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Reductive activation of 5-fluorodeoxyuridine prodrug possessing azide methyl group by hypoxic X-irradiation

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Graphical Abstract

\[ \text{N2-FdUrd} \quad \text{Low toxicity} \quad \text{5-FdUrd} \quad \text{High toxicity} \]
Abstract

We prepared a 5-fluorodeoxyuridine (5-FdUrd) derivative possessing azide methyl group (N₃-FdUrd) as a novel radiation-activated prodrug. The parent antitumor agent, 5-FdUrd, was released efficiently from N₃-FdUrd by hypoxic X-irradiation. On the other hand, the activation of N₃-FdUrd was suppressed upon X-irradiation under aerobic conditions. A biological assay using A549 cells revealed that the cytotoxicity of N₃-FdUrd was significantly enhanced by hypoxic X-irradiation.
Prodrugs that are activated by an external stimulus have been attracting much attention. These prodrugs themselves are nontoxic, but exposure to triggers such as photoirradiation,\(^1\) change in pH\(^2\) or enzymatic reactions\(^3\) bring about changes in the chemical structure of the prodrugs to enable their inherent medical capability.\(^4\) Because of the ease of controlling their functions, stimuli-sensitive prodrugs are prospective high-performing medical agents with mild side effects.

One of the new strategies employed in this area is the utilization of X-irradiation\(^5\) as an external trigger to activate prodrugs. Since chemical reactions triggered by radiation can be controlled spatially and temporary, such prodrugs can be converted to their active forms with exact control of the area, time, and dosage. Recently, we have developed several prodrugs that can be activated by X-irradiation.\(^6\) We prepared prodrugs of the antitumor agents 5-fluorouracil, 5-fluorodeoxyuridine (5-FdUrd), and cytarabine by incorporation of a 2-oxoalkyl group or an indolequinone structure, which are removable by X-ray treatment under hypoxic conditions. A reaction mechanism has been proposed: these substituents undergo reductive activation by reducing species generated by the radiolysis of water\(^7\) to release their parent drug. These prodrugs showed cytotoxicity
towards hypoxic tumor cells upon X-irradiation, while they were nontoxic against aerobic cells, even upon X-irradiation.

Herein, we discuss the design of a novel radiation-activated prodrug of 5-FdUrd possessing azide methyl group (N₃-FdUrd), which is known to be a removable substituent under reducing conditions. We expected that N₃-FdUrd would be activated by reducing species generated from radiolysis of water to form aminal 1 (Figure 1), which would be hydrolyzed in aqueous solution to give 5-FdUrd. We synthesized the N₃-FdUrd and characterized its radiolytic reduction behavior. The radiation-dependent cytotoxicity of N₃-FdUrd using living cells was also assessed.

The strategy for preparation of N₃-FdUrd is summarized in Scheme 1. Diacetyl-5-fluorodeoxyuridine 2 was coupled with chloromethyl methyl sulfide, and then converted to the azide compound 4. Hydrolysis of 4 under basic conditions furnished the desired N₃-FdUrd. N₃-FdUrd had good water solubility as well as 5-FdUrd.

(Figure 1)
Initially, we performed the radiolytic reduction of N₃-FdUrd and monitored the reaction using reversed-phase HPLC.¹¹ We conducted the radiolysis in argon-purged aqueous solutions containing 2-methyl-2-propanol as a scavenger for the oxidizing hydroxyl radicals (OH’). Under these conditions, hydrated electrons (eₐq⁻) and hydrogen atoms (H’) are generated as the major reducing species from the radiolysis of water. As shown in Figure 2, a single new signal appeared during radiolysis under hypoxic conditions, while the starting material, N₃-FdUrd, disappeared. The product was identified as the parent 5-FdUrd, according to the overlapped injection of authentic samples in the HPLC analysis. The G values were estimated to be 159 nmol/J for the formation of the corresponding 5-FdUrd and 336 nmol/J for the decomposition of N₃-FdUrd; thus, 47% of the decomposed N₃-FdUrd was converted to 5-FdUrd upon hypoxic X-irradiation. To verify the reaction mechanism in detail, we next conducted a similar radiolytic reduction of N₃-FdUrd under aerobic conditions. In contrast to the efficient activation of hypoxic irradiation, both formation of 5-FdUrd and decomposition of N₃-FdUrd were
markedly suppressed, as shown in Figure 2b. The G values were 50 nmol / J for the formation of 5-FdUrd and 118 nmol / J for the decomposition of N3-FdUrd. It has been well-characterized that molecular oxygen captures both eaq− and H+.12 Thus, it is most likely that eaq− and H+ are key reactive species for the activation of N3-FdUrd and the formation of 5-FdUrd.

(Figure 2)

We then assessed the cytotoxicity of N3-FdUrd and 5-FdUrd towards A549 cells (human lung adenocarcinoma). A cell viability assay was employed to test the cytotoxicity in vitro. The IC50 value was defined as the concentration required to reduce the viability of the cells by 50%, and was estimated on exposure to N3-FdUrd and 5-FdUrd.13 Figure 3 shows the survival curve of the A549 cells incubated in the presence of drugs at various concentrations in air containing 5% CO2 for 72 h at 37 °C. The IC50 values were 1.47 and 0.047 μM for N3-FdUrd and 5-FdUrd, respectively, indicating that incorporation of an azide methyl group into 5-FdUrd at the N(3)-position is responsible for the decrease
in the cytotoxic effect of 5-FdUrd.

(Figure 3)

The properties of N$_3$-FdUrd described above prompted us to further investigate the effect of X-irradiation on the cytotoxicity (Figure 4A). An aqueous solution of N$_3$-FdUrd was X-irradiated under hypoxic or aerobic conditions, and then the resulting solution was administered to the A549 cells.\textsuperscript{14} In accordance with the suppression of the cytotoxic effects mentioned above, administration of 200 nM N$_3$-FdUrd did not bring about a cytocidal action. We also confirmed that the X-irradiated N$_3$-FdUrd under aerobic conditions did not show any cytotoxic effect, consistent with the results that radiolytic activation is suppressed under aerobic conditions. It is striking that the cell viability was significantly reduced in the presence of hypoxically irradiated N$_3$-FdUrd. These results strongly indicate that 5-FdUrd released from N$_3$-FdUrd upon hypoxic irradiation showed strong cytotoxicity towards A549 cells.
In light of the effect of X-irradiation, further attempts were made to verify the function of N3-FdUrd as a radiation-activated prodrug. We exposed A549 cells to X-rays under hypoxic conditions in the presence or absence of N3-FdUrd and characterized the radiation-dependent cytotoxic effect of N3-FdUrd. As shown in Figure 4B, the cells were viable even in the presence of 100 nM N3-FdUrd without X-irradiation. Although the hypoxic cells showed high viability even upon X-irradiation owing to their resistibility, radiation in the presence of N3-FdUrd significantly enhanced the radiation sensitivity of the A549 cells, resulting in low cell viability. Thus, N3-FdUrd released toxic 5-FdUrd via radiolytic reduction in hypoxic cells, which led to an enhanced cytotoxicity.

In summary, we have synthesized a 5-FdUrd derivative possessing azide methyl group at the N(3)-position (N3-FdUrd) and characterized its properties upon X-irradiation. N3-FdUrd was activated to release 5-FdUrd in a hypoxia-selective manner via reduction by H+ and eaq−, which were generated by the radiolysis of water. A cytotoxicity assay
using A549 cells revealed that N₃-FdUrd showed a lower toxicity. On the other hand, X-irradiation resulted in a formation of 5-FdUrd in hypoxic cells, leading to the enhancement of the radiation sensitivity of A549 cells. Thus, N₃-FdUrd is a promising radiation-activated prodrug with lesser side effects. Further in vivo experiments on N₃-FdUrd, focusing on practical use, are in progress.

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References and Notes


**N3-FdUrd**: Colorless oil; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 2.12–2.18 (2H), 3.57 (ddd, $J = 3.42, 4.88, 12.20$ Hz, 1H), 3.64 (ddd, $J = 3.42, 8.78, 12.20$ Hz, 1H), 3.81 (dd, $J = 3.41, 6.80$ Hz, 1H), 4.24 (m, 1H), 5.15–5.21 (1H), 5.22 (s, 1H), 5.27 (d, $J = 4.40$, 1H), 6.15 (dd, $J = 6.30, 6.30$ Hz, 1H), 8.37 (d, $J = 6.83$ Hz, 1H); $^{13}$C NMR (67.8 MHz, DMSO-$d_6$) $\delta$ 55.8, 60.7, 69.8, 85.6, 87.7, 124.5 (d, $J = 34.4$ Hz), 138.1, 140.9, 148.8, 156.0; FABMS: m/e 302 [(M+H)$^+$]; HRMS calcd for C$_{10}$H$_{13}$N$_5$O$_5$F [(M+H)$^+$] 302.0901, found 302.0900.

Since the large amount of prodrug and products was necessary to monitor the reaction by HPLC, we conducted the experiment using 300 $\mu$M prodrugs and high dose of X-irradiation (up to 960 Gy).

Reducing hydrated electrons and hydrogen atoms are scavenged dissolved O$_2$ ($k = 1.9 \times 10^{10}$ M$^{-1}$ s$^{-1}$ for hydrated electrons, $k = 2.1 \times 10^{10}$ M$^{-1}$ s$^{-1}$ for hydrogen atoms). See ref. 7.

**Assessment of cytotoxicity toward A549 cells**: A549 cells were cultured in
Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were seeded into 96-well plates (2000 cells/well) and cultured at 37 °C in a well-humidified incubator with 5% CO₂ and 95% air (aerobic condition) for 24 hours. The cells were then incubated with the various concentrations of 5-FdUrd or N₃-FdUrd under aerobic conditions for 72 hours, and added with 10 μL of Cell Counting kit-8 (WST-8, DOJINDO, Japan). The plates were further incubated at 37 °C for 30 min and the cell viability assay was performed using Microplate spectrophotometer (BIO-RAD).

(14) **Radiation-induced cytotoxicity of pre-irradiated N₃-FdUrd**: A549 cells were seeded into 96-well plates (2000 cells / well) and incubated at 37 °C for 24 hours under aerobic conditions. An aqueous solution of N₃-FdUrd (0 or 2 μM) was irradiated under aerobic or hypoxic conditions and then added to the cells. Similar to above, we performed cell viability assay to measure an antiproliferative activity (See ref. 13).

(15) **Radiation-induced cytotoxicity of N₃-FdUrd (X-irradiation to cells containing N₃-FdUrd)**: For the hypoxic treatment (< 0.02% of oxygen), the
cells were treated in a Anaeron Pack System (Mitsubishi Gas Chemical Company Inc., Japan) equipped with AnaeroPouch-Anaero (Mitsubishi Gas Chemical Co. Ltd, Japan). The 96-well plates (2000 cells/well) kept under hypoxic conditions using Anaeron Pack System were X-irradiated (0 and 6 Gy) and incubated for 72 hours under aerobic conditions. The cell viability assay was performed as described above (See ref. 13).
Scheme 1. Reagents and conditions: (a) Chloromethylmethylsulphide, NaH, DMF, 2 h, r.t., 78%; (b) NCS, TMSCl, 2 h, r.t., CH₂Cl₂; (c) NaN₃, DMF, 48 h, r.t., 17% (2 steps); (d) NaOH, MeOH, H₂O, 3 h, 0 °C, quant.
Figure 1. Plausible reaction mechanism for the formation of 5-FdUrd upon hypoxic X-irradiation.
Figure 2. (A,B) HPLC profiles for the reductive activation of N$_3$-FdUrd (300 µM) by X-irradiation (0, 240, 480 and 960 Gy). The radiolysis of aqueous solution containing 15 mM 2-methyl-2-propanol was conducted under hypoxic (A) or aerobic (B) conditions. (C) Release of 5-FdUrd from N$_3$-FdUrd (300 µM) in the hypoxic (circle) or aerobic (triangle) radiolysis.
Figure 3. Cytotoxicity of N$_3$-FdUrd (circle) and 5-FdUrd (triangle) against A549 tumor cells. A549 cells were incubated with indicated concentrations of N$_3$-FdUrd or 5-FdUrd under aerobic conditions for 72 h. The cell viability was calculated by means of cell counting kit-8 (WST-8). Results are shown with the mean ± S.D. (n = 4).
**Figure 4.** Radiation-induced cytotoxicity of N3-FdUrd against A549 cells. (A) Aqueous solutions of N3-FdUrd (0 nM: - or 200 nM: +) was X-irradiated (6 Gy) under aerobic or hypoxic conditions, and then administered to cells. (B) A549 cells were cultured in the presence or absence of 100 nM N3-FdUrd, and treated with X-ray (6 Gy) under hypoxic conditions. Results are shown with the mean ± S.D. (n = 3 for (A), n = 4 for (B))(*P < 0.05; NS: not significant).