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Bioorganic & Medicinal Chemistry Letters 15 (2005) 2321–2324
Bioorganic & Medicinal Chemistry Letters

Hypoxia-selective activation of 5-fluorodeoxyuridine prodrug possessing indolequinone structure: radiolytic reduction and cytotoxicity characteristics

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Received 13 October 2004; accepted 1 March 2005

Abstract—We synthesized a 5-fluorodeoxyuridine (**5-FdUrd**) derivative possessing an indolequinone structure (**IQ-FdUrd**) to characterize the radiolytic reduction in aqueous solution and the radiation-activated cytotoxicity against EMT6/KU cells under hypoxic conditions. **IQ-FdUrd** released antitumor agent **5-FdUrd** upon hypoxic, but not aerobic, irradiation with the G value of 0.38×10^{-7} mol J^{-1}. Laser flash photolysis of **IQ-FdUrd** in Ar-purged aqueous solution with dimethylaniline as an electron donor gave rise to a transient absorption spectrum characteristic of semiquinone radical anion, which decayed via second order kinetics. It is most likely that bimolecular disproportionation of intermediate semiquinone radicals occurs to release **5-FdUrd**. **IQ-FdUrd** showed enhanced cytotoxicity against EMT6/KU cells in a radiation dose-dependent manner upon hypoxic irradiation. **IQ-FdUrd** is potentially a prototype compound for new class of radiation-activated antitumor prodrugs that are useful for radiation treatment of hypoxic tumors.

Tumor hypoxia is a major cause for decreased therapeutic effect in the radiation treatment of tumor tissues.[1–5] Hypoxic cells have been recognized to show resistance to ionizing radiation, the biological action of which involves acceleration of oxidation, thus leading to post-irradiation regrowth of tumor.[6] For the radiation therapy of hypoxic tumor cells, there have been various strategies including hyperbaric oxygen therapy,[7,8] hypoxic cell radiosensitizers,[9–12] and hypoxic cytotoxins.[13] A new strategy to overcome tumor hypoxia focuses on the combination of radiotherapy and chemotherapy into prodrugs that are activated to release antitumor agents by hypoxic irradiation.[14,15] Recently, we reported that 5-fluorouracil (**5-FU**) and 5-fluorodeoxyuridine (**5-FdUrd**) derivatives possessing 2-oxoalkyl groups are radiation-activated prodrugs releasing representative antitumor agents **5-FU** and **5-FdUrd**, respectively, to cause effective cytotoxicity against hypoxic tumor cells.[16–20] Mechanistic studies showed that these prodrugs undergo reduction by capturing reducing hydrated electrons (e_{aq}^-) generated by radiolysis of water.[21] This family of prodrugs are radiation-activated only in the hypoxic tumor cells, but not in the aerobic normal cells with sufficient oxygen concentration for trapping e_{aq}^-.

Indolequinones have been identified as the effective eliminating substituents via bioreduction and radiolytic reduction, thus stimulating their application to prodrug development.[22–25] Such a reductive activation of prodrugs with indolequinone derivatives to release drugs accompanies concomitant formation of electrophilic iminium cations like **1**, which potentially invoke DNA alkylation or other cellular damage. In view of these reaction characteristics, antitumor prodrugs possessing an indolequinone structural unit may result in synergic cytotoxicity that originates from both antitumor agents and electrophilic iminium species released upon reductive activation. As an extension of our work on the development of antitumor prodrugs activated by hypoxic irradiation, we synthesized a novel prodrug

Keywords: Prodrug; 5-Fluorodeoxyuridine; Indolequinone; Radiolytic reduction.
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doi:10.1016/j.bmcl.2005.03.013

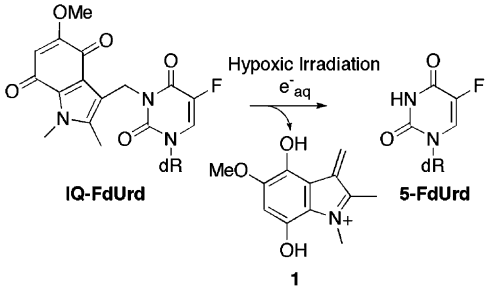
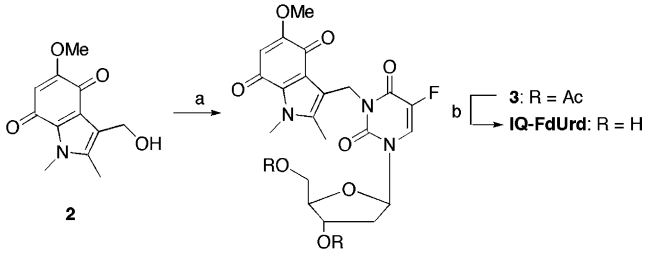


Figure 1. Schematic illustration for activation of **IQ-FdUrd** to release **5-FdUrd** upon hypoxic irradiation.

consisting of **5-FdUrd** and a functional indolequinone structure (**IQ-FdUrd**) as an *N*(3)-substituent (Fig. 1). Under hypoxic conditions, radiolytic reduction of **IQ-FdUrd** occurred exclusively to form **5-FdUrd** and iminium cations, and thereby resulted in the cytotoxicity against hypoxic EMT6/KU cells. Laser flash photolysis studies of **IQ-FdUrd** revealed the formation of semiquinone radical intermediates that undergo bimolecular disproportionation to release **5-FdUrd**.

The synthetic route to **IQ-FdUrd** is outlined in Scheme 1. An indolequinone derivative possessing a primary alcohol **2**[22] was coupled with 2′-deoxy-3′,5′-di-*O*-acetyl-5-fluorouridine[19] into **3** by the Mitsunobu reaction. **IQ-FdUrd** was obtained by hydrolysis of **3** under basic conditions.[26]

We performed one-electron reduction of **IQ-FdUrd** in the radiolysis of Ar-purged aqueous solution containing excess 2-methyl-2-propanol as the scavenger of oxidizing hydroxyl radicals (·OH).[21,27] Figure 2 shows dose–responses for the decomposition of **IQ-FdUrd** and the release of **5-FdUrd** upon hypoxic irradiation. The concentration of **5-FdUrd** increased linearly with increasing radiation dose, along with the more efficient **IQ-FdUrd** decomposition. The *G* values[28] were 0.38×10^{-7} mol J^{-1} for the **5-FdUrd** release and 1.4×10^{-7} mol J^{-1} for the **IQ-FdUrd** decomposition, respectively. Thus, 27% of the decomposed **IQ-FdUrd** was converted to **5-FdUrd** upon hypoxic irradiation. In contrast, the radiolytic decomposition efficiency of **IQ-FdUrd** under aerobic conditions was reduced to one-third the efficiency under hypoxic conditions, in which only trace amounts of released **5-FdUrd** could be detected.[29] It is therefore evident that **IQ-FdUrd** is activated to release



Scheme 1. Reagents and conditions: (a) DEAD, PPh_3, 2′-deoxy-3′,5′-di-*O*-acetyl-5-fluorouridine, THF, 0 °C, 70 min; (b) NaOH, H_2O, MeOH, 0 °C, 1 h, 32% (two steps).

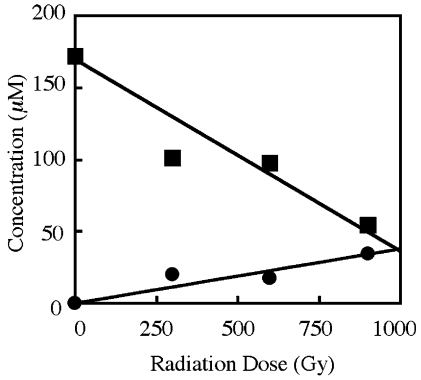


Figure 2. Decomposition of **IQ-FdUrd** (■) and release of 5-FdUrd (●) in the hypoxic radiolysis of aqueous solution containing 2-methyl-2-propanol (17.1 mM).

5-FdUrd via reduction by e^-_{aq} occurring exclusively under hypoxic conditions, similar to the **5-FdUrd** prodrugs possessing 2-oxoalkyl groups.[16–20]

We also conducted laser flash photolysis studies on the reductive activation of **IQ-FdUrd** employing dimethylaniline (DMA) as an electron donor to get further mechanistic insight into the reductive release of **5-FdUrd**.[30] Figure 3a shows the transient absorption spectra created in the 266 nm laser flash photolysis of **IQ-FdUrd** and DMA in phosphate buffer (pH 7.4). The transient appeared 30 μs after laser flash with two absorption maxima at $\lambda_{max} \approx 360$ nm and $\lambda_{max} \approx 460$ nm under hypoxic conditions, which are assigned to semiquinone radical anions[22] and DMA radical cations,[19] respectively, by reference to the previous studies. Neither of the transient absorption spectra could be observed upon laser flash photolysis in the absence of DMA. In contrast to these spectral characteristics under hypoxic conditions, absorption of the semiquinone radical anions disappeared under aerobic conditions even in the presence of DMA, whereas the transient absorption of DMA radical cations remained. These results suggest that the photolysis induces electron ejection from DMA and the resulting hydrated electrons e^-_{aq} reduce **IQ-FdUrd** to release **5-FdUrd** under hypoxic conditions, while molecular oxygen captures e^-_{aq} faster than **IQ-FdUrd** under aerobic conditions. An alternative mechanism by which electronically excited DMA undergoes electron transfer quenching by **IQ-FdUrd** cannot be ruled out at present. As plotted in Figure 3b, the reciprocal of the absorbance at 360 nm of semiquinone radicals generated in the presence of various concentrations of DMA increased linearly with increasing time, indicating that the intermediate semiquinone radicals disappear following the second-order kinetics. It was well documented that indolequinone prodrugs are activated by several mechanisms including one-electron reduction, two-electron reduction, and radical–radical interaction.[22,25] Our observations in the laser flash photolysis strongly indicate that semiquinone radical intermediates generated from **IQ-FdUrd** underwent predominantly bimolecular disproportionation to release **5-FdUrd**. As mentioned above, on the other hand, the selectivity of **5-FdUrd** formation by radiolytic reduction of **IQ-FdUrd**

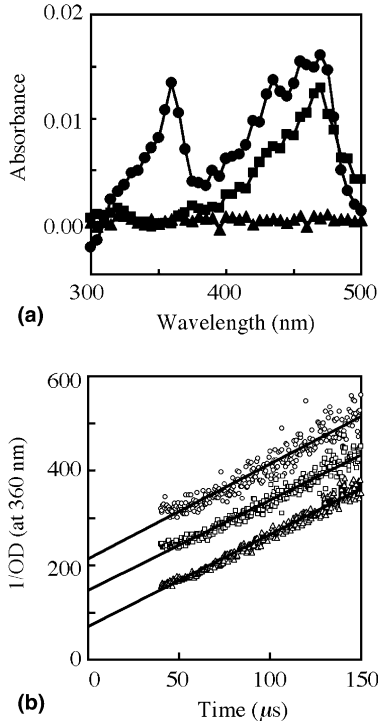


Figure 3. (a) Transient absorption spectra as observed 30 μs after 266 nm laser flash photolysis of 50 μM **IQ-FdUrd** in 10 mM phosphate buffer (pH 7.4) in the presence of 1 μM DMA under hypoxic conditions (●) and aerobic conditions (■), and in the absence of DMA under hypoxic conditions (▲). (b) Plots of the reciprocal of semiquinone radical absorbance at 360 nm against time at several initial DMA concentrations: (○) 1 μM; (□) 1.2 μM; (△) 1.5 μM at pH 7.4.

in hypoxia was just 27%,[31] suggesting that semiquinone radical anions generated via capturing of e^-_{aq} partly underwent unimolecular degradation more efficiently than bimolecular disproportionation under conditions of steady state irradiation at lower dose rate.

For understanding of the biological activity of prodrug, we measured the cytotoxicity of **IQ-FdUrd** against a typical radiation resistant murine tumor cell line of EMT6/KU.[32] The EMT6/KU cells were exposed to varying doses of X-ray in the presence and absence of **IQ-FdUrd** and then incubated at 37 °C for seven days. Cell survival was determined by the colony assay. Figure 4 compares cell survivals after irradiation. Consistent with the radiation chemical reactivity, the cytotoxic effect was significantly enhanced upon X-irradiation up to 4 Gy of the cells in the presence of **IQ-FdUrd** under hypoxic conditions, whereas only minimal cytotoxicity was observed under aerobic conditions. In association with these cellular experiments, the concentration of **5-FdUrd** that will be released upon 4 Gy irradiation under hypoxic conditions is estimated as 150 nM by reference to the G value (0.38×10^{-7} mol J^{-1}). This concentration is much less than the IC_{50} value (the concentration to reduce cellular survival to 50%) of **5-FdUrd** toward EMT6/KU cells that was reported to be 8.1 μM.[33] The significant radiation enhancement of cytotoxicity by **IQ-FdUrd** under hypoxic conditions is therefore attributable to the

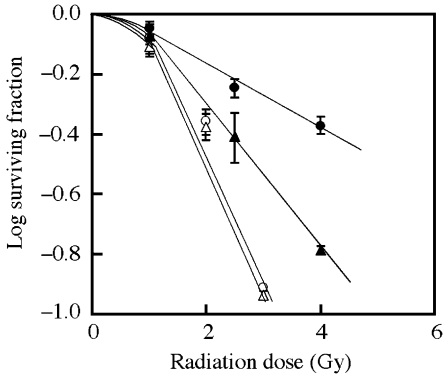


Figure 4. Survival of EMT6/KU cells after (○) aerobic irradiation; (△) aerobic irradiation + 0.2 mM **IQ-FdUrd**; (●) hypoxic irradiation; (▲) hypoxic irradiation + 0.2 mM **IQ-FdUrd**. Error bar represents SE of three to six experiments.

strongly cytotoxic effect of electrophilic iminium cations **1** in addition to released **5-FdUrd**.[34] In contrast, the previously reported eliminating substituent (2-oxoalkyl group) in **5-FdUrd** prodrug[19,20] could not show such a cytotoxicity as in the iminium cations **1**.

In summary, we designed and synthesized a novel radiation-activated prodrug of antitumor agent **5-FdUrd** possessing an indolequinone structure (**IQ-FdUrd**). We confirmed that **IQ-FdUrd** released **5-FdUrd** via one-electron reduction followed by bimolecular disproportionation of intermediate semiquinone radical anions upon hypoxic irradiation, and that the released **5-FdUrd** and electrophilic iminium cations **1** was responsible for the enhanced cytotoxicity against hypoxic EMT6/KU cells. In view of the hypoxia-selective radiation activation and cytotoxicity, **IQ-FdUrd** is potentially a prototype compound for new class of radiation-activated antitumor prodrugs that are useful for radiation treatment of hypoxic tumor cells.

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21. Radiolysis of a diluted aqueous solution at around pH 7.0 produces primary water radicals such as oxidizing hydroxyl radicals (·OH), reducing hydrated electrons (e^-_{aq}) and reducing hydrogen atoms (·H) with the *G* values of $G(\cdot OH) = 2.8 \times 10^{-7}$ mol J^{-1}, of $G(e^-_{aq}) = 2.8 \times 10^{-7}$ mol J^{-1}, and $G(\cdot H) = 0.6 \times 10^{-7}$ mol J^{-1}, respectively.
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26. **IQ-FdUrd**: Mp 202–204 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.85 (d, J = 6.0 Hz, 1H), 6.26 (t, J = 5.7 Hz, 1H), 5.55 (s, 1H), 5.21 (d, J = 3.6 Hz, 2H), 4.57 (br s, 1H), 3.98 (dd, J = 3.0, 6.6 Hz, 2H), 3.92 (br s, 1H), 3.86 (s, 3H), 3.74 (s, 3H), 2.39–2.39 (m, 2H), 2.24 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 178.0, 176.8, 159.2, 156.4 (d, J = 26.3 Hz), 148.8, 136.8, 127.6, 123.5, 123.1, 121.0, 116.0, 106.5, 87.5, 85.3, 69.7, 60.7, 56.4, 38.0, 35.6, 32.0, 9.1; FABMS (NBA) *m/z* 464 $[(M+H)^+]$; FAB-HRMS calcd for $C_{21}H_{22}N_3O_8F$ $[M^+]$ 463.1391, $C_{21}H_{23}N_3O_8F$ $[(M+H)^+]$ 464.1469. Found $[M^+]$ 463.1392, $[(M+H)^+]$ 464.1477.
27. Aqueous solutions of **IQ-FdUrd** (171 μM) containing 2-methyl-2-propanol (17.1 mM) were purged with Ar for 20 min and then irradiated in a sealed glass ampoule at ambient temperature with an X-ray source (5.8 Gy min^{-1}). Immediately after the irradiation, the solution was subjected to HPLC analysis.
28. The number of molecules produced or changed per 1 J of radiation energy absorbed by the reaction system.
29. In the radiolysis up to 900 Gy, **IQ-FdUrd** decomposed 23% under aerobic conditions, whereas 71% under hypoxic conditions. The yield of released **5-FdUrd** was less than 1% upon aerobic irradiation.
30. The laser flash photolysis experiments were carried out with Unisoku TSP-601 flash spectrometer. Aqueous solution of **IQ-FdUrd** (50 μM) and DMA (1 μM) were purged with Ar prior to the laser flash photolysis.
31. Selectivity was defined as percentage ratio of *G* values for formation of **5-FdUrd** and decomposition of starting prodrug.
32. **IQ-FdUrd** (200 μM) was dissolved in Eagle's MEM containing 12.5% fetal bovine serum (FBS). The resulting solution was added into a glass dish containing exponentially growing EMT6/KU cells, followed by incubation at 37 °C for 60 min. The dish was then purged with 95% N_2 + 5% CO_2 for 30 min prior to and during irradiation to establish hypoxic conditions. Irradiation was performed at ambient temperature by an X-ray source (250 kV, 15 mA) with a 0.25 mm Al filter at a dose rate of 5.8 Gy min^{-1}. After the irradiation, solution of **IQ-FdUrd** was aspirated off, and the cells were washed twice with 2 mL of Hank's Balanced Salt solution, trypsinized, counted, diluted, and planted into a 60 mmØ cultural dish in appropriate numbers (200–400), to which 5 mL of MEM plus 12.5% FBS was added. The cells were cultured in an incubator containing 5% CO_2 at 37 °C for 7 days to form the colonies. Seven days after irradiation, colonies were fixed with methanol, stained with diluted Giemsa's dye liquor, and counted to calculate surviving fraction. A colony was defined as containing more than 50 cells.
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34. The indirect effect of exposure to radiation, including the effect of hydroxyl radicals, is represented in the cytotoxicity induced by irradiation in the absence of **IQ-FdUrd**. Therefore, the enhancement of cytotoxicity upon hypoxic irradiation in the presence of **IQ-FdUrd** is mainly attributable to the effect of released **5-FdUrd** and iminium cations.