or 5-FdUrd, and were subsequently subjected to a cell viability assay. The IC_{50} values of each drug were estimated to be 4.66, 1.04, and 0.0124 μM for $FdUrd_2$ A, $FdUrd_2$ B, and 5-FdUrd, respectively. These findings indicate that modification and dimerization of 5-FdUrd by the Oxo-linker can effectively reduce the cytotoxicity of the parent drug as we designed.

The lowered cytotoxicity of 5-FdUrd derivatives possessing the Oxo-linker motivated us to investigate the effect of X-irradiation on drugs' functions. Of the two compounds possessing the Oxo-linker, we chose $FdUrd_2$ A to characterize the radiation-induced enhancement of cytotoxicity because it showed more favorable properties as a radiation-activated prodrug than $FdUrd_2$ B; e.g. the cytotoxicity of $FdUrd_2$ A was lower than that of $FdUrd_2$ B, and $FdUrd_2$ A showed more sensitive reactivity toward radiolytic

Table 1
G-values for X-radiolysis of $FdUrd_2$ A-C (100 μM) and Oxo-FdUrd (100 μM) in aqueous solution containing 2-methyl-2-propanol (5 mM) under hypoxic or aerobic conditions

	Hypoxic conditions		Aerobic conditions	
	Decomposition/nmol J^{-1}	Formation/nmol J^{-1}	Decomposition/nmol J^{-1}	Formation/nmol J^{-1}
$FdUrd_2$ A	319.1	125.9	173.6	14.5
$FdUrd_2$ B	291.0	115.3	177.2	26.2
$FdUrd_2$ C	148.8	<1.0	140.3	1.4
Oxo-FdUrd	312.5	50.9	ND[a]	ND[a]

[a] Not detected.

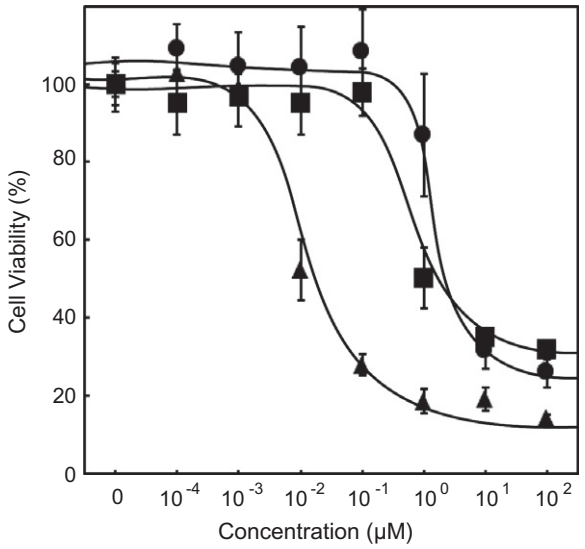


Figure 3. Cytotoxicity of $FdUrd_2$ A (circle), $FdUrd_2$ B (square) and 5-FdUrd (triangle) against A549 tumor cells. A549 cells were incubated with indicated concentration of $FdUrd_2$ A, $FdUrd_2$ B and 5-FdUrd under aerobic conditions. The cell viability was calculated by means of cell counting kit-8 (WST-8). Results are shown with the mean ± S.D. (n = 6).

one-electron reduction. Although the effect of the substituent on the cytotoxicity and sensitivity was unclear, the changes in lipophilicity and electron affinity induced by introduction of methyl group into Oxo-linker may cause differences in these properties. An aqueous solution of $FdUrd_2$ A was X-irradiated under hypoxic or aerobic conditions, and the resulting solution was then administered to A549 cells. As shown in Fig. 4A, X-irradiated $FdUrd_2$ A under aerobic conditions showed a slight cytotoxic effect, which is consistent with the evidence that the efficiency of 5-FdUrd release upon aerobic irradiation was lower than that upon hypoxic irradiation. By contrast, the cell viability was significantly reduced when the hypoxically irradiated $FdUrd_2$ A was administered. These results indicate that the 5-FdUrd released from $FdUrd_2$ A by radiolysis increased the cytotoxicity toward A549 cells. Given that the G values for the formation of 5-FdUrd from $FdUrd_2$ A was 125.9 nmol/J, the amount of 5-FdUrd released upon 120 Gy irradiation is seemed to exceed IC_{50} values of 5-FdUrd against A549 cells.

We next tried the radiolytic activation of $FdUrd_2$ A in the cells. $FdUrd_2$ A was administered to the cells, and then incubated for 12 h. After the incubation, the cells were X-irradiated up to 6 Gy under hypoxic conditions. Although an increase in cytotoxicity by the release of 5-FdUrd was expected, we observed no effect on cytotoxicity or cell viability (Fig. 4B). Given that >10 Gy X-irradiation was required to increase the cytotoxicity of the prodrug in the experiments using preirradiated $FdUrd_2$ A (Fig. 4A), the amount of 5-FdUrd released in the cells by 6 Gy irradiation seemed to be too low to express its original cytotoxicity. We could not increase the radiation dose because of the risk of serious damage to the cells induced by the high radiation dose.

3. Conclusion

We prepared 5-FdUrd derivatives, in which two units of 5-FdUrd were linked by a radiation-sensitive Oxo-linker, and characterized their radiolytic one-electron reduction. $FdUrd_2$ A and $FdUrd_2$ B were activated by X-irradiation to release 5-FdUrd under hypoxic conditions. By contrast, the release of 5-FdUrd was suppressed significantly upon aerobic irradiation because e_{aq}^-, which are key reducing species for activation, were captured by molecular oxygen. A biological assay using A549 cells revealed that pretreatment of $FdUrd_2$ A with X-irradiation caused enhanced cytotoxicity by releasing a sufficient amount of 5-FdUrd. However, increased cytotoxicity was not observed, when the cells were irradiated in the presence of $FdUrd_2$ A under hypoxic conditions. We

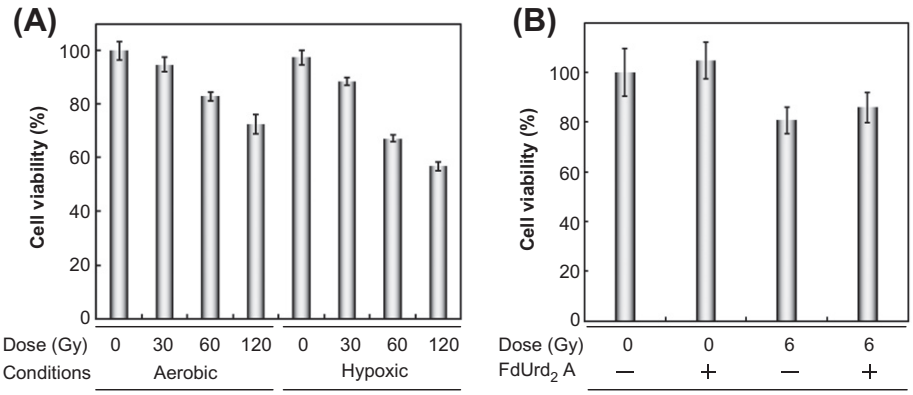


Figure 4. Radiation-induced cytotoxicity of $FdUrd_2$ A against A549 cells. (A) Aqueous solutions of $FdUrd_2$ A (1 μM) was X-irradiated (0, 30, 60 or 120 Gy) under aerobic or hypoxic conditions, and then administered to cells. (B) A549 cells were cultured in the presence (+) or absence (−) of 125 nM $FdUrd_2$ A, and treated with X-ray (0 or 6 Gy) under hypoxic conditions. Results are shown with the mean ± S.D (n = 6 for (A), n = 9 for (B)).

presumed that the amount of 5-FdUrd released from $FdUrd_2$ A in the cells upon low-dose X-irradiation was inadequate for onset of cytotoxicity. Improvements to enhance the efficiency of the radiolytic release of 5-FdUrd are underway.

4. Experimental section

4.1. General procedures

All starting materials and reagents were purchased from Tokyo Kasei Kogyo (Tokyo, Japan), Wako (Tokyo, Japan) and Aldrich Chemical (Milwaukee, WI). All other solvents, purchased from Wako, were GR grade or dry grade and used without further purification. Oxo-FdUrd[20] were synthesized as described previously. The ^{1}H NMR spectra were recorded using a JOEL JNM-AL400 (400 MHz) spectrometer in $CDCl_3$. Coupling constants are given in hertz. ^{13}C NMR spectra were measured with JOEL JMN-AL-400 (100 MHz) spectrophotometer in DMSO-d_6 or $CDCl_3$. The FAB-MS spectra were recorded on a JOEL JMS-SX102A spectrometer, using nitrobenzyl alcohol or glycerol as matrix. The organic reactions were carried out in oven-dried glassware under an argon atmosphere with magnetic stirring. High-Performance liquid chromatography (HPLC) was carried out with a Hitachi HPLC system (L-7455 Diode array detector, L-7300 column oven, L-7100 pump, D-7000 interface). Sample solutions were injected onto a reversed-phase column (Inertsil ODS-3, GL Science Inc.). The solvent mixture of 0.1 M triethylamine acetate (TEAA) at pH 7.0 and 100% acetonitrile was delivered as mobile phase at a flow rate of 0.6 mL or 3.0 mL/min at 40 °C. The column eluents were monitored by the UV absorbance at 260 nm. Rigaku RADIOFLEX-350 was used for X-radiolysis.

4.1.1. 1,3-Bis[5-fluoro-1-(β-2′-deoxy-3′,5′-di-*O*-acetylribofuranosyl)-2,4-dioxopyrimidinyl]-propanone (2A). General procedure for alkylation of 2′-deoxy-3′,5′-di-*O*-acetyl-5-fluorouridine at *N*(3)-position

Sodium hydride (42 mg, 1.12 mmol) was added to a solution of 2′-deoxy-3,5′-di-*O*-acetyl-5-fluorouridine (300 mg, 0.91 mmol) in anhydrous DMF (5 mL) at 0 °C and the mixture was stirred at 0 °C for 15 min. To the resulting mixture was added a solution of 1,3-dibromopropanone (98.1 mg, 0.45 mmol) in anhydrous DMF (761 μL) and the mixture was stirred at ambient temperature for 24 h. The reaction mixture was diluted with saturated ammonium chloride and then extracted with ethyl acetate. The extract was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by column chromatography (SiO_2, 2.5% MeOH/$CHCl_3$) to give **2A** (231 mg, 71%) as a white solid: Mp 140–141 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.09 (s, 6H), 2.12 (s, 6H), 2.14–2.19 (m, 2H), 2.55 (ddd, 2H, J = 16.0, 8.0, 2.0 Hz) 4.25–4.27 (m, 2H) 4.30 (dd, 2H, J = 12.2, 3.4 Hz), 4.38 (dd, 2H, J = 12.2, 3.4 Hz), 4.78 (d, 2H, J = 17.1 Hz), 4.93 (d, 2H, J = 16.6 Hz), 5.18 (ddd, 2H, J = 5.9, 2.9, 2.9 Hz), 6.27 (dd, 2H, J = 6.5, 6.5 Hz), 7.67 (d, 2H, J = 5.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.7, 20.8, 38.0, 47.8, 63.7, 73.9, 82.6, 86.2, 121.9 (d, J = 34.7 Hz), 140.1 (d, J = 241.7 Hz), 148.6, 156.0 (d, J = 26.4 Hz), 170.0, 170.3, 194.8; FABMS (NBA) m/z 715 [(M+H)$^+$]; HRMS calcd for $C_{29}H_{32}F_2N_4O_{15}$ [(M+H)$^+$] 715.1905, found 715.1940.

4.1.2. 1,5-Bis[5-fluoro-1-(β-2′-deoxy-3′,5′-di-*O*-acetylribofuranosyl)-2,4-dioxopyrimidinyl]-2,4-dimethylpropanone (2B)

According to the method detailed for **2A**, the reaction of 2,4-dibromo-3-pentanone (69.4 μL, 0.45 mmol) gave **2B** (65.9 mg, 20%) as a white solid: Mp 85–86 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 1.36 (d, 6H, 6.8 Hz), 2.05 (s, 12H), 2.34–2.39 (m, 4H), 4.16–4.19 (m, 2H), 4.24–4.27 (m, 4H), 5.16–5.20 (m, 2H), 5.36 (q, 2H, J = 4.4 Hz), 6.16 (t, 2H, J = 7.3 Hz), 8.13 (d, 2H, J = 6.3 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.5 (d, J = 100 MHz), 20.7, 20.8, 38.0, 54.6 (d, J = 95.4), 63.7, 73.7, 82.7, 86.2, 122.0 (d, J= 36.4 Hz), 140.0 (d, J = 235.6 Hz), 148.6, 156.2 (d, J = 23.1 Hz), 170.1, 170.3, 197.7; FABMS (NBA) m/z 743 [(M+H)$^+$]; HRMS calcd for $C_{31}H_{36}F_2N_4O_{15}$ [(M+H)$^+$] 743.2218, found 743.2203.

4.1.3. 1,3-Bis[5-fluoro-1-(β-2′-deoxy-3′,5′-di-*O*-acetylribofuranosyl)-2,4-dioxopyrimidinyl]-propane (2C)

According to the method detailed for **2A**, the reaction of 1,3-dibromopropane (45.3 μL, 0.44 mmol) gave **2C** (191.3 mg, 63%) as a white solid: Mp 125–127 °C; ^{1}H NMR (DMSO-d_6, 400 MHz)?δ 1.80–1.85 (m, 2H), 2.04 (s, 6H), 2.05 (s, 6H), 2.30–2.36 (m, 2H), 2.42–2.46 (m, 2H), 3.81 (t, 4H, J = 6.8), 4.16–4.19 (m, 2H), 4.24–4.27 (m, 4H), 5.16–5.20 (m, 2H), 6.17 (t, 2H, J = 5.8 Hz), 8.03 (d, 2H, J = 6.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.7, 20.8, 25.9, 38.0, 39.4, 63.7, 73.8, 82.5, 86.0, 121.2 (d, J = 34.7 Hz), 140.2 (d, J = 234.7 Hz), 149.1, 156.5 (d, J = 25.6 Hz), 170.1, 170.3; FABMS (NBA) m/z 701 [(M+H)$^+$]; HRMS calcd for $C_{29}H_{34}F_2N_4O_{14}$ [(M+H)$^+$] 701.2112, found 701.2122.

4.1.4. 1,3-Bis[5-fluoro-1-(β-2′-deoxyribofuranosyl)-2,4-dioxopyrimidinyl]propanone ($FdUrd_2$ A). General procedure for hydrolysis

To a solution of **2A** (48 mg, 0.068 mmol) in THF (2 mL) and methanol (2 mL) was added aqueous sodium hydroxide (0.1 M, 170 μL, 0.017 mmol) and the mixture was stirred at 0 °C for 5 h. The reaction mixture was concentrated in vacuo and the crude product was purified by column chromatography (SiO_2, 20% MeOH/$CHCl_3$) to give **$FdUrd_2$ A** (37 mg, quant.) as a white solid: Mp 129–131 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 2.12–2.14 (m, 4H), 3.55–3.66 (m, 4H), 3.79–3.82 (m, 2H), 4.23–4.27 (m, 2H), 4.88 (s, 4H), 5.19 (t, 2H, J = 5.4 Hz), 5.27 (d, 2H, J = 4.4 Hz), 6.14 (t, 2H, J = 6.4 Hz), 8.38 (d, 2H, J = 6.8 Hz); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 39.9, 47.9, 60.8, 69.9, 85.6, 87.7, 124.1 (d, J = 34.2 Hz), 139.2 (d, J = 229.4 Hz), 148.5, 155.9 (d, J = 25.8 Hz), 196.9; FABMS (glycerol) m/z 547 [(M+H)$^+$]; HRMS calcd for $C_{21}H_{24}F_2N_4O_{11}$ [(M+H)$^+$] 547.1482, found 547.1501.

4.1.5. 1,5-Bis[5-fluoro-1-(β-2′-deoxyribofuranosyl)-2,4-dioxopyrimidinyl]-2,4-dimethylpropanone ($FdUrd_2$ B)

According to the method detailed for **$FdUrd_2$ A**, the reaction of **2B** (20 mg, 0.027 mmol) gave **$FdUrd_2$ B** (13.3 mg, 87%) as a white solid: Mp 136–140 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 1.35 (d, 6H, J = 6.8 Hz), 2.17 (dd, 4H, J = 11.9, 6.3 Hz), 3.56–3.67 (m, 4H), 3.81 (t, 4H, J = 2.7 Hz), 4.22–4.27 (m, 2H), 5.20 (t, 2H, J = 4.9 Hz), 5.27 (dd, 2H, J = 4.8, 1.6 Hz), 5.35 (q, 2H, J = 6.8 Hz), 6.11 (dd, 2H, J = 10.9, 5.6 Hz), 8.38–8.40 (m, 1H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 13.1 (d, J = 108.6), 40.0, 60.7, 69.7, 85.8, 87.7, 124.3 (d, J = 35.0 Hz), 139.3 (d, J = 228.6), 148.3, 156.0 (d, J = 25.8 Hz), 198.6; FABMS (glycerol) m/z 575 [(M+H)$^+$]; HRMS calcd for $C_{23}H_{28}F_2N_4O_{11}$ [(M+H)$^+$] 575.1795, found 575.1814.

4.1.6. 1,3-Bis[5-fluoro-1-(β-2′-deoxyribofuranosyl)-2,4-dioxopyrimidinyl]propane ($FdUrd_2$ C)

According to the method detailed for **$FdUrd_2$ A**, the reaction of **2C** (26 mg, 0.037 mmol) gave **$FdUrd_2$ C** (20 mg, quant.) as a yellow solid: Mp 105–107 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 1.82 (quint, 2H, J = 7.1 Hz), 2.10–2.16 (m, 4H), 3.54–3.66 (m, 4H), 3.78–3.82 (m, 6H), 4.23 (q, 2H, J = 4.0 Hz), 5.18 (t, 2H, J = 4.0 Hz), 5.25 (d, 2H, J = 4.4 Hz), 6.15 (t, 2H, J = 6.4 Hz), 8.30 (d, 2H, J = 6.8 Hz); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 25.2, 40.0, 48.6, 60.8, 69.8, 85.5, 87.6, 123,5 (d, J = 36.9 Hz), 139.4 (d, J = 227.3 Hz), 148.8, 156.4 (d, J = 25.6 Hz); FABMS (glycerol) m/z 533 [(M+H)$^+$]; HRMS calcd for $C_{21}H_{26}F_2N_4O_{10}$ [(M+H)$^+$] 533.1690, found 533.1683.

4.2. Radiolytic reduction

Aqueous solutions of **$FdUrd_2$ A**, **$FdUrd_2$ B**, and **$FdUrd_2$ C** (100 μM), containing 2-methyl-2-propanol (5 mM), were purged with argon for 10 min and then irradiated in a sealed glass ampoule at ambient temperature with an X-ray source (6.0 Gy min^{-1}). After the X-irradiation, the solution was subjected to HPLC analysis. Since the lower detection limit of the prodrugs and 5-FdUrd by HPLC was micromolar level, we needed large dose of X-irradiation for activation of prodrugs at submilimolar level.

4.3. Assessment of cytotoxicity toward A549 cells

A549 cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotics (mixture of streptomycin, penicillin). The cells were seeded into 96-well plates (1500 cells/well) and cultured at 37 °C in a well-humidified incubator with 5% CO_2 and 95% air (aerobic condition) for 24 h. The cells were then incubated with the various concentrations of **$FdUrd_2$ A**, **$FdUrd_2$ B**, or **5-FdUrd** under aerobic conditions for 72 h, and added with 10 μL of Cell Counting Kit-8 (Dojindo). The plates were further incubated at 37 °C for 30 min and the cell viability assay was performed using Microplate Reader (BIO-RAD).

4.4. Radiation-induced cytotoxicity of pre-irradiated $FdUrd_2$ A

DMEM solutions of **$FdUrd_2$ A** under hypoxic or aerobic conditions were prepared and then irradiated in a sealed glass ampoule at ambient temperature with an X-ray source (6.0 Gy min^{-1}). A549 cells were seeded into 96-well plaets (1500 cells/well) and cultured at 37 °C under aerobic condition for 24 h. The cells were then incubated with irradiated **$FdUrd_2$ A** under aerobic conditions for 72 h. After adding 10 μL of Cell Counting Kit-8 to each well, and the cell viability assay was performed as described above.

4.5. Radiation-induced cytotoxicity of $FdUrd_2$ A (X-irradiation to cells in the presence of $FdUrd_2$ A)

A549 cells were seeded into 96-well plates (1500 cells/well) and incubated at 37 °C for 6 h under hypoxic conditions. The cells were then incubated with **$FdUrd_2$ A** at 37 °C under hypoxic condition for 12 h. For the hypoxic treatment (<0.3% of oxygen), the cells were treated in a hypoxic chamber, $INVIVO_2$ 400 (Ruskinn Technology Ltd). The plates kept under hypoxic conditions using Anaero Pack System (Mitsubishi Gas Chemical Company Inc., Japan) were treated with X-rays at a dose of 6 Gy and incubated for 72 h under aerobic conditions. After adding 10 μL of Cell Couning Kit-8 to each well, and the cell viability assay was performed as described above.

Acknowledgements

This work is partly supported by the Strategic Promotion Program for Basic Nuclear Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

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